The 6th International Conference on Natural Products for Health and Beauty

"*New Frontiers in Natural Products for Health & Longevity*"

January 21-23, 2016

Pullman Raja Orchid Hotel, Khon Kaen, Thailand.

NATPRO 6

Proceeding

Hosted by :

Co-hosted by :

SCHOOL OF MEDICAL SCIENCES UNIVERSITY OF PHAYAO

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Department for Development of Thai Treaditional and Alternative Medcine

Office of TTM Knowledge Fund was established under the Department for Development of Thai Traditional and Alternative Medicine (DTAM), as a revolving fund for any operations related to the protection and promotion of the Thai traditional wisdom, based on the Protection and Promotion of Traditional Thai Medicinal Intelligence Act B.E. 2542.

Office of TTM Knowledge Fund will support the activities related to:

- Surveying, collecting, studying and researching on Thai traditional medicinal wisdom
- Cultivating, producing, processing or propagating medicinal plants
- Advertising on the protection and promotion of Thai traditional medicinal wisdom
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CONFERENCE REPORT

Professor Bungorn Sripanidkulchai

January 21-23, 2016
The 6th International Conference on Natural Products for Health and Beauty (NATPRO6)
Khon Kaen, THAILAND

Dear all delegates and participants.

On behalf of the organizing committee, it is our great pleasure to welcome you to participate in the 6th International Conference on Natural Products for Health and Beauty (NATPRO6) from 21st - 23rd January, 2016 at Pullman Raja Orchid Hotel, Khon Kaen, Thailand.

As initiated by Professor Maitree Suttajit, the 1st NATPRO was successfully organized in 2005 hosted by *Mahasarakham* University, the conference gave the strong foundation on networking of natural product research internationally. After that the NATPRO was continuously organized as a bi-annual event and recognized as a landmark in the calendar for researchers and experts in the field of natural products worldwide. As reported that the number of participants was increased in each conference of the NATPRO2 (2008, hosted by Phayao University), NATPRO3 (2011, hosted by Rangsit University), NATPRO4 (2012, hosted by Chiangmai University) and NATPRO5 (2014, hosted by Prince of Songkla University). The conference provides opportunities for delegates to come together and create network with top-in-the-field researchers, specialists, healthcare professionals, government leaders as well as product and consumer agencies.

This NATPRO6 was successfully organized by the support of four organizations of KKU, including Faculty of Pharmaceutical Science, Faculty of Technology, Center for Research and Development of Herbal of Products (CRD-HHP) and Fermentation Research Center for Value Added Agricultural Products (FerVAAP) , and co-hosted by the School of Medical Service, Phayao University.

New Frontiers in Natural Products for Health and Longevity is the main theme for the 6th NATPRO conference which focuses on the latest research as well as the future outlook of the fields of natural products. The topics include several area of natural product research such as Chemistry of Natural Products, Efficacy of Natural Products, Safety and Regulations on Natural Products, Cosmeceuticals, Nutraceuticals (Functional Foods) and Beverage, Health and Beauty Product Development and Innovation and Consumer Research.

Honorably, we have received the kindest acceptance from outstanding researchers in the natural products field to be the keynote speakers: Professor James Michael Wyss (University of Alabama at Birmingham, USA), Professor Soo-Un Kim (Seoul National University, Korea), Professor M.R. Jisnuson Svasti (Mahidol University, Thailand), Professor Anake Kijjoa (University of Porto, Portugal), Professor Hirota Fujiki (Saitama Cancer Center, Japan), ProfessorSirirurg Songsivilai (National Nanotechnology Center, NASTDA, Thailand), Professor Maitree Suttajit (University of Phayao, Thailand) and several distinguished invited speakers of the 6th NATPRO: Professor Hiroshi Noguchi (University of Shizuoka, Japan), Professor Shizuo Yamada (University of Shizuoka, Japan), Professor Yukihiro Shoyama (Nagasaki International University, Japan), Professor Madalena Pinto (University of Porto, Portugal), Professor Rolf G. Werner (University Tubingen, Germany, Professor Umah Rani Kuppusamy (University of Malaya, Malaysia), Professor Emilia Sousa (University of Porto, Portugal), Professor Fumiyuki Kiuchi (Keio University, Japan), Professor Kinzo Matsumoto (University of Toyama, Japan), Professor Ikuo Saiki (University of Toyama, Japan), Professor Hiroyuki Tanaka

(Kyushu University, Japan), Professor Friedrich Götz (University Tubingen, Germany), Dr. Uwe Scheuring (Boehringer Ingelheim Parma GmbH and Co. KG, Germany), Professor Xu Shen (Shanghai Institute of Materia Medica, Chinese Academy of Sciences, People's Republic of China), Professor Lai Ren (Kunming Institute of Zoology, Chinese Academy of Sciences, People's Republic of China), Dr. Tzung-hsien Lai (Development Center for Biotechnology, Republic of China), Professor Murat Kartal (Bezmialem Vakif University, Turkey), Professor Mosaad A. Abdel-Wahhab (National Research Center, Egypt), Professor Fassouane Abdelaziz (Ecole National de commerce et de Gestion Sattat, Morocco), Professor Amani S. Awaad (Prince Sattam Ben Abdoulazize University, Al-Kharj, KSA), Professor Jaehong Han (Chung-ung University, Korea), Professor Ki Hun Park (Gyeongsang National University, Korea), Associate Professor Gautam Sethi (National University of Singapore, Singapore), Professor Vimon Tantishaiyakul (Prince of Songkla University, Thailand), Professor Weerachai Kosuwon (Khon Kaen University, Thailand), Professor Pawinee Piyachaturawat (Mahidol University, Thailand), Associate Professor Wandee Rungseevijitprapa (Ubon Ratchathani University, Thailand). Professor Supa Hannongbua (Kasetsart University, Thailand), Professor Aranya Manosroi (Chiang Mai University, Thailand), Professor Jiradej Manosroi (Manose Health and Beauty Research Center, Thailand), Professor Supayang Voravuthikunchai (Prince of Songkla University, Thailand) and Associate Professor Neti Waranuch (Naresuan University, Thailand). With the strong supports from scientists all over the world, this two and a half days conference hosted more than 350 participants from 19 countries, including USA, Portugal, Germany, Turkey, Morocco, Egypt, *Kingdom of Saudi Arabia* (*KSA*), Japan, People's Republic of China, Republic of China, Korea, Indonesia, Malaysia, Singapore, Philippines, Kazakhstan, Sri Lanka, France and Thailand. Moreover, there are many innovative and exciting professional and social programs to fulfill this valuable time for all participants.

With the understanding of the significance of natural products research on the impact of herbal products development, several government sectors including the Department of Development of Thai Traditional and Alternative Medicine, National Nanotechnology Center (NANOTEC), the Thailand Research Fund (TRF), Agricultural Research and Development Agency (ARDA) kindly supported this conference.

Furthermore, the social and cultural events ware also supported by the Association of Pharmaceutical Science Alumni of Khon Kaen University.

Lastly, as the chairman of the NATPRO6, I would like to express my appreciation and thankful thought upon all the supports, devotion, great efforts, and enthusiasm from the honor speakers, participants, and the organizing committees. Confidently, the NATPRO6 will engage your time with invaluable experiences, intellectual inspiration, great company, and wonderful memories.

With warm regards,
Professor Bungorn Sripanidkulchai, Ph.D.
Chairman, the 6th NATPRO Organizing Committee.

WELCOME MESSAGE FROM KHON KAEN UNIVERSITY

Associate Professor Dr. Kittichai Triratanasirichai

President, Khon Kaen University

January 21-23, 2016
The 6th International Conference on Natural Products for Health and Beauty (NATPRO6)
Khon Kaen, THAILAND

Dear distinguished guests and privilege delegates.

Thank you all of the organizing committees for the privilege opportunity to give a greeting message to all the honored participants of the conference. On behalf of Khon Kaen University of Thailand, I would like to welcome you all into our city. Khon Kaen is one of the capital cities located in the center of the northeast region, called "E-saan" region, of Thailand which is recognized as the center city for commercial, administrative, beautifully cultural and educational activities. Moreover, Khon Kaen has been a metropolis with the fastest growing which has had a good landscape of the city on safe traveling environment and convenient transportation network as the export center for trade throughout the Indo-China Region. In addition, both Laos and Vietnam have instituted the consulates in Khon Kaen to facilitate the connection and exchanges between the countries. Therefore, Khon Kaen nowadays becomes a charming city for developing the commercial, educational business, and tourism where you can find the diverse array of special themed tour itineraries and delicious local culinary delicacies as well as famous and high quality of Thai silk.

May I take this opportunity to introduce you Khon Kaen University which was established as the major university in the Northeastern part of Thailand in 1964 and become as the largest university of the northeast region and one of the top nine national research universities of Thailand that is a major center of education and technology. This accomplishment has been from the contributions from all institutions and staff under the KKU umbrella throughout half of century, in which, the 50th anniversary of KKU was impressively celebrated in last year. Together, KKU and alliances have continuous learning, supporting and growing to moving toward the ultimate goal to become a leader in medicine including pharmacy, public health and tropical health; tropical agriculture and farming (animal science and plant science), bio-diversity in the Greater Mekong Sub-region; an educational center of the Greater Mekong Sub region.

On behalf of the host, we are so glad to welcome you to the 6th International Conference on Natural Products for Health and Beauty (NATPRO6) themed "New Frontiers in Natural Products for Health & Longevity", I believe that this conference will provide an excellent platform for new innovation and research in the fields of natural products which will bring about the development in all related aspects of chemistry, efficacy, safety and regulations, cosmeceuticals and nutraceuticals, functional foods and beverages, health and beauty product and consumer researches for better quality of life for global citizen. This is one of the most outstanding research area of KKU, throughout the research activities, many patents are delivered, and this year among all universities in Thailand, KKU has been pronounced by the Department of Intellectual Property of Thailand to have the highest number of patents with number of 737.

At present, I have learned that The NATPRO conference is a distinguished bi-annual event in the calendar for researchers and experts in the field of natural products, hereof, the opportunities and stage for creating strong bonding, cooperation, and exchange the knowledge and technology on herbal and healthy product development among universities and academic institutions in various countries and worldwide are provided. Last but not least, faithfully, this conference will be an ideal platform for all delegates to share, learn, and connect to each other for the fullest extended of benefits and progresses for all in term of collaboration, academy, research, health policy and society. Also with my best wishes, all of our honored delegates will have a good time and good experiences in the conference and in exploring the Khon Kaen city.

Sincerely,

Kittichai Triratanasirichai, Ph.D.
President, Khon Kaen University

MESSAGE FROM DEPARTMENT FOR DEVELOPMENT OF THAI TRADITIONAL AND ALTERNATIVE MEDICINE (DTAM)

Dr. Suriya Wongkongkathep

Director–General
Department for Development of Thai Traditional and Alternative Medicine

January 21, 2016
The 6th International Conference on Natural Products for Health and Beauty (NATPRO6)
Khon Kaen, THAILAND

Associate Professor Dr. Kittichai Triratanasirichai, President of Khon Kaen University
Professor Dr. Bungorn Sripanidkulchai, Chairman of the Organizing Committee of the 6th NATPRO
Distinguished guest speakers
Natural Product Researchers and Participants of the 6th NATPRO
Ladies and Gentlemen

On behalf of the Ministry of Public Health of Thailand, I would like to welcome all of you to Thailand and thank you for visiting our country and participate in the 6th NATPRO in Khon Kaen province during January 21-23, 2016.

The Department for Development of Thai Traditional and Alternative Medicine or DTAM, Ministry of Public Health and the Thai Traditional Medical Knowledge Fund are honored to be co-sponsors of the 6th NATPRO, one of the most significant conferences on research and development of natural health and beauty products in Thailand and in Asia in 2016. It is also a great opportunity that the Faculty of Pharmaceutical Sciences, Center of Research and Development of Herbal Health Products and Faculty of Technology of Khon Kaen University co-hosted this conference as these academic and research institutions have been very active in R&D of new health products from Thai medicinal plants.

Even though the weakening of global economic conditions has decreased the growth rates of natural health and beauty products during the past few years, the demand of consumers worldwide for such products still remains high because of health concerns about the safety of synthetic chemicals and general public perception of natural products as safer choice. As such, at the end of 2015, surveys conducted in Thailand by two business schools and the EXIM bank, all predict that health food, health products and natural beauty products businesses and industries will still be on the rise in 2016. In addition, as Thailand is now an aging society moving towards aged society in 2020s, there is a need for health foods and products for the elderly that will help promote healthy aging and longevity.

Therefore, the theme of the 6th NATPRO "New Frontiers in Natural Products for Health & Longevity" is quite appropriate as the participants will have the chance to exchange knowledge and learn about the latest research and the future outlook of natural products, including chemistry, safety and efficacy, cosmeceuticals and nutraceuticals, functional foods and beverages, health and beauty product development and innovation, and consumer researches.

The present government of Thailand, under the leadership of General Prayuth Chan-O-Cha, fully recognizes that value-added herbal health products not only benefit the health of the people but also significantly contribute to the economy of the country from the grass root level up. It is estimated that the value of herbal medicines and health foods, spa and cosmeceutical products accounts for a about 100,000 million baht per year. Therefore, one of the policies of the Public Health Minister, Professor Dr. Piyasakol Sakolsatayadorn, is "*to carry out comprehensive research and development of Thai herbs and Thai traditional medical wisdom for the benefit of Thai economy*".

As a result, the Department for Development of Thai Traditional and Alternative Medicine is therefore tasked by the Government and the Ministry of Public Health to collaborate with all public and private offices involved, such as the Ministry of Agriculture and Cooperatives and the Ministry of Commerce, to formulate "**National Master Plan on the Development of Thai Herbal Products 2017-2021**". The Plan is aimed at systematically push forward the development of Thai herbal products for health and for commercial purposes. The Master Plan will comprise of 4 sub-plans, namely:-

Plan 1 – **Production of potential Thai herbal products** that meet the demands of domestic and international markets

Plan 2 – Development of Thai herbal products **industry** and international **markets** for Thai herbal products

Plan 3 – Promotion of the **use** of herbal products **for therapeutic and health promotion purpose**s

Plan 4 – **Strengthening administrative system** and government policy for sustainable implementation

It is expected that the Master Plan will be finalized and endorsed by the Cabinet in this coming April.

Regarding the use of herbal health products for health care, the government fully supports the use of herbal medicines and Thai traditional medicine and alternative medicine services as one of the twelve service plans of the country's health service system. Thailand's Ministry of Public Health has so far selected 50 traditional medicines and 24 single herbal medicinal products in the National List of Essential Medicines to treat and relieve common minor diseases and symptoms in order to promote self-reliance on health care of Thai people and the country. More items will be added into the list in the future.

Currently, the Ministry supports research and development of new herbal medicinal products for the treatment and prevention of chronic non-communicable diseases that are major health problems of the country. With the financial support from the National Research Council of Thailand, Thai FDA is now formulating plans to assist researchers during the R&D process so that their research outputs will not only support evidence-based medicine but also meet the requirements of Thai FDA and ASEAN market for product registration and licensing.

The 6th NATPRO has convened at a crucial moment of time and serves as a wonderful platform for prominent scientists in the fields of natural products from all over the world to share their latest research results on natural product development. It is hoped that the conference will also provide an excellent opportunity for the participants to build international research collaboration with colleagues and experts from abroad to strengthen R&D in this field even further.

Ladies and gentlemen

Finally, I would like to express my sincere gratitude to the Organizing Committee and the Scientific Committee, the hosts and co-hosts and all those who have worked so hard to make this conference happen. I am also thankful for distinguished speakers and researchers participating in this conference for sharing your valuable findings and experience and contributing to the advancement in the field of natural products development. For the speakers and participants from abroad I hope you have a pleasant stay in Thailand and enjoy you time in Khon Kaen.

Thank you very much.

Dr. Suriya Wongkongkathep
Director –General
Department for Development of Thai Traditional and Alternative Medicine
Ministry of Public Health, Thailand

WELCOME MESSAGE FROM FACULTY OF PHARMACEUTICAL SCIENCES, KHON KAEN UNIVERSITY

Associate Professor Dr. Paiboon Daosodsai,

Dean of Faculty of Pharmaceutical Sciences

January 21-23, 2016
The 6th International Conference on Natural Products for Health and Beauty (NATPRO6)
Khon Kaen, THAILAND

Dear all delegates and participants.

This is an auspicious occasion to welcome all delegates and participants to the 6th International Conference on Natural Products for Health and Beauty (NATPRO6). On behalf of Faculty of Pharmaceutical Sciences, Khon Kaen University, it is so nice that we come back to meet each other again since last year the NATPRO 5th was held in Phuket, Thailand.

NATPRO was established in 2005 by Professor Maitree Suttajit, Mahasarakham University with the aim of building research networking on natural products. NATPRO 2, 3, 4, and 5 were subsequently organized by Naresuan University, Rangsit University Chiang Mai University and Prince of Songkla University in 2008, 2011, 2012 and 2014, respectively. The past conferences have been a successful platform for bringing together researchers of many disciplines with a common interest in learning innovation. After outstanding success of the 5th Conference on Natural Products for Health and Beauty until now the 6th International Conference on Natural Products for Health and Beauty (NATPRO 6) is on the way with the main themed "New Frontiers in Natural Products for Health & Longevity" which will take place on January 21-23, 2016 in Khon Kaen, Thailand. As the host, I would like to invite and welcome delegates, educators and researchers from around the globe to participate the NATPRO6.

It's an opportunity to participate, share ideas and experiences among delegates from different regions. The programs consist of invited an excellent line-up of eminent speakers who will deliver plenary and invited lecture and contributed papers in related Natural Products for Health and Beauty learning and research.

The Faculty of Pharmaceutical Sciences and Khon Kaen University acknowledge the opportunity of developing in teaching and research to be abreast of the rapidly developing and networking on natural products, thus this international conference on natural products was initiated. It is a great privilege that we are honored by all fame professors from many countries as Professor James Michael Wyss (USA), Professor Hirota Fujiki (Japan), Professor Soo-Un Kim (South Korea), Professor Anake Kijjoa (Portugal), Professor M.R. Jisnuson Svasti (Thailand), Professor Sirirurg Songsivilai (Thailand) and Porf.Maitree Sutthajit (Thailand) to be our keynote speakers with 33 invited speakers. At this opportunity, I would like to express our thankful thought and appreciation to all our keynote speakers and Professor Bungorn Sripanidkulchai who is the Chairman of the 6th NATPRO Organizing Committee and all organizers for tremendous efforts and preparation the conference.

Conclusively, I believe that you will find the fullest benefits from informative and interesting keynotes, presentations and posters. Hopefully, you will have an engaging and enjoyable experience in Khon Kaen as well as neighbor provinces which is one of the most popular destination in the northeast of Thailand for visitors around the world.

Sincerely,

Paiboon Daosodsai, Ph.D.
Dean of Faculty of Pharmaceutical Sciences
Khon Kaen University

WELCOME MESSAGE FROM FACULTY OF TECHNOLOGY, KHON KAEN UNIVERSITY

Associate Professor Dr. Pornthap Thanonkeo

Dean of Faculty of Technology
January 21-23, 2016, Khon Kaen, THAILAND

Dear colleagues, ladies, and gentlemen,

On behalf of the organizing committee of the 6th International Conference on Natural Products for Health and Beauty (NATPRO6) held during the period of January 21st - 23rd, 2016 at Pullman Raja Orchid Hotel, Khon Kaen, Thailand. This has been such our great pleasure to welcome you to Thailand and engaging program of the NATPRO6 conference that certainly will provide valuable opportunities for all attendees. Together with warm welcome from all organizing committees, we have received great honor from the outstanding professors from every corner of the globe in the area of health and beauty as our keynote speakers. Throughout 10-year journey, the NATPRO conferences have forged ahead and resulted of fantastic outcomes year by year, strong bonding among involving parties in academic and research, developing of young generation researchers' networks and more social impact outcomes in term of health and beauty. Impressively, simultaneous strengthening the academic and research via inter-professional exchange and collaborations has been also delivered from this continuous annual conference program.

On behalf of Faculty of Technology, Khon Kaen University who appreciably takes part as a host of the NATPRO6, I would like to express the thankful thought upon all supports, encouragement, and contributions from all parties that have made this conference go on with forge relationship, respect and creativity.

Wishfully, you will fruitfully find the warm friendships and excellent opportunities to expand and strengthen your capability in academic and research works in the NATPRO6.

I look forward to welcoming you to this exciting conference and to meeting all of you in person during the conference.

Sincerely,

Associate Professor Pornthap Thanonkeo, PhD.
Dean of Faculty of Technology
Khon Kaen University

Theme of the 6th International Conference on Natural Products for Health and Beauty (NATPRO6)

On behalf of the organizing committee, it is our great pleasure to invite you to participate in the 6th International Conference on Natural Products for Health and Beauty (NATPRO6) from 21st to 23rd January, 2016 at Pullman Raja Orchid Hotel, Khon Kaen, Thailand.

"New Frontiers in Natural Products for Health & Longevity" is the main theme for the 6th NATPRO Conference which focuses on the latest research as well as the future outlook of the fields of natural products.

The Conference Topics for NATPRO6 cover:

- Chemistry of Natural Products
- Efficacy of Natural Products
- Safety and Regulations on Natural Products
- Cosmeceuticals, Nutraceuticals (Functional Foods) and Beverages
- Health and Beauty Product Development and Innovation
- Consumer Research
- Miscellaneous

Venue: Pullman Khon Kaen Raja Orchid Hotel, Khon Kaen, Thailand

Dear Colleagues,

On behalf of the editorial board, it is a great pleasure to publish the e-proceedings of the manuscripts submitted to the 6th International Conference on Natural Products for Health and Beauty (NATPRO6), organizing by 4 hosts of Khon Kaen University including the Faculty of Pharmaceutical Sciences, the Faculty of Technology, the Center for Research and Development of Herbal Health Products and the Fermentation Research Center for Value Added Agricultural, at Pullman Racha Orchid Hotel, Khon Kaen, Thailand, during January 21-23, 2016. The bi-annual NATPRO conference was continuously organized with the regular published proceedings and abstract book. Both the abstract book and proceedings are also available in the CD forms. With the strong support from distinguished scientists from more than 19 countries, this conference received more than 300 abstracts and more than 70 articles. We would like to take this opportunity to thank the organizing and scientific committee and the participants for their enthusiatic contributions. We hope to share the recent discovery of natural product research in the next NATPRO7.

Best regards,

Associate Professor Jomjai Peerapattana

Editor

Chemistry of Natural Products (CNP)
Full Paper

Chemical composition of methanol extract of pilis: An Indonesian forehead transdermal herbal medicine

Kris Herawan Timotius*, Adit Widodo Santoso

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ABSTRACT

Pilis is one of the herbal medicine applied into forehead to recover the healthy condition of especially afterbirth mother. Since the function of pilis depends on its bioactive compounds, then the study on its bioactive compounds is a very important preliminary step for creating new approach in the delivery of the compounds. The objectives of this study were firstly to determine the main chemical bioactive compounds in the methanol extracts of pilis from various producers, and secondly to compare the results with the previous studies of the hexane extract of either pilis or its plant materials. The methanol extracts were analysed with GC-MS. The findings showed that most of the methanol extract of pilis contained eugenol and tumerone as their main components. Tumerone was detected in methanol extract but not in hexane extract of the previous studies.

Keywords: forehead, herbal, jamu, javanese, medicine, pilis, transdermal

INTRODUCTION

As herbal medicine, pilis was wellknown in Java and applied into forehead and temple for treatment of dizziness, hazy vision and eyestrain and blurred eye visions. Pilis was also wellknown for treatment of headache and fever. In our previous publication we used the term topical herbal medicine, but now we use the term transdermal medicine since there is a strong evidence that the substance in pilis were absorbed through skin and distributed in blood stream and give influence on to the brain as a center for headache related problems [1].

The objective of this study was to compare the chemical composition of methanol extracts of pilis from different producers: large jamu factory, local jamu factory, and home-made jamu producers. The differences all of the samples are the producers and the compositions from each pilis samples. The results were also compared with the previous research on the hexane extracts. The analysis was performed by GC-MS.

MATERIALS AND METHODS

Samples

The criteria was we used in Pilis samples selection are product package and the product distribution market. Samples of pilis were obtained from different producers, i.e. large jamu factory, local jamu factory, and home-made jamu producers. Products (pilis) of large jamu industries were distributed nationally. Products of local jamu producers were distributed or wellknown in certain city. Home-made jamu pruducers sell pilis into local market or at home. Total number of the analysed samples was nine.

Methanol Extraction

Samples were obtained from nine producers (large jamu factory, local jamu factory, and home-made jamu producers). Samples of pilis were crushed and powdered or pounded. 100 Grams of the pilis powder of each sample were extracted by methanol using Soxhlet extraction for eight hours. The methanol extracts were concentrated under reduced pressure . The concentrated methanol extracts were then injected to GC-MS.

Chemical Analysis with GC-MS

GC-MS analysis was performed as the previous experiment [1]. The Shimadzu GCMS-QP2010S was used for the analysis with column AGILENT HP, 30 m length, ID 0.25 mm, film 0.25 µm. The carrier gas was helium. Its ionisation was EI at 70 eV. The column oven temperature 70 °C. The injection temperature was 300 °C, Injection mode was splitless. Sampling time was 0.20 min. Flow control Mode: pressure was 13.7 kPa. Total flow was 40.0 mL/min. Column flow was 0.50 mL/min. Split ratio was 73.0. Oven temperature program was with rate 5.00, temparature 70 °C – 300 °C, and hold time was 5-29 min. Start time was 3 min. End time was 80 min. The obtained spectra were identified by comparing with the library NIST12LIB.

RESULTS

Chemical composition of methanol extract of pilis

The components of methanol extract could be divided into three groups, namely main components, minor components, and fatty acids group. The main components of the methanol extract of various pilis were dominated by beta tumerone and eugenol. Both of them were detected in 8 samples.

The minor components were varied significantly among the samples (Table 1). The essential oils components of methanol extracts were varied in local jamu factory and home made jamu producers. The local jamu factory pilis and home made pilis were fresh products, fresher than products from large jamu factory. The components of large jamu factory were less varied than local and home made jamu producers. It is necessary to note that several components were not identified with the available data base library (NIST).

The fatty acid group consisted of lauric acid, stearic acid, palmitic acid, and linoleic acid. Not all pilis has a significant amount of fatty acid. This mean that there were two groups of pilis, i.e. pilis with oil addition and pilis without oil addition.

DISCUSSION

Beta tumerone is a characteristic compound of members of Zingiberaceae, such as *Zingiber officinale*, *Z. montana*, *Curcuma longa* which are used as components of pilis [1]. Tumeric compounds are found in many member of Zingiberaceae. Their function are varied, such as for regeneration of brain cells [2,3], anti-depressant [4], and P-Glycoprotein activities [5]. Beta tumerone was not detected in hexane extracts, and detected as main compound in mathanol extract. This is due to its insolubility in hexane.

Eugenol is an essential oil and could be found in various plant materials. Eugenol has several health effects, such as antioxidant activity, anti-inflammatory action, and antibacterial and antiviral effects [6]. Eugenol is a bioactive compound from cajuput. Usually, Cajuput fruit is used as component of pilis.

The variation of detected components expressed the different formula and quality of the plant meterials used by the producers. The composition was different in terms of the plant materials as well as the quantity and quality of these materials. There were different profil of pilis. Pilis from large jamu factory were different with the local jamu factory or the home made jamu producers.

Fatty acid content could be used as criteria to divide pilis into two groups. Pilis with high content of oil and low content of oils. This is because of the addition of plant oil such as coconut oil. In the previous study with hexane showed also this evident. The large jamu factory added plant oil more than local jamu factory and home made jamu producers. The role of oil is probably important as carrier and comfort feeling for the transdermal process through the skin.

Compared with the previous study with hexane [1,7], methanol extraction as polar extraction showed many similar constituents, namely eugenol, 1,8-cineol, methyl salicylate, anethole, zingeberene, menthol, beta pinene etc. But their variety in methanol was less than in hexane extracts. Several compounds in hexane extract were not detected in methanol extract, such as DMPBD, asaron, shogaol, amyrin.

Table 1. GC-MS-Chemical composition of methanol extract of Pilis (Relative amount %)

	COMPOUNDS	LARGE JAMU FACTORY		LOCAL JAMU FACTORY			HOME BASED JAMU PRODUCER			
		LJG	LMN	OSB	OGT	OHG	HIS	HJJ	HSL	HTJ
Main Components	Eugenol	0.37	4.76	3.70	9.05	n.d.	9.43	19.92	1.13	7.78
	Beta tumerone	7.75	6.28	8.01	n.d.	2.36	0.54	14.11	4.03	8.16
	Vanillin	0.56	2.89	0.66	10.33	n.d.	n.d.	n.d.	n.d.	n.d.
	1,3-Benzenedicarboxylic acid, 4-methyl, dimethyl ester	2.87	17.67	n.d	15.64	n.d.	n.d.	n.d.	n.d.	n.d.
	Zingerone	0.38	9.77	2.32	n.d.	n.d.	0.32	n.d.	n.d.	n.d.
	Methyl salicylate	0.14	n.d.	0.46	n.d.	5.71	n.d.	n.d.	7.78	n.d.
	Germacrone	4.00	n.d.	2.25	n.d.	1.21	n.d.	n.d.	10.36	n.d.
	Zerumbone	n.d.	2.58	n.d.	n.d.	4.75	n.d.	n.d.	6.03	n.d.
	Coumarin	n.d.	2.39	1.02	5.02	0.83	n.d.	n.d.	n.d	n.d.
	Alpha Chamigren	10.29	n.d	3.49	n.d	2.97	n.d.	n.d.	n.d	n.d.
	Ethyl cinnamate	n.d.	7.16	n.d.	n.d.	n.d.	0.47	16.06	n.d	10.38
	2-Furancarbonxaldehyde, 5-(hydroxymethyl)-	n.d.	n.d.	n.d.	n.d.	n.d.	11.14	11.45	1.81	n.d.
Minor Components	Guaiol	0.55	n.d.	2.76	n.d.	n.d	n.d.	n.d.	n.d.	n.d.
	Anethol	2.53	n.d.	n.d.	n.d.	5.09	n.d.	n.d.	n.d.	n.d.
	Alpha, alpha-Dicumyl	2.39	n.d.	n.d.	n.d.	1.34	n.d.	n.d.	n.d.	n.d.
	Alpha tumerone	1.17	n.d.	1.83	n.d.	n.d.	n.d.	6.42	n.d.	n.d.
	3,5-octadiene, 4,5-diethyl	n.d.	5.33	n.d.	4.61	n.d.	n.d.	n.d.	n.d.	n.d.
	9,10-Anthracenediene	n.d.	3.6	n.d.	4.61	n.d.	n.d.	n.d.	n.d.	n.d.
	Phenol, 2,4,6 tris (1,1-dimethyl(ethyl)	n.d.	n.d.	n.d.	n.d	3.29	n.d.	n.d.	1.94	n.d.
	Cyclohexane, 1,1-dimethyl-2,4-bis(1-methylethenyl-,cis	n.d.	n.d.	n.d.	n.d.	n.d	n.d.	13.48	n.d.	3.59
	Camphor	n.d.	n.d.	n.d.	n.d.	n.d	5.17	n.d.	4.42	n.d.
	Neral	n.d.	n.d.	n.d.	n.d.	4.33	n.d.	n.d.	4.44	n.d.
	Citral	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	7.11	n.d.
	4-vinyl-2-methoxy-phenol	n.d.	4.16	1.02	1.86	n.d.	n.d.	n.d.	n.d.	n.d.
	Ar-Curcumene,	2.51	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	1,8 Cineol	n.d.	n.d.	n.d.	n.d	n.d.	4.85	n.d.	n.d.	n.d.
	Tyranton	n.d.	n.d.	0.29	n.d.	n.d.	3.05	n.d.	n.d.	n.d.
	Alpha-Pinene	n.d.	n.d.	n.d.	n.d.	n.d.	10.22	n.d.	n.d.	n.d.
	4-Terpineol	n.d.	n.d.	0.42	n.d	n.d.	3.49	n.d.	n.d.	n.d.
	DMPBD	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	8.86	n.d.	n.d.
Fatty Acids	Lauric acid	n.d.	n.d.	n.d.	n.d	n.d	5.23	0.49	3.12	n.d.
	Stearic acid	n.d.	n.d.	n.d.	n.d.	1.76	n.d.	n.d.	1.97	n.d.
	Oleic acid	17.84	0.82	8.62	3.15	7.21	2.34	0.79	3.89	9.37
	Palmitic acid	2.96	2.05	5.14	12.75	10.53	3.11	1.07	6.76	5.07
	Linoleic acid	n.d.	n.d.	11.57	n.d.	8.72	0.31	n.d.	2.77	9.96
	Total Fatty Acids	20.8	2.87	25.33	15.9	28.22	10.99	2.35	18.51	24.4

Note :

LJG : Large Jamu Factory JAGO Semarang
LMN : Large Jamu Factory MENEER Semarang
OSB : Local Jamu Factory SAMBETAN Klaten
OGT : Local Jamu Factory GENTONG Kudus
OHG : Local Jamu Factory HANGAT Klaten
HIS : Home Based Producer IYAH SEGAR Klaten
HJJ : Home Based Producer JOGJA Jogja
HSL : Home Based Producer SALATIGA Salatiga
HTJ : Home Based Producer TANJUNG Klaten
n.d. : not detected

CONCLUSIONS

Eugenol was the main component in pilis, either in hexane or in methanol extract. Beta tumerone was detected only in methanol extract, not in hexane extract. Two types of pilis was founded, i.e. oily pilis and non oily pilis.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

ACKNOWLEDMENTS

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REFERENCES

1. Timotius KH, Santoso AW. 2015. Major Bioactive Compounds of Pilis Plant Materials: A GC-MS Analysis. Pharmacognosy Communications. 5 (III), 1-6.
2. BioMed Central. 2015. Tumeric compounds boosts regeneration of brain stem cells. 26 September 2014, retrieved 04 May 2015 from http://medicalpress.com/news/2-14=09-tumeric-compound-boosts-regeneration-brain.html
3. Hucklenbroich J, Klein R, Neumaier B, Graf R, Fink GR, Schroeter M, Rueger MA. 2014. Aromatic-tumerone induces neural stem cell proliferation *in vitro* and *in vivo*. Stem Cell Research & Therapy. 5, 100.
4. Liao JC, Tsai JC, Liu CY, Huang HC, Wu LY, Peng WH. 2013. Antidepressant-like activity turmerone in behavioral despair tests in mice. BMC Complement. Altern. Med.13, 299.
5. Yue GGL, Cheng SW, Yu H, Xu ZS, Lee JKM, Hon PM, Lee MYH, Kennelly EJ, Deng G, Yeung SK, Cassileth BR, Fung KP, Leung PC, Lau CBS. 2012. The Role of Turmerones on Curcumin Transportation and P-Glycoprotein Activities in Intestinal Caco-2-Cells. J. Med. Food. 15(3), 242-252.
6. Pavithra B. 2014. Eugenol-A Review. J. Pharm. Sci. & Res. 6(3), 153-154.
7. Timotius KH, Sari IN, Santoso AW. 2015. Oil Composition of Pilis: An Indonesian Forehead Topical Herbal Medicine. Pharmacognosy Communications. 5 (I), 46-51.

Isoprenylated xanthones from the stem bark of *Garcinia dulcis*

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ABSTRACT

Garcinia dulcis Kurz. (Guttiferae) is widely distributed in the southern Thailand. The crude dichloromethane extract of the stem bark of *G.dulcis* exhibited strong antibacterial activity. Study on the chemical constituents of the extract resulted in the isolation of six known isoprenylated xanthones: globuxanthone, 1-*O*-methylglobuxanthone, subelliptenone H, symphoxanthone, 1-*O*-methylsymphoxanthone and subelliptenone D. Their structures were elucidation by spectroscopic methods as well as comparison the data with those previously reported.

Keywords: Guttiferae, *Garcinia dulcis*, xanthones, antibacterial activity

INTRODUCTION

Xanthones are well recognized as chemotaxonomic markers for *Garcinia* genus (Guttiferae). Many xanthones have been reported for several biological activities, including antioxidant, antimalarial, anti-inflammatory, antibacterial and cytotoxic. *Garcinia dulcis*, is widely distributed in the southern Thailand, commonly known as Ma-Phut. Many parts of this plant species have been used in Thai folk medicine. For example, the leaves and seeds have been used against diseases [3] and the stem bark has been used as an anti-inflammatory agent. With the aim of searching for biologically active compounds, the crude extract of the stem bark of *G. dulcis* and subfractions were preliminary testing on antibacterial activity. Subfractions which exhibited the interestingly antibacterial activity against *S. aureus* (MIC 16-32 □g/mL) were found to show the ^{1}H NMR signals corresponded to xanthones with isoprenyl unit. We therefore tried to isolate xanthones from the stem bark of *G. dulcis* especially the isoprenylated xanthones.

MATERIALS AND METHODS

Plant material

The stem barks of *G. dulcis* were collected from Meuang district, Nakhon Srithammarat province in the southern part of Thailand.

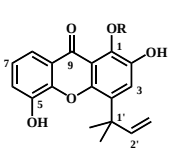
Extraction and isolation

The chopped-dried stem barks of *G. dulcis* (1.5 kg) were extracted with dichloromethane for 30 minutes (2 times) at 35˚C in a sonicator bath. The solution after filtration was evaporated, to give a brown viscous extract (60.15 g). The crude extract was subjected to a quick column chromatography and eluted with gradient of acetone in hexane to acetone. On the basis of their TLC characteristic, fractions which contained the same major compounds were combined to give ten fractions (A-J). Fraction D (MIC 16□□g/mL), E (MIC 32□□g/mL), I and J, which on the ^{1}H NMR spectrum showed the resonances corresponded to isoprenylated xanthone, were further purified. Fraction D (9.12 g) was subjected on sephadex LH-20 with MeOH:CH_2Cl_2 (8:2) to give five subfractions (D1-D5). Subfraction D5 was further purified by column chromatography over silica gel with 15% acetone in hexane to give yellow amorphous solid **1** (10.8 mg), **2** (2.8 mg) and **3** (13.2 mg). A yellow solid **4** (4.5 mg) was isolated from fraction E (15.50 g) by sephadex LH-20 with MeOH:CH_2Cl_2 (8:2) followed by column chromatography using 100% dichloromethane as mobile phase. Fraction I (2.24 g) was purified by

sephadex LH-20 CC with MeOH:CH_2Cl_2 (8:2) to give three subfractions (I1-I3). Subfraction I3 was further purified by column chromatography over silica gel with 2% MeOH in CH_2Cl_2 to give a yellow amorphous solid **5** (3.0 mg). Fraction J (0.79 g) was subjected on sephadex LH-20 and eluted with MeOH:CH_2Cl_2 (8:2) to give four subfractions (J1-I4). The fourth subfraction was repeatedly subjected to sephadex LH-20 using MeOH:CH_2Cl_2 (8:2) to give three subfractions (J4.1-J4.3). A yellow amorphous solid **6** (2.3 mg) was obtained from subfraction J4.3 by column chromatography using 15% acetone in hexane as an eluent.

RESULTS

The stem bark of *G. dulcis* was extracted with dichloromethane. The extract showed activity against *S. aureus* with MIC 32 μg/mL. The active subfractions (MIC 16-32 μg/mL), which on the ^{1}H NMR spectrum showed the resonances corresponded to isoprenylated xanthone, were further purified by column chromatography to give six known isoprenylated xanthones: globuxanthone (**1**), 1-*O*-methylglobuxanthone (**2**), subelliptenone H (**3**), symphoxanthone (4), 1-*O*-methylsymphoxanthone (**5**) and subelliptenone D (**6**).



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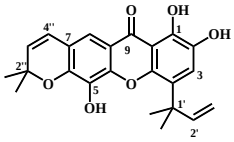
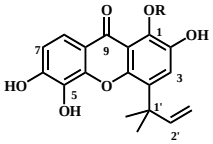
2: R = CH_3



3



4: R = H
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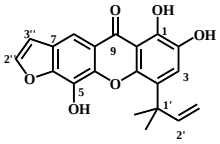

6

Compounds **1**-**6** were obtain as yellow amorphous solids. The UV (MeOH) spectrum showed the λ_{max} (nm) in the range of 203-208, 250-255 and 320-330. The FT-IR (neat) spectrum showed absorption of O-H stretching in the range of 3450-3200 cm^{-1} and C=O stretching in the range of 1650-1615 cm^{-1}.

Globuxanthone (**1**), a yellow amorphous solid. The ^{1}H NMR spectrum showed the resonances of a hydrogen bonded hydroxyl proton 1-OH (δ 12.56), singlet aromatic proton H-3 (δ 7.36) and resonances corresponded to the isoprenyl unit comprise of *gem*-dimethyl protons 1′-$(CH_3)_2$ (δ 1.46), terminal olefinic H-3′ *trans* (δ 5.27, *dd*, *J* = 17.7, 1.5 Hz) and H-3′ *cis* (δ 5.12, *dd*, *J* = 10.8, 1.5 Hz) and a methine proton H-2′ (δ 6.30, *dd*, *J* = 17.7, 10.8 Hz). The cross peaks in the HMBC correlations of H-3 to C-1, C-4a, C-1′ and H-2′, 1′-$(CH_3)_2$ to C-4 were supported the location of H-3 and the isoprenyl group at C-4, respectively. The spectrum further showed the signals with ABM pattern of aromatic protons H-6 at δ 7.25, (*dd*, *J* = 7.8, 1.5 Hz), H-7 at δ 7.18 (*t*, *J* = 7.8 Hz) and H-8 (δ 7.67, *dd*, *J* = 7.8, 1.5 Hz). The most deshielded resonance was assigned for H-8 according to anisotropic effect of the carbonyl group. The HMBC spectrum showed correlations of H-6 to C-8, C-10a; H-7 to C-5, C-8a and H-8 to C-6, C-9, C-10a. Finally, 2-OH and 5-OH were assigned to complete the structure [4].

1-*O*-Methylglobuxanthone (**2**), m.p. 144.0-145.5°C. The ^{1}H and ^{13}C NMR spectrum showed the similar resonances to that of **1**, except for the presence of a methoxy resonance at δ 3.96 instead of a hydrogen bonded hydroxy resonance. The methoxy group was confirmed at C-1 according to the correlation of the methoxy proton and H-3 to C-1. It was then suggested to be methoxy derivative of **1** [6].

Subelliptenone H (**3**), a yellow amorphous solid. The ^{1}H NMR and ^{13}C NMR spectrum indicated that **3** contained a penta-substituted benzene ring B as for **1**. The spectrum further showed the resonances of singlet aromatic proton H-8 (δ 7.38) and singlet of methyl protons 2″-$(CH_3)_2$ (δ 1.47), doublet of *cis*-olefinic protons H-3″ (δ 5.68, *J* = 9.9 Hz) and H-4″ (δ 6.37, *J* = 9.9 Hz), which were typical signals of 2,2-dimethylchromene ring. The cross peaks in the HMBC correlations of H-8 to C-9, C-4″, C-6, C-10a; H-4″ to C-6, C-8, C-2″ and H-3″ to C-7, 2″-$(CH_3)_2$ suggested that the chromene ring was attached to core structure at C-6, C-7 and *ortho* to H-8. Finally, 2-OH and 5-OH were assigned to complete the structure [1].

Symphoxanthone (**4**), m.p. 195.5-196 °C. The ^{1}H NMR spectrum indicated that **4** contained a penta-substituted benzene ring B as for **1**. While ring A was assigned for tetra-substituted benzene ring from two of doublet resonances with a coupling constant of 9.0 Hz at □ 6.98 (H-7) and δ 7.74 (H-8). The HMBC correlations of H-7 to C-5, C-8a and H-8 to C-6, C-9, C-10a supported protons H-7 and H-8, respectively. Finally, 2-OH, 5-OH and 6-OH were assigned to complete the structure [4].

1-*O*-methylsymphoxanthone (**5**), a yellow amorphous solid. The ^{1}H and ^{13}C NMR spectrum showed the similar pattern to that of **4**, with the absence of hydrogen bonded hydroxy signal but the presence of a methoxy proton signal at

δ 3.94. The methoxy group was confirmed at C-1 according to the correlations of the methoxy proton and H-3 to C-1. It was therefor assigned as a methoxy derivative of **4** [5].

Subelliptenone D (**6**), a yellow amorphous solid. The ^{1}H NMR spectrum indicated that **6** contained a penta-substituted benzene ring B and H-8 (□ 8.09) as for **3**. The spectrum further showed signals of *cis*-olefinic protons of furan ring at δ 6.91 (*d*, *J* = 2.1 Hz, H-3′′) and□□ 7.75 (*d*, *J* = 2.1 Hz, H-2′′). The cross peaks in the HMBC correlations of H-8 to C-9, C-3′′, C-6, C-10a; H-3′′ to C-6, C-8 and H-2′′ to C-6, C-7 suggested that H-8 was *peri* to C=O, while furan ring was attached to xanthone at C-6, C-7 and *ortho* to H-8. Finally, 2-OH and 5-OH were assigned to complete the structure [2].

Table 1. ^{1}H NMR spectral data of compounds **1**-**6** (in $CDCl_3$)

Position	δ (multiplicity, J_{Hz})					
	1	**2**	**3**	**4**	**5**	**6**
3	7.36 (*s*)	7.44 (*s*)	7.30 (*s*)	7.38 (*s*)	7.36 (*s*)	7.43 (*s*)
6	7.25 (*dd*, 7.8,1.5)	7.22 (*dd*, 7.8,1.5)	-	-	-	-
7	7.18 (*t*, 7.8)	7.18 (*t*, 7.8)	-	6.98 (*d*, 9.0)	6.87 (*d*, 8.7)	-
8	7.67 (*dd*, 7.8,1.5)	7.73 (*dd*, 7.8,1.5)	7.38 (*s*)	7.74 (*d*, 9.0)	7.68 (*d*, 8.7)	8.06 (*s*)
2′	6.30 (*dd*, 17.7, 10.8)	6.38 (*dd*, 17.7, 10.8)	6.26 (*dd*, 17.4, 10.4)	6.40 (*dd*, 17.7, 10.5)	6.36 (*dd*, 17.4, 10.5)	6.42 (*dd*, 17.4, 10.5)
3′(*trans*)	5.27 (*dd*, 17.7,1.5)	5.30 (*dd*, 17.7,1.5)	5.19 (*dd*, 17.4, 0.9)	5.34 (*dd*, 17.7, 1.5)	5.28 (*dd*, 17.4, 1.5)	5.39 (*dd*, 17.4, 1.5)
3′(*cis*)	5.12 (*dd*, 10.8, 1.5)	5.16 (*dd*, 10.8, 1.5)	5.06 (*dd*, 10.4, 0.9)	5.18 (*dd*, 10.5, 1.5)	5.38 (*dd*, 10.5, 1.5)	5.23 (*dd*, 10.5, 1.5)
2′′			-			7.75 (*d*, 2.1)
3′′			5.68 (*d*, 9.9)			6.91 (*d*, 2.1)
4′′			6.37 (*d*, 9.9)			
1′-$(CH_3)_2$	1.46 (*s*)	1.55 (*s*)	1.49 (*s*)	1.59 (*s*)	1.49 (*s*)	1.56 (*s*)
2′′-$(CH_3)_2$			1.47 (*s*)			
1-OH/OCH_3	12.56 (*s*)	3.96 (*s*)	12.76 (*s*)	12.78 (*s*)	3.94 (*s*)	12.77 (*s*)

Table 2. ^{13}C NMR spectral data of compounds **1**-**6** (in $CDCl_3$)

Position	δ (ppm)					
	1	**2**	**3**	**4**	**5**	**6**
1	145.4	143.2	145.2	145.6	143.3	145.8
2	139.0	145.0	138.6	139.0	133.7	139.0
3	120.8	120.3	120.4	120.0	119.2	120.6
4	125.7	132.1	125.9	125.4	132.0	125.7
4a	146.2	148.1	146.7	145.6	148.1	146.8
5	145.2	145.2	132.3	130.7	130.1	130.4
6	119.9	118.9	145.4	149.4	144.1	145.8
7	124.0	123.5	118.4	112.4	112.4	126.2
8	116.3	116.6	113.3	118.0	118.3	108.7
8a	120.1	122.1	113.6	113.6	115.5	117.3
9	182.9	182.1	182.1	182.1	175.6	183.6
9a	109.0	108.7	108.7	107.9	108.3	108.6
10a	144.8	145.1	145.8	145.1	148.2	142.1
1′	40.2	40.4	40.2	40.3	40.4	40.4
2′	151.4	150.6	149.4	152.2	152.0	151.6
3′(*trans*)	108.5	108.7	109.6	107.9	108.7	108.1
3′(*cis*)	108.5	108.7	109.6	107.9	108.7	108.1
2′′			78.6			147.5
3′′			131.3			107.4
4′′			121.4			
1′-$(CH_3)_2$	27.6	27.3	27.4	27.5	27.4	27.7
2′′-$(CH_3)_2$			28.6			
1-OCH_3		62.1			61.9	

CONCLUSIONS

With the aim of searching for the isoprenylated xanthones from the stem bark *G. dulcis*, globuxanthone, 1-*O*-methylglobuxanthon, subelliptenone H, symphoxanthone, 1-*O*-methylsymphoxanthone and subelliptenone D were isolated. The antibacterial activity of these compounds will be further investigated.

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REFERENCES

1. Iinuma M, Ito T, Tosa H, Tanaka T, Asai F, Shimano R. 1995. Two xanthones with a 1,1-dimethylallyl group in root bark of *Garcinia subelliptica*. Phytochem. 39, 945-947.
2. Iinuma M, Ito T, Tosa H, Tanaka T. 1996. Five new xanthones from *Garcinia dulcis*. J. Nat. Prod. 59, 472-475.
3. Kasahara S, Henmi S. 1986. "Medicine herb index in Indonesia", Jakarta: Eisai Indonesia, pp. 92.
4. Locksley HD, Moore I, Scheinmann F. 1966. Extractives from guttiferae. Part III. The isolation and structure of symphoxanthone and globuxanthone from *Symphonia globulifera* L. J. Chem. Soc. C. 2186-2190.
5. Likhitwittayawuid K, Chanmahasathien W, Ruangrugsi N, Krungkra J. 1998. Xanthones with antimalarial activity from *Garcinia dulcis*. Planta Med. 64, 281-282.
6. Nguyen LH, Harison L. 2000. Xanthones and triterpenoids from the bark of *Garcinia vilersiana*. Phytochemistry. 53, 111-114.

Chemical constituents of malaysian *Uncaria cordata var. ferruginea* and their *in-vitro* antihyperglycemic potential

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ABSTRACT

Continuing our interest in the *Uncaria* genus, the phytochemistry and the *in-vitro* antihyperglycemic potential of Malaysian *Uncaria cordata* var. *ferruginea* were investigated. The phytochemical study of this plant which employed various chromatographic techniques including recycling preparative HPLC led to the isolation of ten compounds with diverse structures comprising three phenolic acids, an iridoid glycoside, two coumarins, three flavonoids and a terpene The constituents were identified as 2-hydroxybenzoic acid or salicylic acid (**1**), 3,4-dihydroxybenzoic acid (**2**), 2,4-dihydroxybenzoic acid (**3**), loganin (**4**) along with 7-hydroxy-6-methoxycoumarin (scopoletin), 3,4-dihydroxy-7-methoxycoumarin, quercetin, kaempferol, taxifolin and β-sitosterol. Structure elucidation of the compounds was accomplished with the aid of 1D and 2D Nuclear Magnetic Resonance (NMR) spectral data, Ultraviolet-Visible (UV-Vis), Fourier Transform Infrared (FTIR) spectroscopy and Mass spectrometry (MS). The antihyperglycemic potential of the plant was measured by its α-glucosidase inhibitory activity. In this assay, the crude methanolic stem extract of the plant and its acetone fraction exhibited strong α-glucosidase inhibition of 87.7% and 89.2%, respectively, while its DCM fraction exhibited only moderate inhibition (75.3%) at a concentration of 1 mg/ml. The IC_{50} values of both fractions were found to be significantly lower than the standard acarbose suggesting the presence of potential α-glucosidase inhibitors. Selected compounds isolated from the active fractions were then subjected to α-glucosidase assay in which 2,4-dihydroxybenzoic acid and quercetin showed strong inhibitory effect against the enzyme with IC_{50} values of 549 and 556 µg/ml compared to acarbose (IC_{50} 580 µg/ml) while loganin and scopoletin only showed weak α-glucosidase inhibition of 44.9% and 34.5%, respectively.

Keywords: *Uncaria cordata,* phytochemistry, antihyperglycemic, α-glucosidase inhibition, phenolic acids, loganin, coumarins, flavonoids

INTRODUCTION

Uncaria (Rubiaceae) is amongst the genera known for its alkaloid content. The majority of alkaloids founds in *Uncaria* are indole and oxindole type [1]. Continuing our interest on the genus, we have reported the isolation of two new heteroyohimbine-type oxindole alkaloids, namely, rauniticine-*allo*-oxindole B and rauniticinic-*allo* acid B along with five of their stereoisomers including four pentacyclic oxindole alkaloids, isopteropodine, pteropodine, uncarine F and isopteropodic acid from Malaysian *Uncaria longiflora* var. *pteropoda* [2-4]. In contrast, the non-alkaloid constituents have not been widely reported from *Uncaria* genus. However, the isolation of flavonoids and other chemical constituents present in *Uncaria* have gained more interest lately [5]. Flavonoids, terpenes, quinovic acid glycosides, and coumarins have been isolated from *Uncaria*. Recently, we have also reported the isolation of a novel flavonoid (-)-2*R*,3*R*-3,5,4'-trihydoxyflavan-[6,7:5",6"]-2"-pyranone, named uncariechin, along with (-)-*epi*afzelechin and (-)-*epi*catechin from the methanol extract of the leaves of *Uncaria longiflora* var. *pteropoda* [6].

Out of 14 species of *Uncaria* available in Malaysia, *Uncaria cordata* var. *ferruginea* is one of the common representatives. This species of *Uncaria* comprises of two major entities namely, *Uncaria cordata* var. *ferruginea and Uncaria cordata* var. *cordata*. A total of eight alkaloids have been reported from the plant including dihydrocorynantheine, corynoxine, corynoxine B, 3-*epi*-β-yohimbine, rhynchophylline, isorhyncophylline, uncarine A and uncaric acid D [1]. Throughout the literature, there has only been one study on the phytochemical of Malaysian *Uncaria cordata* var. *ferruginea* which yielded dihydrocorynantheine from the ethanolic leaf extract of the plant [7]. To date, there has been no further study reported on the non-alkaloid constituents or the biological activities of the plant. Therefore, continuing our interest on the phytochemicals of *Uncaria*, we now report the isolation of three phenolic acids and an iridoid glycoside from the methanolic stems of the plant and the antihyperglycemic potential of selected compounds.

MATERIALS AND METHODS

General

TLC and PTLC were performed using pre-coated aluminium-backed supported silica gel 60 F254 (0.2 mm thickness) and glass supported silica gel 60 F254 (0.5 and 1.0 mm thickness). Column chromatography was carried out using silica gel 60, 70-230 mesh ASTM (Merk 7734). Column chromatography packed with Sephadex LH-20 was carried out and flavonoids were detected on TLC stained with aluminium chloride ($AlCl_3$) reagent. Spots and bands for compounds on TLC, PTLC and radial plates were detected using UV light (254 and 365 nm). Mass spectra were measured on an Agilent 1100 Series Technologies HPLC-TOF LC/MS. The ultraviolet (UV) spectra were obtained in methanol on a Shimadzu UV-Vis 160i. The infrared (IR) data was recorded on a Perkin Elmer model FT-IR spectrometer as KBr disc. The ^{1}H-NMR and ^{13}C-NMR were analyzed on a Bruker 600 Ultrashield NMR spectrometer measured at 600 and 150 MHz or Bruker 500 Ultrashield NMR spectrometer measured at 500 and 125 MHz or Bruker 300 Ultrashield NMR spectrometer measured at 300 and 75 MHz, respectively. Chloroform-*d*, acetone-d_6 or methanol-d_4 was used as solvents.

Plant material

Stems of *Uncaria cordata* var. *ferruginea* was collected from Hutan Pasir Raja, Terengganu in September 2013 and was identified by En. Ahmad Zainudin Ibrahim (Universiti Kebangsaan Malaysia). The voucher specimen (HTBP 4318) were deposited at Herbarium Taman Botani Putrajaya, Malaysia.

Extraction and isolation of compounds 1-4

Successive trituration using hexane, dichloromethane, acetone and methanol on the methanolic stem extract of *U. cordata* var. *ferruginea* (296.5 g) yielded Hex, DCM, acetone and MeOH fractions. The DCM fraction (11.0 g) was further fractionated via column chromatography using Hex, DCM and MeOH with a gradual increase in solvent polarity to yield a total of 15 fractions. Based on its TLC profile observed under UV short wavelength, F12 (3.0 g) was found to contain a potential compound and hence was selected for further isolation and purification. Column chromatography of F12 with Sephadex LH-20 employing $CHCl_3$ and MeOH (9:1) as the solvent system yielded 12 subfractions. Subfractions f9 to f12 were pooled and subjected for further purification by glass PTLC using MeOH:$CHCl_3$ (3:7) to give compound (1). Liquid-liquid partitioning of the acetone fraction (30.5 g) between MeOH and diethyl ether yielded 14.3g of diethyl ether (DE) extract upon evaporation of solvent. The DE extract was subjected to further separation with Sephadex LH-20 using $CHCl_3$:MeOH (9:1) to yield a total of forty fractions. Based on their TLC profiles, subfractions f6 and f7 were pooled and chromatographed by Sephadex LH-20 using $CHCl_3$: MeOH (7:3) as the solvent system to yield four fractions f1-f4. The combined f1 and f2 subfractions were subjected to recycling HPLC via isocratic elution of MeOH and H_2O (1:1) and detected by UV detection at 254 nm. Compounds 2 and 3 were isolated as colorless crystals from f1 while compound 4 was obtained from f2.

α-Glucosidase inhibitory assay

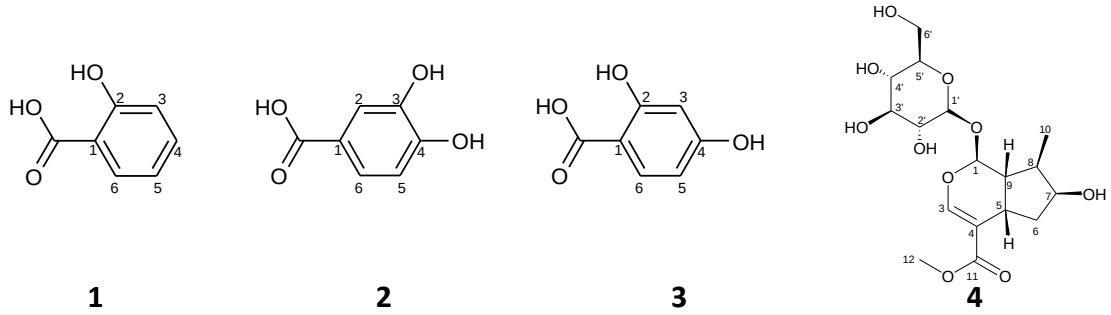
The method for the α-glucosidase inhibitory assay was adopted from Ahmad *et al.* [8] with minor modification. The plant samples (extract/fraction/compound) were prepared in 5% DMSO at concentration of 1000 µg/ml. A series of dilution for each sample were prepared to give a concentration of 1000, 500, 250, 125, 62.5 and 31.3 µg/ml. Then, about 10 µl of sample, 20 µl of α-glucosidase enzyme solution (type 1 from Baker's yeast), phosphate buffer saline (40 µl at pH 6.5) and deionized water (20 µl) were mixed in a 96-well plate. The mixture was incubated at 37 °C. After 10 minutes, 10 µl

of 20 mM *p*-nitrophenyl-α-D-glucopyranoside solution was added into the mixture and the absorbance at time 0 minute was measured at λ 405 nm. Then, the reaction mixture was incubated at 37 °C for 30 minutes and the absorbance was measured. For negative control, the sample was replaced with 5% DMSO and acarbose was used as positive control. Experiments were performed in triplicates with three independent experiments. The percentage inhibitory activity was calculated using the following equation where A is the absorbance of mixture measured at 405 nm:

$$\text{Inhibition (\%)} = \frac{(A_{30\ min} - A_{0\ min})\ \text{control} - (A_{30\ min} - A_{0\ min})\ \text{sample}}{(A_{30\ min} - A_{0\ min})\ \text{control}} \times 100$$

RESULTS

The phytochemistry of Malaysian *Uncaria cordata* var. *ferruginea* led to the isolation of 10 constituents including three phenolic acids (**1**-**3**) and an iridoid glycoside loganin (Compound **4**). This is the first report of the isolation of **1**, **3** and **4** from the *Uncaria* genus [5]. The structures and spectral data of compounds **1**-**4** are given in Figure 1 and 3.1.



1 **2** **3** **4**

Figure 1. Compounds **1**-**4**

Physical and spectral data of compounds 1- 4

2-hydroxybenzoic acid (**1**) White amorphous solid, wt: 2.3 mg (MeOH), mp 154°C (mp lit. 153-154°C). MS *m/z* = 139.0537 $[M+H]^+$, $C_7H_6O_3$; UV (MeOH) λ_{max} nm: 308; IR (KBr) υ_{max} cm^{-1}: 3237 (OH), 1675 (C=O), 1613 (C=C), 759 (C=C-H); ^{1}H NMR (MeOD, 600 MHz) δ ppm : 7.84 (1H, *dd*, *J* = 7.68, 1.68 Hz, H-6), 7.28 (1H, *t*, H-4), 6.78 (1H, *dd*, *J* = 7.86, 1.08 Hz, H-3), 6.80 (1H, *dd*, *J* = 7.20, 1.08 Hz, H-5) ; ^{13}C NMR (MeOD, 150 MHz) δ ppm : 174.8 (C-7), 161.2 (C-2), 132.3 (C-4), 130.2 (C-6), 118.9 (C-1) 117.5 (C-3), 115.7 (C-5) [9].

3,4-dihydroxybenzoic acid (**2**) Transparent needles, wt: 1.5 mg (MeOH), mp 198°C (mp lit. 198-200°C). MS *m/z* = 155. 0494 $[M+H]^+$, $C_7H_6O_4$; UV (MeOH) λ_{max} nm: 293.3; IR υ_{max} cm^{-1}: 3280 (OH), 1655 (C=O); ^{1}H NMR (MeOD, 600 MHz) δ ppm : 7.45 (1H, *d*, *J* = 1.98 Hz, H-2), 7.43 (1H, *dd*, *J* = 8.22, 2.04, H-6), 6.80 (1H, *d*, *J* = 8.22 Hz, H-5); ^{13}C NMR (MeOD, 150 MHz) δ ppm : 169.7 (C-7), 149.7 (C-3), 144.5 (C-4), 123.0 (C-1), 122.4 (C-6), 116.4 (C-2), 114.3 (C-5) [10].

2,4-dihydroxybenzoic acid (**3**) Light yellow crystals, wt: 3.2 mg (MeOH). UV (MeOH) λ_{max} nm: 298.8 ; IR (KBr) υ_{max} cm^{-1}: 3234 (OH), 1674 (C=O), 1602 (C=C), 764 (C=C-H); ^{1}H NMR (MeOD, 600 MHz) δ ppm : 7.45 (1H, *dd*, *J* = 4.38, 1.92 Hz, H-5), 7.44 (1H, *d*, *J* = 1.98 Hz, H-3), 6.82 (1H, *d*, *J* = 8.10 Hz, H-6); ^{13}C NMR (MeOD, 150 MHz) δ ppm : 168.9 (C-7), 150.1 (C-2), 144.7 (C-4), 122.5 (C-3), 121.8 (C-1), 116.4 (C-5), 114.4 (C-6).

Loganin (**4**) Colourless oil, wt: 2.8 mg (MeOH). MS *m/z* = 408.2030 $[M+H]^+$, $C_{17}H_{26}O_{10}$; UV (MeOH) λ_{max} nm: 295, 330; IR υ_{max} cm^{-1}: 3368, 1655, 1420, 1449, 669; ^{1}H NMR (MeOD, 600 MHz) δ ppm : 7.41 (1H, *s*, H-3), 5.29 (1H, *d*, *J* = 4.50 Hz, H-1), 4.67 (1H, *d*, *J* = 7.92 Hz, H-1'), 4.06 (1H, *m*, *J* = 4.44 Hz, H-7), 3.92 (1H, *dd*, *J* = 11.91, 1.74 Hz, H-6'ax), 3.70 (3H, *s*, OCH_3), 3.69 (1H, *dd*, *J* = 11.94, 5.70 Hz, H-6'eq), 3.39 (1H, *m*, H-3'), 3.32 (1H, *m*, H-5'), 3.31 (1H, *m*, H-4'), 3.22 (1H, *m*, H-2'), 3.13 (1H, *m*, H-5), 2.25 (1H, *m*, H-6eq), 2.04 (1H, *m*, H-9), 1.89 (1H, *m*, H-8), 1.64 (1H, *m*, H-6ax), 1.11 (3H, *d*, *J* = 6.96 Hz, CH_3-10); ^{13}C NMR (MeOD, 150 MHz) δ ppm : 168.2 (C=O), 150.7 (C-3), 112.6 (C-4), 98.7 (C-1'), 96.3 (C-1), 70.2 (C-4'), 76.6 (C-3'), 73.7 (C-7), 73.3 (C-2'), 77.0 (C-5'), 61.4 (C-6'), 50.3 (C-13OCH_3), 45.1 (C-9), 41.3 (C-6), 40.8 (C-8), 30.7 (C-5), 12.0 (C-10) [11].

α-Glucosidase inhibitory activities

A preliminary screening of enzyme inhibitory activities was conducted on the crude stem extract as well as DCM and acetone fractions of *U. cordata* var. *ferruginea* in an effort to search for potential α-glucosidase inhibitors. The methanolic stems extract of Malaysian *Uncaria cordata* var. *ferruginea* exhibited high percentage of α-glucosidase inhibition with 87.7% which is consistent to the results reported in an earlier study [8] while the acetone and DCM fraction exhibited strong (89.2%) and moderate inhibition (75.3 %), respectively. The IC_{50} values of both fractions were found to be much lower than the standard acarbose suggesting the presence of potential of α-glucosidase inhibitors (refer Table 1). Subsequent phytochemistry of the acetone fraction yielded 2,4-dihydroxybenzoic acid (2,4-DHBA), quercetin and loganin as well as scopoletin from the DCM fraction which were then assayed against α-glucosidase enzyme.

2,4-DHBA and quercetin showed strong inhibitory effect against the α-glucosidase enzyme with IC_{50} values 549 µg/ml (3.56 mM) and 556 µg/ml (1.84 mM), respectively indicating competitive inhibitory profile resembling that of acarbose (IC_{50} 580 µg/ml or 0.89 mM) which was used as positive control. While there is several literature which support quercetin as a potential α-glucosidase inhibitor [12,13], there has been no specific report on the inhibition activity of 2,4-DHBA. Nevertheless, phenolic acids such as gallic acid, vanillic acid and hydroxybenzoic acid have been reported to demonstrate promising inhibitory effects toward α-glucosidase enzyme [14-17]. In contrast, the iridoid glycoside, loganin showed poor α-glucosidase inhibition (44.9%). This finding supports the moderate α-glucosidase inhibitory activity found for iridoid glycosides against acarbose [18]. Interestingly, scopoletin, the major compound isolated from the DCM fraction also displayed weak α-glucosidase inhibition (34.5%) which agreed with previous findings on the weak α-glucosidase inhibitory activity of coumarins [19]. The inhibitory activities of stem extract, fractions and compounds from *U. cordata* var. *ferruginea* are shown in Table 1. Compounds with high % inhibition and low IC_{50} values are considered to possess anti-hyperglycemic activity as they could help prevent postprandial hyperglycemia by decreasing the rate of carbohydrate degradation to glucose.

Table 1. α-Glucosidase inhibitory activity of stem extract, fractions and compounds from *U. cordata* var. *Ferruginea*

Samples	α-Glucosidase inhibitory* (%)	IC_{50} (µg/ml)	IC_{50} (mM)
Stem Extract	87.7 ± 2.3	102	-
Fractions			
DCM	75.3 ± 2.4	360	-
Acetone	89.2 ± 3.8	200	-
Acarbose	90.9 ± 2.7	580	0.89
Compounds			
2,4-DHBA	78.8 ± 0.4	549	3.56
Quercetin	84.5 ± 0.6	556	1.84
Scopoletin	34.5 ± 0.4	NA	-
Loganin	44.9 ± 0.7	NA	-

*Concentration: 1mg/ml; Values are Means ± SD, n = 3; NA: Not Applicable

4. CONCLUSIONS

Phytochemical study of the methanolic stem extract of Malaysian *Uncaria cordata* var. *ferruginea* has led to the isolation of ten non-alkaloid constituents including three phenolic acids, an iridoid glycoside, two coumarins, three flavonoids and β-sitosterol. In the evaluation of the *in vitro* anti-hyperglycemic potential of the constituents of the plant, 2,4-dihydrobenzoic acid and quercetin isolated from the acetone fraction showed strong α-glucosidase inhibition with IC_{50} values of 549 µg/ml (3.56 mM) and 556 µg/ml (1.84 mM), respectively against acarbose (IC_{50} 580 µg/ml or 0.89 mM) giving further support to their potential as α-glucosidase inhibitors. Loganin and scopoletin showed only weak α-glucosidase inhibition supporting previous reports on weak α-glucosidase inhibitory activities of iridoid glycosides and coumarins.

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REFERENCES

1. Heitzman M, Net CC, Winiarz E, Vaisberg AJ, Hammond GB. 2005. Ethnobotany. phytochemistry and pharmacology of *Uncaria* (Rubiaceae). Phytochem. 66: 5–29.
2. Salim F, Ahmad R. 2010. Isopteropodic acid from Malaysian *Uncaria longiflora* var. *Pteropoda*. WASJ. 10 (11), 1334-1337.
3. Salim F, Ahmad R. 2011. Alkaloids from Malaysian *Uncaria longiflora* var. *Pteropoda*. Biochem. Syst. Ecol. 39, 151–152.
4. Salim F, Ismail NH, Awang K, Ahmad R, 2011. Rauniticine-allo-oxindole B and Rauniticinic-allo acid B, new heteroyohimbine-type oxindole alkaloids from the stems of Malaysian *Uncaria longiflora var. pteropoda*. Molecules. 16, 6541–6548.
5. Andre N, Wang XM, He Y, Pan G, Kojo A, Liu Y. 2013. A review of the occurrence of non-alkaloid constituents in *Uncaria* species and their structure-activity relationships. AJBLS. 1(4), 79-98.
6. Salim F, Zain MM, Ridzuan MSM, Langat M, Mulholland D, Ahmad R. 2013. Flavan-3-ols from the leaves of Malaysian *Uncaria longiflora* var. *pteropoda* (Miq.) Ridsd. Phytochem. Lett. 6 (2), 236 - 240.
7. Kam TS, Lee KH, Goh SW. 1992. Alkaloid distribution in Malaysian *Uncaria*. Phytochem. 31, 2031-2034.
8. Ahmad R, Hashim HM, Noor ZM, Ismail NH, Salim F, Lajis NH, et al. 2011. Antioxidant and antidiabetic potential of Malaysian *Uncaria*. Research Journal of Medicinal Plants. 5, 587-595
9. Banday JA, Mir FA, Farooq S, Qurishi MA, Koul S, Razdan TK. 2012. Salicylic acid and Methyl gallate from the roots of *Conyza canedensis*. IJCAS. 3(2):1305-1308.
10. Yu Y, Gao H, Tang Z, Song X, Wu L. 2006. Several Phenolic Acids from the Fruit of *Capparis spinosa*. Asian. J. Tradit. Med. 1, 3-4.
11. Morel LJdF, Baratto DM, Pereira PS, Contini SHT, Momm HG, Bertoni BW, et al. 2011. Loganin production in *Palicourea rigida* H.B.K. (Rubiaceae) from populations native to Brazilian Cerrado. J. Med. Plants. Res. 5 (12), 2559-2565.
12. Wei JF, Zhang YB, Kang WY. 2012. Antioxidant and a-glucosidase inhibitory compounds in Lysimachia clethroides. AJPP. 6(46), 3230-3234.
13. Yin Z, Zhang W, Feng F, Zhang Y, Kang W. 2014. α-Glucosidase inhibitors isolated from medicinal plants. Food. Sci. Hum. Wellness. 3, 136-174.
14. Wansi JD, Lallemand MC, Chiozem DC, AToze FA, Mbaze LM, Khan SN, et al. 2007. a-Glucosidase inhibitory constituents from stem bark of *Terminalia superba* (Combretaceae). Phytochem. 68, 2096–2100.
15. Mbaze LC, Poumale HM, Wansi JD, Lado JA, Khan SN, Iqbal CM, et al. 2007. α-Glucosidase inhibitory pentacyclic triterpenes from the stem bark of *Fagara tessmannii* (Rutaceae). Phytochem. 68 , 591–595.
16. Wan C, Yuan T, Cirello AL, Seeram NP. 2012. Antioxidant and α-glucosidase inhibitory phenolics isolated from highbush blueberry flowers. Food. Chem. 135, 1929-1937.
17. Jabeen B, Riaz N, Saleem M, Naveed MA, Ashraf M, Alam U, et al. 2013. Isolation of natural compounds from *Phlomis stewartii* showing a-glucosidase inhibitory activity. Phytochem. 96, 443–448.
18. Hua J, Qi J, Yu BY. 2014. Iridoid and phenylpropanoid glycosides from *Scrophularia ningpoensis* Hemsl. and their α-glucosidase inhibitory activities. Fitoterapia. 93, 67–73.
19. Kato A, Minoshima Y, Yamamoto J, Adachi I, Watson A, Nash RJ. 2008. Protective Effects of Dietary Chamomile Tea on Diabetic Complication. Agric. Food Chem. 56, 8206–8211.

Development of chromatographic fingerprint of Tongkat Ali (*Eurycoma longifolia*) roots using online solid phase extraction-liquid chromatography (SPE-LC)

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ABSTRACT

Eurycoma longifolia or commonly known as Tongkat Ali is attracting interest due to its aphrodisiac and vitality effects. In this study, a chromatographic fingerprint was developed using online solid phase extraction-liquid chromatography (SPE-LC) approach for quality control of the root extract. Pressurized liquid extraction (PLE) technique was applied prior to online SPE-LC using polystyrene divinyl benzene (PSDVB) and C_{18} columns. The influence of mobile phase and column switching on the chromatographic fingerprint were optimized. Validation of the developed online SPE-LC was studied based on eurycomanone. Linearity was in the range of 5 to 50 µg mL^{-1} (r^2 = 0.997). Relative standard deviation of retention time and peak area of eurycomanone was 0.1 % and 3.2 %, respectively showing a good precision. The developed online SPE-LC method applied to Tongkat Ali root samples from various sources showed that chromatographic fingerprint offers an efficient approach to quality consistency of herbal product.

Keywords: Chromatographic fingerprint, eurycomanone, *Eurycoma longifolia*, online SPE-LC

INTRODUCTION

Herbal formulation is gaining popularity worldwide for health promotion and adjuvant therapy. *Eurycoma longifolia* has its unique characteristic in enhancing sexual prowess and virility making this species as one of the most popular herbal plants [1,2]. As the demand of this plant is increasing globally [3], a fast and reliable method in determining its quality is required.

Quality control is crucial in ensuring its safety and efficacy. Thus, a comprehensive evaluation of the chemical profile of a sample, instead of only a single compound [4] is essential. Chromatographic fingerprint technology has gained increasing attention and has been accepted by leading organizations such as the World Health Organisation (WHO), Federal Drug Administration (FDA) and the British Herbal Medicine Association [5]. A fingerprint is a specific profile or pattern which chemically represents a sample, based on the detected compounds [6, 7].

In obtaining the fingerprint of herbal plants, the steps include extraction (soaking [2], Soxhlet [8], PLE [9]), clean up commonly using SPE [10] followed by gas chromatography (GC) [11] or high performance liquid chromatography (HPLC) [5]. In this study, online solid phase extraction liquid chromatography (SPE-LC) method was developed in obtaining comprehensive fingerprint compounds in Tongkat Ali (*Eurycoma longifolia*) roots after extraction using PLE.

MATERIALS AND METHODS

Chemicals, reagents and materials

Eurycomanone reference compound was purchased from Chromadex (Irvine, California). Acetonitrile (ACN) and methanol (MeOH) of HPLC grade were purchased from Merck (Darmstadt, Germany). Methanesulfonic acid (MSA) was purchased from Merck Schuchardt (Hohenbrunn, Germany). Roots of *Eurycoma longifolia* from various sources were obtained from local suppliers in Malaysia.

Sample preparation

Pressurised Liquid Extraction (PLE) was performed on a Dionex ASE 350 accelerated solvent extractor (Thermo Scientific Ltd. Camberly, Surrey, UK). Dried root of *Eurycoma longifolia* was accurately weighed (2 g) and mixed with an equal amount of diatomaceous earth, then transferred to a 34 mL PLE stainless steel extraction cell for extraction at 100°C for 30 minutes [12].

Online solid phase extraction liquid chromatography (SPE-LC)

Online SPE-LC analysis was performed on a Dionex Ultimate 3000 Liquid Chromatography system equipped with degasser, quaternary delivery system, an auto-sampler, column oven and a diode array detector (DAD). Online SPE-LC was done using polystyrene divinyl benzene (PSDVB) column (5µm, 4.6 x 50 mm), while all the chromatographic separation was carried out on a C_{18} column (5µm, 4.6 x 250 mm). The column temperature was maintained at 37°C in an oven and injection volume was 100 µL. Data acquisition was performed by Chromeleon software.

The method involved four major steps: sample loading, clean up, elution of extract and LC separation achieved using 2 pumps (left and right) operated simultaneously. The left pump controlled the first three steps while the right pump was applied for separation of analytes. Solvents delivered by left pump were 95% MSA and 5% ultrapure water, while those of the right pump were ultrapure water (A), methanol (B) and acetonitrile (C). Both pumps were operated simultaneously.

RESULTS AND DISCUSSION

Optimization of online SPE-LC conditions

A reverse phase-high performance liquid chromatography (RP-HPLC) is a predominant technique for the separation of eurycomanone in *Eurycoma longifolia* [9] after clean up using SPE C_{18} [10]. In this study fingerprint chromatogram of *Eurycoma longifolia* root extract by online SPE-LC technique was developed using PSDVB as the SPE column and C_{18} as the separating column. Methanesulfonic acid (MSA) (95 %) and ultrapure water (5 %) were used as eluting solvent for SPE. Various gradient elution compositions of mobile phase comprising ultrapure water (A), methanol (B) and acetonitrile (C) were studied. A decrease in the composition of ultrapure water with an increase of ACN (70%A and 30%C) improved the separation of the compounds (Figure 1a, b). The quality of the fingerprint chromatogram was further improved by optimizing the column switching time, defined as the time set for elution of analytes from the SPE column. Column switching time will affect the removal of interferences from the matrix [13]. Optimizing column switching time is important at optimum HPLC gradient elution program in producing better separation of the analytes [14]. An increase in the number of separated compounds was observed with column switching between 12-18 minutes (Figure 1c).

Method validation of online SPE-LC

In order for an analytical method to be employed and regarded as reliable, method validation was carried out. In this study, standard solution of eurycomanone (50 µg mL^{-1}) was used in the determination of accuracy and precision of the developed method. High recovery (90.6%) of eurycomanone was obtained and ten replicates of standard eurycomanone gave low percent RSD (3.7%). Analysis of five *Eurycoma longifolia* root extracts showed good repeatability (2.5% RSD). Good reproducibility was supported by principal component analysis (PCA) of 34 selected compounds (based on peak areas) from the fingerprint chromatogram. Three replicates of each *Eurycoma longifolia* root extract clustered close together while distinct separation between the six types of *Eurycoma longifolia* root samples were observed (Figure 2).

Linearity study was conducted using individual standard of eurycomanone with concentration ranges from 5 to 50 µg mL^{-1}. Linear relationship of the correlation coefficients (R^2=0.997) was obtained with linear regression equation y= 0.0476x + 1.5654. The limit of detection (LOD) and quantification (LOQ) were identified by injecting a series of reference standard of eurycomanone until the signal-to-noise (S/N) ratio was 3 and 10 for LOD and LOQ, respectively. LOD and LOQ of eurycomanone were 2.7 µg mL^{-1} and 9.1 µg mL^{-1}, respectively.

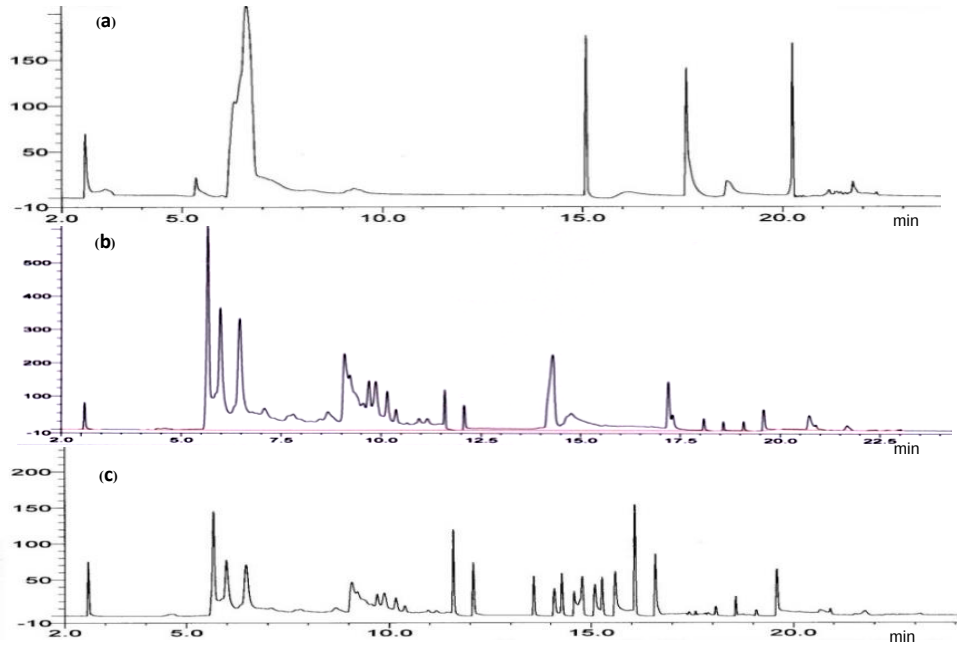

Figure 1. Chromatographic fingerprint of *Eurycoma longifolia* roots (a) 0-6.0 min: 80% A :20% C; 6.0-16.0 min: 50% A:50% B; 16.0-25.0 min; 70% B: 30%C (b) 0-5.7 min: 70%A :30%C ; 5.7-8.5 min gradient elution to 30%A :70%C; 8.5-16.0 min: gradient elution 30%A:70%B to 60%A :40%B; 16.0-19.0 min: gradient elution 50%B :50% C to 10%B: 90%C; 19.0-25.0 min; isocratic 10% B: 90% C. (c) conditions same as (b) with column switching between 12-18 minutes.

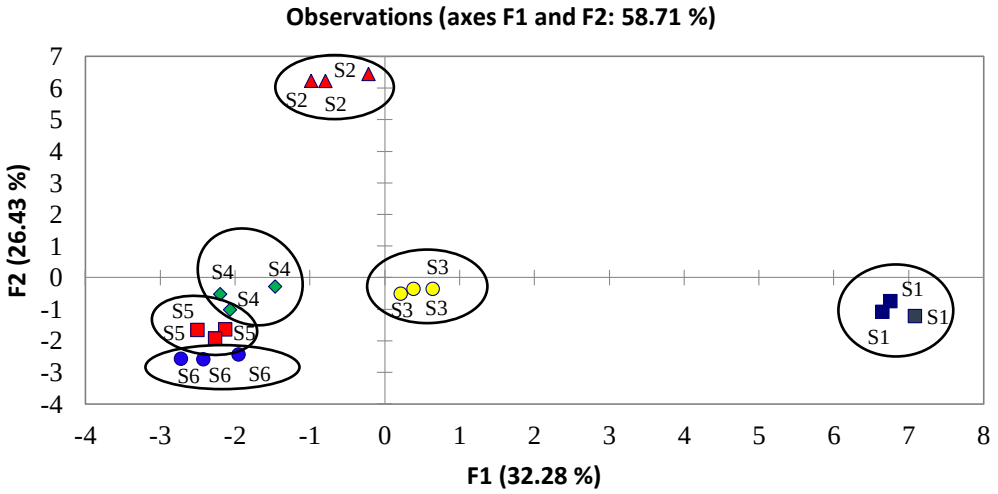

Figure 2. PCA plot on triplicate fingerprint chromatogram of *Eurycoma longifolia* roots samples.

Online SPE-LC fingerprint of *Eurycoma longifolia* root samples

Fingerprint chromatograms of five *Eurycoma longifolia* root samples using online SPE-LC are shown in Figure 3. The fingerprint chromatograms obtained visibly showed similarities and differences among the *Eurycoma longifolia* roots.

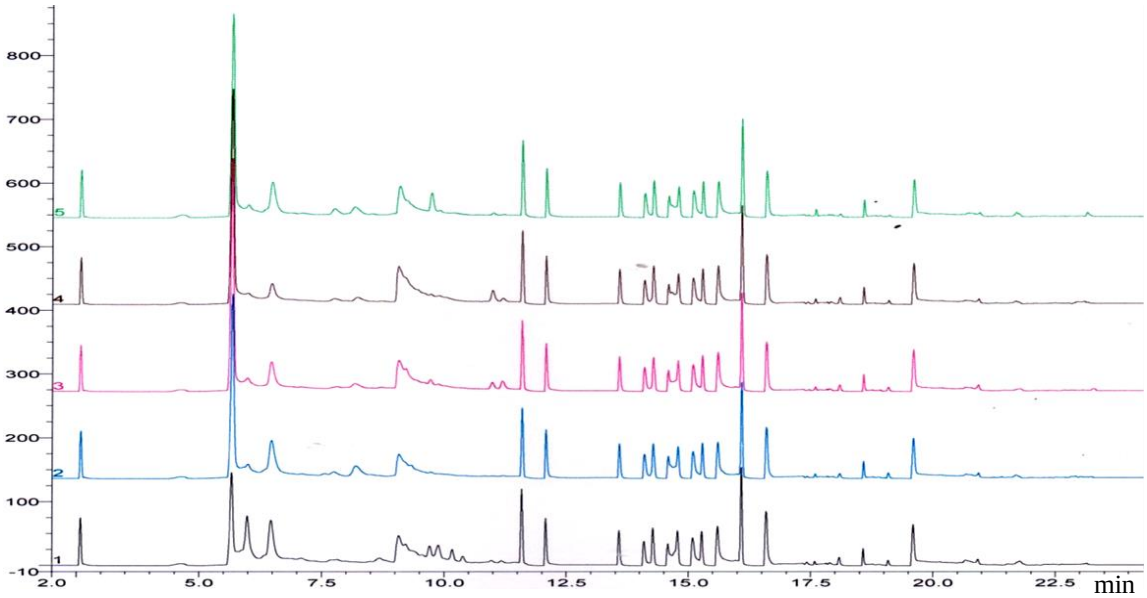

Figure 3. Chromatograms of selected *Eurycoma longifolia* roots

CONCLUSIONS

A fast, reliable and comprehensive chromatographic fingerprint of *Eurycoma longifolia* root was obtained using online SPE-LC method after extraction using PLE. Optimization of the mobile phase compositions and column switching time produced fingerprint chromatogram with good precision, accuracy and reproducibility. The fingerprint chromatogram of *Eurycoma longifolia* root from various sources showed similarities and differences in the chemical profiles, thus can be a promising approach for quality control application.

ACKNOWLEDGMENTS

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REFERENCES

1. Zanoli P, Zavatti M, Montanari C, Baraldi M. 2009. Influence of *Eurycoma longifolia* on the copulatory activity of sexually sluggish and impotent male rats. J. Ethnopharmacol. 126, 308-313.
2. Mohamad M, Ali MW, Ripin A, Ahmad A. 2013. Effect of extraction process parameters on the yield of bioactive compounds from the roots of *Eurycoma longifolia*. J. Technology (Sciences & Engineering). 60, 51-57.
3. Bhat R, Karim AA. 2010. Tongkat Ali (*Eurycoma longifolia* Jack): A review on its ethnobotany and pharmacological importance. 81, 669-679.
4. Liang X, Xie P, Chan K. 2004. Quality control of herbal medicines. J. Chromatogr. B. 812, 53-70.
5. Fan X, Cheng Y, Ye Z, Lin R, Qian Z. 2006. Multiple chromatographic fingerprinting and its application to the quality control of herbal medicines. Analytica Chimica Acta. 555, 217-224.
6. Alaerts G, Dejaegher B, Smeyers-Berbeke J, Vander-Heyden Y. 2010. Recent developments in chromatographic fingerprints from herbal products: Set-up and data analysis. Comb. Chem. High Throughput Screen. 13, 900-922.
7. Tistaert C, Dejaegher B, Vander-Heyden Y. 2011. Chromatographic separation techniques and data handling methods for herbal fingerprints: A review. Anal. Chim. Acta. 690, 148-161.

8. Khari N, Aisha AFA, Ismail Z. 2014. Phase high performance liquid chromatography for the quantification of eurycomanone in *Eurycoma longifolia* Jack (Simaroubaceae) extracts and their commercial products. Trop. J.Pharm. Res. 13(5), 801-807.
9. Peres VF, Saffi J, Melecchi MIS, Abad FC, Martinez MM, Oliveira EC, Jacques RA, Caramao EB 2006. Optimization of pressurised liquid extraction of *Piper gaudichaudianum* Kunth leaves. J. Chromatogr. A. 1105, 148-153.
10. Liang Z, Li K, Wang X, Ke Y, Jin Y. 2012. Combination of off-line two-dimensional hydrophilic interaction liquid chromatography for polar fraction and two-dimensional hydrophilic interaction liquid chromatography × reversed-phase liquid chromatography for medium-polar fraction in a traditional chinese medicine. J. Chromatogr. A. 1224, 61-69.
11. Gong F, Liang YZ, Xie PS, Chau FT. 2003. Information theory applied to chromatographic fingerprint of herbal medicine for quality control. J. Chromatography A. 1002, 25-40.
12. Osman R, Saim N, Saaid M, Zaini NN. An experimental design approach for the extraction of eurycomanone from Tongkat Ali (*Eurycoma longifolia*) roots using pressurised liquid extraction (PLE). Malaysian J. Anal. Sci. article in press.
13. Ramos CF, Satinsky D, Solich P. 2014. New method for the determination of carbamate and pyrethroid insecticides in water samples using on-line SPE fused core column chromatography. Talanta. 129, 579-585.
14. Kuklenyik Z, Ye X, Reich JA, Needham LL, Calafat AM. 2004. Automated offline and online solid phase extraction methods for measuring isoflavones and lignans in urine. J. Chromatogr. Sci. 42, 495-500.

Purification of policosanol from crude sugarcane wax obtained from supercritical CO_2 extraction

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ABSTRACT

Policosanol (PCs) are the mixture of the long chain fatty alcohols which can be extracted from solid waste (filter cake), the by-product in the process of sugar production. The pure form of PCs, especially octacosanol, has been reported to show the efficiency of low-density lipoprotein (LDL) cholesterol-lowering properties in human. In this study, crude sugarcane wax was initially extracted from supercritical CO_2 technique to be used as starting material for our further purification. In general, crude sugarcane wax contains many impurities such as phytosterols, chlorophyll and other chemicals. Our study aims to find the best solvent of choice and a proper solvent system in order to give the highest yield with less impurities of purified policosanol. The chemical profiles of solid and supernatant from experiments were determined by using gas chromatography–mass spectrometry (GC/MS) and be quantified based on the reference standards of policosanol. The results from this study will be used as a guidance to support the policosanol extraction processes in a laboratory scale while the pilot scale extraction of policosanol is in progress and will be reported elsewhere.

Keywords: Sugarcane, Crude sugarcane wax, Solvent extraction, Purification, Policosanol

INTRODUCTION

Policosanol extracted from sugarcane (*Saccharum officinarum L.*) represents a group of long chain (C_{20}-C_{36}) aliphatic alcohols [1]. Research studies show substantially beneficial effects of policosanol to human health. Policosanol supplement can usually be found over the counter in drug market [2]. By taking dosages of policosanol 2 to 20 mg per day, it can reduce up to 30% of cholesterol level, especially low density lipoprotein cholesterol (LDL-C) in blood circulation [3]. The main component of policosanol is octacosanol (60-70%), followed by hexacosanol (4.5-10%) [4]. Recently, octacosanol ($C_{28}H_{58}O$) increases more attention in the research subject and normally founded in vegetable wax such as sugarcane peels (219±3 mg/kg) wheat straw (140±5 mg/kg) and bee wax-brown (2.0±0.1) [2]. In this study, crude sugarcane wax obtained from filter-press mud by Supercritical CO_2 extraction was further purified by different solvent systems in order to obtain high yielding of policosanol and to remove impurities such as phytosterols, chlorophylls and etc.

MATERIALS AND METHODS

Crude sugarcane wax extraction using solvents

Crude sugarcane wax extracted from supercritical CO_2 technique was further purified by various solvents i.e. polar protic solvent (i.e. water, ethanol, and isopropanol), polar aprotic solvent (i.e. acetone and ethyl acetate), weak polar solvent (i.e. toluene) and non-polar solvent (i.e. hexane). Crude sugarcane wax (2 g) was placed into Erlenmeyer flask and dissolved in 40 mL of ethanol. The mixture was stirred and heated until the solid wax completely dissolved. The resulting solution was allowed to cool down to precipitate at room temperature. The slurry mixture was filtered by filter papers and the solid was collected, air-dried and weighting. The above experiments were repeated with other remaining solvents except water. For water, the mixture of crude wax (2 g) and water (40 mL) was autoclaved. The resulting solid (wax) and supernatant was subjected to determine the purities and the content of policosanol by gas chromatography–mass spectrometry (GC/MS).

Standards and Samples preparation for GC/MS

3 Mg of policosanol standards i.e. docosanol (C_{22}), tricosanol (C_{23}), tetracosanol (C_{24}), hexacosanol (C_{26}), heptacosanol (C_{27}), octacosanol (C_{28}) and triacontanol (C_{30}) were weighted into microtubes 1.5 mL and dissolved in 100 µL of analytical grade chloroform, then mixed with vortex mixer. The dilutions were made with chloroform in the ratio of 1:2, 1:5, 1:20 and 1:50, and directly injected to GC/MS instrument.

For sample preparation, residual solid (10 mg) precipitated by solvents purification of crude sugarcane wax was diluted with chloroform (100 µl) and directly injected to GC/MS instrument. The GC/MS conditions are as follow:

Determination of phytosterols and content of policosanol

The composition of policosanol extracts were analyzed on a Agilent 7683B GC/MS system. A capillary column DB-5HT (30 m x 0.25 mm x 0.25 µm) used for analysis. Oven temperature was programmed from 50 °C to 300 °C, at 10 °C /min, and maintained at 300 °C for 15 min, Helium was used as carrier with a flow rate of 54 mL/min. The inlet temperature was 300 °C. GC/MS parameters were as follows: MS transfer line 280 °C, ion source 230 °C, and MS quadrupole 150 °C. The ionization energy was 70 eV. The scan range and rate were 50-600 amu and 2 scans/s, respectively. The injection volume was 5 µL with 1:10 split ratio. The calibration curves were obtained by injecting the standard solutions with concentrations ranging from 10 to 50 mg/mL. Data analysis was carried out by using Enhanced software. The PC compositions of the samples were identified by direct comparison of their chromatographic retention times and mass spectra with those of authentic compounds. The peaks were also confirmed with NIST/EPA/NIH Mass Spectral Library (Version 2.0).

RESULTS

Solvents with a wide range of dielectric constants, polar protic solvents such as water (78.5) and isopropanol (20.1), polar aprotic solvents such as ethanol (24.3), acetone (20.7), and ethyl acetate (6.02), weak polar solvent such as toluene (2.38), and non-polar such as hexane (1.88) were selected for this study. The yields obtained from the precipitation by various solvent systems were collected from each experiment. Water extraction obtained the highest yield than other solvents (79.31% w/w), but phytosterols and other purities cannot be removed from crude wax. Other polar solvents i.e. ethanol, isopropanol, acetone and ethyl acetate and non-polar solvents (hexane) used in this study are capable of removing phytosterols and some impurities. Crude sugarcane wax comprised three phytosterols i.e. campesterol, stigmasterol, and beta-sitosterol. In our study, policosanol composition was mainly found to be a mixture of tetracosanol (C_{24}), hexacosanol (C_{26}), octacosanol (C_{28}), and with less concentration of docosanol (C_{22}), tricosanol(C_{23}), heptacosanol (C_{27}), and triacontanol(C_{30}) according to the peak areas comparing to the standards. As shown in Figure 1A, crude sugarcane waxes contained many compounds, policosanol (peaks a, b, c, and d) with the presence of phytosterols (peaks e, f, and g) and other impurities. Figure 1B is an example of GC/MS profile after the recrystallization using ethyl acetate as a solvent. Most of impurities were removed from crude sugarcane wax and policosanol compositions were enriched with the majority of octacosanol.

Each policosanol composition concentrations were calculated on the basis of the peak area based on the calibration curve of each of the 3 standards used (Figure 2). The use of ethyl acetate, acetone and isopropanol were selected as the good solvents of choice that can be eliminated the impurities for the first solvent screenings. Policosanol

contents in crude wax, and solid waxes which were extracted by ethyl acetate, acetone and isopropanol as shown in Table 1 and compared with crude sugarcane wax as shown in Table 2. In fact, a larger amount of phytosterols were efficiently removed by using ethyl acetate, acetone and isopropanol. Moreover, for other solvents i.e. ethanol and hexane, phytosterols cannot be removed completely and a larger amount of purified solid wax still remained (no data shown). Toluene and water were failed for wax purification, due to their non-polar molecules. Toluene can dissolved the crude sample completely. [6], thus the crude wax cannot be precipitated out of the system. Water, a high polarity solvent, cannot differentiate the crude wax compositions; hence phytosterol and chlorophyll cannot be removed from crude wax after recrytallization. Although phytosterols were still presented using ethyl acetate as an extractor (but less than other solvents), ethyl acetate was shown to be the best solvent of choice to obtain the higher yield and higher purity of policosanol. The purity of the solvent purification can be improved by repeating the purification step with the same solvent.

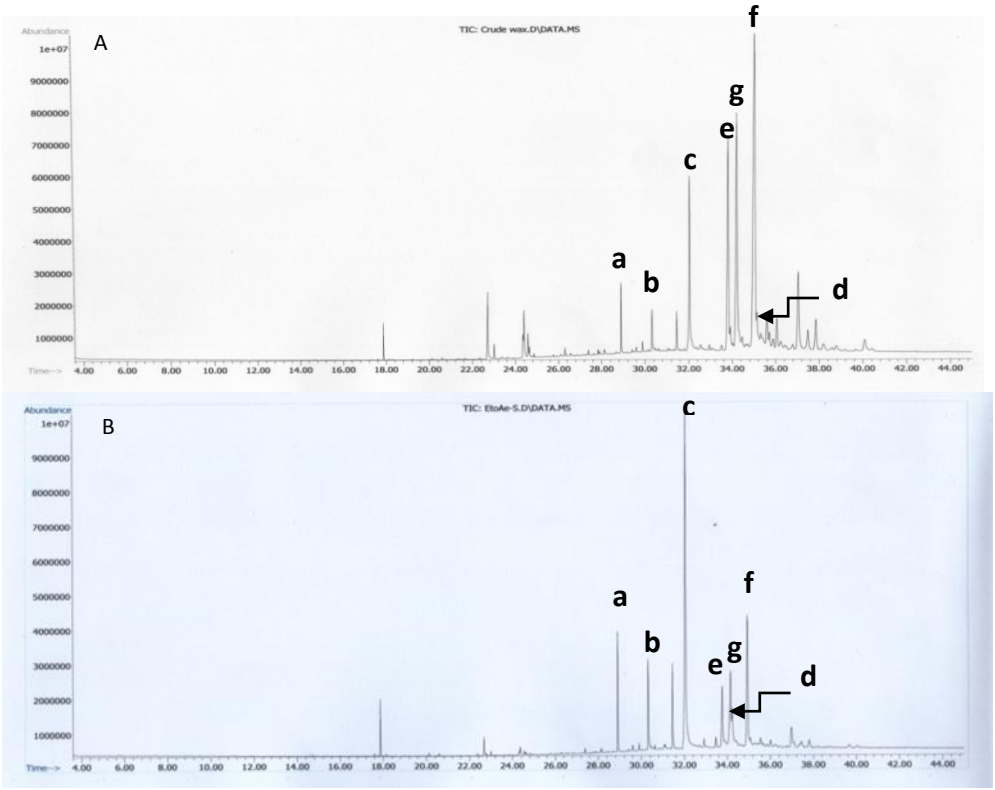

Figure 1. Chromatogram for crude sugarcane wax (A) and solid wax from ethyl acetate extraction (B)
Notes: a, tetracosanol; b, hexacosanol; c, octacosanol; d, triacontanol; e, campesterol; f, stigmasterol; g, beta-sitosterol

Table 1. Policosanol composition from GC-MS analysis of solid wax

Extraction	Amount of policosanol contents from GCMS (mg/3mg)			Amount of policosanol in crude wax (%W/W)		
	C_{24}	C_{26}	C_{28}	C_{24}	C_{26}	C_{28}
Crude sugarcane wax	0.32	0.16	1.99	10.67	5.33	66.23
Ethyl acetate extraction	0.29	0.12	1.94	9.67	4.00	64.67
Acetone extraction	0.22	0.12	1.53	7.33	4.07	51.00
Isopropanol extraction	0.25	0.12	0.98	8.33	4.13	32.67

Table 2. Yield of policosanol contents in solid waxes after solvent extraction compared to the amount in crude wax.

Solvent Extraction	C_{24} (%)	C_{26} (%)	C_{28} (%)
Ethyl acetate	90.63	75.00	97.63
Acetone	68.75	76.25	77.00
Isopropanol	78.13	77.50	49.32

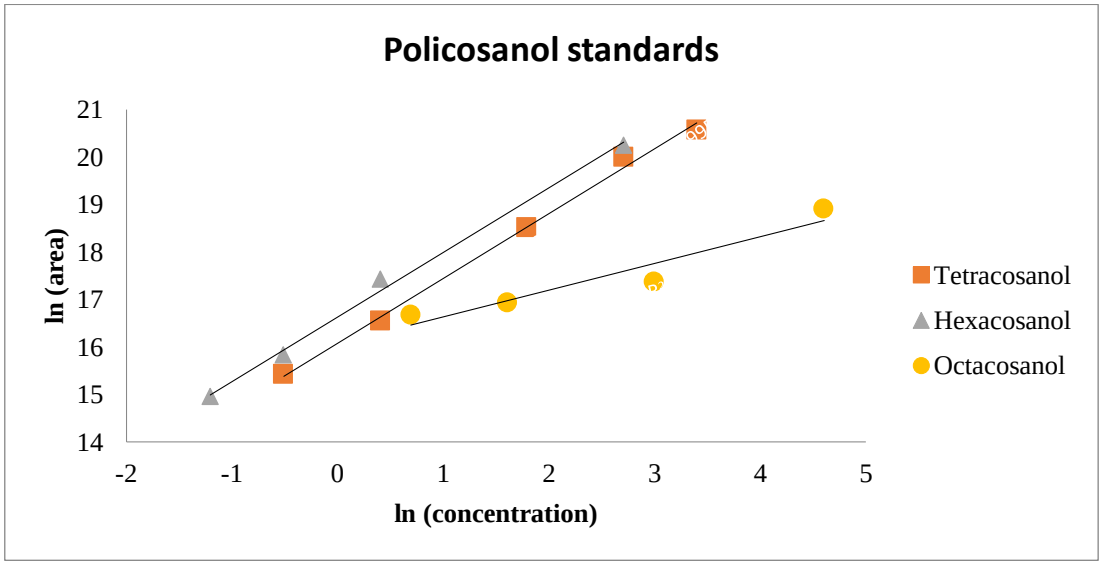

Figure 2. Standard curves for tetracosanol, hexacosanol, and octacosanol.

CONCLUSIONS

Our research showed that ethyl acetate extraction had many advantages over the original report using acetone as a solvent in terms of purer policosanol contents and higher yield of waxes obtained. The yield of tetracosanol (C_{24}), hexacosanol (C_{26}) and octacosanol (C_{28}) extracted from crude wax using ethyl acetate, which were better than that of acetone and isopropanol, were 90.63, 75.00 and 97.63, respectively. The purity of the solvent purification can be improved by repeating the purification step with the same solvent and utilized the mixed solvents of ethyl acetate and acetone that might be more effective solvent system than single solvent for wax extraction to remove the chlorophylls and a number of impurities.

ACKNOWLEDGEMENTS

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REFERENCES

1. Cherif AO, Ben MM, Boukhchina S, Pepe C, Kallel H. 2010. Comparison of the concentrations of long-chain alcohols (policosanol) in three Tunisian Peanut Varieties (*Arachis hypogaea L.*). J. Agric. Food Chem. 58, 12143-12148.
2. Irmak S, Dunford NT, Milligan J. 2006. Policosanol contents of beeswax, sugar cane and wheat extracts. Food Chem. 95(2), 312-318.
3. Varady KA, Wang Y, Jones PJH. 2003. Role of policosanols in the prevention and treatment of cardiovascular disease. Nutrition Reviews. 61(11), 376-83.
4. Mar R. 2000. Policosanol. Drugs of the future. 25, 569-86.
5. Ou S, Zhao J, Wang Y, Tian Y, Wang J. 2012. Preparation of octacosanol from filter mud produced after sugarcane juice clarification. *LWT-Food* Sci Technol. 45(2), 295-298.
6. Marco A, Miguel R, Claudio M, Rogério A, Andre V. 2014. Analysis of phytosterols in plants and derived products by gas chromatography. Austin J. 5, 1-4.

Physicochemical properties of defatted rambutan (*Nephelium lappaceum*) seed flour on alkali treatment

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ABSTRACT

Rambutan seeds were subjected to SC-CO_2 extraction at 35 MPa, 45°C to obtain defatted rambutan seed flour. Its physicochemical properties before and after treated with 0.075 M NaOH solution were investigated. In general, alkali-treated flour with less protein, fat and amylose contents had a significant increment in bulk density, swelling power, water absorption capacity, emulsion capacity and stability, but a reduction in turbidity, solubility, and oil absorption capacity. Pasting property studies showed peak viscosity, breakdown, setback and final viscosity increased significantly, but pasting temperature decreased for alkali-treated flour. With the alkali treatment, least gelation concentration decreased from 14 to 8 g/100 ml. The apparent viscosity increased significantly for alkali-treated flour with increasing solid content from 10 to 25 g/100 ml. These results showed most of the physico-chemical properties of the defatted flour were different after the alkaline solution.

Keywords: Rambutan (*Nephelium lappaceum*), flour, physicochemical properties, alkali treatment

INTRODUCTION

Rambutan (*Nephelium lappaceum*) seeds, a fruit waste, contain high amounts of fat (14-41%) and carbohydrate (28-46%) [1-3]. Although largely unexploited, they could be an alternative source for vegetable fat and processed to flour and starch as food ingredients. During recent years, an increased interest in the study of fat from rambutan seeds has been focused mainly on the physicochemical properties [1-3]. These investigations suggest possibilities of rambutan seed fat in confectionary products. However, there is a lack of information on flour derived from rambutan seeds for exploiting its potential use in human food products.

Defatting of rambutan seeds has been performed using a hexane solvent [1-3], whereas toxic solvent residue is undesirable and of concerned for food applications. Supercritical carbon dioxide (SC-CO_2) has been proved as an effective solvent in the extraction of several seed oils [4] and acceptable for use in food processing industry [5].

Aqueous alkaline, such as sodium hydroxide (NaOH), is commonly used in the production of starches in the food industry. Alkaline treatment has been applied to alter physicochemical properties of starches from different botanical sources. For instance, the alkaline treatment affected characteristics of sago, potato and corn starches depending on steeping time [6].

In our pervious study we have applied SC-CO_2 for extracting fat and fractionating oil from rambutan seeds [7-8]. We reported that defatted rambutan seed flour had the nutritional potential as compared to the commercial all-purpose wheat flour [7]. In present study, our objectives were (i) to characterize physicochemical properties of defatted rambutan seed flour and (ii) to determine the effect of alkali treatment on the flour properties.

MATERIALS AND METHODS

Preparation of defatted rambutan seed flour

Defatted rambutan seed flour was prepared by SC-CO_2 extraction of ground rambutan seeds (about 100 g dry basis) at 35 MPa, 45 °C using Speed SFE instrument (Applied Separations Inc., PA, USA) for 44 h. After grinding the defatted seeds to a fine powder, the defatted flour was sieved through a 100 mesh-sieve, stored in a sealed plastic bag until use.

Alkaline treatment

Defatted rambutan seed flour sample (500 g) was suspended in 5 L of NaOH solution (0.3% w/v) under constant stirring for 4 h. The suspension was centrifuged at 5000 rpm for 30 min, and the supernatant was discarded. The slurry was mixed with distilled water and centrifuged again. The washing procedure was repeated five times keeping pH 8.0-9.0. The remaining sediment was oven-dried over night at 50 °C, ground and then screened using a 100 mesh-sieve. It was stored in a sealed plastic bag until use.

Chemical composition

Moisture, fat, protein, and ash contents were determined following the AOAC methods [9]. Amylose content was determined in compliance with the guidelines described in the Thai Agricultural Standard (TAS) number 4000-2003 on Thai Hom Mali rice [10].

Color and turbidity

The color of samples was measured using a Chroma Meter (Model CR-400, Konica Minolta, Japan). Turbidity was determined by measuring the absorbance of suspension (2% w/v), which was adjusted the suspension pH to 7.0, at 640 nm against a water blank with a Jenway UV-visible spectrophotometer (Model 6405, Jenway Limited, Essex, UK).

Bulk density, water and oil absorption capacity

Bulk density was determined using a 10 ml graduated cylinder with 1 g sample. The cylinder was carefully tapped until each sample was leveled out. The bulk density was expressed as g/ml. For water absorption, suspensions (10%w/v) were prepared with distilled water and then centrifuged. The supernatant was discarded, and the wet sediment was weighed. Similarly for the determination of oil adsorption, grape seed oil was used. The water and oil adsorption were determined as g of water or oil adsorbed per g of the sample on a dry-weight basis.

Solubility, swelling power and emulsion properties

Suspensions (1% w/v) were first prepared with distilled water, heated at 85 °C for 30 min, followed by centrifugation at 5000 rpm for 30 min. The supernatant was transferred into an aluminum can and dried at 105 °C in an oven overnight. The wet sediment was weighed. Solubility was calculated as the weight of dried supernatant divided by the initial weight of dry sample, reported as g/100g. Swelling power was defined as the weight of the wet sediment to the initial weight of dry sample.

To determine emulsion properties, samples (1.0 g) were suspended in distilled water (6 ml) and grape seed oil (6 ml) was added. The dispersed samples were mixed using a vortex mixer for 5 min and then centrifuged at 5000 rpm for 30 min. Emulsion capacity was the ratio of the volume of emulsified layer to the whole emulsion volume.

After preparation of the test samples, they were homogenized at 3,400 rpm for 2 min, heated to 85 °C for 15 min, followed by centrifugation as previously described. The emulsion stability was determined as the volume of emulsified layer to that of the heated emulsion.

Pasting properties

Pasting properties of samples were determined using a rapid visco analyzer (RVA-TecMaster, Newport Scientific, Australia) with a paddle rotated at a constant 160 rpm. Sample dispersions of 16 g/100 ml were equilibrated at 50 °C for 1 min, heated from 50 to 95 °C in 5 min, maintained at 95 °C for 2.5 min, cooled to 50 °C in 4 min, and held at 50 °C for 2 min. Pasting properties such as the pasting temperature, peak time, peak viscosity, final viscosity, breakdown and setback were obtained.

Least gelation concentration

Suspensions of 2, 4, 6, 8, 10, 12, 14 and 16% (w/v) were prepared in test tubes with 5 ml distilled water, heated for 1 h at 95 °C in a water bath, followed by cooling to 10 °C. The gelation results were expressed as no (-), complete (+) or partial (±) gelling and the least gelation concentration was the lowest value at which a complete gelling occurred.

Apparent viscosity

Samples of 5, 10, 15, 20 and 25% (w/v) were prepared with distilled water. All samples were measured for apparent viscosity at 27 ± 1 °C using a Brookfield DV-III Ultra viscometer (Brookfield Engineering Laboratories, Inc., MA, USA) with a T-bar spindle at a rotating speed of 100 rpm.

Statistical analysis

Data were statistically analyzed using the SPSS software (version 13.0). Differences were considered signi-ficant at $p < 0.05$.

RESULTS AND DISCUSSION

Table 1 shows chemical composition of defatted rambutan seed flour, before and after alkali treatment. The alkali solution could partially remove protein, fat and amylose contents with the percentage reduction of 9.1, 24.9 and 6.0%, respectively. The ash content of alkali-treated flour was significantly higher than that of untreated flour ($p < 0.05$). The percentage yield of treated flour after the washing process was average of 60.4%. From this result, a single step of 4 h alkaline treatment was less effective in removing protein and fat from the defatted flour. Thus, a multi-step treatment with alkali and duration of treatment would be factors influencing preparation of rambutan starch as should be further investigated.

Table1. Chemical composition (g/100g) for untreated and alkali-treated defatted rambutan seed flours

Sample	Protein	Fat	Ash	Amylose
Untreated	10.46 ± 0.05a	6.64 ± 0.31a	1.54 ± 0.01b	19.09 ± 0.04a
Treated	9.50 ± 1.01b	4.99 ± 0.01b	1.87 ± 0.13a	17.94 ± 0.02b

Means with different letters in same column differ significantly ($p < 0.05$), n =2.

The Hunter color parameters, water activity and turbidity for untreated and alkali-treated defatted rambutan seed flours are presented in Table 2. Untreated flour showed a higher value of lightness and lower value of chroma compared to the treated flour. The color values indicated that alkali-treated flour was darker white with increased yellowness. It is possible that alkali-treated flour contained some absorbed material, although some of the soluble components were removed during water washing, resulting in its higher ash content (Table 1). The treated flour showed a lower turbidity value than did untreated flour. This result might be due to lower protein, fat and amylose contents (Table 1), decreasing the light absorption.

Table 2. Hunter color values (L*, a*, b*), chroma, and turbidity of untreated and alkali-treated defatted rambutan seed flours

Sample	Hunter color values			Chroma	Turbidity
	L*	a*	b*		(ABS)
Untreated	92.14 ± 0.24a	4.09 ± 0.04b	9.26 ± 0.12b	10.13 ± 0.10b	2.78 ± 0.01a
Treated	90.26 ± 0.16b	4.84 ± 0.01a	11.14 ± 0.32a	12.15 ± 0.29a	2.11 ± 0.01b

Means with different letters in same column differ significantly ($p < 0.05$), n =3.

Table 3 shows bulk density, water and oil absorption capacity values for untreated and alkali-treated defatted rambutan seed flours. A greater bulk density for alkali-treated flour indicated a better packing than untreated flour. For absorption capacities, alkali-treated flour showed a greater water absorption but a lower oil absorption than untreated flour. This can be explained as Na^+ ions absorbed in the flour's internal structure, which would increase hydrophilic tendency by electrostatic interaction with the hydroxyl groups of water [6]. As a result, more amounts of water could be accessed into the structure, increasing in water absorption. A higher oil absorption capacity was attributed to a physical entrapment of oil associated with protein [11].

Table 3. Bulk density, water and oil absorption capacity of untreated and alkali-treated defatted rambutan seed flours

Sample	Bulk density (g/ml)	Water absorption (g/g)	Oil absorption (g/g)
Untreated	0.36 ± 0.01b	2.56 ± 0.01b	1.41 ± 0.04a
Treated	0.65 ± 0.01a	3.90 ± 0.04a	1.25 ± 0.05b

Means with different letters in same column differ significantly ($p < 0.05$), n =3.

RVA pasting profiles of untreated and alkali-treated defatted rambutan seed flours are shown in Figure 1. The corresponding data are presented in Table 4. Untreated flour displayed a very low rise in viscosity during a constant 95 °C, followed by a fairly constant viscosity until the end of RVA run. A higher pasting temperature (89 °C) but lower peak viscosity (1056 cP), breakdown (86 cP), final viscosity (1244 cP) and setback (273 cP) were obtained. The pasting viscosity developed for untreated flour suggested the possible existence of cross-links, which was more resistant to shear during heating and cooling [12].

Alkali-treated flour displayed a similar viscosity profile to a typical V-type (results not shown) with a lower pasting temperature (68 °C), but greater peak viscosity (3055 cP), breakdown (647 cP), final viscosity 4050 cP) and setback (1643 cP). Alkali-treated flour was less resistant to shear and heat, as evidenced by significant increase in peak viscosity and breakdown. This could be attributed to the presence of OH^- ions that might have increased hydration by weakening the bonding within cross-links [6]. Increase in the viscosity on cooling (high setback value) for alkali-treated flour could be due to the tendency of reassociation [13].

Table 4. Viscosity parameters of untreated and alkali-treated defatted rambutan seed flours

Sample	Pasting temperature (°C)	Peak time (min)	Peak viscosity (cP)	Breakdown (cP)	Final viscosity (cP)	Setback (cP)
Untreated	89	7	1056	86	1244	275
Treated	68	6	3055	647	4050	1643

Determinations carried out in triplicates.

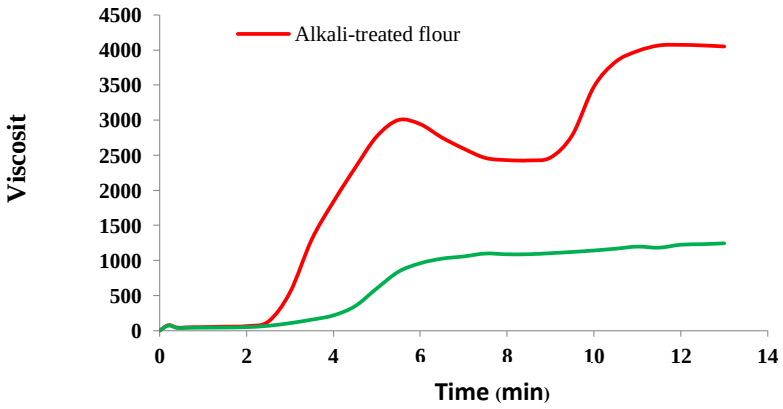

Figure 1. Viscosity of untreated and alkali-treated defatted rambutan seed flours

Solubility, swelling power and emulsion properties of untreated and alkali-defatted rambutan seed flours are given in Table 5. Untreated flour showed a higher solubility value than did alkali-treated flour, presumably due to some of soluble components could be presented before being removed by alkali treatment. A higher swelling power of alkali-treated flour might be attributed to the weakened bonding within cross-links, thus allowing the ability to swell freely as compared to its untreated flour. The increase in emulsion capacity and emulsion stability after alkali treatment reflected the presence of alkali ions that enhanced water absorption, pasting and swelling power of the treated flour, which could contribute to these observed results.

Table 5. Solubility, swelling power and emulsion properties of untreated and alkali-defatted rambutan seed flours

Sample	Solubility (g/100g)	Swelling power (g/g)	Emulsion properties	
			Capacity (ml/100 ml)	Stability (ml/100 ml)
Untreated	17.69 ± 0.31a	10.64 ± 0.20b	47.69 ± 1.54b	34.55 ± 1.38b
Treated	13.99 ± 0.78b	13.84 ± 0.68a	61.22 ± 1.94a	51.79 ± 0.89a

Means with different letters in same column differ significantly ($p < 0.05$), n =3.

Gelation properties observed for untreated and alkali-treated rambutan seed flours at different concentrations (2-16 g/100 ml) are shown in Table 6. Untreated flour began gelling at 10 g/100 ml, with complete gelling at 14 g/100 ml, while treated flour showed a complete gelling at 8 g/100 ml. The reduction in gelation concentration after alkali treatment was attributed to partial reassociation to form gel [13].

Table 6. The least gelation concentrations of untreated and alkali-defatted rambutan seed flours

Sample	Concentration (g/100 ml)							
	2	4	6	8	10	12	14	16
Untreated	–	–	–	–	±	±	+	+
Treated	±	±	±	+	+	+	+	+

Determinations carried out in duplicates. No gelling (–), complete gelling (+) or partial gelling (±).

Apparent viscosity of untreated and alkali-treated rambutan seed flours at solid contents of 5-25 g/100 ml are shown in Figure 2. Untreated flour displayed a first increase in viscosity at 15 g/100 ml and reached a plateau at 25 g/100 ml, whereas a detectable increase in viscosity of alkali-treated flour was at or above 10 g/100 ml. This result agreed with the experiment of least gelation concentrations, which was indicative that the alkali ions were mainly responsible for the increase in apparent viscosity.

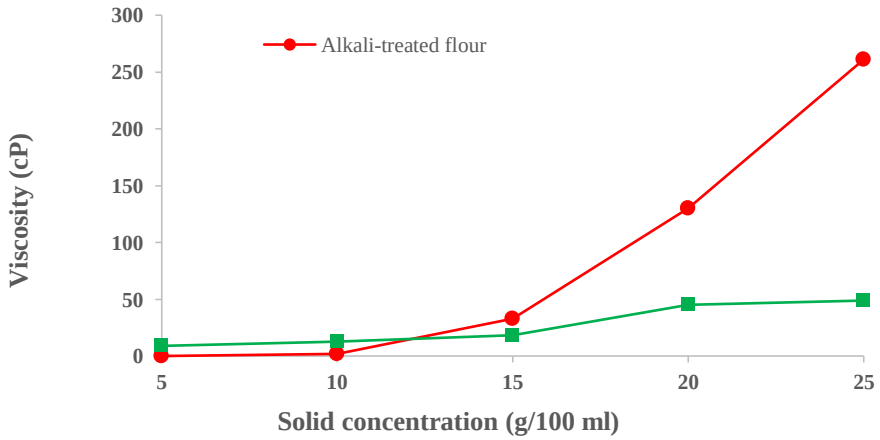

Figure 2. Apparent viscosity of untreated and alkali-treated rambutan seed flours

CONCLUSION

Our results showed that physicochemical properties of defatted rambutan seed flour were affected by alkaline treatment. The overall changes were increase in bulk density, swelling power, water absorption capacity, emulsion capacity and stability, but decrease in turbidity, solubility and oil absorption capacity. Alkali treated flour showed significant increase in peak viscosity, breakdown, setback and final viscosity with reduction in pasting temperature. The alkali treatment resulted in the decrease in least gelation concentration, but increase in apparent viscosity. This study represents a preliminary attempt for further investigation on rambutan starch.

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REFERENCES

7. Sirisompong W, Jirapakkul W, Klinkesorn U. 2011. Response surface optimization and characteristics of rambutan (*Nephelium lappaceum*) kernel fat by hexane extraction. LWT – Food Sci. Technol. 44, 1946-1951.
8. Solís-Fuentes JA, Camey-Ortíz G, Hernández-Medel MDR, Pérez-Mendoza F, Durán-de-Bazúa C. 2010. Composition, phase behavior and thermal stability of natural edible fat from rambutan (*Nephelium lappaceum*) seeds. *Bioresource Technology*, 101, 799-803.
9. Yanty NAM, Marikkar JMN, Long K, Ghazali HM. 2013. Physicochemical characterization of the fat from red-skin rambutan (*Nephelium lappaceum*) seed. J. Oleo Sci. 62(6), 335-343.
10. Reverchon E, Macro IDe. 2006. Supercritical fluid extraction and fractionation of natural matter. J. Supercrit. Fluids. 38, 146-166.
11. Mohamed RS, Mansoori GA. 2002. The use of supercritical fluid extraction technology in food processing. *Food Technology Magazine*, June, available at https://www.uic.edu/labs/trl/1.OnlineMaterial/SCEin FoodTechnology.pdf
12. Nadiha MZN, Fazilah A, Bhat R, Karim AA. 2010. Comparative susceptibilities of sago, potato and corn starches to alkali treatment. Food Chem. 121, 1053-1059.
13. Eiamwat J, Wanlapa S, Sematong T, Reungpatthanapong S, Phanthanapatet W, Hankhuntod, N, Kampruengdet S. 2015. Rambutan (*Nephelium lappaceum*) seed flour prepared by fat extraction of rambutan seeds with SC-CO_2. Isan J. Pharm. Sci. 10 (Suppl.), 138-146.
14. Thiensong B, Eiamwat J, Natpinit P. 2015. Composition and physicochemical characteristics of rambutan seed oil extracted by supercritical carbon dioxide. In proceedings of food innovation asia conference. Bangkok, Thailand, 18-19 June, 348-353.
15. AOAC. Official method of analysis. 16th ed., Association of Official Analytical Chemists; Arlington, VA; 2000.
16. Thai Agricultural Standard. Thai Hom Mali Rice. Guideline No.: TAS 4000-2003. Available at http://www.acfs.go.th/standard/download/eng/Thai_Hom_Mali.pdf
17. Siddiq M, Ravi R, Harte JB, Dolan KD. 2010. Physical and functional characteristics of selected dry bean (*Phaseolus vulgaris L.*) flours. LWT – Food Sci. Technol. 43, 232-237.
18. Taggart P. 2004. Starch as an ingredient: manufacture and implication. In: Elliason AC, editor. *Starch in Food.* England: CRC Press; 363-392.
19. Adebowale KO, Lawal OS. 2003. Microstructure, physicochemical properties and retrogradation behavior of Mucuna bean (*Mucuna pruriens*) starch on heat moisture treatments. Food Hydrocolloid. 17, 265-272.

Flavonoids form *Friesodielsia desmoides*

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ABSTRACT

Phytochemical study of the methanol extract from the dried flowers of *Friesodielsia desmoides*, resulted in the isolation of two known flavanones, (2*S*)-8-formyl-5,7-dihydroxyflavanone (1) and (2*S*)-5,7-dihydroxy-6-methylflavanone (2). The constituents were isolated and purified by preparative reversed-phase HPLC using linear gradient of water and methanol. Their structures were determined on the basis of spectroscopic analyses. The antioxidant activity of the isolated flavanones was assessed by 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical bioassay, using butylated hydroxytoluene (BHT) as a standard. Compound 2 showed antioxidant activity with the IC_{50} value of 8.09 μg/mL which was stronger than the standard BHT (IC_{50} 13.68 μg/mL).

Keywords: Annonaceae; *Friesodielsia desmoides*; flavonoids

INTRODUCTION

Friesodielsia is a genus of Annonaceae, with about 60 species found in Africa and Asia [1]. The first phytochemical report on the genus was made in 1960, in which alkaloids were isolated [2]. Chemical investigation on some species of the genus *Friesodielsia* led to the isolation of hexahydro-xanthenic derivatives [3], alkaloids [4], flavonoids [3-6], phenylpropanoids [4], terpenoids [4] and bisabolene sesquiterpenes [5]. No phytochemical studies on *F. desmoids* have been reported.

MATERIALS AND METHODS

General experimental procedures

IR spectra were recorded on a ATR FT-IR spectrometer (Agilent Technologies Cary 630). UV spectra were recorded on UV-VIS spectrophotometer (UV-1700 PharmaSpec SHIMADZU). The ^{1}H and ^{13}C NMR spectra were recorded with 300 MHz Bruker spectrometer. Chemical shifts (□) are quoted in parts per million (ppm) from an internal standard tetramethylsilane (TMS). The TSP HPLC system was used for separation purposes with Lichrospher®100 RP-18 endcapped column (Ø 25x250 mm, particle size 5 µm, Merck). For thin-layer chromatography (TLC), aluminium sheets of silica gel 60 F_{254} (20x20 cm, layer thickness 0.2 mm, Merck) was used for analytical purposes. The compounds were visualized under ultraviolet light. All solvents for extraction and chromatography were distilled at their boiling ranges prior to use except for MeOH for HPLC separation which was analytical grade (Merck). 1,1-Diphenyl-2-picrylhydrazyl radical (DPPH radical), and butylated hydroxy toluene (BHT) were also purchased from Merck (Germany).

Plant materials

The flowers of *F. desmoids* were collected from Trang Province, Thailand. They were dried by a hot air oven at 60°C, and then ground into fine powder with a blender. The plant was identified by Dr. Natthaya Choosingh Van Beem and the specimen was deposited at the Department of Biology, Faculty of Science, Thaksin University.

Extraction and Isolation

The dried flowers (120 g) of *F. desmoids* were macerated with methanol for 7 days at room temperature. The filtrate was collected through a filter paper and the marc was re-soaked in methanol twice. The combined filtrate was evaporated to dryness under reduced pressure to give a brown gum (8.37 g). The methanol extract was separated by preparative reversed-phase HPLC with gradient solvent of CH_3OH-H_2O in the presence of 0.1% trifluoroacetic acid (TFA) to afford 12 fractions based on TLC characteristics. Fraction 6 was further purified by the same method as the methanol extract to give 6 subfractions. Subfraction 5 gave **1** (10.5 mg) by crystallization. Fraction 8 was crystallized to give **2** (12.0 mg).

Antioxidant activity assays

The DPPH radical scavenging activity of the isolated compounds was analysed by a modified method of Chu [7], using BHT as a standard. Initially, 0.2 mL of sample (dissolved in 95% ethanol) was mixed with 4 mL of 0.004% w/v DPPH in 95% ethanol. The reaction mixture was incubated at 28°C in a dark room for 30 min. The control contained all reagents except the extract while 95% ethanol was used as a blank. The scavenging activity against DPPH radical was determined by measuring the absorbance at 517 nm with spectrophotometer. All experiments were measured in triplicate. The inhibition of DPPH radical was calculated as a percentage of radical scavenging following equation.

$$\% \text{ Radical scavenging} = [(A_{control}-A_{sample})/A_{control}] \times 100$$

Where $A_{control}$ is the absorbance of the control and A_{sample} is the absorbance of the sample or ascorbic acid.

The results were expressed as the concentration of the sample or BHT which scavenged DPPH radicals by 50% (IC_{50}).

Structural Identification

Compound 1; Yellow needle; mp. 165-166°C; $[\alpha]_D^{29}$ –55.8 (c 0.32, $CHCl_3$); UV (CH_3OH) λ_{max} (logε) 329 (2.15), 300 (2.12), 265 (1.52), nm; FT-IR (ATR) ν_{max} : 3400, 2845, 2751, 1613, 1154 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 12.74 (1H, s, 7-OH), 12.56 (1H, s, 5-OH), 10.09 (1H, s, 8-CHO), 7.46 (5H, s, H-2′, H-3′, H-4′, H-5′, H-6′), 6.04 (1H, s, H-6), 5.59 (1H, dd, J = 13.2, 3.3 Hz, H-2), 3.16 (1H, dd, *J* = 17.4, 13.2 Hz, H_a-3), 2.92 (1H, dd, *J* = 17.4, 3.3 Hz, H_b-3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 195.1 (C-4), 191.2 (8-CHO), 170.8 (C-7), 169.3 (C-5), 166.6 (C-8a), 137.1 (C-1′), 129.4 (C-4′), 129.1 (C-3′, C-5′), 126.1 (C-2′, C-6′), 104.5 (C-8), 101.8 (C-4a), 97.5 (C-6), 80.5 (C-2), 42.6 (C-3)

Compound 2; colorless powder; mp. 225-226°C; $[\alpha]_D^{29}$ –88.2 (c 0.05, $CHCl_3$); UV (CH_3OH) λ_{max} (logε) 342 (2.27), 313 (1.51), 264 (0.93) nm; FT-IR (ATR) ν_{max} : 3411, 1649, 1509, 1428, 1352, 1256, 1164, 1120, 1031, 838 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ: 11.79 (1H, s, 5-OH), 7.43 (5H, m, H2′-6′), 6.01 (1H, s, H-8), 5.43 (1H, dd, *J* = 12.3, 3.2 Hz, H-2), 3.02 (1H, dd, *J* = 17.1, 12.3 Hz, H_a-3), 2.82 (1H, dd, *J* = 17.1, 3.2 Hz, H_b-3), 2.01 (3H, s, CH_3); ^{13}C NMR (75 MHz, $CDCl_3$): δ 190.7 (C-4), 167.2 (C-7), 165.4 (C-5), 163.9 (C-8a), 136.4 (C-1′), 130.5 (C-4′), 129.1 (C-3′, C-5′), 126.9 (C-2′, C-6′), 103.0 (C-8), 102.8 (C-4a), 102.6 (C-6), 80.3 (C-2), 43.7 (C-3), 6.0 (CH_3)

RESULTS

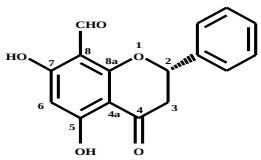
Identification of the flavonoids

The methanol extract of the flowers of *F. desmoids* was separated by preparative reversed-phase HPLC to afford two flavones, (2*S*)-8-formyl-5,7-dihydroxyflavanone (**1**) and (2*S*)-5,7-dihydroxy-6-methylflavanone (**2**). The compounds were identified by comparison of their physical and spectroscopic data with those reported in the literature.

Compound (**1**) was obtained as yellow needles. The IR spectrum showed absorption bands at 3400 (OH stretching), 2845, 2751 (aldehyde C-H stretching), 1613 (C=O stretching) and 1154 (C-O stretching) cm^{-1} whereas the UV spectrum exhibited absorption bands at 329, 300 and 265 nm. The ^{1}H NMR displayed signals of one aromatic singlet at δ 6.04 (H-6), a singlet of five protons at δ 7.46, assignable to monosubstituted phenyl ring (ring B), and an ABX system at δ 3.16 (1H, dd, *J* = 17.4,13.2 Hz, H_a-3), 2.92 (1H, dd, *J* = 17.4, 3.3 Hz, H_b-3) and 5.59 (1H, dd, J = 13.2, 3.3 Hz, H-2) for C-ring of flavanone. The ^{1}H NMR spectrum of **1** also showed an aldehyde proton at δ 10.09, two hydrogen bonded phenolic hydroxyls at □ 12.74 and 12.56. The ^{13}C NMR spectrum displayed characteristic signals for one monosubstituted aromatic ring (ring B) at 137.1 (C-1′), 129.4 (C-4′), 129.1 (C-3′, C-5′), and 126.1 (C-2′, C-6′), one pentasubstituted aromatic ring (ring A) at

δ_c 170.8 (C-7),169.3 (C-5), 166.6 (C-8a), 104.5 (C-8), 101.8 (C-4a), and 97.5 (C-6), one aldehyde carbon at δ_c 191.2 (8-CHO), one oxygenated methine carbon at δ_c 80.5 (C-2), one methylene carbon at δ_c 42.6 (C-3) and one carbonyl carbon at δ_c 195.1 (C-4). Compound **1** was an optical rotation ($[\alpha]_D^{29}$) -55.8 which nearly observed as (2*S*)-8-formyl-5,7-dihydroxyflavanone ($[\alpha]_D^{27}$ -52.06) as well as their melting point and spectral data [6]. It concluded that the absolute stereochemistry at the C-2 position was *S* configuration. Compound **1** was thus assigned as (2*S*)-8-formyl-5,7-dihydroxyflavanone.

Compound (**2**) was obtained as colorless power. It showed similar IR (ν_{max} 3411 (OH stretching), 1649 (C=O stretching) and 1164 (C-O stretching) cm^{-1} and UV (λ_{max} 342, 313 and 264 nm) data to those of **1**, indicating the presence of flavanone skeleton. The ^{1}H NMR data had signals of one aromatic singlet at □□6.01 to H-8, a multiplet of five protons at □ 7.43, and an ABX system at δ_H 5.43 (1H, dd, J = 12.3, 3.2 Hz, H-2), 3.02 (1H, dd, *J* = 17.1,12.3 Hz, H_a-3), and 2.82 (1H, dd, *J* = 17.1,3.2 Hz, H_b-3) for C-ring of flavanone. The ^{1}H NMR spectrum of **1** also showed one hydrogen bonded phenolic hydroxyl at δ_H 11.79 and singlet of methyl protons at δ_H 2.01 (CH_3). These data together with the absence of an aldehyde proton suggested that the methyl group was substituted in the ring-A of compound **1** instead of the formyl group. The ^{13}C NMR spectrum displayed characteristic signals of one monosubstituted aromatic ring (ring B) at 136.4 (C-1′), 130.5 (C-4′), 129.1 (C-3′, C-5′), and 126.9 (C-2′, C-6′), one pentasubstituted aromatic ring (ring A) at δ_c 165.4 (C-5), 102.6 (C-6), 167.2 (C-7), 163.9 (C-8a), 103.0 (C-8), and 102.8 (C-4a), one methyl carbon at δ_c 6.0, one oxygenated methine carbon at δ_c 80.3 (C-2), one methylene carbon at δ_c 43.7 (C-3) and one carbonyl carbon at δ_c 190.7 (C-4). Compound **2** was assigned to be the same absolute configuration at the C-2 as found in compound **1** according to the optical rotation. Compound **2** was identified as (2*S*)-5,7-dihydroxy-6-methylflavanone [8,9].



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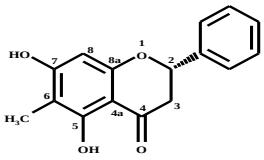

2

DPPH radical scavenging effect

Compounds **1** and **2** were evaluated for antioxidant activity by the DPPH radical scavenging assay. When antioxidants reacted with DPPH radical, the purple solution became yellow. The degree of discoloration indicated the radical scavenging activity of the antioxidants. Compound **2** showed antioxidant activity with the IC_{50} value of 8.09 µg/mL which was stronger than the standard BHT (IC_{50} 13.68 µg/mL), while compound **1** showed antioxidant activity with the IC_{50} value of 38.09 µg/mL.

CONCLUSIONS

Two known flavanones were isolated from the methanol extract of flowers of *F. desmoids*. Flavanones **1** and **2** were assigned as (2*S*)-8-formyl-5,7-dihydroxyflavanone and (2*S*)-5,7-dihydroxy-6-methyl-flavanone, respectively, by comparison of the physical and spectral data with those reported in the literature. Compound **2** showed antioxidant activity with the IC_{50} value of 8.09 µg/mL which was stronger than the standard BHT (IC_{50} 13.68 µg/mL).

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REFERENCES

20. Le Thomus, A. 1969. Flore du Gabon., 16, 240.
21. Perry L.M. 1980. Medicinal Plants of East and Southeast Asia: Attributed Properties and Uses. Medicinal Plants of East and South-east Asia, 20.
22. Richomme P, Sinbandhit S, David B, Had AHA, Bruneton J. 1990. Oxymitrone and 9a-O-methyl-oxymitrone, new hexahydroxanthenic derivatives from oxymitra kingii. J. Nat. Prod. 53, 294-297.
23. Achenbach H, Hemrich H. 1991. Alkaloids, flavonoids and phenylpropanoids of the west african plant oxymitra velutina. Phytochem. 30, 1265-1267.
24. Fleischer TC, Waigh RD, and Waterman PG. 1996. Bisabolene sesquiterpenes and flavonids from Friesodielsia enghiana. Phytochem. 44, 315-318.
25. Prawat U, Phupornprasert D, Butsuri A, Sala, AW, Boonsri S, Tuntiwachwuttikul P. 2012. Flavonoids from friesodielsia discolor. Phytochem. Lett. 5, 809-813.
26. Chu YH, Chang CL, Hsu HF. 2000. Determination of DPPH free radical scavenging activity: Application of artificial neural networks. J. Sci. Food Agric. 80, 561-566.
27. Pavan FR, Leite CQF, Coelho RG, Coutih ID, Honda NK, Cardoso CAL, Vilegas W, Leite SRA, Sato DN. 2009. Flavanoids from dalea elegans: Chemical reassignment and determination of kinetics parameters related to their anti-tyrosinase activity. Quim. Nova. 32, 1222-1226.
28. Mayer R. 1990. Flavonoids from *Cleistocalyx o perculatus*. Phytochem. 29, 1340-1342.

Transformation of epieudesmins into a new substance through nitration reaction

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ABSTRACT

This study modified epieudesmins using a nitration reaction at a temperature range of 60-65 °C for 3 hours to obtain a new substance with increased solubility. The structure of the resulting substance was investigated by means of FTIR NMR and MS. The FTIR spectrum of the new substance showed a broad transmittance band at 3450 cm^{-1} that is assigned to O-H stretching. The transmittance region at 1383 cm^{-1} and 837 cm^{-1} represent the specific peaks of the NO_2 sym stretching and C-N stretching frequency, respectively. The ^{1}H-NMR spectrum data was the presence of signals at δ 0.63 (m, H-2), 1.76 (m, H-3) and 2.63 (m, H-4), respectively. The proton signals of -OCH_3 groups disappeared in the spectrum due to the -OCH_3 groups having been replaced with the -OH groups. The -OH group was induced with the -NO_2 groups and became the -OH^+ group and the new substance was cyclic. The carbon signals of this cyclic in the ^{13}C-NMR spectrum displayed signals at δ 18.0 (C_2), 24.84 (C_3) and 26.01 (C_4). Moreover, the carbon signals of aromatic disappeared at δ 100-160 ($C_{1'}$-$C_{5'}$) due to the aromatic being replaced with the -NO_2 and -OH groups. The mass spectra showed a peak at m/e 257 indicating that the new substance was the 3,4- dihydro-5,8-dinitro-2H-chromene-6,7-diol. But, unfortunately, the substance was not active against human hepatocarcinoma (HepG2), MCF7-breast cancer, NCI-H187-small cell lung cancer, or KB-Oral cavity cancer and was non-cytotoxic against Vero cells.

Keywords: *Mitrephora sirikitiae,* lignans, epieudesmin, nitration reaction, anticancer activity

INTRODUCTION

The epieudesmins are lignans that have been isolated from the leaves of *Manolia coco* [1], *Acorus calamus* [2], *Monarda fistulosa* [3] and *Mitrephora sirikitiae* [4]. Epieudesmins are also found in the root of *Zanthoxylum armatutum* [5] and the bark of *Persea Kurzii* kosterm [6]. The epieudsdesmins have been shown to possess anti-inflammatory activity [5], anti-oxidant activity [7], and anti-fungal activity against basidiomycetes [8]. The epieudesmins have been reported to have anti-neoplastic activity against several human cancer cell lines such as human primary pancreatic adenocarcinoma (BXPC-3), human glioblastoma (SF268), human non-small cell lung cancer (NCL-H460), colon cancer cells (KM20L2), human prostate carcinoma (DU-145) and breast cancer (MCF-7) [2]. The properties of anticancer drugs include water and blood solubility for efficient transport into cells. But the epieudesmins are poorly water soluble due to their furofuran lignan structure. Previous studies have not reported modification of epieudesmin to increase solubility and anticancer activity. Therefore, in the current study, epieudesmins' structure was modified using the nitration reaction method to increase solubility and the resulting substance's anticancer properties were tested.

MATERIALS AND METHODS

Extraction and purification of epieudesmin from the leaves of *Mitrephora sirikitiae*

255 g of dried leaves of *Mitrephora sirikitiae* were ground into a fine powder which was then percolated with hexane at room temperature for three days. The solvent was evaporated using a rotary evaporator to produce a crude extract. Epieudesmins were extracted from the crude extract and were purified by column chromatography with the solvent of 20%v/v ethyl acetate and hexane. 2.04 g of epieudesmins were obtained from the extraction and purification.

Synthesis of epieudesmin with nitration reaction

The epieudesmins (0.3 g) were dissolved in concentrated sulfuric acid (12 ml). 24 ml. of nitrating agents (a mixture of 12 ml of concentrated nitric acid and 12 ml of concentrated sulfuric acid) were poured slowly into this solution in the temperature range of 60-65 °C for 3 hours. The solution was neutralized with concentrated sodium hydroxide and distilled water was added to stop the reaction and dissolve salts in the solution. Thin layer chromatography (TLC) was used to analyze the solution in the solvent system of ethyl acetate and hexane (6:4) and column chromatography was used to purify the solution to get one substance. The solvent in the solution was eliminated by rotary evaporator to get the new substance.

Characterization of substance

Fourier transform infra-red spectra was measured using Model spectrum one, Perkins Elmer Connecticut USA. The product was ground with KBr powder and the FTIR spectra were scanned from 4000 - 400 cm^{-1} at a resolution of 4.0 cm^{-1}. The structure was analyzed with ^{1}H-NMR and ^{13}C-NMR on a 500 MHz Varian using INOVA spectrometer. Mass spectrometer was measured using Esquire 3000 plus, Agilent Technology for the characterization of molecular mass.

Anticancer activities

The product was tested for cytotoxicity against human hepatocarcinoma (HepG2) ATCC HB-8065, the anti-Cancer (MCF7-breast cancer), the anti-cancer (NCI-H187-Small cell lung cancer) and the anti-Cancer (KB-Oral cavity cancer) by Resazurin Microplate assay (REMA), as well as for cytotoxicity against Vero cells (African green monkey kidney) by Green Fluorescent Protein (GFP)-based assay.

RESULTS

Epieudesmin has very poor water-solubility, making it cannot soluble in blood and effect to develop as a good anticancer drug. Generally, anticancer drugs are water-insoluble, delaying their clinical effectiveness [9]. Then, this work modified epieudesmins using a nitration reaction at a temperature range of 60-65 °C for 3 hours to obtain a new substance with increased solubility. The epieudesmins and a new substance were analyzed by TLC using ethyl acetate and hexane (6:4) system which have the R_f values of 0.65 and 0.33, respectively. The trend of R_f value of substance had a lower than epieudesmin due to the structure was increased with the hydroxyl groups. The FTIR spectrum of the substance showed a broad transmittance band at 3450 cm^{-1} that is assigned to O-H stretching. The transmittance region at 1383 cm^{-1} and 837 cm^{-1} represents the specific peaks of the NO_2 sym stretching and C-N stretching frequency, respectively. Moreover, the ^{1}H-NMR spectra of the substance showed the proton signals of cycles at δ 0.63 (m, H-2), δ 1.76 (m, H-3) and δ 2.63 (m, H-4) respectively. The ^{1}H-NMR of -OCH_3 signals disappeared in the spectra due to the -OCH_3 group which were replaced with the -OH groups. The ^{13}C-NMR spectra of the substance displayed carbon signals of cycles at δ 18.0 (C_2), 24.84 (C_3) and 26.01 (C_4), respectively. Furthermore, the aromatic signals disappeared at 100-160 ppm due to the aromatics being replaced with the -NO_2 and -OH groups. Moreover, the -OH groups were induced with -NO_2 groups to become the -OH^+ group and the cycles of the substance are shown in figure 1. The mass spectra of the substance showed a peak at m/e 257 indicating that the new substance was 3,4- dihydro-5,8-dinitro-2H-chromene-6,7-diol with related structure. But , unfortunately, The percent of survival cells (HepG2, MCF7, NCI-H187 and KB) were observed to be 106.83%, 102.35%, 100.2% and 118.51%, respectively for the concentrated substance of 5.56 µg/ml compared to that of ellipticine (positive control) of HepG2, NCI-H187 and KB cells were observed to be 22.16%, 45.76% and 34.01%, respectively, that of doxorubicin (positive control) of MCF7 cells was observed to be 59.76% by using the REMA assay. In the Green Fluorescent Protein (GFP)-based assay, the percent of survival cells of Vero cells at the same concentration was observed to be 100.65% compared to that of ellipticine was observed to be 3.69%. The anticancer activities of the substance were not against human hepatocarcinoma (HepG2) ATCC HB-8065, MCF7-breast cancer, NCI-H187-Small cell lung cancer, or KB-Oral cavity cancer and the substance showed non-cytotoxicity against Vero cells (African green monkey kidney). Although, the substance had increased the solubility but the modified epieudesimin through nitration reaction cannot effect to increase the anticancer activity.

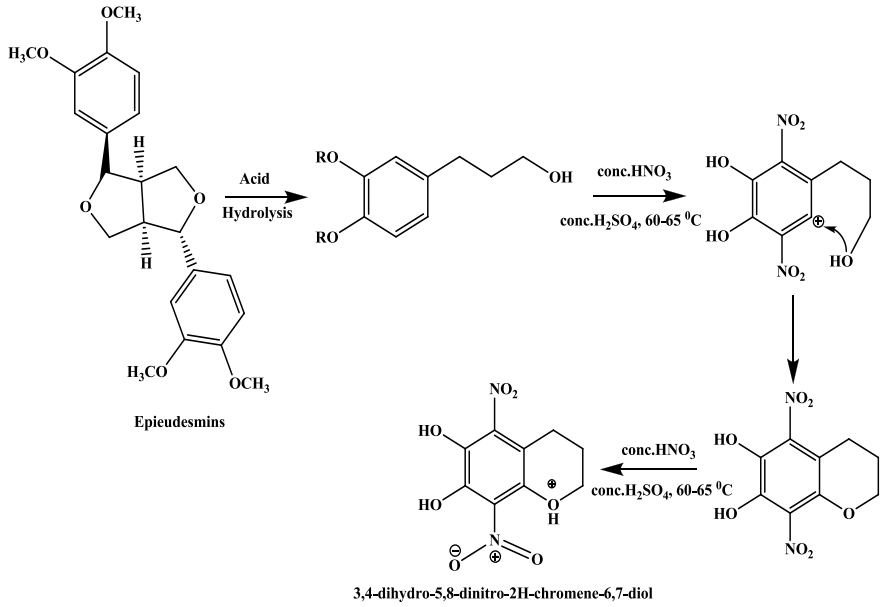

Figure 1. Nitration reaction of epieudesmin

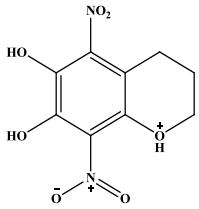

Figure 2. The new substance of 3,4-dihydro-5,8-dinitro-2H-chromene-6,7-diol

CONCLUSIONS

The epieudesmins were synthesized by nitration reaction to obtain the 3,4- dihydro-5,8-dinitro-2H-chromene-6,7-diol substance at the temperature range of 60-65 °C, for 3 hours. The substance was soluble in water but anticancer activities were not demonstrated by the 3,4- dihydro-5,8-dinitro-2H-chromene-6,7-diol.

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REFERENCES

1. Yu H, Chen CC, Shieh BJ. 1998. Two new constituents from the leaves of *Magnolia coco*. J. Nat. Prod. 61, 1017-1019.
2. Balakumbahan R, Rajamani K, Kumanan K. 2010. *Acorus calamus*: An overview. J. Med. Plants Res. 4(25), 2740-2745.
3. Weber M, Knoy C, Kindscher K, Brown RCD, Niemann S, Chapman J. 2007. Identification of medicinally active compounds in prairie plants by HPLC coupled to electron impact-mass spectrometry. Am. Lab. 39(12), 9-11.
4. Katekunlaphan T, Chalermmglin R, Niyasom C, Rukachisririkul T, Chalermglin R. 2014. Chemical constituents from the leaves of *Mitrephora sirikitiae*. In: Rukachisririkul V, Tansakuk C, Kanokmedhakul S, editors. Science and Technology towards ASEAN Development. Proceedings of the 40th congress on science and technology of Thailand (STT40); 2014 December 2-4; Hotel Pullman Khon Kaen Raja Orchid. Khon Kaen: The science society of Thailand under the patronage of his majesty the king, 229-232.

5. Guo T, Deng YX, Xie H, Yao CY, Cai CC, Pan S, et al. 2011. Antinociceptive and anti-inflammatory activities of ethyl acetate fraction from *Zanthoxylum armatum* in mice. Fitoterapia. 82, 347-351.
6. Denrungruang P. 1988. Some chemical constituents of the bark of Yaang Bong. Thesis for Chemistry, Master Degree, Kasetsart University.
7. Ghimire BK, Seong ES, Kim EH, Ghimeray AK, Yu CY, Ghimire BK, et al. 2011. A comparative evaluation of the antioxidant activity of some medicinal plants popularly used in Nepal. J. Med. Plants Res. 5(10), 1884-1891.
8. Timngim M, Rajviroongit S. 2009. Isolation and structure elucidation of components from *Cinnamomum glaucescens*. The international congress for innovation in chemistry, 2009 May 3-6; Jomtien Palm Beach Hotel & Resort Pattaya. Chonburi: Ministry of education PERCH-CIC, p. 289.
9. Chadha R, Kapoor VK, Thakur D, Kaur R, Arora P, Jain DVS. 2008. Drug carrier systems for anticancer agents: a review. J. Sci. Ind. Res. 67, 185-197.

Preliminary phytochemical and antimicrobial evaluations of *Myriopteron extensum* (Wight) K.Schum. and *Telosma minor* Craib fruit extracts

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ABRTRACT

The objectives of this study were to test for significant phytochemicals and antimicrobial activities of indigenous plants *Myriopteron extensum* (Wight) K.Schum. and *Telosma minor* Craib. The dichloromethane extracts of fruit parts from both plants contained terpenoids, whereas the methanolic extracts showed the presence of diterpenoids, terpenoids and saponins as major components. The methanolic extracts were further tested against *Staphylococcus aureus, Bacillus subtilis*, *Pseudomonas aeruginosa, Escherichia coli* and *Candida albicans*. Inhibition of *E. coli* was observed from the *Myriopteron extensum* (Wight) K.Schum. extract in milligram concentrations. Additionally, *E. coli, C. albicans* and *S. aureus* were inhibited by the *Telosma minor* Craib extract in the same range of concentrations. These results revealed a potential discovery of antimicrobial agents from the easily found plants in Thailand.

Keywords: *Staphylococcus aureus*, *Candida albicans*, Cha-aim Tao, Kha chon

INTRODUCTION

Drugs are essential for health care system all over the world. Even though various drugs are available, novel medications are still urgently needed. Plants, especially, have shown a dominant role in providing herbal drugs for the treatment of a broad spectrum of diseases [1]. Therefore, our research interest focuses on the evaluation of bioactive phytochemicals from plants widely grown in Thailand. The indigenous plants, *Myriopteron extensum* (Wight) K.Schum. ("Cha-aim Tao" in Thai) and *Telosma minor* Craib ("Kha chon" in Thai) were selected for this study. Flowers of *Telosma minor* Craib are commonly consumed as side dishes, whereas fruits of *Myriopteron extensum* (Wight) K.Schum. are used in Thai cooking. Several bioactivities were reported from different parts of these plants such as *Myriopteron extensum* (Wight) K.Schum. stem [2-3]. However, no information about the fruit part was found.

In order to fully investigate putative biologically active compounds from both plants, the fruit parts should be evaluated. In this work, we test for the major phytochemicals and antimicrobial activities of dichloromethane and methanolic extracts.

MATERIALS AND METHODS

Plant Materials and Extraction

Both plants were collected during January – March 2015 from Moo.3 Tambon Nong Sang, Kaeng Khro District, Chaiyaphum Province, Thailand. The fruit parts were cleaned and cut into small pieces before being dried at a room temperature. After drying, they were ground to obtain 500 g powder of each part. Dichloromethane (1.5 L) and methanol

(1.5 L) were used to extract the materials at room temperature for 7 days. The materials were filtered and the solvents were removed in vacuum to yield dichloromethane extract 28.03 g (5.61%) and methanol extract 30.12 g (6.02%) of *Myriopteron extensum* (Wight) K.Schum and dichloromethane extract 30.13 g (6.03%) and methanol extract 37.83 g (7.57%) of *Telosma minor* Craib.

Phytochemical Tests

Detection of carbohydrates

Benedict's test: 0.1 g of the sample was mixed with 5 ml of distilled water in a test tube and gently boiled for 10 minutes. The cool solution was filtered. Filtrates were treated with 1 ml of benedict's solution and heated gently about 5 minutes. Orange red precipitate indicated the presence of reducing sugars [4].

Iodine test: 0.1 g of the sample was mixed with 5 ml of distilled water in a test tube and gently boiled for 10 minutes. The cool solution was filtered and treated with 2-3 drops of iodine solution. A dark blue coloration indicated the presence of the carbohydrates [5].

Detection of saponins

Foam test: 0.1 g of the sample was mixed with 5 ml of distilled water in a test tube and gently boiled for 10 minutes. The cool solution was filtered and shaken vigorously. The formation of stable foam for 10 minutes indicated the presence of saponins [5].

Detection of phenolic compounds

Ferric chloride test: 0.1 g of the sample was added to 5 ml of distilled water in a test tube and gently boiled for 10 minutes. The cool solution was filtered and treated with 2-3 drops of 2% ferric chloride solution. Formation of blue-green or black coloration indicated the presence of phenolic compounds [6].

Detection of anthraquinones

Borntrager's test: 0.1 g of the sample was treated with 5 ml of chloroform. The mixture was shaken for 5 minutes and then filtered. The filtrate was shaken with 2 ml of 10% ammonia solution. A pink, red, orange or violet color in the aqueous layer after shaken indicated the presence of free anthraquinones [7].

Modified Borntrager's test (for combined anthracene derivatives): 0.1 g of the sample was boiled with 5 ml of 10% hydrochloric acid for 5 minutes. The hot solution was filtered into a test tube, cooled and extracted gently with 3 ml of chloroform. The chloroform layer was separated and treated with 2 ml of 10% ammonia solution. Formation of rose-pink or orange color in the ammonia layer indicated the presence of anthraquinones [7].

Detection of flavonoids

Shinoda's test: 0.1 g of the sample was added to 2 ml of concentrated hydrochloric acid and a few magnesium chips were added. A pink or red color indicated the presence of flavonoids [7].

Detection of tannins

Gelatin test: 0.1 g of the sample was added to 5 ml of distilled water in a test tube and gently boiled for 10 minutes. The cool solution was filtered and treated with 2-3 drops of 1% gelatin solution. Formation of white precipitate indicated the presence of tannins [8-9].

Ferric chloride test: 0.1 g of the sample was added to 5 ml of distilled water in a test tube and gently boiled for 10 minutes. The cool solution was filtered and treated with 2-3 drops of 2% ferric chloride solution. Formation of blue-green or black coloration indicated the presence of tannins [10].

Lead acetate test: 0.1 g of the sample was mixed with 5 ml of distilled water in a test tube and gently boiled for 10 minutes. The cool solution was filtered and treated with 2-3 drops of 10% lead acetate solution. Formation of gray precipitate indicated the presence of tannins [10-11].

Detection of Alkaloids

Wagner's reagent: 0.1 g of the sample was added to 5 ml of distilled water in a test tube and gently boiled for 10 minutes. The cool solution was filtered and treated with 2-3 drops of Wagner's reagent. Formation of reddish-brown precipitate indicated the presence of alkaloids [12].

Detection of diterpenoids

Copper acetate test: 0.1 g of the sample was mixed with 5 ml of distilled water in a test tube and gently boiled for10 minutes. The cool solution was filtered and treated with 2-3 drops of 10% copper acetate solution. Formation of emerald green color indicated the presence of diterpenoids [12].

Detection of triterpenoids

Salkowski's test: 0.1 g of the sample was treated with 5 ml of chloroform and then filtered. The filtrate with 2 ml concentrated sulphuric acid was shaken and allowed to stand. Appearance of reddish brown color indicated the presence of triterpenoids [13].

Antimicrobial Tests [14-15]

Paper disc preparation

Plant extracts were dissolved in dimethyl sulfoxide to make a solution of 100 mg/ml after sterilization by filtration through 0.45-micron membrane filter, the solutions were distributed to 6.14 millimeter paper discs equivalent to 0.5, 1.0, 1.5 and 2.0 mg.

Antibacterial Tests

Inoculum preparation

Overnight colonies of testing bacteria were suspended in steriled normal saline solution (0.85% NaCl). Bacterial suspensions were standardized to McFarland 0.5 Standard prior to plate inoculation. Bacterial inoculation was carried out by 3D swab onto Mueller Hinton agar plates and let stand for five minutes.

Antibacterial assays

Discs containing different concentrations of plant extracts were placed onto Mueller Hinton agar plates simultaneously with ones of dimethyl sulfoxide as a negative control. Mueller Hinton agar plates were incubated at 37°C for 24-48 h. Inhibition zones were subsequently measured.

Antifungal Tests

Inoculum preparation

Fungal suspension were standardized with McFarland 0.5 Standard prior to plate inoculation. Then, 50 µl of the *Candida albicans* suspension were spread onto the Mueller Hinton agar plates. After incubated at 30°C for 24 - 48 h, the colonies were counted. Then inoculation was carried out by 3D swab onto Mueller Hinton agar plates and let stand for five minutes.

Antifungal assay

Discs containing different concentrations of plant extracts were placed onto Mueller Hinton agar plates simultaneously with ones of dimethyl sulfoxide as a negative control. Mueller Hinton agar plates were incubated at 30°C for 24-48 h. Inhibition zones were subsequently measured.

RESULTS

The dried fruits of *M. extensum* and *T. minor* were extracted with methanol and dichloromethane. The results of phytochemical analysis of *M. extensum* and *T. minor* extracts are shown in Table1. The methanolic extracts of both plants contained saponin, diterpinoids and terpenoids in equal amounts. Diterpeniods, terpenoids and carbohydrates with reducing sugars were found in the dichloromethane extract of *M. extensum*. Whereas, the dichloromethane extract of *T. minor* showed only the presence of terpenoids in a high quantity. These results indicated that the methanolic extracts of both plant materials possessed slightly higher numbers of phytochemicals than the dichloromethane extracts. Therefore, the methanolic extracts were further tested against several microorganisms. The results in terms of antimicrobial activities are exhibited in Table 2.

Table 1. Phytochemical analysis of the dichloromethane and the methanolic extracts.

Plants / Phytochemical analysis		Methaolic extracts		Dichloromethane extracts	
		M. extensum	*T. minor*	*M. extensum*	*T. minor*
Saponins		+++	++	-	-
Phenolic compounds		-	-	-	-
Flavonoids		-	-	-	-
Tanins	Gelatin	-	-	-	-
	Lead acetate	-	-	-	-
Alkaloids		-	-	-	-
Antraquinone	Borntrager's test	-	-	-	-
	Modified Borntrager's test	-	-	-	-
Diterpenoids		++	++	+	-
Triterpenoids		++	++	++	+++
Carbohydrates	Benedict	-	-	+	-
	I_2 solution	-	-	-	-

(-) = Negative test, (+) = Weakly positive test, (++) = Positive test, (+++) = Strongly positive test

The methanolic extracts with 0.5 mg, 1.0 mg, 1.5 mg and 2.0 mg concentrations were tested against gram-positive bacteria (*S. aureus* and *B. subtilis*), gram-negative bacteria (*P. aeruginosa* and *E. coli*) and fungi (*C. albicans*). The disc diffusion method showed only the detectable zones of inhibition when testing 1.5 mg and 2.0 mg of the *M. extensum* extract with *E. coli*. However, the zone of inhibition was observed in the plate containing *E. coli* when only 0.5 mg of the *T. minor* extract were used. Moreover, the antimicrobial activities against *S. aureus* and *C. albicans* of the *T. minor* extract were found with 2.0 mg and 1.5 mg concentrations, respectively. No inhibition was observed when testing the extracts with *B. subtilis* and *P. aeruginosa*. Interestingly, the *T. minor* extract contained possible broad spectrum antibiotics, whereas the *M. extensum* extract provided a specific inhibition with *E. coli*.

Table 2. Antimicrobial activities of the methanolic extracts against Gram positive bacteria (*Staphylococcus aureus and Bacillus subtilis*), Gram negative bacteria (*Pseudomonas aeruginosa and Escherichia coli*) and Fungi (*Candida albicans*).

Volume (mg)	Average zone diameter of inhibition (mean ± SD;mm)					Negative control
	Gram-positive bacteria		Gram-negative bacteria		Fungi	
	S. aureus	*B. subtilis*	*P. aeruginosa*	*E. coli*	*C. albicans*	
M. extensum						
0.5	-	-	-	-	-	6.14
1.0	-	-	-	-	-	6.14
1.5	-	-	-	12.42 ± 0.68	-	6.14
2.0	-	-	-	13.58 ± 0.58	-	6.14
T. minor						
0.5	-	-	-	12.62 ± 0.61	-	6.14
1.0	-	-	-	12.14 ± 0.63	-	6.14
1.5	-	-	-	13.60 ± 0.84	12.40 ± 0.37	6.14
2.0	12.74 ± 0.50	-	-	14.10 ± 0.34	12.54 ± 0.64	6.14

Zone of inhibition measured in millimeter (mm), (-) = No activity was observed.

The methanolic extracts with 0.5 mg, 1.0 mg, 1.5 mg and 2.0 mg concentrations were tested against gram-positive bacteria (*S. aureus* and *B. subtilis*), gram-negative bacteria (*P. aeruginosa* and *E. coli*) and fungi (*C. albicans*). The disc diffusion method showed only the detectable zones of inhibition when testing 1.5 mg and 2.0 mg of the *M. extensum* extract with *E. coli*. However, the zone of inhibition was observed in the plate containing *E. coli* when only 0.5 mg of the *T. minor* extract were used. Moreover, the antimicrobial activities against *S. aureus* and *C. albicans* of the *T. minor* extract were found with 2.0 mg and 1.5 mg concentrations, respectively. No inhibition was observed when testing the extracts with *B. subtilis* and *P. aeruginosa*. Interestingly, the *T. minor* extract contained possible broad spectrum antibiotics, whereas the *M. extensum* extract provided specific inhibition with *E. coli*.

CONCLUSION

In conclusion, the methanolic and the dichloromethane extracts of *M. extensum* and *T. minor* dried fruits were screened for bioactive phytochemicals. The methanolic extracts of both plants contained saponins, diterpenoids and terpenoids as major phytochemicals. In contrast, the dichloromethane extracts mainly contained terpenoids as important phytochemicals. The methanolic extracts were investigated for potentials antimicrobial activities. The best inhibition was observed when testing the *T. minor* fruit extract against *S. aureus*, *E. coli* and *C. albicans*. Further isolation of the extracts will be carried out to fully elucidate structures of the antimicrobial compounds. These data lead to a possible finding of novel antimicrobial agents.

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REFERENCES

1. Cragg GM, Grothaus PG, Newman DJ. 2009. Impact of Natural products on developing new anti-cancer agents. Chem. Rev. 109, 3012-3043.
2. Soonthornchareonnon N, Ubonopas L, Kaewsuwan S, Wuttiudomlert M. 2004. Lupinifolin, a bioactive flavanone from *Myriopteron extensum* (Wight) K. Schum. Stem. Thai J of Phytopharm. 11, 19-28.
3. Rakariyatham N, Teerawutgulrag A, Chanwitheesuk A. 2005. Screening of antioxidant activity and antioxidant compounds of some edible plants of Thailand. Food Chem. 92, 491-497.
4. Yadav RNS, Agarwala M. 2011. Phytochemical analysis of some medicinal plants. J. of Phytol. 3, 10-14.
5. Fatima ZS, Meriem B, Samira S, Muneer MSA. 2012. Phytochemical Screening and identification of some compounds from Mallow. J. Nat. Prod. Plant Resour. 2, 512-516.
6. Ghongade R. 2013. Phytochemical Analysis of *Citrus Karna* fruit. Int. J. Pharm. Bio. Sci. 4, 1162-1167.
7. Yusuf AZ, Zakir A, Shemau Z, Abdullahi M, Halima SA. 2014. Phytochemical analysis of the methanol leaves extract of *Paullinia pinnata* linn. J. Pharmacognosy Phytother. 2, 10-16.
8. Gutal VS. 2011. Phytochemical Analysis of the Indian Medicinal Plant *Argyreia involucrate*. Int. J. Res. Pharm. Bio. Sci. 2, 1778-1782.
9. Tongco JVV, Aguda RM, Razal RA. 2014. Proximate analysis, phytochemical screening, and total phenolic and flavonoid content of Philippine bamboo *Schizostachyum lumampao*. J. Chem. Pharm. Res. 6, 709-713.
10. Godghate A, Sawant R, Sutar A. 2012. Phytochemical analysis of ethanolic extract of roots of *Carissa Carandas* Linn. RASAYAN J. Chem. 5, 456-459.
11. Sawant RS, Godghate AG. 2013. Preliminary phytochemical analysis of leaves of *Tridax Procumbens* Linn. Int. J. Environ. Sci. Tech. 2, 388-394.
12. Tiwari P, Kumar B, Kaur M, Kaur G, Kaur H. 2011. Phytochemical screening and Extraction: A Review. Int. Pharm. Sci. 1, 98-106.

13. Ayoola GA, Coker HAB, Adesegun SA, Adepoju-Bello AA, Obaweya K, Ezennia EC, Atangbayila TO. 2008. Phytochemical screening and antioxidant activities of some selected medicinal plants used for malaria therapy in southwestern nigeria. J. Pharm. Res. 7, 1019-1024.
14. Boonmee A, Chantra S, Suwancharoen S. 2015. Phytochemical and antibacterial activity of jellyfish curing extract. KKU Sci. J. 43, 106-115.
15. Marie B. Coyle. 2005. Disk diffusion testing. Manual of antimicrobial susceptibility testing. American society for microbiology. Departments of laboratory medicine and microbiology. University of Washington. USA, 39-52.

Xanthones from the green branch of *Garcinia dulcis*

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ABSTRACT

Investigation of the chemical constituents in dichloromethane extract from green branch of *Garcinia dulcis* resulted in the isolation of three xanthones: garciniaxanthone E (**1**), 2,5-dihydroxy-1-methoxyxanthone (**2**) and 2,6-dihydroxy-1,5-dimethoxyxanthone (**3**). Their structures were elucidated by analysis of spectroscopic data and comparison of the NMR data with those reported previously. Their antibacterial activities were also examined.

Keywords: *Garcinia dulcis*, Clusiaceae, Xanthone

INTRODUCTION

Garcinia dulcis (Clusiaceae) is an evergreen tree, 7-10 meters tall, widely distributed in Southern Thailand, where it is commonly known as "Ma-phut". Xanthones are the most characteristic secondary metabolite constituents of *G. dulcis*, and more than 30 compounds of this type have been isolated and characterized from the various parts of this plant [1-9]. The biological effects of xanthones in this plant are diverse and include antioxidant, antibacterial, anti-inflammatory, anti-androgens and cytotoxic activities [6,10-11]. Previous phytochemical investigation on the branch of *G. dulcis* led to the identification of only one xanthone and no information on antibacterial activity of xanthone [9]. We thus investigated the antibacterial activity of crude extract of *G. dulcis* green branch and found that the crude dichloromethane extract exhibited a significant antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) SK1 with a minimum inhibition concentration (MIC) of 128 µg/mL. In this preceding paper, we describe the isolation and structural elucidation of compounds **1**-**3** from the dichloromethane of *G. dulcis* green branch.

MATERIALS AND METHODS

General Experimental Procedures

Melting points were obtained on a Fisher-Johns Melting Point Apparatus. Infrared (IR) spectra were determined on a Perkin–Elmer 783 FTS 165 FT-IR spectrometer and were recorded in wave number (cm^{-1}). Ultraviolet (UV) absorption spectra were determined by using MeOH on a Shimadzu UV-160A spectrophotometer and principle bands (λ_{max}) were recorded as wavelengths (nm) and log ε in methanol solution. *Nuclear magnetic resonance (NMR)* spectra were measured on Bruker FT-NMR Ultra Shield™ 300 and 500 MHz spectrometers at the Department of Chemistry, Faculty of Science, Prince of Songkla University. ^{1}H and ^{13}C NMR spectra were measured at 300 and 75 MHz or 500 and 125 MHz, respectively. Chemical shifts (δ) are recorded in parts per million (ppm) in $CDCl_3$, DMSO-d_6 and acetone-d_6 containing TMS as an internal standard (δ 0.00) and coupling constant (J) are expressed in hertz (Hz). Column chromatography (CC) was performed with silica gel 100 (70–230 Mesh ASTM, Merck) and Sephadex LH-20 (Amersham Biosciences, Sweden). Thin-layer chromatography (TLC) was performed on silica gel GF_{254} (20×20 cm, layer thickness 0.2 mm, Merck) and compounds were detected under UV (254 nm) fluorescence. All solvents were of spectroscopic grade or distilled from glass prior to use.

Plant Material

The green branch of *G. dulcis* was collected from Songkhla Province in the southern part of Thailand, in April 2013. The voucher specimen (Coll. No. 02, Herbarium No. 0012652) has been deposited at the Herbarium of the Biology Department, Faculty of Science, Prince of Songkla University, Thailand.

Extraction and Isolation

The air-dried and powdered green branch of *G. dulcis* (1.0 kg) was extracted with dichloromethane (8.0 L×2, 48 h each) at room temperature, and the extract was concentrated under vacuum condition. Then, the dichloromethane extract (32.89 g) was partitioned between CH_2Cl_2 and 5% NaOH. After worked up, the aqueous layer (8.53 g) and CH_2Cl_2 layer (23.78 g) were obtained. The aqueous layer (8.53 g) was subjected to a silica gel QCC and eluted with a gradient of CH_2Cl_2-acetone (100:0 to 0:100) with increasing polarity to give seven combined fractions (1-7) based on analyzing by TLC using CH_2Cl_2-MeOH (98:2) as solvents systems. Fraction 4 (1.54 g) was submitted to Sephadex LH-20 CC and eluted with MeOH to yield six fractions (E1–E6). Fraction E3 (189.9 mg) was further isolated by silica gel CC with CH_2Cl_2-acetone (98:2) to yield an orange solid **1** (9.4 mg). Fraction 5 (2.23 g) was fractionated by silica gel CC employing a gradient of CH_2Cl_2-acetone (93:7 to 20:80) resulting in eleven fractions (F1-F11). Fractions F6 (114.4 mg) was further purified by Sephadex LH-20 CC with MeOH to give a yellow solid **2** (5.8 mg). Fractions F8 (770.3 mg) was further purified by Sephadex LH-20 CC eluting with MeOH to yield yellow solid **2** (4.5 mg) and yellow gum **3** (8.2 mg).

Garciniaxanthone E (1): Orange solid, m.p. 212-213 °C; UV (MeOH) λ_{max} (log ε): 254 (4.23) and 328 (3.92) nm; IR (neat) ν_{max}: 3375 (O-H stretching) and 1648 (C=O stretching) cm^{-1}; ^1H and ^{13}C NMR data, see Table 1.

2,5-dihydroxy-1-methoxyxanthone (2): Yellow solid, m.p. 194-195 °C; UV (MeOH) λ_{max} (log ε): 258 (4.30) and 374 (3.42) nm; IR (neat) ν_{max}: 3275 (O-H stretching) and 1604 (C=O stretching) cm^{-1}; ^1H and ^{13}C NMR data, see Table 1.

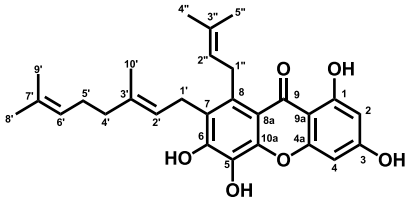
2,6-dihydroxy-1,5-dimethoxyxanthone (3): Yellow gum; UV (MeOH) λ_{max} (log ε): 240 (3.76), 252 (3.76) and 310 (3.34) nm; IR (neat) ν_{max}: 3200 (O-H stretching) and 1606 (C=O stretching) cm^{-1}; ^1H and ^{13}C NMR data: Table 1.

Antibacterial assay

The procedure for antibacterial assay was performed by modified colorimetric broth microdilution method [14-16]. The test substances were dissolved in DMSO (Merck, Germany). The working solutions (10 mg/mL) and the bacterial inocula were prepared by diluting with Mueller-Hinton broth (MHB) in the ratio of 1:25 and 1:200 (~$7.5x10^5$ CFU/mL), respectively. Triplicate 50 µL of test diluted solutions and 50 µL of bacterial inocula were added to sterile microtiter plates. The final concentration of test substances in microtiter plates were 200 µg/mL. Plates were incubated at 35 °C (15 hours) and then 20 µL of 0.09% resazurin was added into each well. Finally, plates were further incubated at 35 °C (3 hours) for complete incubation (adapted from Sarker *et al.*, 2007) [15]. *Staphylococcus aureus* ATCC25923, methicillin-resistant *Staphylococcus aureus* (MRSA) SK1 (isolated from a clinical specimen, Songklanakarin Hospital), *Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853 were used as test strain. Standard drugs were used as positive control including vancomycin (MIC 1 µg/mL) for *S. aureus* and MRSA, gentamicin (MIC 0.5 µg/mL) for *E. coli* and *P. aeruginosa*.

RESULTS AND DISCUSSION

The dichloromethane extract of *G. dulcis* green branch was sequentially partitioned with 5% NaOH and CH_2Cl_2. The aqueous layer was then purified by repeated chromatography, which led to the isolation of three xanthones: garciniaxanthone E (**1**), 2,5-dihydroxy-1-methoxyxanthone (**2**) and 2,6-dihydroxy-1,5-dimethoxyxanthone (**3**) [12-13]. All isolates were characterized by spectroscopic methods and compared with those previously published data.



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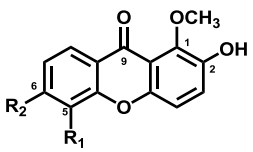

2: R_1 = OH, R_2 = H
3: R_1 = OMe, R_2 = OH

Compound **1** was an orange solid, m.p. 212-213 °C. The UV spectrum showed maximum absorption bands at 254 and 328 nm. The IR spectrum showed the absorption bands of O-H stretching at 3375 cm^{-1} and C=O stretching at 1648 cm^{-1}. The ^{1}H NMR spectrum (Table 1) showed the presence of a chelated hydroxyl group (*δ* 13.69, *s*, 1-OH) and *meta*-coupled aromatic protons (*δ* 6.25, *d*, *J* = 2.4 Hz, H-2; *δ* 6.42, *d*, *J* = 2.4 Hz, H-4). Proton 1-OH correlated to C-1 (*δ* 164.2), C-2 (*δ* 97.8), C-9a (*δ* 102.9); H-2 correlated to C-4 (*δ* 92.9), C-9a (*δ* 102.9); and H-4 correlated to C-2 (*δ* 97.8), C-4a (*δ* 156.7), C-9a (*δ* 102.9). The spectrum further showed signal of a prenyl group (*δ* 4.17, *d*, *J* = 5.7 Hz, H-1"; *δ* 5.16, *br t*, *J* = 5.7 Hz, H-2"; *δ* 1.73, *s*, H-4"; *δ* 1.85, *s*, H-5") and a geranyl group (*δ* 3.54, *d*, *J* = 6.3 Hz, H-1'; *δ* 5.17, *br t*, *J* = 6.3 Hz, H-2'; *δ* 2.06, *br t*, 7.2, H-4'; *δ* 2.12, *m*, H-5'; *δ* 5.14, *br t*, *J* = 7.2 Hz, H-6'; *δ* 1.68, *s*, H-8'; *δ* 1.62, *s*, H-9'; *δ* 1.85, *s*, H-10'). Owing to the low field chemical shift of methylene protons H-1" (*δ* 4.17), the prenyl group then was placed nearby the carbonyl group (*δ* 182.5). The geranyl side chain was located at C-7 according to the HMBC correlations (Figure 1) of H-1" (*δ* 4.17) and H-2' (*δ* 5.17) to C-7 (*δ* 125.3). To fulfill the structure, three hydroxyl groups were placed at C-3 (*δ* 164.4), C-5 (*δ* 129.9) and C-6 (*δ* 149.3). Thus, compound 1 was identified to be 1,3,5,6-tetrahydroxy-7-(3,7-dimethyl-2,6-octadienyl)-8-(3-methyl-2-butenyl)xanthone which was known as garciniaxanthone E [12].

Compound 2 was a yellow solid, m.p. 194-195 °C. The UV spectrum showed maximum absorption bands at 258 and 374 nm. The IR spectrum showed the absorption bands of O-H stretching at 3275 $cm^{-1}$and C=O stretching at 1604 cm^{-1}. The ^{13}C NMR spectrum (Table 1) displayed 13 carbons (*δ* 114.1, *δ* 116.3, *δ* 116.6, *δ* 119.7, *δ* 122.8, *δ* 123.1, *δ* 123.4, *δ* 145.0, *δ* 145.1, *δ* 145.9, *δ* 146.0, *δ* 150.7, *δ* 176.5) of a xanthone skeleton and a methoxy group (*δ* 62.3). The ^{1}H NMR spectrum (Table 1) showed the resonances of *ortho*-coupled aromatic protons at *δ* 7.39 (*d*, *J* = 9.3 Hz, H-3) and *δ* 7.32 (*d*, *J* = 9.3 Hz, H-4); and a methoxy group at *δ* 4.01 (*s*, 1-OCH_3). These protons were assigned for H-3, H-4 and 1-OCH_3 from its correlations (Figure 1) of H-3 to C-1 (*δ* 145.0), C-2 (*δ* 145.9), C-4a (*δ* 150.7); H-4 to C-2 (*δ* 145.9), C-4a (*δ* 150.7), C-9a (*δ* 116.3); and 1-OCH_3 to C-1 (*δ* 145.0). The remaining resonances appearing as an ABM system at *δ* 7.27 (*dd*, *J* = 8.1, 1.5 Hz, H-6), *δ* 7.16 (*t*, *J* = 8.1 Hz, H-7) and *δ* 7.75 (*dd*, *J* = 8.1, 1.5 Hz, H-8). The most deshielded resonance (*δ* 7.75) was proposed for H-8 according to anisotropic effect of the carbonyl group (C-9). The assignments of H-6, H-7 and H-8 were supported by ^{3}J correlation of H-6 to C-8 (*δ* 116.6), C-10a (*δ* 145.1); H-7 to C-5 (*δ* 146.0), C-8a (*δ* 123.1); and H-8 to C-6 (*δ* 119.7), C-9 (*δ* 176.5), C-10a (*δ* 145.1) on HMBC experiment. To fulfill the structure, the hydroxyl groups were placed at C-2 (*δ* 145.9) and C-5 (*δ* 146.0). It thus was identified as 2,5-dihydroxy-1-methoxyxanthone [12].

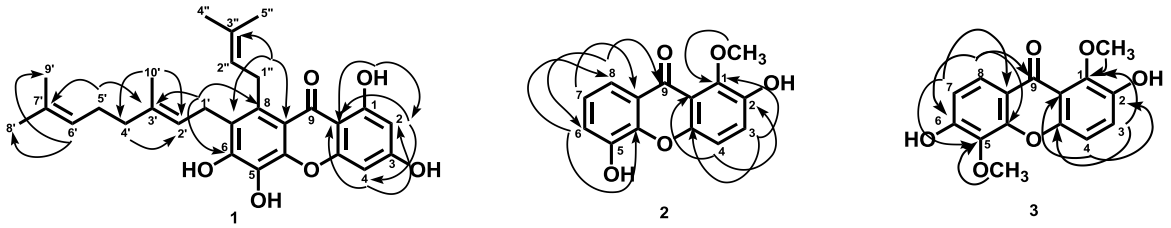

Figure 1. Selected HMBC correlations for **1**, **2** and **3**

Compound **3** was a yellow gum. The UV spectrum showed maximum absorption bands at 240, 252 and 310 nm. The IR spectrum showed the absorption bands of O-H stretching at 3200 $cm^{-1}$and C=O stretching at 1606 cm^{-1}. The ^{13}C NMR spectrum (Table 1) showed signals of a carbonyl carbon (*δ* 175.3), twelve aromatic carbons (*δ* 112.8, *δ* 113.7, *δ* 116.0, *δ* 116.6, *δ* 121.8, *δ* 122.2, *δ* 133.9, *δ* 145.0, *δ* 145.9, *δ* 149.7, *δ* 150.7, *δ* 154.6) and two methoxy groups (*δ* 61.5, *δ* 62.3). The ^{1}H NMR spectrum (Table 1) showed doublet of *ortho*-coupled protons H-3 and H-4 at *δ* 7.35 and *δ* 7.26 (*J* = 9.0 Hz); doublet of *ortho*-coupled protons H-7 and H-8 at *δ* 6.96 and *δ* 7.93 (*J* = 9.0 Hz); and singlet of methoxy groups 1-OCH_3 and 5-OCH_3 at *δ* 4.02 and *δ* 4.05. The HMBC spectrum (Figure 1) showed correlations of 1-OCH_3 to C-1 (*δ* 145.0); H-3 to C-1 (*δ* 145.0), C-4a (*δ* 150.7); H-4 to C-2 (*δ* 145.9), C-9a (*δ* 116.0); 5-OCH_3 to C-5 (*δ* 133.9); H-7 to C-5 (*δ* 133.9), C-8a (*δ* 116.6); H-8 to C-6 (*δ* 154.6), C-9 (*δ* 175.3); confirming their locations. This compound was 2,6-dihydroxy-1,5-dimethoxyxanthone [13].

All isolated compounds were tested for their antibacterial activity against *Staphylococcus aureus* ATCC25923, methicillin-resistant *Staphylococcus aureus* (MRSA) SK1, *Escherichia coli* ATCC25922 and *Pseudomonas aeruginosa* ATCC27853, with Vancomycin and Gentamicin as the standard drugs. They showed no antibacterial activity against those bacterial strains at 200 μg/mL.

Table 1. ^{1}H and ^{13}C NMR data of compound **1**-**3**

Position	**1**[a]		**2**[b]		**3**[c]	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
	164.2 (C)	-	145.0 (C)	-	145.0 (C)	-
1	97.8 (CH)	6.25 (1H, *d*, *J* = 2.4)	145.9 (C)	-	145.9 (C)	-
2	164.4 (C)	-	122.8 (CH)	7.39 (1H, *d*, *J* = 9.3)	121.8(CH)	7.35 (1H, *d*, *J* = 9.0)
3	92.9 (CH)	6.42 (1H, *d*, *J* = 2.4)	114.1 (CH)	7.32 (1H, *d*, *J* = 9.3)	113.7 (CH)	7.26 (1H, *d*, *J* = 9.0)
4	156.7 (C)	-	150.7 (C)	-	150.7 (C)	-
4a	129.9 (C)	-	146.0 (C)	-	133.9 (C)	-
5	149.3 (C)	-	119.7 (CH)	7.27 (1H, *dd*, *J* = 8.1, 1.5)	154.6 (C)	-
6	125.3 (C)	-	123.4 (CH)	7.16 (1H, *t*, *J* = 8.1)	112.8 (CH)	6.96 (1H, *d*, *J* = 9.0)
7	134.1 (C)	-	116.6 (CH)	7.75 (1H, *dd*, *J* = 8.1, 1.5)	122.2 (CH)	7.93 (1H, *d*, *J* = 9.0)
8	111.0 (C)	-	123.1 (C)	-	116.6 (C)	-
8a	182.5 (C=O)	-	176.5 (C=O)	-	175.3 (C=O)	-
9	102.9 (C)	-	116.3 (C)	-	116.0 (C)	-
9a	145.5 (C)	-	145.1 (C)	-	149.7 (C)	-
10a	24.2 (CH_2)	3.54 (2H, *d*, *J* = 6.3)	-	-	-	-
1'	123.0 (CH)	5.17 (1H, *br t*, *J* = 6.3)	-	-	-	-
2'	134.8 (C)	-	-	-	-	-
3'	39.5 (CH_2)	2.06 (2H, *br t*, *J* = 7.2)	-	-	-	-
4'	26.4 (CH_2)	2.12 (2H, *m*)	-	-	-	-
5'	124.2 (CH)	5.14 (1H, *br t*, *J* = 7.2)	-	-	-	-
6'	130.8 (C)	-	-	-	-	-
7'	24.9 (CH_3)	1.68 (3H, *s*)	-	-	-	-
8'	16.8 (CH_3)	1.62 (3H, *s*)	-	-	-	-
9'	15.6 (CH_3)	1.85 (3H, *s*)	-	-	-	-
10'	28.1 (CH_2)	4.17 (2H, *d*, *J* = 5.7)	-	-	-	-
1"	124.6 (CH)	5.16 (1H, *br t*, *J* = 5.7)	-	-	-	-
2"	129.7 (C)	-	-	-	-	-
3"	24.9 (CH_3)	1.73 (3H, *s*)	-	-	-	-
4"	17.4 (CH_3)	1.85 (3H, *s*)	-	-	-	-
5"	-	13.69 (1H, *s*)	-	-	-	-
1-OH	-	-	62.3 (CH_3)	4.01 (3H, *s*)	62.3 (CH_3)	4.02 (3H, *s*)
1-OCH_3	-	-	-	-	61.5 (CH_3)	4.05 (3H, *s*)
5-OCH_3						

[a] Data were measured in acetone-d_6 at 300 MHz

[b] Data were measured in $CDCl_3$ + DMSO-d_6 at 300 MHz

[c] Data were measured in $CDCl_3$ + DMSO-d_6 at 500 MHz

CONCLUSIONS

Three xanthones, garciniaxanthone E (**1**), 2,5-dihydroxy-1-methoxyxanthone (**2**) and 2,6-dihydroxy-1,5-dimethoxyxanthone (**3**), were isolated from the dichloromethane extract of *G. dulcis* green branch. This is the first report of these three xanthones in *G. dulcis*.

ACKNOWLEDGEMENTS

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REFERENCES

1. Iinuma M, Tosa H, Ito T, Tanaka T, Riswan S. 1996. Three new benzophenone-xanthone dimers from the root of *Garcinia dulcis*. Chem. Pharm. Bull. 44(9), 1744-1747.
2. Iinuma M, Ito T, Tosa H, Tanaka T, Riswan S. 1996. Five New Xanthones from *Garcinia dulcis*. J. Nat. Med. 59(5), 472-475.
3. Kosela S, Hu L-H, Yip S-C, Rachmatia T, Sukri T, Daulay TS, et al. 1999. Dulxanthone E: a pyranoxanthone from the leaves of *Garcinia dulcis*. Phytochem. 52(7), 1375-1377.
4. Kosela S, Hu LH, Rachmatia T, Hanafi M, Sim KY. 2000. Dulxanthones F-H, three new pyranoxanthones from *Garcinia dulcis*. J. Nat. Med. 63(3), 406-407.
5. Ito C, Miyamoto Y, Nakayama M, Kawai Y, Rao KS, Furukawa H. 1997. A novel depsidone and some new xanthones from Garcinia species. Chem. Pharm. Bull. 45(9), 1403-1413.
6. Deachathai S, Mahabusarakam W, Phongpaichit S, Taylor WC. 2005. Phenolic compounds from the fruit of *Garcinia dulcis*. Phytochem. 66(19), 2368-2375.
7. Deachathai S, Mahabusarakam W, Phongpaichit S, Taylor WC, Zhang Y-J, Yang C-R. 2006. Phenolic compounds from the flowers of *Garcinia dulcis*. Phytochem. 67(5), 464-469.
8. Deachathai S, Phongpaichit S, Mahabusarakam W. 2008. Phenolic compounds from the seeds of *Garcinia dulcis*. Nat. Prod. Res. 22(15), 1327-1332.
9. Harrison LJ, Leong L-S, Leong Y-W, Sia G-L, Sim K-Y, Tan HT-W. 1994. Xanthone and flavonoid constituents of *Garcinia dulcis* (Guttiferae). Nat. Prod. Lett. 5(2), 111-116.
10. Pang X, Yi T, Yi Z, Cho SG, Qu W, Pinkaew D, et al. 2009. Morelloflavone, a biflavonoid, inhibits tumor angiogenesis by targeting rho GTPases and extracellular signal-regulated kinase signaling pathways. Cancer Res. 69(2), 518-525.
11. Shakui T, Iguchi K, Ito T, Baba M, Usui S, Oyama M, et al. 2014. Anti-androgenic activity of hydroxyxanthones in prostate cancer LNCaP cells. Fitoterapia. 92, 9-15.
12. Minami H, Takahashi E, Kodama M, Fukuyama Y. 1996. Three xanthones from *Garcinia subelliptica*. Phytochem. 41(2), 629-633.
13. Niu S-L, Li Z-L, Ji F, Liu G-Y, Zhao N, Liu X-Q, et al. 2012. Xanthones from the stem bark of *Garcinia bracteata* with growth inhibitory effects against HL-60 cells. Phytochem. 77, 280-286.
14. Clinical and Laboratory Standards Institute (CLSI). 2012. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically; approved standard. CLSI document MO7-A9. Clinical and Laboratory Standards Institute: Wayne.
15. Sarker SD, Nahar L, Kumarasamy Y. 2007. Microtitre plate-based antibacterial assay incorporating resazurin as an indicator of cell growth, and its application in the *in vitro* antibacterial screening of phytochemicals. Methods. 42(4), 321-324.
16. Phongpaichit S, Rungjindamai N, Rukachaisirikul V, Sakayaroj J. 2006. Antimicrobial activity in cultures of endophytic fungi isolated from *Garcinia* species. FEMS Immunol. Med. Microbiol. 48(3), 367-372.

Stilbenoid constituents from *Myristica fragrans* Houtt.

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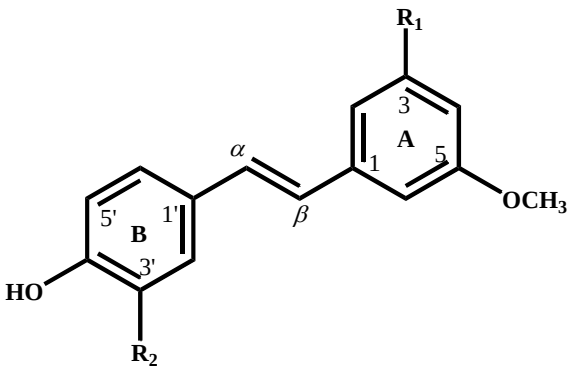
ABSTRACT

This work was aim to phytochemical investigation and structure elucidation of compounds isolated from *Myristica fragrans* (Myristicaceae). The preparative-HPLC separation of the methanolic wood extract had led to the isolation of three stilbene derivatives. The isolated stilbenes were exhaustingly determined by the analyses of 1D and 2D NMR spectroscopic techniques together with comparison of the literatures reported. Their structures were assigned to be *trans*-resveratol (pterostilbene), 3′,4-dihydroxy-3,5′-dimethoxystilbene and *trans*-4-[2-(3′,5′-dimethoxyphenyl)ethenyl]-1,2-benzenediol.

Keywords: Myristicaceae; *Myristica fragrans*; stilbenoids; phytochemical this purpose.

INTRODUCTION

Myristica fragrans, commonly known as nutmeg tree, is a medium to large evergreen tree belonging to the family Myristicaceae usually growing to 5-13 meters in high. It was wide-spread throughout the tropical countries [1] including Thailand which was locally known as luk jan [2]. It was widely used as a traditional medicine for a broad range of pharmacological properties such as a stomachic, stimulant, carminative, aphrodisiac, headaches, appetite, diarrhea, rheumatism, muscle spasm, nausea, fever and anti-angiogenic [1-6]. It was also beneficially valuable for its antioxidant [1,4,7-8,10], antibacterial [2,7-9], antifungal [6-7] and anti-inflammatory [1,6] activities. This plant has been revealed that the main chemical constituents were lignans [6-11]. In this work we have reported the isolation and structure elucidation of the stilbene derivatives obtained from the methanolic woods extract of *M. fragrans*. The preparative-HPLC separation led to the isolation of three stilbenoid compounds (Figure 1). Based on the spectroscopic data together with comparison to those reported in the literatures, the isolated compounds were elucidated as *trans*-resveratol (pterostilbene) (**1**) [12], 3′,4-dihydroxy-3,5′-dimethoxystilbene (**2**) [13] and *trans*-4-[2-(3′,5′-dimethoxyphenyl)ethenyl]-1,2-benzenediol (**3**) [14].



1: R_1 = OCH_3; R_2 = H

2: R_1 = OH; R_2 = OCH_3

3: R_1 = OCH_3; R_2 = OH

MATERIALS AND METHODS

General Methods

Melting points (mp.) were measured by a Fisher-Johns apparatus and were uncorrected. The FT-IR spectra were recorded using an ATR technique with a Cary 630 FT-IR spectrophotometer (Agilent Technologies). The UV spectra were obtained from an UV-1700 spectrophotometer (SHIMADZU). The NMR spectra were recorded by a FT-NMR Bruker Avance 300 MHz spectrometer using TMS as the internal standard (□ 0.00 ppm). The preparative-HPLC separation was carried out using the Thermo Separation Product (TSP) P2000 system with Lichrospher® 100 RP-18 endcapped column (Ø 25x250 mm, particle size 5 μm, Merck). For thin-layer chromatography (TLC), aluminium sheets of silica gel 60F_{254} and silica gel 60 RP-18 F_{254}S (20x20 cm, layer thickness 0.2 mm, Merck) were used for analytical purposes. The compounds were visualized under ultraviolet light. For extraction and chromatography procedures, the solvents were distilled at their boiling ranges before use except for HPLC separation (analytical grade MeOH, Merck).

Plant Material

The woods of *M. fragrans* were collected from Trung Province in Southern Thailand in June 2013. The identification was made by Dr. Natthaya Choosingh van Beem and the voucher specimen has been deposited at the Department of Biology, Faculty of Science, Thaksin University, Thailand.

Extraction and Isolation

The air-dried woods of *M. fragrans* (0.5 kg) were chopped and successively macerated with MeOH twice (each for 3 days) at room temperature. After evaporation to dryness, the crude MeOH extract (17.4 g) was obtained as a dark brown solid. This extract (1.5 g) was subjected to HPLC separation using linear gradient solvent manner of H_2O-MeOH. The elution was conducted from 50% MeOH/H_2O up to MeOH in 100 min, then eluted with MeOH for a further 60 min at the flow rate of 4.5 mL/min to give 80 fractions (2 min each). After combining with the similar TLC characteristics, eight fractions (M1 – M8) were obtained. Fraction M4 (105.6 g) was repeatedly purified by preparative-HPLC using linear gradient solvent manner of H_2O-MeOH at the flow rate of 4.5 mL/min. The elution was conducted from 70% MeOH/H_2O up to MeOH in 100 min, then eluted with MeOH for a further 30 min to yield compounds **2** (5.9 mg) and **3** (14.2 mg) as brownish gums. Fraction M5 (256.6 g) was purified by preparative-HPLC using linear gradient solvent manner of H_2O-MeOH at the flow rate of 4.5 mL/min. The elution was conducted from 70% MeOH/H_2O up to MeOH in 60 min, then eluted with MeOH for a further 40 min to yield a brownish gum of compound **1** (44.3 mg).

Spectroscopic data of Compounds 1-3

Compound 1: brownish gum; UV (MeOH) λ_{max} nm (log ε) 206 (4.02), 306 (3.85); FT-IR (ATR) ν_{max} (cm^{-1}) 3370 (OH stretching), 1585 and 1510 (C=C stretching in aromatic); ^1H and ^{13}C NMR data see Table 1.

Compound 2: brownish gum; UV (MeOH) λ_{max} nm (log ε) 210 (3.92), 320 (3.56); FT-IR (ATR) ν_{max} (cm^{-1}) 3420 (OH stretching), 1605 and 1515 (C=C stretching in aromatic); ^1H and ^{13}C NMR data see Table 1.

Compound 3: brownish gum; UV (MeOH) λ_{max} nm (log ε) 215 (4.01), 358 (3.62); FT-IR (ATR) ν_{max} (cm^{-1}) 3450 (OH stretching), 1612 and 1524 (C=C stretching in aromatic); ^1H and ^{13}C NMR data see Table 1.

RESULTS

Chemical investigation of the constituents from the MeOH woods extract of *M. fragrans* had led to the isolation of three stilbene derivatives, *trans*-resveratol (pterostilbene) (**1**), 3□,4-dihydroxy-3,5□-dimethoxystilbene (**2**) and *trans*-4-[2-(3□,5□-dimethoxyphenyl)ethenyl]-1,2-benzenediol (**3**). Their structures were elucidated by the analyses of spectroscopic data, especially 1D and 2D NMR techniques. Compound **1** was obtained as a brown gum. The UV spectrum displayed absorption bands at 206 and 306 nm suggestive for a conjugated chromophore. The IR spectrum showed the O-H stretching absorption bands at 3370 cm^{-1} and the C=C stretching in aromatic ring at 1585 and 1510 cm^{-1}. The ^1H NMR spectrum (Table 1) exhibited two olefinic proton signals resonated at δ 7.00 (*d*, J = 16.5 Hz) and 6.68 (*d*, J = 16.5 Hz) assignable for the □ and □ protons of stilbene. The large coupling constant was suggested for the *trans*-configuration. The spectrum showed further *ortho* coupled aromatic signals with integration of two protons each at δ 7.36 (*d*, J = 8.4 Hz) and

6.80 (*d*, J = 8.4 Hz) indicating the 1,4-disubstituted benzene ring B. The remaining aromatic protons resonated at *δ* 6.64 (*d*, J = 2.4 Hz, 2H) and 6.38 (*t*, J = 2.4 Hz) were assigned for a symmetrical 1,3,5-trisubstituted ring A. The ^{1}H NMR spectrum displayed additionally a singlet signal at *δ* 3.81 attributable for the two methoxyl groups. The HMBC experiment exhibited the correlations of protons H-2□/H-6□ (*δ* 7.36) of ring B to the □-carbon (C-□) of stilbene at *δ* 128.7 which was confirmed the connection between the C-□ and C-1□ of ring B. These data suggested that the □-carbon (C-□) was linked to the aromatic ring A at the C-1 (*δ* 128.7). The ^{3}J HMBC correlation of the aromatic protons H-2/H-6 (*δ* 6.64) of ring A with the C-□ at *δ* 126.4 was clearly supported this structure assignment. Two methoxyl groups (*δ* 3.81) were located at the C-3 and C-5 of ring A according to their NOESY cross-peaks with the H-2/H-6 (*δ* 6.64) and H-4 (*δ* 6.38). The hydroxyl substituent at the C-4□ of ring B implied from the low-field carbon chemical shift (*δ* 155.5). Based on the spectroscopic data and comparison with the literature reported, compound **1** was elucidated to be *trans*-resveratol (pterostilbene) [12].

Table 1. ^{1}H and ^{13}C NMR spectral data of compounds **1**-**3**

No.	**1**[a]		**2**[b]		**3**[c]	
	□$_H$ (mult., J_{Hz})	□$_C$	□$_H$ (mult., J_{Hz})	□$_C$	□$_H$ (mult., J_{Hz})	□$_C$
□	7.00 (*d*, 16.5)	128.7	7.08 (*d*, 16.2)	129.0	7.04 (*d*, 16.5)	128.9
□	6.86 (*d*, 16.5)	126.4	6.96 (*d*, 16.2)	126.2	6.80 (*d*, 16.5)	126.5
1	-	139.7	-	140.2	-	140.1
2	6.64 (*d*, 2.4)	104.4	6.63 (*d*, 2.0)	105.8	6.68 (*d*, 2.1)	104.5
3	-	160.8	-	160.7	-	161.4
4	6.38 (*t*, 2.4)	99.6	6.34 (*t*, 2.0)	100.0	6.32 (*t*, 2.1)	99.7
5	-	160.8	-	159.2	-	161.4
6	6.64 (*d*, 2.4)	104.4	6.63 (*d*, 2.0)	103.4	6.68 (*d*, 2.1)	104.5
1□	-	129.9	-	130.1	-	130.4
2□	7.36 (*d*, 8.4)	128.0	7.25 (*d*, 2.2)	110.3	6.95 (*d*, 2.0)	114.2
3□	6.80 (*d*, 8.4)	115.6	-	147.5	-	143.6
4□	-	155.5	-	148.2	-	143.7
5□	6.80 (*d*, 8.4)	115.6	6.80 (*d*, 8.4)	115.1	6.79 (*d*, 9.0)	115.8
6□	7.36 (*d*, 8.4)	128.0	7.04 (*dd*, 8.4,2.2)	120.6	6.70 (*dd*, 9.0,2.0)	119.6
3-OCH_3	3.81 (*s*)	55.3	-	-	3.76 (*s*)	55.4
5-OCH_3	3.81 (*s*)	55.3	3.77 (*s*)	56.2	3.76 (*s*)	55.4
3□-OCH_3	-	-	3.86 (*s*)	56.0	-	-
3□-OH	-	-	-	-	9.10 (*s*)	-
4□-OH	-	-	-	-	8.92 (*s*)	-

[a]recorded in $CDCl_3$; [b]recorded in Acetone-d6; [c]recorded in DMSO-d_6

Compound **2** was obtained as a brown gum. The UV spectrum displayed absorption bands at 206 and 306 nm and the IR spectrum showed the O-H stretching absorption bands at 3420 cm^{-1} and the C=C stretching in aromatic ring at 1605 and 1515 cm^{-1} suggesting the same skeleton to those of compound **1**. The ^{1}H NMR spectrum (Table 1) revealed the similar resonances as those of compound **1** including the *trans* □ and □ protons of stilbene at *δ* 7.08 (*d*, J = 16.2 Hz) and 6.96 (*d*, J = 16.2 Hz) and 1,3,5-trisubstituted benzene ring A from the signals at *δ* 6.63 (*d*, J = 2.0 Hz, 2H) and 6.34 (*t*, J = 2.0 Hz). The differences were the aromatic proton signals in ring B resonated at *δ* 7.25 (*d*, J = 2.2 Hz), 7.04 (*dd*, J = 8.4, 2.2 Hz) and 6.80 (*d*, J = 8.4 Hz) attributable to 1,2,4-trisubstituted benzene instead for the 1,4-disubstituted ring in compound **1** as well as the two singlet methoxyl groups at *δ* 3.86 and 3.77. The HMBC experiment showed the cross peaks of proton H-2□ (*δ* 7.25) of ring B and the methoxyl group (*δ* 3.86) to the carbon at *δ* 147.5 (C-3□) indicating the placement of this methoxyl group at the C-3□. These data concluded that the methoxyl group at the C-3 in the aromatic ring A of compound **1** was replaced by the hydroxyl group leaving the other methoxyl group (*δ* 3.86) at the C-5 (*δ* 159.2). The NOESY spectral data exhibited the correlations of the H-2/H-6 (*δ* 6.63) to 5-OCH_3 (*δ* 3.77) and H-2□ (*δ* 7.25) to 3□-OCH_3 (*δ* 3.86) supporting these conclusions. The HMBC spectrum was completely confirmed the structure assignment. From the spectroscopic data compound **2** was clearly determined as 3□,4-dihydroxy-3,5□-dimethoxystilbene together with comparison to the literature reported [13].

Compound **3** was also obtained as a brown gum. The UV and IR spectra showed the similar absorptions to those of compound **1** suggesting the same chromophore. The ^{1}H NMR spectrum (Table 1) revealed the similar resonances as those

of compound **1** except for the aromatic protons in ring B. The signals exhibited for the resonances of 1,2,4-trisubstituted benzene ring from the signals at δ 6.95 (*d*, J = 2.0 Hz, H-2′), 6.79 (*d*, J = 9.0 Hz, H-5′) and 6.70 (*dd*, J = 9.0, 2.0 Hz). The down-field shift of the C-3′ and C-4′ resonated at δ 143.6 and 143.7, respectively, in accordance with the presences of two singlet hydroxyl signals at δ 9.10 and 8.92 indicated the position of these hydroxyl groups at the C-3′ and C-4′. The HMBC spectrum was fully confirmed these assignments. From the spectroscopic data as well as comparison to the previous report, compound **3** was assigned as 3′,4-dihydroxy-3,5′-dimethoxystilbene [14].

CONCLUSIONS

The reversed phase preparative-HPLC separation from the methanol wood extract of *M. fragrans* was performed by the TSP P2000 pump system using linear gradient solvents of H_2O-MeOH. The spectroscopic analysis of the isolated constituents was identified as *trans*-resveratol (pterostilbene) (**1**), 3′,4-dihydroxy-3,5′-dimethoxystilbene (**2**) and *trans*-4-[2-(3′,5′-dimethoxyphenyl)ethenyl]-1,2-benzenediol (**3**).

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REFERENCES

1. Cuong TD, Hung TM, Na MK, Ha DT, Kim JC, Lee D, et al. 2011. Inhibitory effect on NO production of phenolic compounds from *Myristica fragrans*. Bioorg. Med. Chem. Lett. 21, 6884-6887.
2. Chung JY, Choo JH, Lee MH, Hwang JK. 2006. Anticariogenic activity of macelignan isolated from *Myristica fragrans* (nutmeg) against *Streptococcus mutans*. Phytomedicine.13, 261-266.
3. Merzoukia A, Ed-derfoufi F, Molero Mesa J. 2000. Contribution to the knowledge of Rifian traditional medicine. II: Folk medicine in Ksar Lakbir district (NW Morocco). Fitoterapia. 71, 278-307.
4. Piaru SP, Mahmud R, Abdul Majid AMS, Mahmoud Nassar ZD. 2012. Antioxidant and antiangiogenic activities of the essential oils of *Myristica fragrans* and *Morinda citrifolia*. Asain. Pac. J. Trop. Med. 294-298.
5. Ram A, Lauria P, Gupta R, Sharma VN, 1996. Hypolipidaemic effect of *Myristica fragrans* fruit extract in rabbits. J. Ethnophamlacology. 55, 49-53.
6. Cao GY, Xu W, Yang XW, Gonzalez FJ, Li F. 2015. New neolignans from the seeds of *Myristica fragrans* that inhibit nitric oxide production. Food. Chem. 173, 231-237.
7. Gupta AD, Bansal VK, Babu V, Maithil N. 2013. Chemistry, antioxidant and antimicrobial potential of nutmeg (*Myristica fragrans* Houtt). J. Genet. Eng.Biotechnol. 11, 25-31.
8. Sulaiman SF, Ooi KL. 2012. Antioxidant and anti food-borne bacterial activities of extracts from leaf and different fruit parts of *Myristica fragrans* Houtt. Food Control. 25, 533-536.
9. Shahidi Bonjar GH. 2004. Antibacterial screening of plants used in Iranian folkloric medicine. Fitoterapia. 75, 231-235.
10. Kwon HS, Kim MJ, Jeong HJ, Yang MS, Park KH, Jeong TS, et al. 2008. Low-density lipoprotein (LDL)-antioxidant lignans from *Myristica fragrans* seeds. Bioorg. Med. Chem. Lett. 18, 194-198.
11. Nguyen PH, Thu Le TV, Kang HW, Chae J, Kim SK, Kwon K, et al. 2010. AMP-activated protein kinase (AMPK) activators from *Myristica fragrans* (nutmeg) and their anti-obesity effect. Bioorg. Med. Chem. Lett. 20, 4128-4131.
12. Fuendjiep V, Wandji J, Tillequin F, Mulholland DA, Budzikiewicz H, Fomum ZT, et al. 2002. Chalconoid and stilbenoid glycosides from *Guibourtia tessmanii*. Phytochemistry. 60, 803-806.
13. Silayo A, Ngadjui BT, Abegaz BM. 1999. Homoisoflavonoids and stilbenes from the bulbs of *Scilla nervosa* subsp. *rigidifolia*. Phytochemistry. 52, 947-955.
14. Ma ZJ, Li X, Li N, Wang JH. 2002. Stilbenes from *Sphaerophysa salsula*. Fitoterapia. 73, 313-315.

Phenolics from the leaves of *Artocarpus elasticus*

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ABSTRACT

Artocarpus elasticus (Moraceae) has been used for traditional medicine in South east Asia. This plant is found widely in the southern part of Thailand. The acetone extract of the leaves showed significant cytotoxicity. Further chemical investigation of the acetone extract resulted in the isolation of isochavicinic acid, elastichalcone B, carpachromene and cycloartocarpesin. Their structures were characterized by analyses of spectroscopic data. Isochavicinic acid was isolated from *Artocarpus* genus for the first time.

Keywords: *Artocarpus elastics*, dihydrochalcones, prenylated flavonoids, Moraceae

INTRODUCTION

Artocarpus elasticus (Moraceae) is a traditional plant in Indonesia, Malaysia and Thailand. In Indonesia, The bark has been used for the treatment of back ache [1]. The leaves have been used for cure tuberculosis. The wood, bark and root were previously chemical investigated to contain chalcones, dihydrochalcones, flavonoids, arylbenzofurans and terpenoids. Some of the isolated compounds showed moderate cytotoxic activity and good antioxidant activity [2, 3]. However a few chemical constituents in the leaves were reported. Moreover, this plant species are found wide in the southern part of Thailand, locally known as "ka-ok" and we found that the acetone extract of the leaves show significant cytotoxicity. We therefore further investigated the chemical constituents of the leaves of *A.elasticus*.

MATERIALS AND METHODS

General methods

Column chromatography was completed with silica gel 100 (70-230 Mesh ASTM, Merck) or SephadexTM LH-20 (Amersham Biosciences, Sweden). Quick column chromatography performed with silica gel 60 (230-400 Mesh ASTM, Merck). Aluminum sheets of silica gel 60 F254 (layer thickness 0.2 mm, Merck) were used for thin-layer chromatography (TLC) and the compounds were visualized under ultraviolet light. Solvents for extraction and chromatography were distilled at their boiling ranges prior to use. Melting points were recorded in ^{o}C on a digital Electrothermal Melting Point Apparatus (Electrothermal 9100). Ultraviolet spectra were measured with SPECORD S100 spectrophotometer (Analytik, Jena, Germany). The IR spectra were measured with an FTS 165 FT-IR Perkin-Elmer spectrophotometer (Perkin-Elmer, Shelton, USA). ^{1}H- and ^{13}C-Nuclear magnetic resonance spectra were recorded on an FT-NMR Bruker Ultra ShieldTM 300 MHz spectrometer or 500 MHz spectrometer at Department of Chemistry, Faculty of Science, Prince of Songkla University. Spectra were recorded in $CDCl_3$ and acetone-d_6, were recorded as δ value in ppm down field from TMS (internal standard δ 0.00).

Plant Material

The leaves of *A. elasticus* (Moraceae) were collected from Prince of Songkla University, Hat Yai, Songkhla Province in the southern part of Thailand, in May 2012. Identification was compared with the specimen (A. Yanya 1Phang-

nga:Kuraburi 2/4/2009) deposited in the Herbarium of the Department of Biology, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla, Thailand.

Extraction and isolation of the leaves of *A. elasticus*

Ground-dried leaves of *A. elasticus* (3.0 kg) was immersed in acetone (10 litters) at room temperature for three days (twice). A dark yellow viscous (88.0 g) was obtained after removal of solvent (A). The acetone extract was dissolved in hexane to give hexane soluble (B) (24.12 g) and hexane insoluble (C) (63.88 g) fractions. The hexane insoluble fraction was further dissolved in CH_2Cl_2 to give CH_2Cl_2 soluble (D) (11.68 g) and CH_2Cl_2 insoluble fractions (E) (52.2 g).

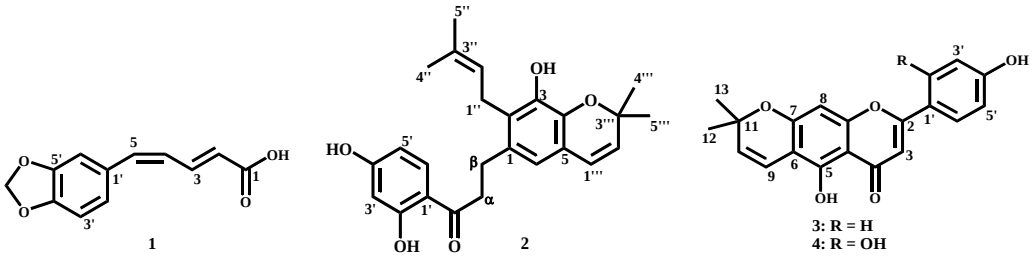
The dichloromethane soluble fraction D (11.68 g) was subjected to quick column chromatography over silica gel 60H and eluted with acetone:hexane (9:1) to MeOH:hexane (1:3) to afford 19 fractions (D1-D19).

Fraction D12 (690.0 mg) was subjected to column chromatography over silica gel 60H and using acetone:hexane (1:9 to 3:7) as eluent. Sixteen fractions (D12A-D12P) were obtained after combination base on TLC characteristic. Fractions D12M-D12P were each further purified over sephadex LH-20 and eluted with MeOH to give pure yellowish gum of **1** (3.7 mg), yellowish gum of **2** (2.7 mg) and yellow solid of **3** (3.2 mg).

Fraction D19 (530.0 mg) was fractionated over sephadex LH-20 and using MeOH as eluent yielding yellow solid of **4** (3.5 mg)

RESULTS

The acetone extract from the leaves of *A.elasticus* was purified by chromatographic technique resulting in the isolation of 4 phenolic compounds. They were recognized as isochavicinic acid (**1**), elastichalcone B (**2**), carpachromene (**3**) and cycloartocarpesin (**4**).



Compound 1 was found yellowish gum (3.7 mg). Its UV spectrum displayed maximum adsorption at λ_{max} nm (log ε) 340 (3.94), 208 (3.99). The IR spectrum showed the characteristic adsorption band at υ_{max} (cm^{-1}) 3428 and 1630 for O-H stretching and C=O stretching, respectively. The ^{13}C NMR spectrum showed the resonances of a carbonyl group (δ 165.5, C-1), a methylene carbon (δ 101.2, CH_2O_2), seven methine carbons (δ 142.5, C-3; 138.3, C-5; 125.3, C-4; 122.4, C-2'; 120.0, C-2; 108.1, C-3; 105.7, C-6') and three quaternary carbons (δ 148.2, C-4'; 148.1, C-5'; 131.0, C-1'). The ^{1}H NMR spectrum showed the resonances of 1,3,4-trisubstituted protons at δ 6.88 (*dd*, *J* = 8.1, 1.5 Hz, H-2'), δ 6.77 (*d*, *J* = 8.1 Hz, H-3') and δ 6.97 (*d*, *J* = 1.5 Hz, H-6'). A substituent on benzene ring was proposed for a methylenedioxy group (δ 5.97, *s*, CH_2O_2) which on HMBC experiment, the methylenedioxy proton correlated to C-4' (δ 148.2) and C-5' (δ 148.1). The ^{1}H NMR spectrum further showed the resonances of four of methine olefinic protons at δ 6.43 (*d*, *J* = 14.1 Hz, H-2), δ 7.40 (*ddd*, *J* = 14.1, 8.1, 2.4 Hz, H-3), δ 6.74 (*d*, *J* = 8.1 Hz, H-4) and δ 6.75 (*d*, *J* = 2.4 Hz, H-5). The COSY spectrum displayed correlations of H-2 to H-3; H-3 to H-2, H-4; H-4 to H-3, H-5, and the HMBC spectrum showed correlation of H-3 to carbonyl carbon (C-1, δ 165.5) suggested an acid side chain. In addtion, a coupling constant of 14.1 Hz indicated trans configuration of C-2 and C-3. The side chian was place at C-1' due to H-4 showed correlation to C-1' (δ 131.0); H-2'/H-6' correlation to C-5 (δ 138.3), H-5 to C-2' (δ 122.4) and C-6' (δ 105.7) in HMBC experiment. The NMR property and the assigned structure of **1** was in agreement with isochavicinic acid [4]

Compound 2 (2.7 mg) was obtained as yellow viscous liquid. The UV spectrum displayed the maximum adsorption bands at λ_{max} nm (log ε) 315 (3.60), 276 (3.83), 213 (4.28). The IR spectrum exhibited the maximum adsorption bands for hydroxyl group and a carbonyl group at υ_{max} (cm^{-1}) 1629 and 3226. The ^{13}C NMR displayed 25 carbons; a carbonyl carbon (δ

204.0, C=O); four methyl carbons (δ 2x28.0, C-4''' and C-5'''; 25.6, C-4'' and 17.8, C-5''); three methylene carbons (δ 39.5, C-α; 27.2, C-β; 25.3, C-1''); seven methine carbons (δ 132.1, C-6'; 129. 9, C-2'''; 122.8, C-2''; 121.9, C-3'''; 117.7, C-6; 107.8, C-5'; 103.4, C-3'') and ten quaternary carbons (δ 165.1, C-2'; 163.1, C-4'; 142.4, C-3; 137.5, C-4; 131.8, C-3''; 131.5, C-1; 126.4, C-2, 118.7, C-5; 113.5, C-1'; 77.1, C-4'''). The ^{1}H NMR showed the signals of methylene proton at δ 3.12 (*dd*, *J* = 8.7, 6.6 Hz, H-α) and δ 2.93 (*dd*, *J* = 8.7, 6.6 Hz, H-β), isolated aromatic proton at δ 6.42 (*s*, H-6), 1,2,4-trisubstituted benzene ring at δ 6.38 (*d*, *J* = 2.4 Hz, H-3'), δ 6.34 (*d*, *J* = 8.7, 2.4 Hz, H-5') and δ 7.60 (*d*, *J* = 2.4 Hz, H-6'), singlet resonance of a hydroxyl proton (2'-OH) at δ 12.80. The HMBC correlations of H-α to C-1 (δ 131.5) while H-β to C-2 (δ 126.4), C-6 (δ 117.7) suggested the α- and β-methylene proton of dihydrochalcone skeleton [3]. In HMBC spectrum H-3' correlated to C-1' (δ 113.5) and C-5' (δ 107.8); H-5' correlated to C-1' (δ 113.5) and C-3' (δ 103.4) and H-6' correlated to C=O (δ 203.7), C-2' (δ 165.1) and C-4' (δ 163.1) confirmed the assignment of aromatic protons H-6', H-5' and H-3'. The ^{1}H NMR also exhibited the resonances of prenyl group including methylene protons δ 3.37 (*d*, *J* = 6.6 Hz, H-1''), methine proton at δ 5.14 (*t*, *J* = 6.6 Hz, H-2''), and methyl protons at δ 1.72 (*s*, H-4'') and δ 1.66 (*s*, H-5''). The presence of 2,2-dimethylchromene ring was indicated from the resonances of methine protons at δ 6.30 (*d*, *J* = 9.9 Hz, H-1''') and δ 5.56 (*d*, *J* = 9.9 Hz, H-2'''), and methyl protons at δ 1.44 (*s*, 6H, H-4''' and H-5'''). The HMBC correlation of H-2'' to C-2 (δ 126.4), and H-1'' to C-1 (δ 131.5), C-3 (δ 142.4) determined that the prenyl group was at C-2. While the HMBC correlations of H-1''' to C-4 (δ 137.5), C-6 (δ 117.7), and H-6 to C-4 (δ 137.5), C-1''' (δ 121.9) suggested that the chromene ring was at C-4 and C-5. The spectral data of **2** was in agreement with that of elastichalcone B [2]

Compound 3 (3.2 mg) was a yellowish gum. The UV spectrum showed maximum adsorption at λ_{max}nm (log ε) 342 (3.95), 306 (4.00), 231 (4.01), 209 (4.03). The IR spectrum showed the stretching of hydroxyl group and carbonyl group at υ_{max} (cm^{-1}) 3226 and 1629, respectively. The ^{13}C NMR spectrum of **3** showed the resonances of carbons: a carbonyl carbon (δ 182.3, C-4), two methyl canbons (δ 27.6x2, C-12 and C-13), eight methine carbons (δ 3x128.1, C-10; C -2' and C-6'; 2x115.7, C-3' and C-5'; 114.8, C-9; 102.9, C-3; 94.4, C-8) and nine quarternary carbons (δ 164.2, C-2; 161.2, C-4'; 159.1, C-7; 157.0, C-8a; 156.4, C-5; 122.4, C-1'; 2x104.8, C-4a; 77.6; C-11). The ^{1}H NMR spectrum exhibited the resonances of a hydrogen bonded hydroxyl group (δ 13.49, 5'-OH), methine olefinic proton (δ 6.73, H-3), singlet aromatic proton (δ 6.55, H-8), *para*-disubstituted aromatic protons (δ 8.01, *d*, *J* = 8.7, H-2' and H-6'; δ 7.08, *d*, *J* = 8.7, H-3' and H-5'). The correlation of H-3 to C=O (δ 182.3), C-2 (δ 164.2), C-1' (δ 122.4); H-2'/H-6' to C-2 (δ 164.2), C-4' (δ 161.2), and H-3'/H-5' to C-1' (δ 122.4) were observed in the HMBC. Compound **3** was flavonoid with 1,2,3,4,5-pentasubstituted- and *para*-disubstituted benzene rings, including a hydroxyl group at C-4'. 2,2-Dimethylchromene ring was detected from the characteristic signals at δ 6.72 (*d*, *J* = 9.9 Hz, H-9), δ 5.82 (*d*, *J* = 9.9 Hz, H-10) and δ 1.52 (*s*, H-12 and H-13). The HMBC correlation of H-9 to C-5 (δ 156.4), C-7 (δ 159.1), C-11 (δ 77.6), and H-10 to C-6 (δ 104.8) confirmed the orientation of a chromene ring at C-6 (δ 104.8) and C-7 (δ 159.0) position. Correlations of H-8 to C-4a (δ 104.8) and C-6 (δ 104.8) were observed in the HMBC spectrum. The assigned structure of **3** was in agreement with carpachromene [5]

Compound 4 (3.5 mg) was a yellow solid (m.p. 234-235°C). The UV spectrum showed maximum adsorption at λ_{max} nm (log ε) 352 (3.74), 286 (4.13), 208 (3.46). The IR spectrum showed the stretching of hydroxyl group and carbonyl group at υ_{max} (cm^{-1}) 3380 and 1605, respectively. The ^{13}C NMR spectrum showed of **4** the resonances of 20 carbons: a carbonyl carbon (δ 182.5, C-4), two methyl canbons (δ 27.5x2, C-12 and C-13), seven methine carbons (δ 130.0, C-6'; 128.3, C-10; 114.9, C-9; 108.2, C-5'; 107.5, C-3; 103.5, C-3'; 94.4; C-8) and ten quarternary carbons (δ 162.2, C-2; 162.0, C-2'; 159.4, C-7; 158.6, C-4'; 157.0, C-8a; 109.5, C-1'; 2x104.7, C-4a and C-6; 77.6; C-11). The ^{1}H NMR spectrum exhibited a intramolecular hydrogen bonded hydroxyl group 5'-OH (δ 13.58), methine olefinic proton H-3 (δ 7.12), singlet aromatic proton H-8 (δ 6.45) and 2,2-dimethylchromene ring (δ 6.65, *d*, *J* = 10.1 Hz, H-9; δ 5.54, *d*, *J* = 10.1 Hz, H-10; 1.43, *s*, H-12 and H-13) at C-6 and C-7 as for **3**. A 1,2,4-trisubstituted benzene ring C was indicated form the doublet at δ 7.84 (*J* = 8.7 Hz, H-6'); doublet of doublet at δ 6.54 (*J* = 8.7, 2.7 Hz, H-5') and doublet at δ 6.66 (*J* = 2.7 Hz, H-3'). The assignment of 1,2,4-trisubstituted was confirmed by HMBC correlation of H-3'/H-5' correlated to C-1' (δ 109.5) and H-6' to C-2' (δ 162.0) and C-4' (δ 158.6). Two hydroxyl groups were further proposed at C-2' and C-4' according to the HMBC correlations of H-6' to oxy carbons C-2' (δ 162.0) and C-4' (δ 158.6). The assigned structure of **4** was in agreement with cycloartocarpesin [2]

CONCLUSIONS

The chemical investigation of the leaves of *A.elasticus* resulted in the isolation of one benzene derivative (isochavicinic acid), one dihydrochalcone (elastichalcone B), and two prenylated flavonoids (carpachromene and cycloartocarpesin). The isolated compounds will be further tested for cytotoxicity.

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REFERENCES

1. Musthapa I, Juliawaty DL. Syah MY, Hakim HE, Latip J, Ghisalberti LE. 2009. An Oxepinoflavone from *Artocarpus elasticus* with Cytotoxic Activity Against P-388 Cells. Arch. Pharm. Res. 32(2), 191-194.
2. Ramli F, Rahmani M, Kassim N, Hashim MN, Sukari AM, Akim MA, et al. 2013. New diprenylated dihyrochalcones from leaves of *Artocarpus elasticus*. Phytochem. Lett. 6, 582–585.
3. Wang Y, Xu K., Lin L, Pan Y, Zheng X. 2007. Geranyl flavonoids from the leaves of *Artocarpus altilis*. Phytochem. 68, 1300–1306.
4. Hamiduzzaman Md, Hasan MC, Azam ZATM. 2012. Isochavicinic Acid and Steroids from *Gomphrena globosa L.* J. Pharm. Sci. 11(1), 79-81.
5. Zheng Z, Cheng K, To TJ, Li H, Wang M. 2008. Isolation of tyrosinase inhibitors from *Artocarpus heterophyllus* and use of its extractas antibrowning agent. Mol. Nutr. Food Res. 52, 1530 – 1538.

Biflavonoids from the green branch of *Garcinia dulcis*

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ABSTRACT

Biflavonoids possess a wide range of biological activities. The study on biflavonoids in the green branch of *Garcinia dulcis* resulted in the isolation of four known biflavonoids: dulcisbiflavonoid A (**1**), amentoflavone (**2**), volkensiflavone (**3**) and morelloflavone (**4**). The structure identifications of all the isolates were achieved by analysis of the UV, IR and NMR data and comparison the data with those previously reported.

Keywords: *Garcinia dulcis*, Guttiferae, biflavonoids

INTRODUCTION

Biflavonoids possess a wide range of biological activities such as anti-inflammatory [3], antioxidant [8], anti-HIV [5], antitumor [6] and antimicrobial [4]. Biflavonoids has been found in family Anacardiaceae, Theaceae, Ochnaceae, Selaginellaceae, Aristolochioideae, Guttiferae, Euphorbiaceae, Thymelaeaceae, Gentianaceae and Ginkgoaceae. With the aim of searching for bioactive compounds from Thai plant, we are thus interested in study on the biflavonoids *Garcinia* genus (Guttiferae). Twenty-nine species of *Garcinia* are distributed in Thailand. *Garcinia dulcis* grows wide in the south of Thailand. Biflavonoids has been isolated from stem bark, leaves, twig, root, heartwood, fruits, flowers and seeds of this plant species. To continue our search for biflavonoids. We are thus interested in further study on the biflavonoids from the green branch of *G. dulcis*.

MATERIALS AND METHODS

Plant material

The green branches of *G. dulcis* were collected from Meuang district, Nakhon Srithammarat province in the southern part of Thailand.

Extraction and isolation

The chopped-dried green branches of *G. dulcis* (9 kg) was extracted with dichloromethane and acetone (2 times x 3 days) at room temperature and then filtered. Acetone was removed by evaporation to give a brown viscous acetone extract (178.3 g). The acetone extract was fractionated by a quick column chromatography using silica gel 60H as the stationary phase and gradiently eluted with CH_2Cl_2, CH_2Cl_2-MeOH. Fraction which showed the similar TLC chromatograms were combined and evaporated under pressure to yield twelve fractions (A-L). Fraction H (6.5 g) was chromatographed on CC using CH_2Cl_2:MeOH (7:3) and CH_2Cl_2:MeOH (6:4) as eluent to give twelve fractions (H1-H12). Fraction H6 was crystallized to give a pale yellow powder of **1** (6.5 mg). Purification of fraction H9 by CC on sephadex LH-20 and crytallizated gave yellow powder of **2** (3.2 mg). Fraction H10 (30.5 mg) was subjected to CC silica gel and eluted with CH_2Cl_2:MeOH (6:4) to yield five fractions (H10.1-H10.5). Fraction H10.4 (9.7 mg) was further purified by CC on sephadex LH-20 and eluted with MeOH to give a pale yellow solid of **3** (4.7 mg). Fraction I was further separated by CC on sephadex LH-20 and eluted with MeOH followed by CC silica gel and gradiently eluted with CH_2Cl_2:MeOH (7:3) to CH_2Cl_2:MeOH (3:7) to give **4** (3.0 g) as a yellow solid.

Table 1. ^{1}H NMR and ^{13}C NMR spectral data of compounds **1**-**4**

Position	δ_C(C-Type)				δ_H (multiplicity, J_{Hz})			
	1*	**2****	**3****	**4***	**1***	**2****	**3****	**4***
2	164.4	168.9	82.0	81.4	-	-	5.75 (*d*, 12.0)	5.73 (*d*, 12.0)
3	103.5	108.2	48.7	49.1	6.82 (*s*)	6.39 (*s*)	4.65 (*d*, 12.0)	4.92 (*d*, 12.0)
4	182.1	188.9	196.5	196.7	-	-	-	-
4a	103.5	109.2	102.3	102.1	-	-	-	-
5	161.9	166.7	164.4	164.3	-	-	-	-
6	99.3	104.2	95.8	95.8	6.16 (*d*, 1.8)	6.12 (*d*, 2.1)	5.95 (*s*)	5.99 (*s*)
7	164.5	168.9	167.2	167.0	-	-	-	-
8	94.5	98.9	96.8	96.7	6.43 (*d*, 1.8)	6.25 (*d*, 2.1)	5.97 (*s*)	6.06 (*s*)
8a	157.8	162.6	163.5	163.3	-	-	-	-
1′	121.6	126.9	128.8	128.6	-	-	-	-
2′	128.0	132.3	128.7	128.7	7.87 (*d*, 2.1)	7.67 (*dd*, 8.7, 2.1)	6.99 (*d*, 8.4)	7.17 (*d*, 8.4)
3′	119.0	120.9	115.3	114.9	-	7.05 (*d*, 8.7)	6.41 (*d*, 8.4)	6.41 (*d*, 8.4)
4′	157.8	163.9	157.0	157.8	-	-	-	-
5′	112.0	124.8	115.3	114.9	-	-	6.41 (*d*, 8.4)	6.41 (*d*, 8.4)
6′	129.8	136.1	128.7	128.7	7.76 (*d*, 2.1)	7.76 (*d*, 2.1)	6.99 (*d*, 8.4)	7.17 (*d*, 8.4)
7′	21.9				3.30 (*m*)			
8′	122.4				5.23 (*t*, 6.6)			
9′	132.5				-			
10′	18.2				1.74 (*s*)			
11′	26.0				1.74 (*s*)			
2″	163.8	168.9	164.3	164.1	-	-	-	-
3″	102.9	107.7	103.4	102.7	6.77 (*s*)	6.39 (*s*)	6.23 (*s*)	6.60 (*s*)
4″	182.7	187.4	182.2	182.1	-	-	-	-
4a″	104.4	109.6	104.2	103.6	-	-	-	-
5″	158.4	166.3	161.0	163.9	-	-	-	-
6″	112.0	104.2	99.0	99.1	-	6.41 (*s*)	6.25 (*s*)	6.24 (*s*)
7″	160.0	166.2	161.6	162.1	-	-	-	-
8″	104.2	108.4	101.2	101.0	-	-	-	-
8a″	153.3	159.8	156.0	155.7	-	-	-	-
9″	22.6				3.30 (*m*)			
10″	122.8				5.42 (*t*, 7.2)			
11″	131.1				-			
12″	26.0				1.63 (*s*)			
13″	18.3				1.74 (*s*)			
1″′	121.9	126.7	122.5	121.5	-	-	-	-
2″′	128.6	132.7	128.8	113.7	7.52 (*d*, 8.7)	7.31 (*d*, 8.7)	7.45 (*d*, 8.7)	7.45 (*d*, 2.4)
3″′	116.18	121.6	116.5	161.0	6.70 (*d*, 8.7)	6.62 (*d*, 8.7)	6.86 (*d*, 8.7)	-
4″′	161.4	165.8	161.0	150.1	-	-	-	-
5″′	116.2	121.6	116.5	116.6	6.70 (*d*, 8.7)	6.62 (*d*, 8.7)	6.86 (*d*, 8.7)	6.92 (*d*, 8.4)
6″′	128.5	132.7	128.7	119.8	7.52 (*d*, 8.7)	7.31 (*d*, 8.7)	7.45 (*d*, 8.7)	7.27 (*dd*, 8.4, 2.4)
5-OH					12.96 (*s*)	12.67 (*s*)	12.20 (*s*)	12.30 (*s*)
5″-OH					13.38 (*s*)	12.86 (*s*)	12.20 (*s*)	13.10 (*s*)

*^{1}H and ^{13}C NMR spectral data of **compound 1** and **compound 4** in DMSO-d_6

^{1}H and ^{13}C NMR spectral data of **compound 2 and **compound 3** in $CDCl_3$+DMSO-d_6

Compound 1

Melting point: 233-234 ˚C. UV (MeOH) λ_{max} (nm): 339, 269 and 203. FT-IR (neat) ν_{max} (cm^{-1}): 3420 (O-H stretching), 1653 (C=O stretching). ^{1}H and ^{13}C NMR spectral data, see Table 1.

Compound 2

Melting point: 236-238 ˚C. UV (MeOH) λ_{max} (nm): 333, 269 and 202. FT-IR (neat) ν_{max} (cm^{-1}): 3402 (O-H stretching), 1649 (C=O stretching). ^{1}H and ^{13}C NMR spectral data, see Table 1.

Compound 3

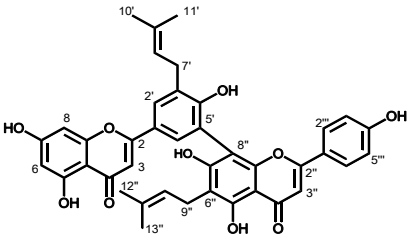
Melting point: 305-307 ˚C. UV (MeOH) λ_{max} (nm): 344 and 289. FT-IR (neat) ν_{max} (cm^{-1}): 3215 (O-H stretching), 1641 (C=O stretching). ^{1}H and ^{13}C NMR spectral data, see Table 1.

Compound 4

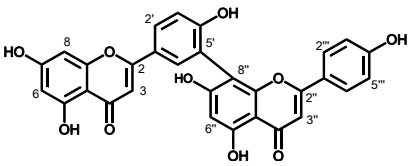
Melting point: 247-250 ˚C. UV (MeOH) λ_{max} (nm): 339, 322 and 292. FT-IR (neat) ν_{max} (cm^{-1}): 3215 (O-H stretching), 1622 (C=O stretching). ^{1}H and ^{13}C NMR spectral data, see Table 1.

RESULTS

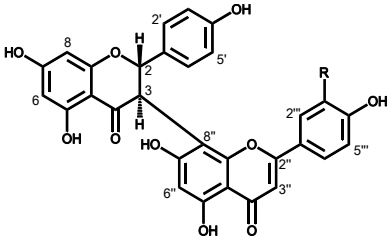
The crude acetone extract from the green branch of *G. dulcis* were subjected to a quick column and column chromatography over silica gel and sephadex LH-20 to give four biflavonoids including dulcisbiflavonoid A (**1**), amentoflavone (**2**), volkensiflavone (**3**) and morelloflavone (**4**). Their structures were elucidated mainly by 1D and 2D NMR spectroscopic data. The physical properties and spectroscopic data were also compared with the reported values.



1



2



3 R= H

4 R= OH

Compound 1 is a pale yellow solid. The ^{1}H NMR spectrum showed resonances of two of chelated hydroxyl protons at δ 12.96 (5''-OH) and δ 13.38 (5-OH), and two of methine olefinic protons at δ 6.82 (H-3) and δ 6.77 (H-3''), indicating flavonylflavone core structure. The ^{1}H NMR spectrum showed the resonances of a *meta* aromatic protons H-6 and H-8 at δ 6.16 and δ 6.43 (*d*, *J* = 1.8 Hz, each), *meta* aromatic protons H-2′ and H-6′ at δ 7.87 and δ 7.76 (*d*, *J* = 2.1 Hz, each) and *para* disubstituted aromatic protons H-2'''/H-6''' at δ 7.52, H-3'''/H-5''' at δ 6.70 (2H each, *d*, *J* = 8.7 Hz). A prenyl group at C-3′ was indicated from the resonances of methylene protons at δ 3.30 (*m*, H-7′), methine olefinic proton at δ 5.23 (*t*, *J* = 6.6 Hz, H-8′) and methyl protons at δ 1.74 (*s*, H-10′, H-11′) together with the HMBC correlations of H-7′ to C-2′ (δ 128.0) and C-4′ (δ 157.8). While a prenyl group at C-6″ was indicated from the resonances of methylene proton at δ 3.30 (*m*, H-9″), methine olefinic proton at δ 5.42 (*t*, *J* = 7.2 Hz, H-10″) and methyl protons at δ 1.63, 1.74 (2*s*, H-12″, H-13″), together with the HMBC correlations of H-9″ to C-5″ (δ 158.4). In addition, the HMBC correlations of H-6′ to C-8'' suggested that the linkage of two flavone units was form between C-5′ and C-8''. Thus compound **1** was assigned to be 8-(5-(5,7-dihydroxy-4-oxo-4*H*-chromen-2-yl)-2-hydroxy-3-(3-methylbut-2-enyl)phenyl)-5,7-dihydroxy-2-(4-hydroxy-phenyl)-6-(3-methyl but-2-enyl)-4*H*-chromen-4-one which was known as dulcisbiflavonoid A [7].

Compound 2 was yellow solid. The ^{1}H NMR spectrum exhibited the signals of a chelated hydroxyl protons 5-OH and 5''-OH (δ 12.67 and δ 12.86), methine olefinic protons H-3 and H-3″ (δ 6.39), *meta* aromatic protons H-6 and H-8 (δ 6.12 and 6.25, *J* = 2.1 Hz), and *para*-disubstituted aromatic protons H-2″/H-6″ and H-3″/H-5″ (δ 7.31 and δ 6.62) as for **1**. The spectrum further showed the resonances of singlet aromatic proton H-6″ at δ 6.41, and aromatic proton H-2′, H-3′ and H-6′ at δ 7.67 (*dd*, *J* = 8.7, 2.1 Hz), δ 7.05 (*d*, *J* = 8.7 Hz) and δ 7.76 (*d*, *J* = 2.1 Hz). The HMBC correlations of H-6′ to C-8'' suggested that the linkage of two flavone units was form between C-5′ and C-8''. Compound **2** was then identified as 8-(5-(5,7-dihydroxy-4-oxo-4*H*-chromen-2-yl)-2-hydroxyphenyl)-5,7-dihydroxy-2-(4-hydroxyphenyl)-4*H*-chromen-4-one or known as amentoflavone [1].

Compound 3 was a pale yellow solid. The ^{1}H and ^{13}C NMR spectrum displayed duplicated signals (in a ratio of 1:0.67), suggesting the existence of two conformers of flavonylflavanone bioflavonoids at room temperature [7]. The major conformer (Table 1) showed the resonances of a chelated hydroxyl protons 5-OH and 5''-OH at δ 12.20, *meta* aromatic protons H-6 and H-8 at δ 5.95 and δ 5.97, *trans* methine protons H-2 and H-3 at δ 5.75 and δ 4.65 (J = 12.0 Hz) and two *para*-disubstituted aromatic protons H-2′/H-6′ at δ 6.99, H-3′/H-5′ at δ 6.41 (2H each, *d*, J = 8.5 Hz) and H-2‴/H-6‴ at δ 7.45), H-3‴/H-5‴ at δ 6.86 (2H each, *d*, J = 8.7 Hz), singlet aromatic protons H-6'' at δ 6.25, and methine olefinic protons H-3″ at δ 6.23 (s). The HMBC correlation of H-3 to C-8a″ allowed the two units of flavonoids connected via C-3 and C-8″. Compound **3** was then assigned as (2*S*,3*R*)-2,3-dihydro-5,5′,7,7′-tetrahydroxy-2,2′-bis(4-hydroxyphenyl)-[3,8″-Bi-4*H*-1-benzopyran]-4,4″-dione or known as volkensiflavone [2].

Compound 4 is a yellow solid. The ^{1}H NMR spectrum (Table 1) showed the similar pattern to those of compound **3** except for the presence of H-2‴ (δ 7.45, *d*, J = 2.4 Hz), H-5‴ (δ 6.92, *d*, J = 8.4 Hz) and H-6‴ (δ 7.27, *dd*, J = 8.4, 2.4 Hz) revealed a 1,3,4-trisubstituted benzene ring in **4** instead of a 1,4-disubstituted benzene in **3**. The HMBC spectrum also showed the correlation of H-3 to C-8a″. Compound **4** was then assigned for 5,7,4′,5″,7″,3″,4″-heptahydroxyl-[3,8″]-flavonylflavanone which was known as morelloflavone [9].

CONCLUSIONS

The investigation indicated that the green branch of *Garcinia dulcis* contained dulcisbiflavonoid A (**1**), amentoflavone (**2**), volkensiflavone (**3**) and morelloflavone (**4**). These biflavonoids were previously reported in the stem bark, leaves, twig, root, heartwood, fruits, flowers and seeds of this plant species.

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REFERENCES

1. Cao SG, Sim KY, Goh SH. 1997. Biflavonoids of *Calophyllum venulosum*. J. Nat. Prod. 60, 1245-1250.
2. Cotterill PJ, Scheinmann F, Puranik GS. 1977. Phenolic compounds from the heartwood of *Garcinia indica*. Phytochem. 16, 148-149.
3. Gil B, Sanz MJ, Terencio MC, Gunasegaran R, Paya M, Alcaraz MJ. 1997. Morelloflavone, a novel biflavonoid inhibitor of human secretory phospholipase A_2 with anti-inflammatory activity. Biochem. Pharmacol. 53, 733-740.
4. Kuete V, Azebaze AGB, Mbaveng A, Nguemfo EL, Tshikalange ET, Chalard P, et al. 2011. Antioxidant, antitumor and antimicrobial activities of the crude extract and compounds of the root bark of *Allanblackia floribunda*. Pharm. Biol. 49, 57-65.
5. Lin YM, Anderson H, Flavin MT, Pai YH, Mata-Greenwood E, Pengsuparp T, et al. 1997. In vitro anti-HIV activity of biflavonoids isolated from *Rhus succedanea* and *Garcinia multiflora*. J. Nat. Prod. 60, 884-888.
6. Pang X, Yi T, Yi Z, Cho SG, Qu W, Pinkaew D, et al. 2009. Morelloflavone, a biflavonoid, inhibits tumor angiogenesis by targeting rho GTPases and extracellular signal-regulated kinase signaling pathways. Cancer. Res. 69,518-525.
7. Saelee A, Phongpaichit S, Mahabusarakam W. 2015. A new prenylated biflavonoid from the leaves of *Garcinia dulcis*. Nat. Prod. Res. 29, 1884-1888.
8. Stark TD, Losch S, Wakamatsu J, Frank O, Hofmann T, Balemba OB. 2015. UPLC-ESI-TOF MS-based metabolite profiling of the antioxidative food supplement *Garcinia buchananii*. J. Agri. Food. Chem. 63, 7169-7179.
9. Waterman PG, Crichton EG. 1980. Xanthone and biflavonoids from *Garcinia densivenia* stem bark. Phytochem. 19, 2723-2726

Cytotoxicity activity and phytochemical studies of some iridoids isolated from the rhizomes of Thunbergia laurifolia Lindl.

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ABSTRACT

Thunbergia laurifolia Lindl. (Acanthaceae, Thai name: Rang Chuet), a vine distributed in Southeast Asia, is described in traditional medicines for protection against dietary and environmental toxicants. Its high concentrations extract has reported to decrease in cell viability, cell growth, and clonogenic cell survival appeared to be strongly correlated with cell cycle perturbations when applied to MCF-7 breast cancer cells. This study aims to isolate some secondary metabolites and evaluate the cytotoxicity activity on the human colorectal adenocarcinoma cells. The pulverized, oven-dried at temperature 50°C (539.64 g) was successively extracted by Accelerated Solvent Extraction (ASE) using methanol solvent at temperature 100 °C. The extracts were evaporated to dryness (68.26 g) and fractionated by column chromatography, using a gradient solvent system of *n*-hexane-EtOAc, EtOAc, EtOAc-MeOH and MeOH with increasing amounts of the more polar solvents. Sub-fraction was subjected to two repeated column chromatography, eluted under isocratic condition following by sephadex LH-20. The structure of the isolated natural product was elucidated by comparison of its NMR with those of previously iridoid and confirmed by extensive 2D NMR spectral analysis. The results revealed the isolated compounds having the presence of cyclopentanopyran ring, corresponding to a C-9, the typical signals at δ 6.90 ppm. (H-3) and δ 4.06 ppm. (H-1) of iridoid skeleton. Furthermore, the methanol extracts from *T. laurifolia* showed the cytotoxicity activity on the human colorectal adenocarcinoma cells, (49.049%, SD 1.248) in this experiment. Further works will focus on biological activity investigation for anti-colon cancer agent from the isolated pure compounds from this plant species.

Keywords: *Thunbergia laurifolia*, Acanthaceae, iridoid, Human colorectal adenocarcinoma cells nanoparticle

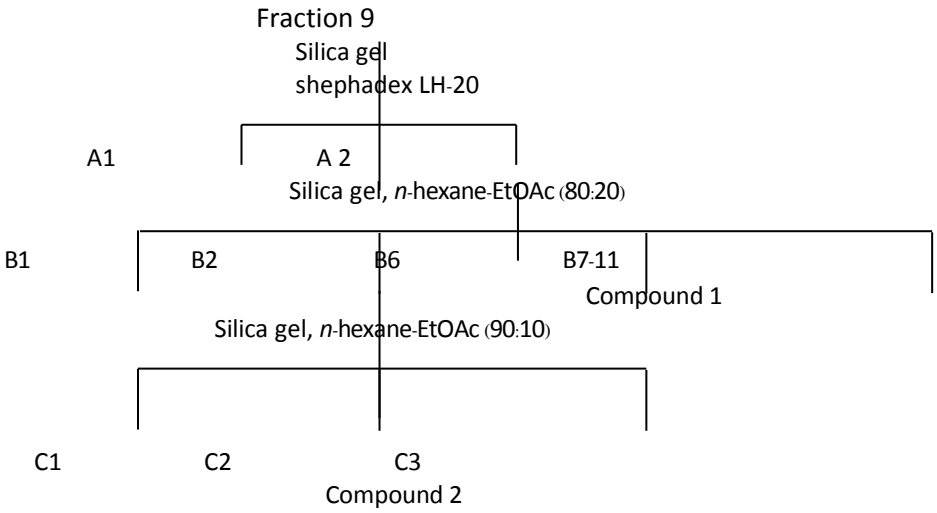
INTRODUCTION

Thunbergia laulifolia Lindl., commonly known in Thai as Rang Chuet (RC), belongs to the botanical family of Acanthaceae. *Thunbergia laurifolia* leaves are opposite, heart-shaped with serrated leaf margin and taper to a pointed tip. Flowers are not scented and borne on pendulous inflorescences [1]. The hermaphrodite flower is trumpet-shaped with a short broad tube, white outside and yellowish inside. The corolla is pale blue in colour with 5–7 petals, one larger than the others. For centuries, herbal remedies have been used to treat infections, ailments and diseases. They are often consumed in the form of tea, i.e. an infusion of dried parts (leaves, flowers, seeds, roots and barks) steeped in boiling water. Herbal teas have been gaining popularity in recent years and a great variety is sold in health food stores. It has been used in Thailand as a natural remedy for centuries. *Thunbergia Laurifolia* Linn. (TL) is one of the most familiar plants in Thai traditional medicine that is used to treat various conditions, including cancer. However, the antitumor activity of TL or its constituents has never been reported at the molecular level to support the folklore claim. The present study was designed to investigate the antitumor effect of an aqueous extract of TL in human breast cancer cells and the possible mechanism of action. An aqueous crude extract was prepared from dried leaves of TL. Folin-Ciocalteu colorimetric assays wereused to determine the total phenolic content. Antiproliferative and cell cycle effects were evaluated in human breast

adenocarcinoma MCF-7 cells by MTT reduction assay, cell growth inhibition, clonogenic cell survival [2]. The advent of NMR spectroscopy has made it progressively easier to determine of natural products, thus access to ^{1}H-NMR and ^{13}C NMR spectra of iridoid has made it possible to determine their substitution patterns, due to their interesting biological activities prompted us to investigate its chemical constituents in order to isolate bioactive constituents from this plant. However, establishing the relative configuration at the cyclopentanopyran ring centres (C-6, C-7, C-8 and C-9) in substituted iridoid has been difficult by spectroscopy alone, mostly due to the flexibility of the ring [1], the typical signals at H-3 and of iridoid skeleton.

MATERIALS AND METHODS

The rhizomes of *T. laurifolia* (539.64 g) were pulverized, oven-dried at temperature 50 °C and successively extracted by Accelerated Solvent Extraction (ASE) using methanol solvent, at temperature 100 °C, gave 12.6 % yield The extracts were evaporated to dryness under reduced pressure at temperature ca. 40 °C to give the MeOH extract (dark brownish sticky mass, 68.26 g). The MeOH extract (68.26 g) was fractionated by column chromatography (Merck silica gel 60, 0.063-0.200 mm, 500 g), using a gradient solvent system of n-hexane-EtOAc, EtOAc, EtOAc-MeOH and MeOH with increasing amounts of the more polar solvents. The eluates were examined by TLC and the 12 groups of eluting fractions were obtained as (Fraction 1-12). Fraction 9 was a combination of fractions that had similar chemicals constituent on the TLC. This fraction was chromatographed by sephadex LH-20 column, eluting with MeOH to give 2 sub-fractions (A1-ans A2). The sub-fraction A2 was collected and chromatographed over silica gel and eluted under isocratic condition using n-hexane-EtOAc (80:20) as the solvent system to afford 11 sub-fractions (B1-B11). The fraction B6 was re-fractionation sephadex and silica gel using n-hexane-EtOAc (80:20), gave **compound 1** as 0.40 % Fraction B2 was re-chromatographed on silica gel, using n-hexane-EtOAc (90:10) as a solvent system to give three fractions (C1-C3). The fraction C2 has been re-fractionation by using the same procedure by using n-hexane-EtOAc (80:20) as the solvent, gave **compound 2** as 18.8 % as shown in Scheme 1.



Scheme 1. Shows the fractionation flow chart of Fraction 9 isolated from the MeOH Extract of *Thunbergia laulifolia* Lindl rhizomes

The **compounds 1and 2** were submitted to ^{1}H NMR, ^{13}C NMR and 2D NMR, the results has elucidated by comparison of its NMR data with those of previously report. The methanol extract was evaluated its biological activity on the human colorectal adenocarcinoma cells.

RESULTS AND CONCLUSIONS

The methanol extract of *T. laurifolia* rhizomes were purified by chromatographic technique led to the isolation to give compound**1** and compound **2**. Compound **1** was obtained as white solid. The ^{1}H- and ^{13}C-NMR spectra ($CDCl_3$) indicated the presence of one olefinic proton at δ 6.90 (1H, *J* = 8.1, 2.4 Hz, H-3), δ 4.06 (1H, *J* = 7.8 Hz, H-1), methylenes [δ 2.65 (2H, *m*, H-7'), 1.80 (2H, *m*, H-6'), 1.44-1.7 (2H, *m*, H-1'-6') and, methane proton δ 2.50 (1H, *J*=18.6, 8.2, H-9), δ 2.03 (1H, *J*= 10.2, 3.0 H-5) and 1.45 (2H, *m*, H-7), two tertiary methyls [δ 0.76 (3H, *s*, H-11) and 0.79 (3H, *s*, H-12)] and methyl proton δ 0.89 (3H, *s*, H-10). The presence of two carbonyl signals at δ 193.1 and 206.4 was observed in the ^{13}C-NMR data. The quaternary signal presented at δ 193.1 of oxygen atom from the molecular formula indicated the presence of carbonyl group in each compound, in both cases; this was assigned to C-4 due to the significant difference in the chemical shifts C-4. The advent of NMR spectroscopy has made it progressively easier to determine of natural products, thus access to ^{1}H-NMR and ^{13}C NMR spectra of iridoid has made it possible to determine their substitution patterns. However, establishing the relative configuration at the cyclopentanopyran ring centres (δ 53.4 C-6, δ 38.7 C-7, δ 27.9 C-8 and δ 41.7 C-9) in substituted iridoid has been difficult to be identified by spectroscopy alone, mostly due to the flexibility of the ring [2], the typical signals at H-3 of iridoid skeleton [3]. The estimated structure of compound 1, is shown in **Figure 1**. The ^{1}H-NMR spectrum of compound 2 in $CDCl_3$, shown in **Table 1** its chemical shift was similar to those of compound 1, the significant difference of which was the absence of the methoxyl signal at δ 3.49 ppm., correlated to the ^{13}C-NMR signals at δ 56.1. However, the absolute configuration of the compound 2 is now during in the identification process to be established from the existing data.

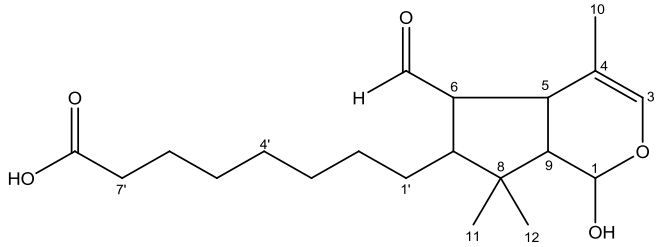


Figure 1. Shows the structure of compound 1

Structural Elucidation/Structural Characterization

Phytochemical investigation of *Thunbergia laulifolia* Lindl. rhizomes led to the isolation of two compounds. The structures of the compounds were elucidated by NMR spectroscopic techniques.

Biological Activities

The methanol extracts from *T. laurifolia* showed the cytotoxicity activity on the human colorectal adenocarcinoma cells, (49.049%, SD 1.248). The two pure compounds isolated from the methanol extract will be submitted for their cytotoxicity activity on the human colorectal adenocarcinoma cells test and investigation for anti-colon cancer in the future works.

CONCLUSIONS

Extraction of *Thunbergia laurifolia* Lindl. rhizomes by ASE using methanol as solvent gave methanol extract, submitted to the cytotoxicity activity on the human colorectal adenocarcinoma cells, resulted in 49.049% with SD 1.248. The isolation of the methanol extract by sephadex LH-20 and silica gel in open column chromatography using a gradient solvent system of n-hexane and ethyl acetate to give compound 1 and compound 2, identified their structures by NMR techniques revealed as the iridoid derivatives. Further works will continued on the cytotoxicity activity on the human colorectal adenocarcinoma cells and anti-colon cancer of the isolated two compounds.

Table 1. shows chemical shifts (ppm.) of ^{1}H- and ^{13}C-NMR Data of Compound **2** , in $CDCl_3$

^{1}H-NMR	**^{13}C-NMR**
0.68, (*S*)	14.2
0.78, (*S*)	21.6
0.85, (*S*)	33.4
1.02 (*m*)	39.2
1.1 (*m*)	55.6
1.3 (*dd*, 8.8), 1.68 (*m*)	39.2
1.17 (*dd*, 13.3, 4.0), 1.38 (*br d*, 13.1)	42.0
1.5, 1.57 (*m*)	19.2
1.68 (m)	24.0
1.83 (br *d*, 1.8)	55.4
1.97, 2.38 (*m*)	37.7
2.68 (*d*, 17.3), 2.98 (*m*)	32.6
3.49 (*S*)	56.1
4.35 (*d*, 14.7),	107.4
4.8 (*d*, 4.97)	107.2
5.48 (*m*)	102.0
6.7 (*br s*)	142.6

ACKNOWLEDGEMENTS

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REFERENCES

1. Damtoft S, Jensen S R, Nielsen B J. 1981. ^{13}C and ^{1}H-NMR spectroscopy as a tool in the configurational analysis of iridoid glucosides. Phytochem. 20, 2717-2732.
2. Chan E W C, Eng S Y, Tan Y P, Wong Z C, Lye P Y and Tan L N. 2012. Antioxidant and sensory properties of thai herbal teas with emphasis on *Thunbergia laurifolia* Lindl. Chiang Mai J. Sci. 39, 599-609
3. Damtoft S, Frederiksen L B and Jensen S R. 1994. Alatoside and thunaloside, two iridoid glucosides from *Thunbergia Alata*. Phytochem. 35, 1259-1261.

Extraction and characterization of Tamarind seeds polysaccharides (TSP) as drug delivery from *Tamarind indica* L.

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ABSTRACT

Tamarind seeds polysaccharide (TSP), natural polysaccharides, was extracted from tamarind seeds, using in pharmaceutical, textile and food industries as mucoadhesive polymer. This work aims to extract TSP from tamarind seeds from three sources and characterized its physical and chemical properties. Kernel powder of tamarind seeds were prepared slurry as a clear solution and kept it aside for an overnight and then centrifuged at 6000 rpm. for 20 mins. to separate all the foreign matter. The supernatant liquid was separated and poured into excess of 95% ethanol solvent with continuously stirred. Precipitates obtained were collected and dried in the oven and then stored the dried polymer of TSP in a desiccator. The dried TSP was analyzed by ^{1}H NMR, FT-IR and SEM including other techniques involved. The results showed TSP from tamarind seeds taken from paddy farmland (A), a waste from the export tamarind juice industry (B) and the export tamarind powder industry(C) gave their percent yields as 31.55%, 17.30% and 53.65%, respectively. The FT-IR spectra displayed peaks at 3,351.95 cm^{-1}, 2,920.76 cm^{-1}, 1,018.85 $cm^{-1}$and 555.16 cm^{-1}. The ^{1}H NMR resonated peaks between δ 3.50-4.20 ppm. of polysaccharides and SEM exhibited its shape and surface. Further works will focus on quantitative analysis, biological activity and drug delivery system of TSP.

Keywords: Tamarind seed polysaccharide (TSP), *Tamarind indica* L., natural polysaccharides

INTRODUCTION

Natural polymer or gum has been used in preparation of release and controlled release types of dosage form, because of its great properties such as biodegradable, nontoxic, biocompatible in nature and swells when it comes in contact with the aqueous media. Tamarind gum, natural polymer, is highly viscous, mucoadhesive and biocompatible in nature, it can be used for oral controlled release administration, ocular drug delivery system and in sustained release of drug delivery system designs and dosage forms (1). Tamarind seeds polysaccharides (TSP), natural branched polysaccharides extracted from Tamarind seeds of *Tamarind indica* L., belonging to the Leguminosae family (2). TSP composed of (1□4) β-D-glucan backbone substituted with side chains of α-D-xylopyranose and β-D-galactopyranosyl linked (1□2)- α-D-xylopyranose linked (1□6) to glucose residues (3) (Figure 1). Chemicals constituents of TSP represent as glucose, xylose and galactose in the ratio of 2.80:2.25:1.00 (4). TSP, regards as a galactoxylloglucan, has various properties using in textile, food, and pharmaceutical industry and is rich in a high molecular weight polysaccharide (~65-72%) with the molecular weight of 700-880 kDa (5). The TSP constitutes of 65%of tamarind seed composition. (6), the seed oil are major in fatty acids such as palmitic, oleic, linoleic and eicosanoic acids, the highest concentration belonging to linoleic acid and palmitic acid, presenting in the percent of 36-49% and 14-20%, respectively. The TSP possesses of various features, attractive candidate as a vehicle for ophthalmic medicaments (7). The mixture of TSP and hyaluronic acid are employed as artificial tears for ophthalmic application in the eye dry syndrome (8). TSP is a novel mucoadhesive polymer, can be used in the drug delivery system for the ocular administration of hydrophilic and hydrophobic antibiotics (9). Therefore, this work aims to extract TSP from tamarind seeds of three sources and characterized its physical and chemical properties for the future drug delivery system development.

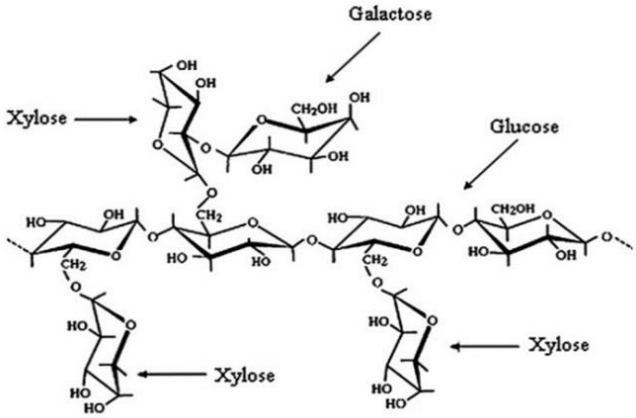

Figure 1. Structure of tamarind seed polysaccharides (TSP)

MATERIALS AND METHODS

TSP extraction procedure

Tamarind seed preparation

Tamarind seeds taken from paddy farmland (A), with pulse tissue, were separated their pulse from the seeds by hand, the seeds were washed by tap water and dried in oven, 100^0C for 30 mins. The seeds were let to cool down in room temperature and were slightly ground in 0.5-1 min. by a blender to separate the brown peels from the kernel seeds. The kernel seeds were grounded into powder by a blender.

TSP extraction

The powder weight of 20 grams, were added by 200 ml of cold distilled water to prepare the slurry. The slurry obtained was poured into 800 ml of boiling distilled water, and then boiled for 20 mins. on a hot plate, obtaining a clear solution and it was kept aside overnight. The thin clear solution was further centrifuged at 6000 rpm. for 20 mins. to separate all the foreign matter. The supernatant liquid was separated and poured into excess of 95% ethanol solvent with continuously stirred. The obtained precipitants were collected using a stainless sieve, dried in the oven at temperature 50°C for 4 hrs. The dried polymer stored in a desiccator. In the same ways, tamarind seed powder, a waste from the export tamarind juice industry (B) and the export tamarind powder industry (C) were extracted in the procedure mentioned above. Moreover, only tamarind seeds taken from paddy farmland (A), was extracted by Accelerated Solvent Extraction (ASE) using methanol as a solvent, following by ethanol solvent, at temperature 100^0C for 30 mins. to give methanol extract 7.51% and ethanol extract 3.31%.

Materials

Tamarind seeds taken from paddy farmland (A) were bought from Mr. Winai Jattanakul, Phetchaboon province, Thailand, a waste from the export tamarind juice industry (B) were bought from P. Prateeptong Best Foods Company Limited, Samutsongkram province, Thailand and the export tamarind powder industry (C) was given by G.M. Ichihara (Thailand) Company Limited, Pathumthani province Thailand. The extraction using methanol and ethanol in AR grade, RCI Labscan Asia, supplied in Thailand.

Methods

Tamarind seeds taken from paddy farmland (A) were extracted by Accelerated Solvent Extraction (ASE) instrument: DIONEX/USA, model ASE350, USA. Dried the TSP extracted by oven: Termaks, Shine Engineer International Limited. Structure of TSP analyzed by NMR instrument: Bruker, AVANCEISS 400, Switzerland.

Characterization of TSP

TSP samples from the three sources were subjected to FT-IR spectroscopy in a Fourier-transform infrared spectrophotometer (Spectrum 100, Perkin Elmer, USA) in a range of 4500–500 cm^{-1} as KBr pellets. The electron photomicrographs of TSP were recorded using scanning electron microscope (JEOL, JSM-6610 LV, Inc., USA). The samples were mounted on stub containing double adhesive carbon tape and recorded at an accelerating voltage of 15 kV.The TSP from the three sources was submitted for ^{1}H NMR, 400 MH_Z in D_2O solvent.

RESULTS AND DISCUSSION

The tamarind seeds taken from paddy farmland (A) were separated the brown peels from the kernel seeds by a blender and separated the seeds by using plastic sieve. The kernel powder in the quantity of 20 grams from the seeds taken from paddy farmland (A), a waste from the export tamarind juice industry (B) and the export tamarind powder industry (C) were extracted and precipitated, gave the dried Tamarind seed polysaccharides (TSP) as shown in Table1. TSP (C) was the highest % yield which was nearly a double amount of TSP (A) while TSP (B) was the lowest percentage compared to the others.

Table1. Tamarind Seed Polysaccharides (TSP) extracted from the three sources.

Methods	**Paddy farmland (A)**	**Waste from the export tamarind juice industry (B)**	**Export tamarind powder industry(C)**
Weight (grams)	20.00	20.00	20.00
Supernatant (grams)	173.75	202.50	208.00
TSP Weight (grams)	6.31	3.46	10.73
% yield	31.55	17.30	53.65

The tamarind seeds taken from paddy farmland (A) was submitted to extract by methanol solvent by Accelerated Solvent Extraction (ASE), gave methanol extract (TS1) and further identify its chemicals constituents by ^{1}H NMR technique, resulted in TSP in the chemical shift region of δ 3.00-4.20 ppm, methyl and methylene groups at δ 0.45-2.00 ppm. (Figure 2A) While the ethanol extract (TS2), resonated a mixture of methyl linoleate and triacylglycerol. (Figure 2B) The tamarind seeds powder should be extracted with methanol solvent in order to give TSP as its chemicals shift resonated at δ 3.00-4.20 ppm, but it needed to further separate alkyl groups at δ 0.45-2.00 ppm. to yield a pure TSP. On the other hand, when it extracted with ethanol solvent, gave only fatty acid derivatives and presented none of TSP.

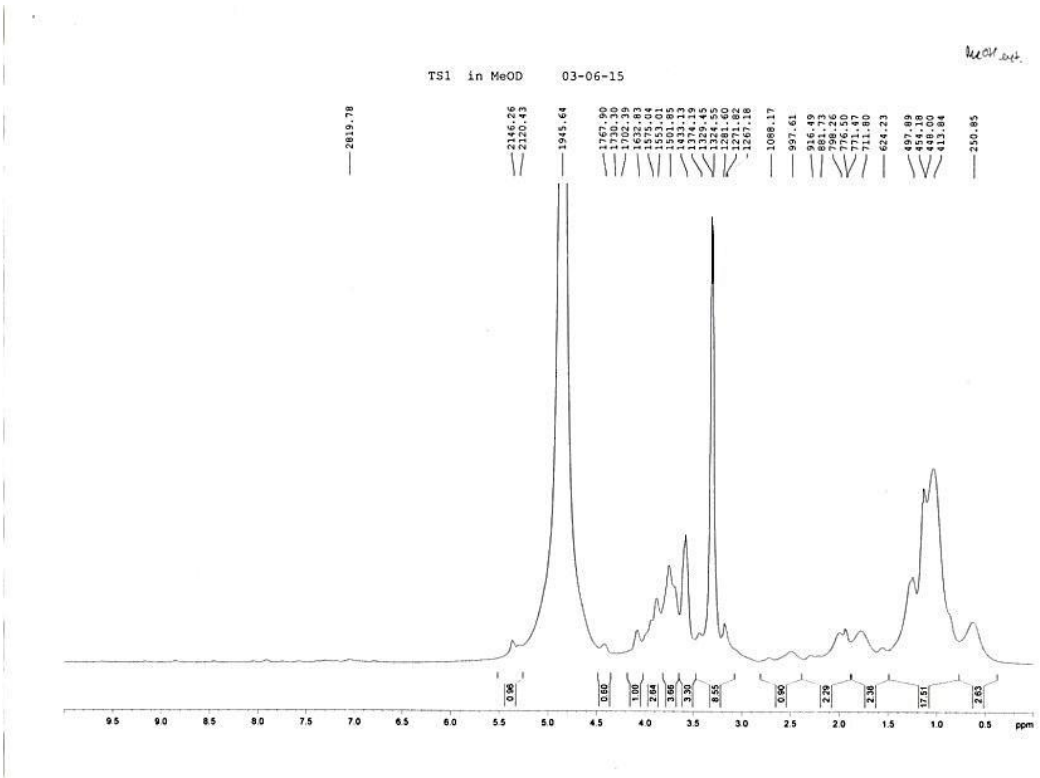

Figure 2A. ^{1}H NMR spectrum of TS1 extracted from (A), in MeOD

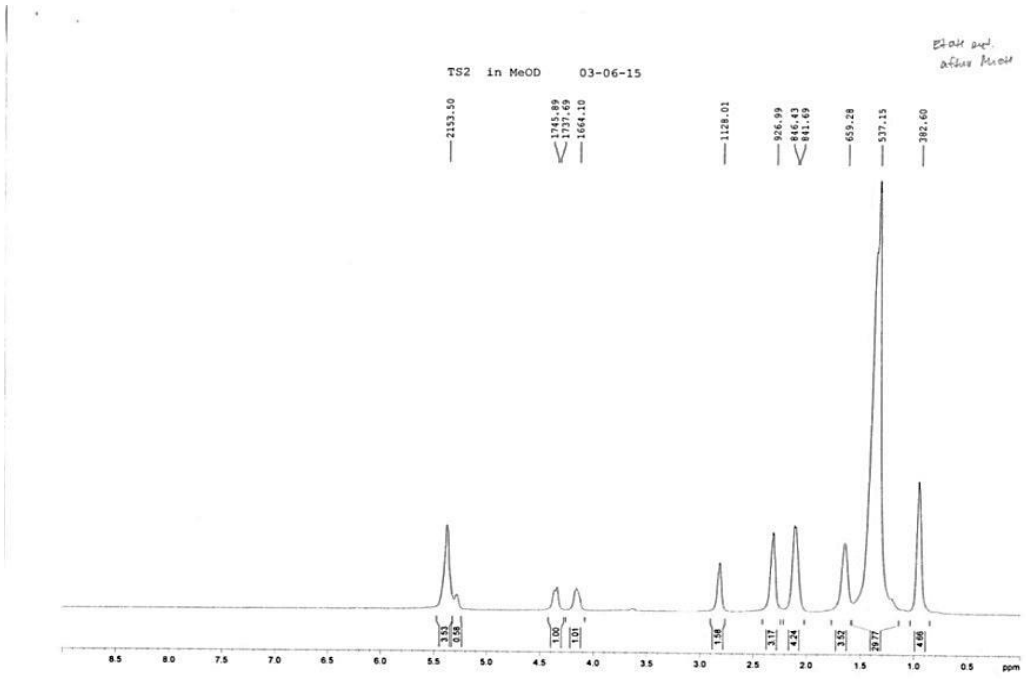

Figure 2B. ^{1}H NMR spectrum of TS2 extracted from (A), in MeOD

The Infrared spectra (IR) of TSP, were extracted from the tamarind seeds taken from paddy farmland (A) (Figure 3A), a waste from the export tamarind juice industry (B) (Figure 3B), and the export tamarind powder industry (C) (Figure 3C). TSP displayed a characteristic broad peak at 3,351.95 cm^{-1}, 3,355.85 cm^{-1} and 3,357.46 cm^{-1}, respectively, representing hydroxyl (OH) stretching of glucan backbone. Peaks at 2,920.76 cm^{-1}, 2,923.36 cm^{-1} and 2,920.99 cm^{-1}, respectively, attributed to C-H stretching of alkanes. Peaks appeared at 1,018.85 cm^{-1}, 1,016.20 cm^{-1} and 1,016.13 cm^{-1} , respectively, represented to (C-O-C) stretching of cyclic ether. Peaks at 555.16 cm^{-1}, 556.41 cm^{-1} and 555.75 cm^{-1}, respectively, confirmed for their the OH bending.

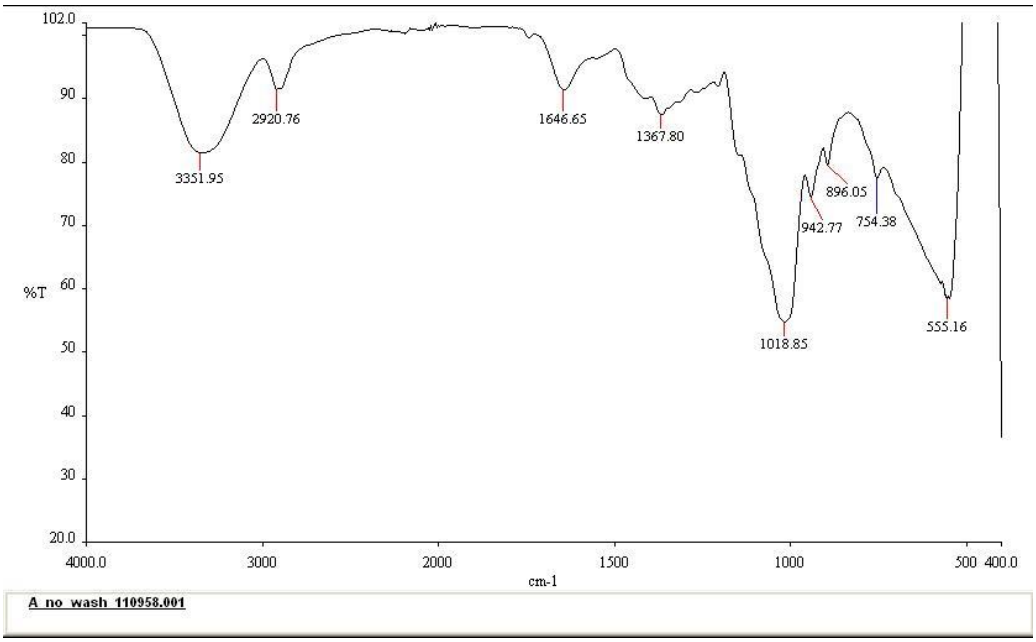

Figure 3A. IR spectrum of TSP from (A)

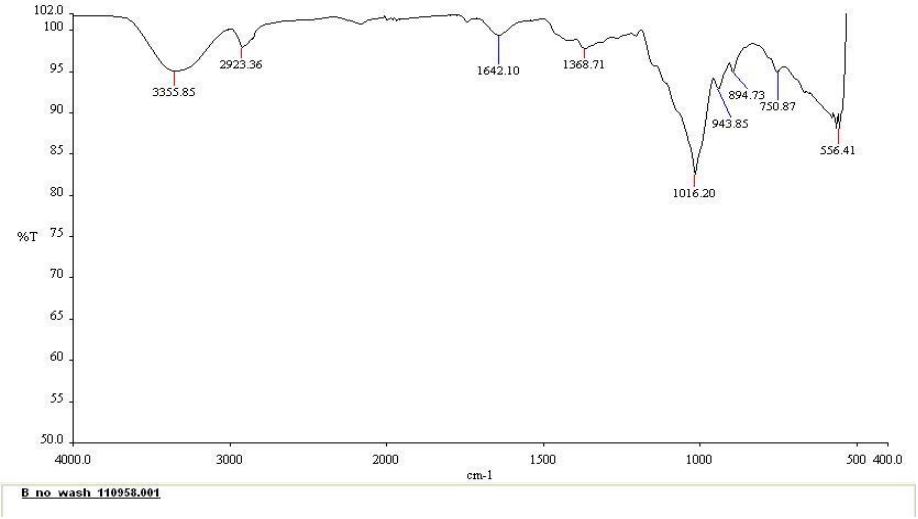

Figure 3B. IR spectrum of TSP from (B)

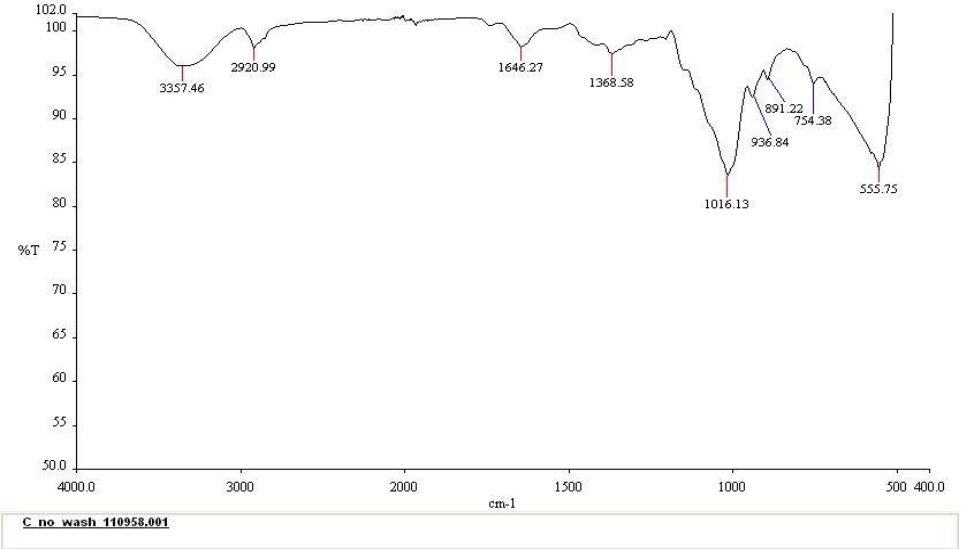

Figure 3C. IR spectrum of from (C)

The SEM showed the shape and surface, examined under scanning electron microscope of TSP extracted from The tamarind seeds taken from paddy farmland (A) (Figure 4A), a waste from the export tamarind juice industry (B) (Figure 4B) , and tamarind powder from the export tamarind powder industry (C) (Figure 4C) revealed that TSP (A) surface (1) was rougher than TSP (B) and TSP (C) while TSP (C) surface was the smoothest but exhibited the roughest cross section (2) compared to TSP (A) and TSP (B), representing that TSP (A) give an exceptional result than TSP (B) and TSP (C) as TSP (A).

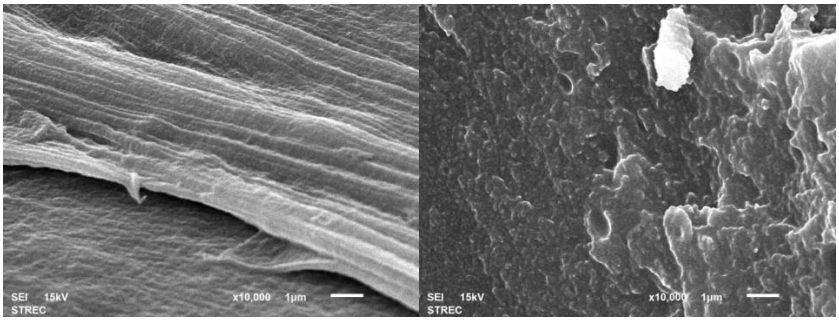

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Figure 4A. Scanning electron micrographs (SEM) spectrum of TSP from (A)

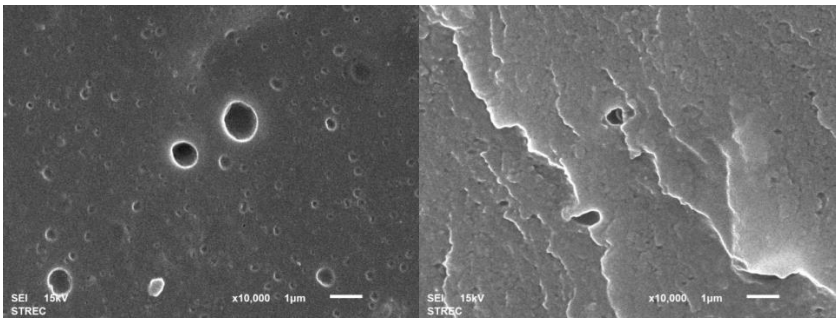

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Figure 4B. Scanning electron micrographs (SEM) spectrum of TSP from (B)

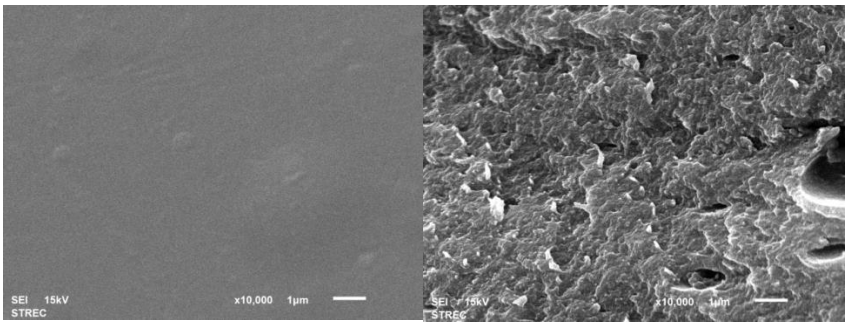

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Figure 4C. Scanning electron micrographs (SEM) spectrum of TSP from (C)

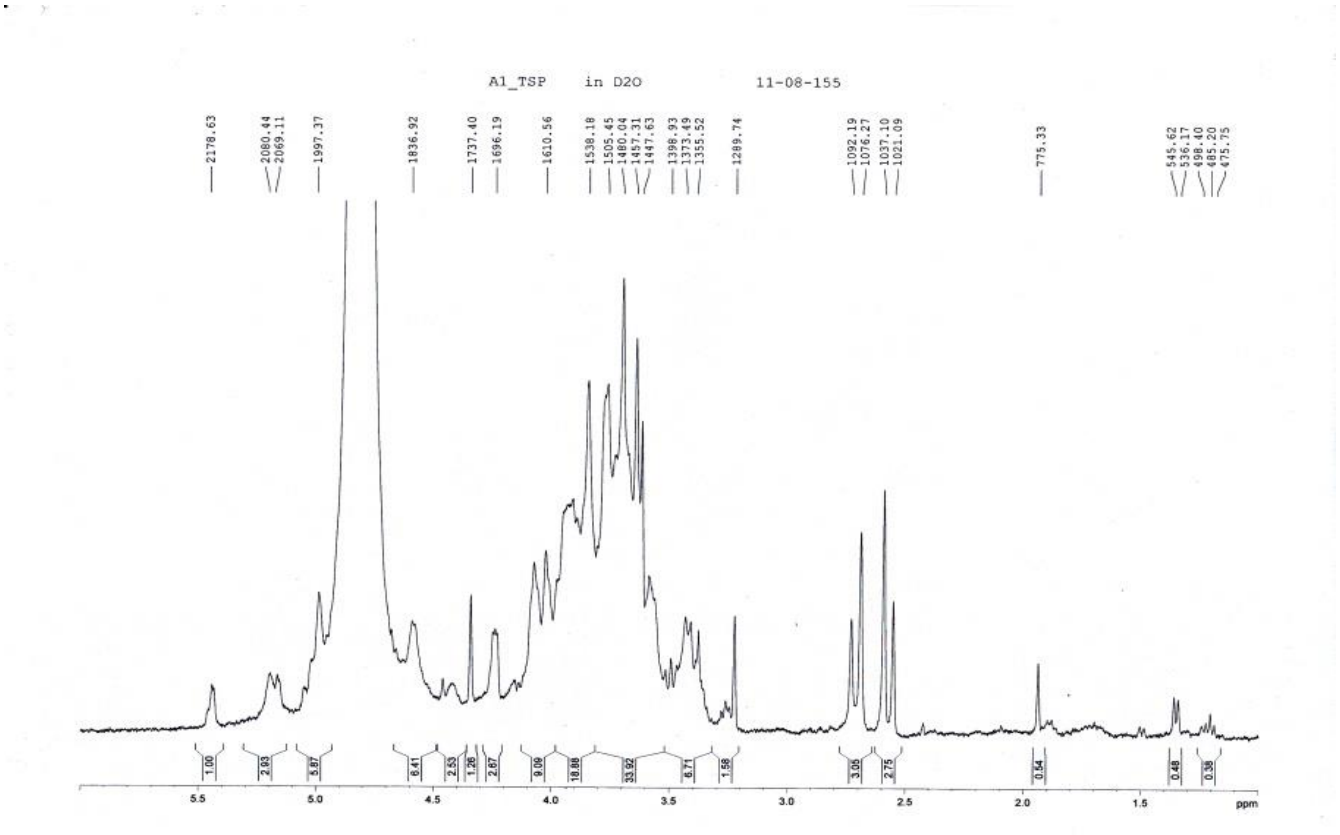

Figure 5A. ^{1}H NMR spectrum of TSP extracted from (A), in D_2O

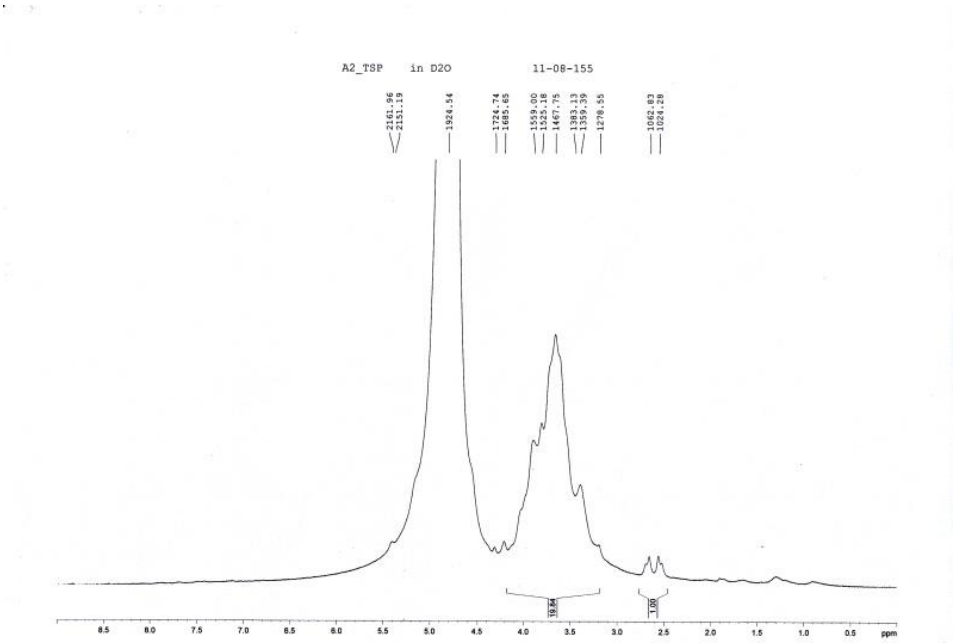

Figure 5B. ^{1}H NMR spectrum of TSP extracted from (B), in D_2O

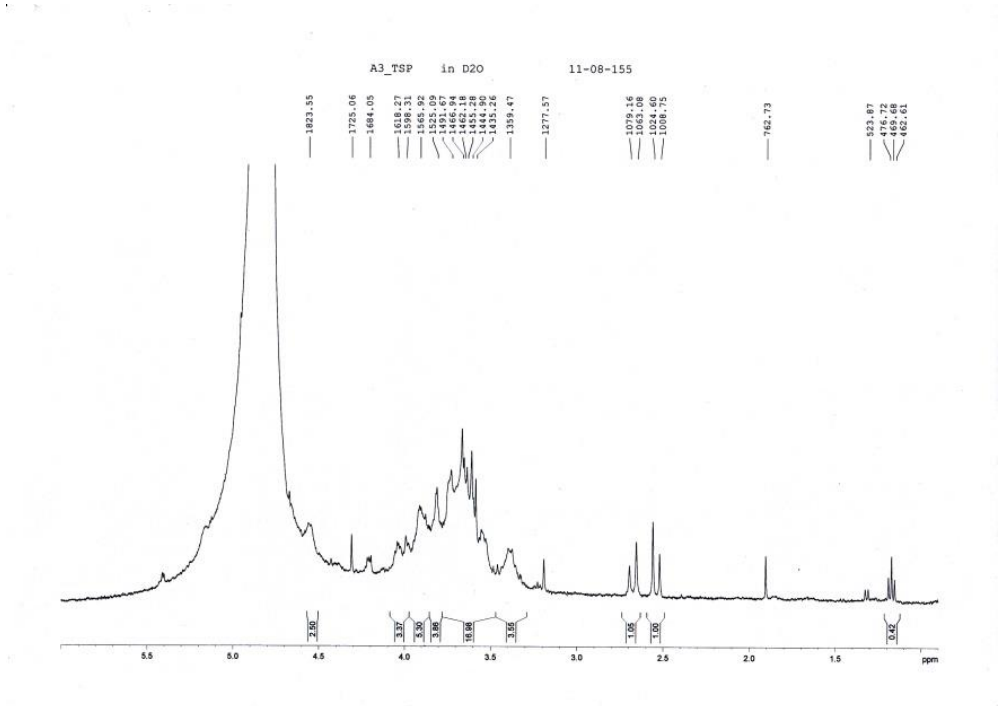

Figure 5C. ^{1}H NMR spectrum of TSP extracted from (C), in D_2O

The ^{1}H NMR illustrated the TSP extracted from tamarind seeds taken from paddy farmland (A) (Figure 5A), exported tamarind juice industry (B) (Figure 5B), and tamarind powder from the exported tamarind powder industry (C) (Figure 5C) exhibited the typical bands and peak characteristic of polysaccharides between chemical shifts at δ 3.4-4.1 ppm. The signals of TSP(C) presented its chemicals shift; similarly to the TSP (A) while those of the TSP (B) resonated only polysaccharides with none of α -residue of doublet signal at δ 5.1- δ 5.25 ppm. and absent of the singlet signal at δ 1.96 ppm., relating to methyl groups of rhamnose.

The ^{1}H NMR spectra of mucilage indicated certain sugars composition such as δ 3.65-3.55ppm. can be attributed to OH and CH group of mannose. The signals between δ 3.90-3.50ppm., displayed CH_2 group of arabinose. The singlet signal at δ 1.96 ppm., is related to methyl groups and the proton linked to C-6 (δ 3.65 and δ 3.70 ppm.) of rhamnose and C-4 (δ 3.98- δ 4.28 ppm.) presented to galactose. The anomeric protons have been assigned to α -residue of doublet signal at δ 5.1- δ 5.25 ppm. The mucilage signals resonated at δ 4.02 and δ 3.84 ppm. were illustrated to H-1 of glucose. The ^{1}H NMR spectra showed the crowded region between δ 3.00 ppm. to δ 5.00 ppm. of typical of polysaccharides and confirm for the presence of many similar sugar residues. The signals presented between δ 3.20- δ 4.30 ppm. can be assigned to non-anomeric protons (H_2-H_6) whereas signals between δ 4.30 ppm. to δ 4.80 ppm and δ 4.90 to δ 5.50 ppm. can be suggested for α –anomeric and β-anomeric protons, respectively. The ^{1}H NMR demonstrated of mucilage, possesses properties that useful for pharmaceutical and can be used for effective controlled release drug delivery from the designed matrix system.

CONCLUSION

TSP extracted from the export tamarind powder industry (C) resulted in the higher percent yield than the TSP from The tamarind seeds taken from paddy farmland (A) and a waste from the export tamarind juice industry (B), respectively. The TSP characterized of their physical and chemical properties displayed that tamarind seeds powder taken from paddy farmland (A) possessed a mixture of methyl linoleate and triacylglycerol in ethanol extract extracted by ASE, while its methanol extract yielded TSP as its chemicals shift resonated at δ 3.00-4.20 ppm. Therefore, the tamarind seed powder should be suitable extracted with methanol than ethanol solvent to yield TSP. The IR showed the characteristic of stretching and bending glucan backbone of TSP while the scanning electron micrographs (SEM) of TSP (A) exhibited an exceptional result than TSP (B) and TSP (C) which TSP (A) of mucoadhesive potential, considering for further product development. The FT-IR and ^{1}H NMR confirmed the presence of glucan backbone, non-reducing sugars and demonstrated of mucilage. The TSP from (A) could be reasonable for drug delivery system more than TSP from (C) and (B) as it gave the highest signals of the TSP. Further works, will focus on TSP mucohesive strength, their biological activities, toxicity evaluations and design for controlled release drug delivery system.

REFERENCES

1. Singh R, Malviya R, Kumar Sharma P. 2011. Extraction and Characterization of Tamarind Seed Polysaccharide as a Pharmaceutical Excipient. Pharm. J. 3(20)
2. Kaur H, Yadav S, Ahuja M, Dilbaghi N. 2012. Carboxy methyl tamarind seed kernel of Biological Macromolecules, 50, 833-839
3. Jana s, Das A, Nayak AK, Sen KK, Basu SK. 2013. Aceclofenac-loaded chitosan-tamarind seed polysaccharide interpenetrating polymeric network microparticles. Colloids. Surf. B. Biointerfaces, 105, 303-309.
4. Goyal P, Kumar V, Sharma P. 2007. Carboxymethylation of Tamarind kernel powder. Carbohydr. Polym. 69, 251-255.Kaur H, Yadav S, Ahuja M, Dilbaghi N. 2012. Synthesis, characterization and evalution of thiolated tamarind seed polysaccharide as a mucoadhesive polymer. Carbohydr. Polym. 90,1543-1549.
5. Chandramouli Y, Firoz S, Vikram A, Mahitha B, Rubia Yasmeen B, Hemanthpavankumar K. 2012. Tamarind seed polysaccharide (TSP) – An Adaptable Excipient for Novel Drug Delivery System. IJPPDR. 2, 57-63.
6. Sahoo S, Sahoo R, et al. 2010. Tamarind Seed Polysaccharide: A Versatile Biopolymer for Mucoadhesive Applications. JPBMS. 8, 20
7. Uccello-Berretta G, Balzano F, Vanni L, Sansò M. 2013. Mucoadhesive properties of tamarind-seed polysaccharide/ hyaluronic acid mixture: A nuclear magnetic resonance spectroscopy investigation. . Carbohydr. Polym. 91, 568-572.
8. Singh, P.P. 1973. Oxalic acid content of Indian foods, *Quarterly* Plant Materials Vegetables. 22, 335, Chemical Adstracts, 79: 135560.1973, 22, 335.

Secondary metabolites and their biological activities in Indonesian soft corals of the genus *Lobophytum*

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ABSTRACT

Marine soft corals have been proven to be rich sources of bioactive secondary metabolites characterized by uncommon structural features and potent bioactivities. In the continuation of our search for bioactive compounds from Indonesian soft corals, we have been recently studying the chemical composition and biological activities of two soft corals of the genus *Lobophytum*. This organism was collected from the Selayar Islands (South Sulawesi). The present study aimed to investigate the antioxidant, antibacterial and antiplasmodium activities as well as cytotoxicity of the *n*-hexane, ethyl acetate, *n*-butanol, and aqueous fractions from a crude extract of *Lobophytum* sp. The antioxidant activity was performed by the DPPH (1,1-diphenyl-2-picryl hydrazyl) radical scavenging method. All fractions from the crude extract of *Lobophytum* sp were examined for their cytotoxicity using the brine shrimp lethality bioassay and heme polymerization inhibitory activity assay for antimalarial activity. It was found that the ethyl acetate, *n*-butanol and aqueous fractions exhibited heme polymerization inhibitory activity with IC_{50} values of 11.7, 14.3 and 12 µg/mL, respectively, while the *n*-butanol fraction showed moderate antioxidant activity and cytotoxicity and 93 µg/mL, respectively. This is a new report of antiplasmodium activity substances derived from *Lobophytum* sp.

Keywords: *Lobophytum* sp., cytotoxicity, antioxidant, antiplasmodium

INTRODUCTION

Soft corals belonging to the family Alcyoniidae are characterized by a great variety of colors, shapes, and sizes and they are by far the most dominant reef dwelling octocorals in the Indo-West Pacific. These organisms are known to produce a wide array of secondary metabolites, particularly diterpenoids, sesquiterpenoids, steroids and other chemical compounds, often characterized by uncommon structural features. It has been estimated that the percentage of new metabolites discovered from soft corals represents up to 22% of the total new marine natural products reported from 2010 to 2011. A number of natural products isolated from soft corals have demonstrated they are of great biomedical interest having antiviral, anti-tumor, anti-inflammatory and antifouling properties [1-3]. Since 1997-2014, more than 20 publications have reported on the bioactive compounds from Indonesian soft corals such as *Cladiella* sp., *Lobophytum* sp. and *Sinularia* sp.

As part of our continuing research program aimed at the discovery of bioactive metabolites from marine organisms, we have recently been studying the chemical composition of marine invertebrates from the Indonesian coast, considered to be one of the richest biodiversity hot spots in the ocean. In this context, we have started the analysis of a specimen of *Lobophytum* sp. collected from the island of Selayar (South Sulawesi).

Soft corals belonging to the genus *Lobophytum* (Alcyoniidae) have been shown to be a rich source of macrocyclic cembranoids and their cyclized derivatives [4-18] commonly described as defensive substances against predators such as other corals and fishes [19,20]. Some of these metabolites are of considerable interest and merit continuous attention due to their unique structures and significant biological activities, including anti-tumor, anti-viral, and anti-inflammatory properties [4-18]. In this study, we examined the antioxidant, antibacterial and antiplasmodium activity as well as cytototoxicity of fractions (*n*-hexane, ethyl acetate, *n*-butanol, and water) from the crude extract of *Lobophytum* sp.

MATERIALS AND METHODS

Animal Material

Specimens of *Lobophytum* sp. (500 g wet weight) were collected in April 2015 from the island of Selayar (South Sulawesi) at a depth of 3 m. Each sample of *Lonophytum* sp. was rinsed with sea water and immediately kept in ice. After being sent to the laboratory, immediate storage in the freezer was necessary to reduce any possible degradation. A voucher sample was deposited at the Research Center for Oceanography-LIPI.

Extraction

Colonies of *Sinularia* sp. were homogenized and repeatedly extracted with MeOH:DCM (1:1) at room temperature and the obtained combined material (10.7 g) was partitioned with *n*-hexane (non-polar), ethyl acetate (semi polar), *n*-BuOH (polar) and residual aqueous fractions, also coded NH (3.4 g), EA (0.3 g), NB (0.28) and RA (6.9 g), respectively. Each of fractions was subjected to preliminary phytochemical screening and heme polymerization inhibitory activity, antioxidant and cytotocixity.

Phytochemical Screening

All fractions were subjected to a preliminary phytochemical screening test for the presence of the following secondary metabolites utilizing the standard conventional protocol described by Harborne (1987) [21].

DPPH Radical Scavenging Assay

All fractions from the crude extract of *Lobophytum* sp. were prepared in different concentrations, ranging from 20 to 200 µg/ml for each sample and analyzed in triplicate. The methanol solution of the fraction of the tested sample (500 µL) were added to DPPH solution (1 mL) in 96-well plates and incubated in the dark for 30 min. Lower absorbance values were read at 517 nm using the microplate reader Infinite® 200 PRO (Tecan Austria GmbH).

The reference standard compound being used was butylated hydroxytoluene (BHT) and the experiment was done in triplicate. The IC_{50} value of the sample, which is the concentration of a sample required to 50% inhibit of the DPPH free radical, was calculated using the Log dose inhibition curve [22]. Lower absorbance of the reaction mixture indicated a higher free radical activity [23]. The percent DPPH scavenging effect was calculated using the following equation:

$$\text{DPPH scavenging effect (\%) or Percent inhibition} = [(A_0 - A_1) / A_0] \times 100\%$$

Where A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of a test or standard sample [24].

Brine shrimp lethality bioassay

The cytotoxic activity of all fractions from the crude extract were evaluated using the Brine shrimp lethality bioassay method with different concentrations (50, 100, 200 µg/mL). Each concentration was made in triplicate. The brine shrimp eggs were placed in 1 L of sea water, aerated for 48 h at 37 °C to hatch. After 48 h, ten brine shrimp eggs were placed in a small container filled with sea water. The numbers of survivors were counted after 24 h. Larvae were considered dead if they did not exhibit any internal or external movement during several seconds of observation. The larvae did not receive food. To ensure that the mortality observed in the bioassay could be attributed to bioactive compounds, and not to starvation, we compared the dead larvae in each treatment to the dead larvae in the control.

Heme polymerization inhibitory activity assay

Heme polymerization inhibitory activity (HPIA) assay was conducted using a method developed by Bassilico et al. [25]. A total of 100 mL solution of 1 mM hematin in 0.2 M NaOH was put into a 96-well micro-culture plate, and then a 50 mL assay solution with various concentrations, ranging from 0.3125 to 20 mg/mL, was added into each well. Glacial acetate acid (50 mL, pH 2.6) was added to the microculture to initiate a heme polymerization reaction. The microculture was then incubated at 37 ºC for 24 h to obtain perfect polymerization. After the period of incubation, the microculture was centrifuged and the resulting deposits were washed three times using 200 mL of dimethyl sulfoxide (DMSO). The solution of 0.1 M NaOH (200 mL) was subsequently added to the deposits in each well of microculture. Absorbance values were read at 405 nm using a microplate reader, Infinite® 200 PRO (Tecan Austria GmbH). The value of heme polimerization inhibitory activity was expresses in IC_{50}. Aquadest and chloroquine were used for negative and positive control, respectively. The percentage inhibition of heme polymerization was calculated by the formula:

$$\% \text{ Inhibition} = [(\beta\text{-hematin}_0 - \beta\text{-hematin}_1) / \beta\text{-hematin}_0] \times 100\%$$

β-hematin$_0$ = Concentration of negative control
β-hematin$_1$ = Concentration of fraction test

RESULTS

The phytochemical analysis of all fractions from the crude extract of *Lobophytum* sp were presented in Table 1. The chemical analysis of all fractions indicated the presence of alkaloids, steroids, triterpenoids, flavonoids, saponins, terpenoids, and phenols.

Table 1. Phytochemical analysis of all fractions from the crude extract of *Lobophytum* sp

Chemical constituents	Fractions			
	n-Hexane (NH)	*n*-Butanol (NB)	Ethyl acetate (EA)	Aqueous (RA)
Alkaloids	+	-	+	+
Steroids	+	+	+	-
Flavanoids	+	+	+	-
Saponins	-	-	+	-
Terpenoids	+	+	+	-
Phenols	+	+	+	-
Tannins	-	-	-	-

+: present and -: absent

The identification of these chemical constituents showed the medicinal importance of *Lobophytum* sp. Fractions isolated by using different solvents such as *n*-hexane, ethyl acetate, *n*-butanol and water of *Lobophytum* sp. were evaluated to detect secondary metabolites. Terpenoids and steroids were found in the *n*-hexane, ethyl acetate and *n*-butanol fraction. Several reports are available on terpenoids from the soft coral genus lobophytum, which exhibited a high potential for biological activities such as anti-inflammatory, antimicrobial and antiviral activity. Most of the isolated terpenoids were diterpenoids or cembranoid compounds [26], which were found in high concentrations (up to 5% dry weight) in soft corals and possibly had chemical defense role against predators such as fish as well as microorganisms and other corals [27-28].

DPPH Radical Scavenging Assay

The DPPH radical scavenging method is the most popular and widely used method for screening the free radical scavenging ability of compounds. This assay is sensitive and easy to perform and offers a rapid way to screen radical scavenging activity compared to other methods. DPPH is a stable radical, with a strong absorption maximum at 517 (purple color) in the UV spectrum [29]. The hydrogen atom or electron donation abilities of the corresponding extracts/fractions were measured from the bleaching of the purple-colored methanol solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) [30]. The results of the free radical scavenging potential of all fractions tested by DPPH method are presented in Figure 1. The *n*-butanol fraction showed the highest DPPH radical scavenging activity (IC_{50} = 150 µg/mL) compared to other fractions. This assay provides information on the reactivity of different fractions with a stable free radical.

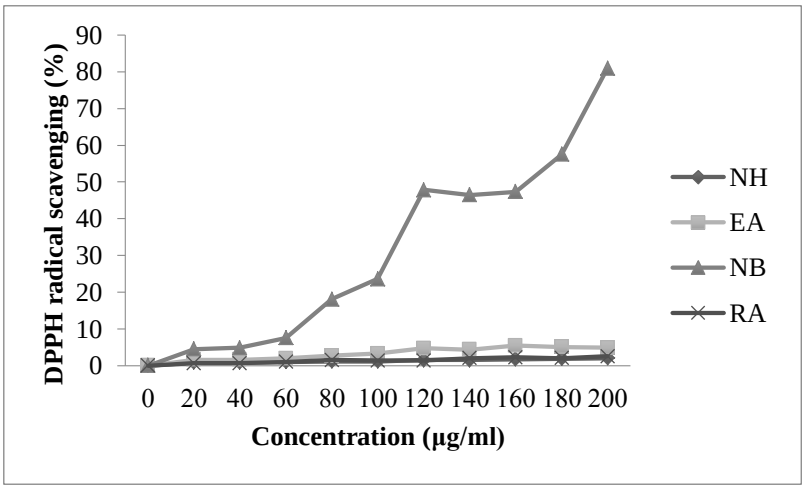

Figure 1. Radical scavenging potential of fractions by DPPH method at different concentrations (µg/mL)

Brine shrimp lethality bioassay

The brine shrimp lethality bioassay was used to predict the cytotoxic activity [31-33] of the *n*-hexane, ethyl acetate, *n*-butanol and aqueous fractions from the crude extracts. For the experiment, 3 mg of each of the extracts was dissolved in DMSO and solutions of varying concentrations (50, 100 and 200 µg/mL) were obtained by the serial dilution technique using simulated seawater. The solutions were then added to the pre-marked vials containing 10 live brine shrimp nauplii in 5 ml simulated seawater. After 24 h, the vials were inspected using a magnifying glass and the number of surviving nauplii in each vial was counted [34]. Plotting the log of concentration (log C) versus the percent mortality (% Mortality) for all test samples showed an approximate linear correlation (Figure 2). From the graphs, the median lethal concentration (LC_{50}), the concentrations at which 50% mortality of brine shrimp nauplii occurred were determined.

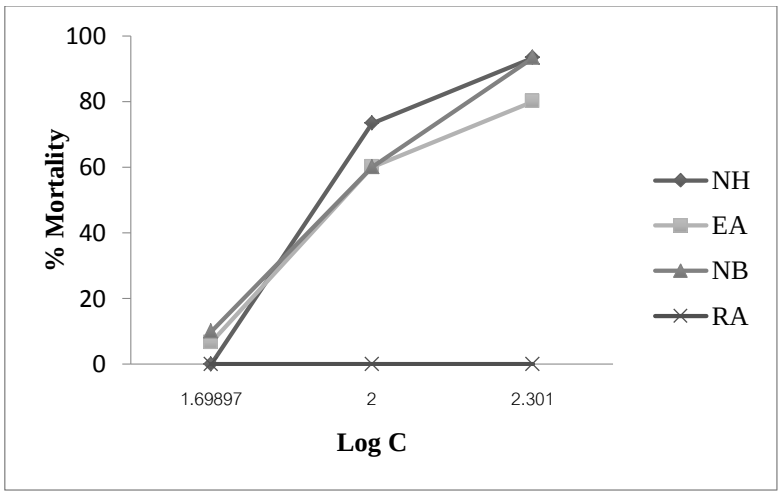

Figure 2. Plot of log concentration of *n*-hexane (NH) ethyl acetate (EA), *n*-butanol (NB) and aqueous (RA) fraction versus percent of shrimp mortality after 24 h of exposure.

The LC_{50} values of the *n*-hexane, ethyl acetate and *n*-butanol crude extract of *Lobophytum* sp. (Table 2 and 3) were found to be 123, 109 and 93 µg/mL, respectively. The degree of lethality was directly proportional to the concentration of the extract. Maximum mortalities (93.33%) were observed at a concentration of 200 ppm in both *n*-hexane and *n*-butanol (Table 2). From the LC_{50} values, it can be concluded that the *n*-butanol fraction has more potent cytotoxic compounds than the other fractions. Moreover, the crude extract or fractions resulting in LC_{50} values less than 100 µg/mL were considered significantly active and indicated the presence of potent bioactive compounds for further investigation.

Table 2. Effect of *n*-hexane, ethyl acetate, *n*-butanol and aqueous fraction of the crude extract of *Lobophytum* sp., on brine shrimp

Fraction	Concentration (µg/mL)	Log C	% Mortality	Probits	LC_{50} (µg/mL)
NH	50	1.699	0	0	
	100	2	73.33	5.61	123.069
	200	2.301	93.33	6.48	
EA	50	1.699	6.667	3.45	
	100	2	60	5.25	109.409
	200	2.301	80	5.84	
NA	50	1.699	10	3.72	
	100	2	60	5.25	92.742
	200	2.301	93.33	6.48	
RA	50	1.699	0	0	
	100	2	0	0	0
	200	2.301	0	0	

Table 3. The result cytotoxic activity of *n*-hexane (NH), Ethyl Acetate (EA), *n*-butanol (NH) and aqueous (RA) fraction on brine shrimp

Fraction	**LC_{50} (µg/mL)**	**Regression equation**	**R^2**
NH	123.069	y = 10.76x – 17.49	0.848
EA	109.409	y = 3.969x – 3.093	0.921
NB	92.742	y = 4.584 – 4.018	0.996
RA	0	0	0

Heme polymerization inhibitory activity assay

Heme polymerization is a mechanism in changing Ferriprotoporfirin IX (FPIX) which is toxic to Plasmodium. Heme polymerization occurs within the food vacuole of *P. falciparum*. FPIX is formed when Plasmodium degrade hemoglobin as a source of nutrients. FPIX is polymerized into hemozoin, a non-toxic malarial pigment. A polymer identical to haemozoin, β-hematin, can be obtained in vitro from haematin at acidic pH, appears to retain the chemical, spectroscopic and biological properties of the native pigment [35]. The heme polymerization inhibitory activity of a compound is directly related to its potential as an antimalarial [36]. Among all fractions, the ethyl acetate and aqueous fractions were the most active fractions in inhibiting heme polymerization with IC_{50} values of 11.7 and 12 µg/mL. When compared with the IC_{50} value of the positive control (chloroquine), the ethyl acetate (EA) had a lower value which showed that the activity of EA on heme polymerization inhibition was greater than chloroquine. According to Baelsman et al. [37], a compound could be considered to have heme polymerization inhibitory activity if it has heme polymerization inhibitory IC_{50} values smaller than the limit of chloroquine diphosphate, (37.5 mM or 12 mg/mL). Thus, the ethyl acetate and aqueous fractions displayed heme polymerization inhibitory activity.

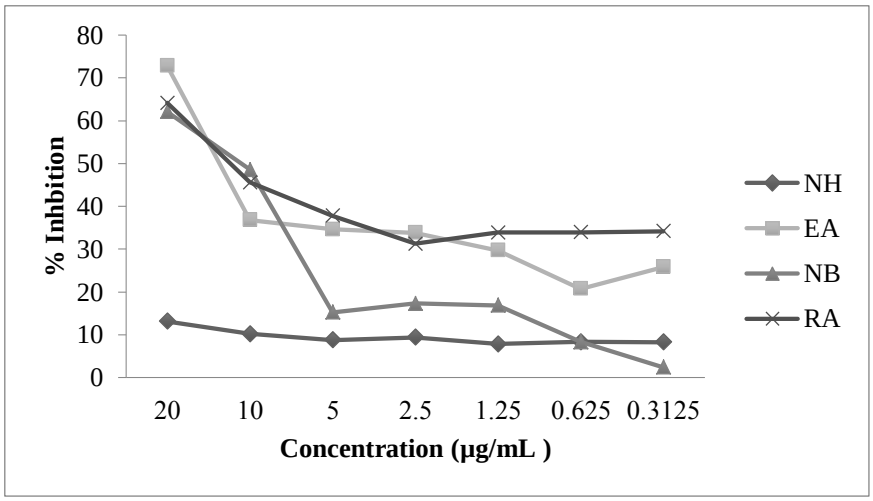

Figure 3. Heme polymerization inhibitory activity assay of fraction from the crude extract of *Lobophytum* sp.

CONCLUSIONS

This study provides information on antioxidant, antibacterial and antiplasmodium activities as well as the cytotoxicity of all fractions from the crude extract of *Lobophytum* sp. Among all fractions, the *n*-butanol fraction showed moderate antioxidant activity and cytotoxicity with IC_{50} values of 150 and 93 µg/mL, respectively. The ethyl acetate, *n*-butanol and aqueous fractions exhibited heme polymerization inhibitory activity with IC_{50} values of 11.7, 14.3 and 12 µg/mL, respectively. This is a new report of antiplasmodium activity substances derived from *Lobophytum* sp. The chemical analysis of all fractions indicated the presence of alkaloids, steroids, triterpenoids, flavonoids, saponins, terpenoids and phenols. alkaloids, steroids, triterpenoids, flavonoids, saponins, terpenoids and phenols.

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REFERENCES

1. Blunt JW, Copp BR, Keyzers RA, Munro MH, Prinsep MR. 2013. Marine natural products. Nat. Prod. Rep. 30, 237–323.
2. We WC, Sung PJ, Duh CY, Chen BW, Sheu JH, Yang NS. 2013. Anti-inflammatory activities of natural products isolated from soft corals of Taiwan between 2008 and 2012. Mar. Drugs. 11, 4083-4126.
3. Putra MY**,** Ianaro A, Panza E, Bavestrello G, Cerrano C, Fattorusso E, Taglialatela-Scafati O. 2012. Sinularioside, a triacetylated glycolipid from the Indonesian soft coral Sinularia sp., is an inhibitor of NO release. Bioorg. Med. Chem. Lett. 22, 2723–2725.
4. Bonnard I, Jhaumeer-Laulloo SB, Bontemps N, Banaigs B, Aknin M. 2010. New lobane and cembrane diterpenes from two Comorian soft corals. Mar. Drugs. 8, 359–372.
5. Wanzola M, Furuta T, KohnoY, Fukumitsu S, Yasukochi S, Watari K, Tanaka C, Higuchi R, MiyamotoT. 2010. Four new cembrane diterpenes isolated from an Okinawan soft coral Lobophytum crassum with inhibitory effects on nitric oxide production. Chem. Pharm. Bull. 58, 1203–1209.
6. Cheng SY, Wen ZH, Chiou SF, Hsu CH, Wang SK, Dai CF, Chiang MY, Duh CY. 2008. Durumolides A–E, anti-inflammatory and antibacterial cembranolides from the soft coral Lobophytum durum. Tetrahedron. 64, 9698–9704.
7. Zhang W, Krohn K, Ding J, Miao ZH, Zhou XH, Chen SH, Pescitelli G, Salvadori P, Kurtan T, Guo YW. 2008. Structural and stereochemical studies of α-methylene-γ-lactone-bearing cembrane diterpenoids from a South China Sea soft coral Lobophytum crassum. J. Nat. Prod. 71, 961–966.

8. Chao CH, Wen ZH, Wu YC, Yeh HC, Sheu JH. 2008. Cytotoxic and anti-inflammatory cembranoids from the soft coral Lobophytum crassum. J. Nat. Prod. 71, 1819–1824.
9. Chen SH, Huang H, Guo YW. 2008. Four new cembrane diterpenes from the Hainan soft coral Lobophytum sp. Chin. J. Chem. 26, 2223–2227.
10. Tseng YJ, Wen ZH, Hsu CH, Dai CF, Sheu JH. 2011. Bioactive cembranoids from the Dongsha Atoll soft coral Lobophytum crassum. Bull. Chem. Soc. Jpn. 84, 1102–1106.
11. Quang TH, Ha TT, Minh CV, Kiem PV, Huong HT, Ngan NTT, Nhiem NX, Tung NH, Tai BH, Thuy DTT Song SB, Kang HK, Kim YH. 2011. Cytotoxic and anti-inflammatory cembranoids from the Vietnamese soft coral Lobophytum laevigatum. Bioorg. Med. Chem. 19, 2625–2632.
12. Hong JY, Boo HJ, Kang JI, Kim MK, Yoo ES, Hyun JW, Koh YS, Kim GY, Maeng YH, Hyun, CL, Chang wY, Kim YH, Kim YR, Kang HK. 2012. (1S,2S,3E,7E,11E)-3,7,11,15-cembratetraen-17,2-olide, a cembrenolide diterpene from soft coral Lobophytum sp., inhibits growth and induces apoptosis in human colon cancer cells through reactive oxygen species generation. Biol. Pharm. Bull. 35, 1054–1063.
13. Cheng SY, Chen PW, Chen HP, Wang SK, Duh CY. 2011. New cembranolides from the Dongsha Atoll soft coral Lobophytum durum. Mar. Drugs. 9, 1307–1318.
14. Lin ST, Wang SK, Duh CY. 2011 Cembranoids from the Dongsha Atoll soft coral Lobophytum crassum. Mar. Drugs. 9, 2705–2716.
15. Hegazy MEF, Su JH, Sung PJ, Sheu JH. 2011. Cembranoids with 3,14-ether linkage and a secocembrane with bistetrahydrofuran from the Dongsha Atoll soft coral Lobophytum sp. Mar. Drugs. 9, 1243–1253.
16. Kao CY, Su JH, Lu MC, Hwang TL, Wang WH, Chen JJ, Sheu JH, Kuo YH, Weng CF, Fang LS, Wen ZH, Sung PJ. 2011. Lobocrassins A–E: New cembrane-type diterpenoids from the soft coral Lobophytum crassum. Mar. Drugs. 9, 1319–1331.
17. WangSK, Duh CY. 2012 New cytotoxic cembranolides from the soft coral Lobophytum michaelae. Mar. Drugs. 10, 306–318.
18. Zhao M, Yin J, Jiang W, Ma M, Lei X, Xiang Z, Dong J, Huang K, Yan P. 2013. Cytotoxic and Antibacterial Cembranoids from a South China Sea Soft Coral, *Lobophytum* sp. Mar. Drugs. 11, 1162-1172.
19. YamadaK, Ryu K, Miyamoto T, Higuchi R. 1997. Bioactive terpenoids from octocorallia. 4. Three new cembrane-type diterpenoids from the soft coral *Lobophytum schoedei*. J. Nat. Prod. 60, 798–801.
20. Uchio Y, Eguchi S, Kuramoto J, Nakayama M, Hase T. 1985. Denticulatolide, an ichthyotoxic peroxide-containing cembranolide from the soft coral *Lobophytum denticulatum*. Tetrahedron Lett. 26, 4487–4490.
21. Harborne JB. 1987. Phytochemical Methods. Chapman and Hall Ltd, London.
22. Shekhar TC, Anju G. 2014. Antioxidant Activity by DPPH Radical Scavenging Method of *Ageratum conyzoides* Linn. Leaves. A. J. Ethno. 1 (4), 244-249.
23. Koleva II, van Beek TA, Linssen JP, de Groot A, Evstatieva LN.2002. Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. Phytochem. Anal. 13, 8-17.
24. Achola KJ, Munenge RW. 1998. Bronchodilating and uterine activities of Ageratum conyzoides extract. Pharm. Bio. 36(2), 93-96.
25. Basilico N, Pagani E, Monti D, Olliaro P, Taramelli D. 1998. A microtitre-based method for measuring the haem polymerization inhibitory activity (HPIA) of antimalarial drugs. J. Antimicrob. Chemother. 42 (1), 55–60.
26. Blunt JW, Copp BR, Keyzers RA, Munro MH, Prinsep MR. 2012. Marine natural products. J. Nat Prod Rep. 29,144-222.
27. Gross H, Kehraus S, Nett M, König GM, Beil W, Wright AD. 2003. New cytotoxic cembrane based diterpenes from the soft corals *Sarcophyton cherbonnieri* and *Nephthea* sp. Org Biomol Chem. 1, 944-949.
28. Coll JC. 1992. The chemistry and chemical ecology of octocorals (Coelenterata, Anthozoa, Octocorallia. Chem. Rev. 92, 613-631.
29. Phang CW, Malek SNA, Ibrahim H, Wahab NA. 2011. Antioxidant properties of crude and fractionated extracts of *Alpinia mutica* rhizomes and their total phenolic content. Afr. J. Pharm. Pharmacol. 5(7). 842-852.
30. Rauf A, Uddin G, Khan H, Siddiqui BS, Arfan M, Yousuf M, Hussain A. 2014. Analgesic and antioxidant activity of crude extracts and isolated fractions of aerial parts of Hedera helix L. JSM chem. 2(2), 1012.
31. Meyer BN, Ferrigni NR, Putnam JE, Jacobsen LB, Nichols DE, McLaughlin JL. 1982. Brine shrimp: A convenient general bioassay for active plant constituents. Planta Medica. 45, 31–34.

32. McLaughlin JL, Rogers LL. 1999. The use of biological assays to evaluate botanicals. Drug. Inf. J. 32, 513.
33. Apu AS. Muhit MA, Tareq SM, Pathan AH, Jamaluddin ATM, Ahmed M. 2010. Antimicrobila activity and Brine shrimp lethality bioassay of the leaves extract of *Dillenia indica* Linn. J. Young Pharm. 21(1), 50-53.
34. Middleton P, Stewart F, Al-Qahtani S, Egan P, O'Rourke C, Abdulrahman A, Byres M, Middleton M, Kumarasamy Y, Shoeb M, Nahar L, Delazar A, Sarker SD, et al. 2005. Antioxidant, antibacterial activities and general toxicity of *Alnus glutinosa, Fraxinus excelsior* and *Papaver rhoeas*. Iranian J. Pharma. Res. 2: 81–86.
35. Bassilico N, Pagani E, Monti D, Olliaro P and Taramelli D. A. 1998. Microtitre based Method for Measuring the Haem Polimerization Inhibitory Activity (HPIA) of Antimalarial Drugs. J. Antimicrob. Chemother. 55-60
36. Turaley R, Susidarti RA, Wijayanti MA. *In vivo* Antiplasmodial of the most active fraction and its compound of Kapur Leaves (*Harmsiopanax aculeatus* Harms) Extract against *Plasmodium berghei*. Tropical Med. J. 1 (2). 131-140.
37. Baelmans, R., Deharo, E., Muñoz, V., Sauvain, M., and Ginsburg, H. 2000. Experimental conditions for testing the inhibitory activity of chloroquine on the formation of beta-hematin. Exp. Parasitol., 96 (4), 243–248.

Optimization of microwave-assisted extraction of anti-oxidant compound from coffee berry pulp using response surface methodology

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ABSTRACT

Coffee is one of the most widely consumed beverages in the world. During the coffee beans production process, the coffee berry pulp (CBP) was discarded as the main remaining wastes. CBP exhibits various biological properties such as antibacterial, antioxidant, and anticarcinogenic activities. This study aimed to investigate the optimum condition for extract the antioxidant compounds from the remaining waste of coffee beans production by using response surface methodology (RSM). A central composite design was used to monitor the effects of extraction solvent (0 – 80% ethanol in water), liquid-to-solid ratio (10:1 – 30:1) and power of microwave (180 – 900 watt) on antioxidant activity. The antioxidant property was determined by using ferrous reducing power (FRAP) assay. The optimum extraction condition of reducing component was obtained as 39.45% ethanol with 22:1 liquid-to-solid ratio by microwave-assisted with 540 watt showed the reducing power at 0.472 mg AAE/ml. A triplicate experiment was set up to confirmation of the value at optimal extraction condition. The experimental values agree with the predicted with a relative error less than 5%. The results indicated that this condition could be used to extract the antioxidant compounds from coffee berry pulp for utilizing in foods and cosmetic applications.

Keywords: Coffee berry pulp, Microwave assisted, Reducing power, Response surface methodology

INTRODUCTION

Coffee berry is one of economic plant in Chiang Rai, Thailand. During coffee beans production process, ripeness coffee berry was harvested and soaked in water for 24 hours for eliminated CBP which is the main remaining waste. Then, the seed was further subjected to roasting process. Therefore, CBP should be reused for add-up value and consequently reducing the environmental problem of waste. The main component in CBP is phenolic compounds which is the most common water-soluble antioxidant compounds [1]. The antioxidant activities of compounds were attributed to their redox properties, which allow them to act as reducing agents, hydrogen donators and singlet oxygen quenchers, as well as their metal chelating abilities [2].

There are many factors such as solvent type, solvent concentration, extraction time, temperature, liquid-to-solid ratio and particle size that may significantly influence the extraction efficacy. Various novel extraction techniques have been developed for extraction of some active compounds from plant. Microwave-assisted extraction (MAE) is a relatively new extraction technique that combines microwave and traditional solvent extraction. Application of microwaves for heating the solvents and plant tissues in extraction process can increases the kinetic of extraction [3]. Response surface methodology (RSM) is an effective statistic technique for optimizing extraction processes [4], which influenced by several factors. RSM not only defines the effects of the independent variables, but also generates a mathematical model [5].

The aim of this study was to derive the optimum condition for microwave-assisted extraction of reducing compounds from coffee berry pulp by RSM. Power of microwave, ethanol concentration, and liquid-to-solid ratio were designed as parameters to optimize the maximum of reducing content.

MATERIALS AND METHODS

Plant materials

Coffee berry pulp (CBP) was obtained as waste product from a manufacturing of coffee bean production, Chiangrai, Thailand. The sample was dried in air at 40 °C for 24 hours and ground to powder (0.5 mm). CBP (1 g) was extracted with aqueous ethanol in a designed concentration (0, 40 and 80 % v/v), the ratio of liquid-to-solid material (10:1, 20:1 and 30:1) and power of microwave (180, 540, 900 watt) at room temperature for 2 hours.

Determination of ferrous reducing power activity

Reducing power of sample were determined by the modified method of Thakam & Saewan [6]. Each 25 µl of sample or ascorbic acid (a positive control), 25 µl of 0.1 M phosphate buffer (pH 7.2) and 50 µl of 1% potassium ferricyanide (w/v) were added to 96-well microplate. After incubation at 37ºC for 60 minutes, 25 µl of 10% trichloroacetic acid (w/v) and 100 µl of DI water were added. Then, the absorbance was measured at 700 nm (A1). After 25 µl of 0.1% ferric chloride (w/v) was added to the mixture and the absorbance was measured again (A2). The reducing power of samples was determined by compared with the standard ascorbic acid calibration curve and expressed as ascorbic acid equivalents (mg AAE/mL).

Experimental design and statistical analyzes

Response surface methodology (RSM) with Box-Behnken experiment was applied for determining optimal condition for extract reducing compound from CBP at 3 levels of 3 factors with 16 runs, from 12 factorial experiments and 4 central-points performed to estimate the errors. Microwave power (X_1), ethanol concentration (X_2) and liquid-to-solid ratio (X_3) were independent variables studied to optimize antioxidant content. The responses function (Y) was partitioned in to linear, quadratic and interactive components. The variables were coded according to the following equation:

$$Y = b_0 + \sum_{i=1}^{3} b_i X_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{i=1}^{2} \sum_{m=i+1}^{3} b_{im}\, x_i X_m$$

Where Y is the dependent variable
b_0 is the model constant of the intercept
b_i, b_{ii} and b_{im} are the model coefficients for the linear, quadratic and interaction term, respectively.
X_i is independent variable

Analysis of the experimental design data and calculation of predicted responses were carried out using Design Expert software (Version 7.1.6, Stat-Ease, Inc., USA). The model adequacies were checked in terms of the R^2 values and adjusted R^2. Analysis of variance (ANOVA) was employed to determine the significance of the models. Verification of optimized conditions and predicted values were done in triplicate to confirm the validity of the model.

RESULTS

The reducing power assay determines the electron-donating capacity of an antioxidant. The presence of reducer causes the conversion of the Fe^{3+}/ferricyanide complex (yellow color) to the ferrous form (green and blue color) may serve as a significant indicator of its antioxidant capacity. The antioxidant activity has been reported to be concomitant with development of reducing power [2]. The extraction of reducing component from CBP was optimized through the RSM approach. The effect of microwave power (180, 540 and 900 watt), concentrations of ethanol in water (0, 40 and 80%) and liquid-to-solid ratios (10:1, 20:1 and 30:1) on reducing power was investigated. The result showed that the best combination of response function for antioxidant extraction was 540 watt, 40% ethanol and 20:1 ratio as shown in Table 1. The microwave power (X_1) and ethanol concentration (X_2) was factor that highly affect to reducing power activity, while the liquid-to-solid ratio (X_3) were not affect to antioxidant activity. The interaction between X_1 and X_2 has significantly effect on reducing power. While, interaction between X_1 and X_3 and X_2 and X_3 did not affect to reducing power. The quadatic of X_1^2 and X_2^2 showed higher effect on reducing power than the quadatic of X_3^2. (Table 2).

Model summary statistics output showed that quadratic model gave the highest value of the R^2 and adjusted R^2 when compared to the other models. For quadratic versus 2FI, the *P* Value obtained was less than 0.0005 which shows significance. So the quadratic model was selected to analysis of ANOVA for the regression equation of reducing power. The value of R^2 was 0.9947 which relatively high value which implies that more than 95% of experimental data can be explained by the model. The adjusted R^2 value corrects the R^2 value which was 0.9867. The results indicated high correlation between observed and the predict value. A very low value of coefficient of variance (0.71 %) clearly indicated very high degree of precision and reliability of the experimental values. Moreover, the value of residuals was greater than -2 and less than 2 which showed smaller residuals. The data showed that the residuals followed the fitted line fairly closely with the outline that mean its followed normal distribution well as majority of the data points.

The RSM was successfully employed to optimize condition of CBP extraction. The optimal condition was performed by using numerical optimization. The Design Expert software used searches for a combination of factor levels that simultaneously satisfy the requirements placed on each responses and factor. The optimal values of the selected variables were obtained by solving the regression equation. The reducing power was obtained by the following equation:

$$Ferrous\ reducing\ power\ (mg\ AAE/mL) = +0.29 + 3.37 \times 10^{-4}X_1 + 1.66 \times 10^{-3}X_2 + 3.74 \times 10^{-3}X_3 - 2.78 \times 10^{-7}X_1X_2 - 6.25 \times 10^{-7}X_1X_3 + 3.13 \times 10^{-6}X_2X_3 - 2.57 \times 10^{-7}X_1^2 - 1.36 \times 10^{-5}X_2^2 - 8.50 \times 10^{-5}X_3^2$$

After calculation by Design Expert software, the optimal conditions of antioxidant extraction was 39.45% ethanol with 22:1 liquid-to-solid ratio by microwave-assisted with 540 watt showed the highest reducing power 0.472 mg AAE/ml. Three-dimensional response surface plots are presented in Figure 1. The experimental values agree with the predicted from the regression model with a relative error less than 5%. The verification value for reducing power obtained within 95% of predicted values which clearly showed that the model can be used to optimize reducing compound extraction from CBP.

Table 1. Responses surface design for combination of the independent variables and experiment data

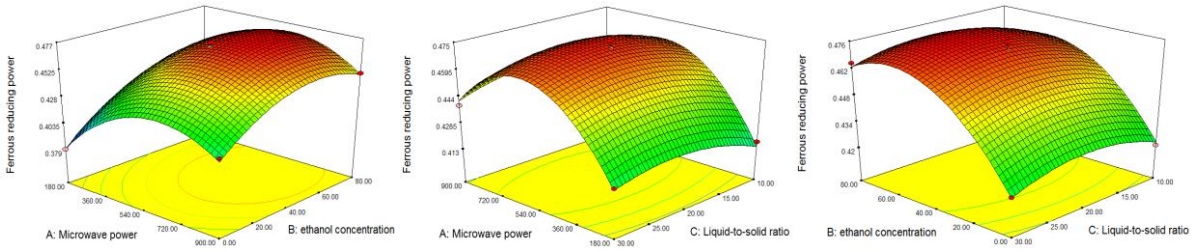
Treatment	Independent variables			Ferrous reducing power (mg AAE/mL)
	Microwave power (X_1)	Ethanol concentration (X_2)	Liquid-to-solid ratio (X_3)	
1	180	0	20	0.379
2	180	40	10	0.416
3	180	40	30	0.421
4	180	80	20	0.424
5	540	0	10	0.420
6	540	0	30	0.422
7	540	40	20	0.471
8	540	40	20	0.470
9	540	40	20	0.470
10	540	40	20	0.475
11	540	80	10	0.458
12	540	80	30	0.465
13	900	0	20	0.417
14	900	40	10	0.443
15	900	40	30	0.439
16	900	80	20	0.446

Table 2. Analysis of variance (ANOVA) for the regression equation of reducing power

Source	Sum of squares	df	Mean square	F Value	P Value	Remarks
Model		1.378E-003	1	1.378E-003	< 0.0001	***
X_1	3.003E-003	1	3.003E-003	304.11	< 0.0001	***
X_2	0.30	1	0.30	285.48	< 0.0001	***
X_3	0.250E-005	1	1.250E-005	1.27	0.3035	
X_1X_2	6.400E-005	1	6.400E-005	6.48	0.0437	*
X_1X_3	2.025E-005	1	2.025E-005	2.05	0.2021	
X_2X_3	6.250E-006	1	6.250E-006	0.63	0.4566	
X_1^2	4.422E-003	1	4.422E-003	447.82	< 0.0001	***
X_2^2	1.892E-003	1	1.892E-003	191.62	< 0.0001	***
X_3^2	2.890E-004	1	2.890E-004	29.27	0.0016	**
Residual	5.925E-005	6	9.875E-006			
Lack of fit	4.225E-005	3	1.408E-005	2.49	0.2371	

$R^2 = 0.9947$ $R^2_{Adj} = 0.9867$ CV % = 0.71

* significant at 95%; ** significant at 99%; *** significant at 99.9%;



(a) (b) (c)

Figure 1. Response surface graphs for the effects of microwave power, ethanol concentration and liquid-to-solid ratio on total antioxidant content: (a) microwave power (X_1) and ethanol concentration (X_2); (b) microwave power (X_1) and liquid-to-solid ratio (X_3); (c) ethanol concentration (X_2) and liquid-to-solid ratio (X_3).

A triplicate experiment was set up to validate with the optimized condition. Ferrous reducing power of the coffee berry pulp extract under optimal condition was also investigated. The experimental values (0.458± 0.021 mg AAE/ml) agree with the predicted (0.472 mg AAE/ml) from the regression model with a relative error less than 5%. The verification value of ferrous reducing power activities obtained is within 95% of predicted values which clearly showed that the model can be used to optimize the extraction of reducing compound from coffee berry pulp.

CONCLUSIONS

The RSM was successfully employed to optimize the extraction and several experimental parameters (powers of microwave, ethanol concentration, and liquid-to-solid ratio) have been evaluated. The results showed that the operation microwave power, ethanol concentration showed significant effects on extraction of reducing compounds from coffee berry pulp. The best combination of response function for antioxidant extraction was 39.45% ethanol with 22:1 liquid-to-solid ratio by microwave-assisted with 540 watt. The results from this study are helpful for utilization and extraction of important phytochemicals from of coffee berry pulp.

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REFERENCES

1. Macheix JJ, Fleuriet A. 1998. Antioxidant acids in fruits. Rice-Evans CA, Packer L, eds. Flavonoids in Health and Disease. New York: Marcel Dekker, Inc., 35 - 59.
2. Chanda S, Dave R. 2009. In vitro models for antioxidant activity evaluation and some medicinal plants possessing antioxidant properties: An overview. Afr. J. Microbiol. Res. 3, 981□996.
3. Delazar A, Nahar L, Hamedeyazdan S, Sarker SD. 2012. Microwave-assisted extraction in natural products isolation. Methods Mol. Biol. 864, 89 - 115.
4. Bezerra MA, Santelli RE, Oliveira EP, Villar LS, Escaleira LA. 2008. Response surface methodology (RSM) as a tool for optimization in analytical chemistry. Talanta. 76, 965 - 977.
5. Liu W, Yu Y, Yang R, Wan C, Xu B, Cao S, 2010. Optimization of total flavonoid compound extraction from Gynura medica leaf using response surface methodology and chemical composition analysis. Int. J. Mol. Sci. 11, 4750 - 4763.
6. Thakam A, Saewan N. 2012. Chemical composition of essential oil and antioxidant activities of *Curcuma petiolata* Roxb. rhizomes. Adv. Mat. Res. 506, 393 - 396.
7. Ballesteros L, Teixeira J, Mussatto S. 2014. Selection of the solvent and extraction conditions for maximum recovery of antioxidant antioxidant compounds from coffee silverskin. Food Bioprocess Tech. 7, 1322 - 1332.
8. Kreicbergs V, Dimins F, Mikelsone V, Cinkmanis I, 2011. Biologically active compounds in roasted coffee. 6th Baltic Conference on Food Science and Technology. Jelgava, Latvia, 110 - 115.

Extraction of tyrosinase from Thai edible mushrooms by aqueous two-phase system

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ABSTRACT

Tyrosinases from *Auricularia auricular, Thaeogyroporus porentosus* and *Volvariella vovacea* were extracted by using aqueous two-phase system (ATPS). Effects of polyethylene glycol and salt were investigated. Tyrosinase was expected to partitioning to the PEG rich top phase. The *T. porentosus* provided the higher tyrosinase activity than *A. auricularia* and *V. volvacea*. The highest tyrosinase activity of 110.8% with 2.6 fold purification was obtain from the *T. porentosus* crude extract in which the condition of 18% PEG-2000 and 14% $MgSO_4$. The ATPS condition of 21% PEG-6000 and 14% $MgSO_4$ provided the highest yield of tyrosinase from *A. auricularia* at 106.03%, while the condition of 18% PEG-6000 and 22% $MgSO_4$ showed the highest enzymatic yield (49.6%) from the *V. volvacea*. Due to the highest tyrosinase obtained, the top phase of the *T. porentosus* was further determined its optimal pH and temperature. The tyrosinase from *T. porentosus* showed the highest activity at pH 6-8 and temperature at 60°C. This study showed preliminary information of tyrosinase extraction and its property for further industrial application of hair darkening product.

Keywords: Aqueous two-phase system, *Auricularia auricular*, *Thaeogyroporus porentosus,* Tyrosinase, *Volvariella volvacea*

INTRODUCTION

Tyrosinase, also known as polyphenol oxidase, is a copper-containing enzyme, which is widely distributed in microorganisms, animals and plants, responsible for melanin production in mammal and enzymatic browning in plant. Tyrosinase has been proposed to desire the method in research of tyrosinase inhibitors or depigment agents in cosmetic field. Its activity has attracted interest for use in the synthesis of L-DOPA, a preferred drug for the treatment of Parkinson's disease [8]. It has wide variety of useful application, such as treatment of aqueous phenols in waste water [4]. Biphasic systems formed by two polymers or a polymer and a salt in water can be used for separation of cells, membranes, viruses, protein, nucleic acid and other biomolecules. The partitioning between two phases is dependent on the surface properties and conformation of the materials and also on the composition of the two-phase system. Aqueous two-phase system (ATPS) has been proven to be a useful tool for analysis of biomolecular and cellular surface and their interactions, fractionation of cell populations, product recovery in biotechnology and so forth. Because ATPS are easily scalable and also able to hold high biomass load in comparison with other separation techniques, the application that has attracted most interest so far has been the large-scale recovery of protein from crude feedstock. As chemicals constitute the major cost factor for large-scale system, use of easily recyclable phase components and the phase system generated by a single phase chemical in water are being studied [3]. Mushrooms have been well known to be great nutritional and pharmaceutical food. The mushrooms contain high amount of tyrosinase making most of them appear as dark or fast generate browning reaction. There has been commercial tyrosinase extracted from mushrooms. However, the commercial mushroom tyrosinase is expensive. Therefore this work was aimed to extract the tyrosinase from *Auricularia auricular* (Aa), *Volvariella vovacea* (Vv) and *Thaeogyroporus porentosus* (Tp).

MATERIALS AND METHODS

Crude enzyme extract

All fresh mushroom samples were cleaned with tapped water and kept in a freezer at -20°C. The sample was blended with 50 mM phosphate buffer pH 6.8 on the ratio of 1:15 (w/v). The mixture was then filtered and centrifuged. The volume of supernatants were measured, recorded and stored at -20°C for the next experiment.

Tyrosinase activity assay

Tyrosinase activity was determined by spectrophotometric method, using L-DOPA as a substrate as reported earlier [11]. Reaction mixture contained, 0.3 mL of L-DOPA solution and 1.6 ml phosphate buffer pH 6.8 and 0.1 mL of crude enzyme extract. The extract, the mixture was incubate for 15 min then measured the absorption at 475nm.

Protein determination

Protein concentration of the sample was determined by using the Bradford method [1] and BSA was used as a standard.

Aqueous two phase systems

Tyrosinase from sample mushroom was extracted by ATPS. ATPS was performed in 15 mL centrifuge tubes according to the method of Nisawang et al. [7] with slight modification. Effect of PEG and salts were studied. Fifty percentage of crude extract were used in the ATPS. The mixture was mixed thoroughly for 5 min using a vortex mixer. Phase separation was achieved by centrifugation for 15 min. The protein content in the obtain fractions was determined. The partitioning parameters were calculated as; specific activity (SA), purification factor (PF), and %Yield. The phase that gave the highest tyrosinase recovery was chosen for characterization.

Determination of pH profile

Optimum pH was determined by measuring tyrosinase activity at room temperature in the different pHs range 3-11 buffers (sodium citrate pH 3, sodium acetate pH 4, 5, phosphate buffer pH 6, 6.8, 7, Tris-HCL pH 8, 9, sodium phosphate pH 10, 11). L-DOPA was use as substrate for tyrosinase activity and incubated for 15 min then measured the absorption at 475nm.

Determination of thermal profile

Tyrosinase activity was determined at different temperatures (20-90°C) with phosphate buffer pH 6.8, L-DOPA was use as substrate for tyrosinase activity and incubated for 15 min then measured the absorption at 475 nm.

RESULTS

Tyrosinase activity obtain from three edible fungi

Crude extract was prepared by blending mushrooms powder with phosphate buffer pH 6.8 at the ratio of 1:15 (w/v).The Tp showed the highest tyrosinase activity at 3,441 U followed by the Vv.

Table 1. Total protein, total activity and specific activity of 1 g of dried edible mushrooms.

Cultivar	Volume (ml)	Total protein (mg)	Total activity (U)	Specific Activity (U/mg)
Thaeogyroporus porentosus (Tp)	5	2.385	3441.65	722.05
Volvariella vovacea (Vv)	5	3.225	983.35	152.35
Auricularia auricular (Aa)	5	0.995	158.35	79.9

Effect of PEG by ATPS

The tyrosinase partitioning by the ATPS containing different molecular weight (2000, 4000 and 6000) and concentration (15, 18 and 21% w/w) of PEG was studied. As shown in Table 2, partitioned tyrosinase strongly depended on PEG.

Table 2. Effect of PEG on partitioning of tyrosinase from mushrooms by ATPS at constant 18% $(NH_4)_2SO_4$

Mushroom extract	PEG	Specific activity	Yield (%)
Thaeogyroporus porentosus (Tp)	PEG2000-15%	413.89	70.394
	PEG2000-18%	517.94	74.82
	PEG2000-21%	466.56	67.28
	PEG4000-15%	446.38	57.44
	PEG4000-18%	418.4	53.58
	PEG4000-21%	389.17	48.56
	PEG6000-15%	515.02	65.07
	PEG6000-18%	453.931	66.03
	PEG6000-21%	477.49	64.87
Volvariella vovacea (Vv)	PEG2000-15%	202.44	43.92
	PEG2000-18%	208.06	48.65
	PEG2000-21%	215.20	53.14
	PEG4000-15%	190.69	33.77
	PEG4000-18%	186.67	40.52
	PEG4000-21%	170.26	47.06
	PEG6000-15%	203.47	37.07
	PEG6000-18%	207.53	46.74
	PEG6000-21%	239.02	51.68
Auricularia auricular (Aa)	PEG2000-15%	46.90	20.18
	PEG2000-18%	43.89	23.99
	PEG2000-21%	40.98	19.77
	PEG4000-15%	65.47	35.73
	PEG4000-18%	52.86	28.22
	PEG4000-21%	48.695	26.67
	PEG6000-15%	52.78	25.81
	PEG6000-18%	57.32	25.84
	PEG6000-21%	50.54	36.76

For the Tp, The PEG 15% provided the condition that the highest tyrosinase yield at 70.394% was obtained. Higher molecular weight of PEG at 4000-6000 gave lower tyrosinase recovery from the Tp. This result was similar to that of the Vv. The PEG 2000 also provided higher yield of enzyme than the PEG 4000-6000. In contrast to the Aa, higher MW of PEG, the higher recovery yield of tyrosinase. It can be seen that difference in tyrosinase recovery. The PEG 2000 18%, PEG 2000 21% and PEG 6000 21% showed the highest tyrosinase yield from the Tp, Vv and Aa respectively.

Effect of Salt by ATPS

The effect of types and concentrations of salts on the tyrosinase partitioning parameter are show in Table 3 Salts are frequently used in ATPS to improve the partitioning of the target molecules between the phases [5-6, 9]. The addition of salt to the aqueous PEG solution led to arrangement of ordered water molecules around the PEG molecule because of

the water structure breaking effect [6]. From the result each salt $(NH_4)_2SO_4$, $MgSO_4$ and K_2HPO_4 at each concentration (14, 18 and 22% w/w) was mix with the PEG that gave the highest yield in the above studied. $MgSO_4$ was selective to use in the system because can provided the highest activity of tyrosinase from *Thaeogyroporus porentosus*

Table 3. Effect of salts on tyrosinase partitioning from mushrooms by ATPS

Mushroom extract	PEG	Salt	Specific activity	Yield (%)
Thaeogyroporus porentosus (Tp)	PEG2000-18%	$(NH_4)_2SO_4$-14%	1683.21	85.21
		$(NH_4)_2SO_4$-18%	1605.63	69.42
		$(NH_4)_2SO_4$-22%	1661.90	56.22
		$MgSO_4$-14%	3422.00	87.89
		$MgSO_4$-18%	2064.00	79.56
		$MgSO_4$-22%	1438.55	72.01
		K_2HPO_4-14%	1003.00	57.13
		K_2HPO_4-18%	881.05	52.06
		K_2HPO_4-22%	699.51	38.33
Volvariella vovacea (Vv)	PEG6000-18%	$(NH_4)_2SO_4$-14%	337.76	36.38
		$(NH_4)_2SO_4$-18%	229.65	39.59
		$(NH_4)_2SO_4$-22%	178.28	40.75
		$MgSO_4$-14%	985.50	37.56
		$MgSO_4$-18%	644.90	35.95
		$MgSO_4$-22%	1442.00	39.27
		K_2HPO_4-14%	226.95	20.88
		K_2HPO_4-18%	206.40	26.53
		K_2HPO_4-22%	163.96	29.83
Auricularia auricular (Aa)	PEG6000-21%	$(NH_4)_2SO_4$-14%	188.89	104.20
		$(NH_4)_2SO_4$-18%	162.64	70.36
		$(NH_4)_2SO_4$-22%	100.25	85.25
		$MgSO_4$-14%	1314.45	121.25
		$MgSO_4$-18%	748.55	93.67
		$MgSO_4$-22%	1211.39	81.68
		K_2HPO_4-14%	136.05	43.36
		K_2HPO_4-18%	80.63	41.68
		K_2HPO_4-22%	84.30	62.99

Determination of pH profile

The effect of pH (3-11) on the activity of crude extract was measured and reported as a relative sigma tyrosinase activity. (Figure 1) Optimum pH for the activity of both tyrosinase was searched with L-DOPA as the substrate. It is worth to mention that at pH more than 8 the L-DOPA was usually tanned this could be due to autooxidized of L-DOPA towards alkaline also the enzyme an optimum for both tyrosinase are at higher pH values (>8.5) which were not interesting for this reaction because L-DOPA is more unstable at higher pH value (very alkaline) [2]. The pH range of 3-8 was chosen as a compromise between an optimal enzyme activity and the stability of L-DOPA The enzyme obtain from Tp and sigma tyrosinase had their optimum pH at 7, 6 in order

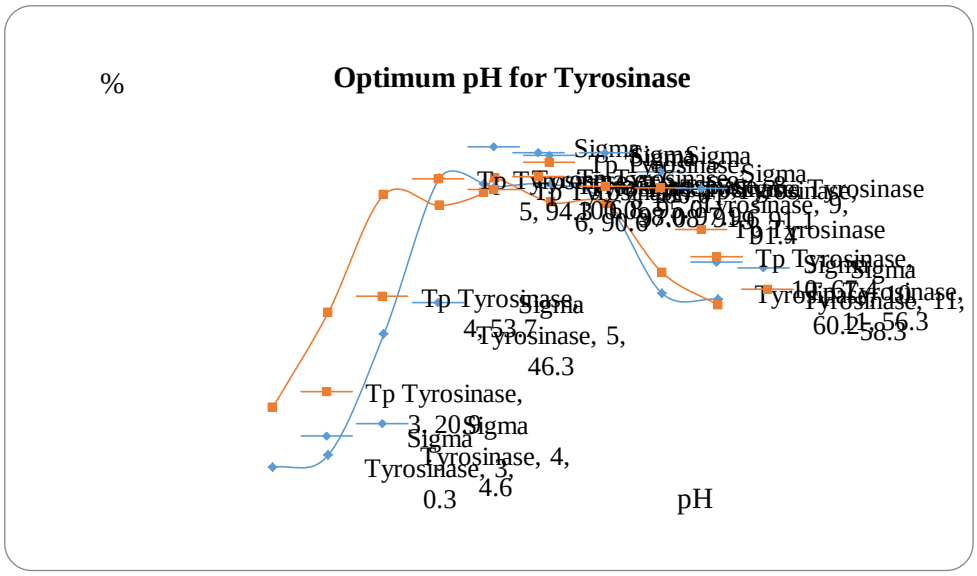

Figure 1. Effect of pH on tyrosinase activity of *Thaeogyropous porentosus* and Sigma Tyrosinase

Determination of Temperature profile

Tp tyrosinase extract and sigma tyrosinase were determined at different temperatures (20, 25, 30, 37, 40, 45, 50, 55, 60, 70, 80 and 90°C) with phosphate buffer pH 6.8, L-DOPA were use as substrate. The highest activity of Sigma tyrosinase found at 40°C and the highest activity of Sigma tyrosinase found at 60°C. Temperature higher than 60°C, drastically decreased the tyrosinase activity.

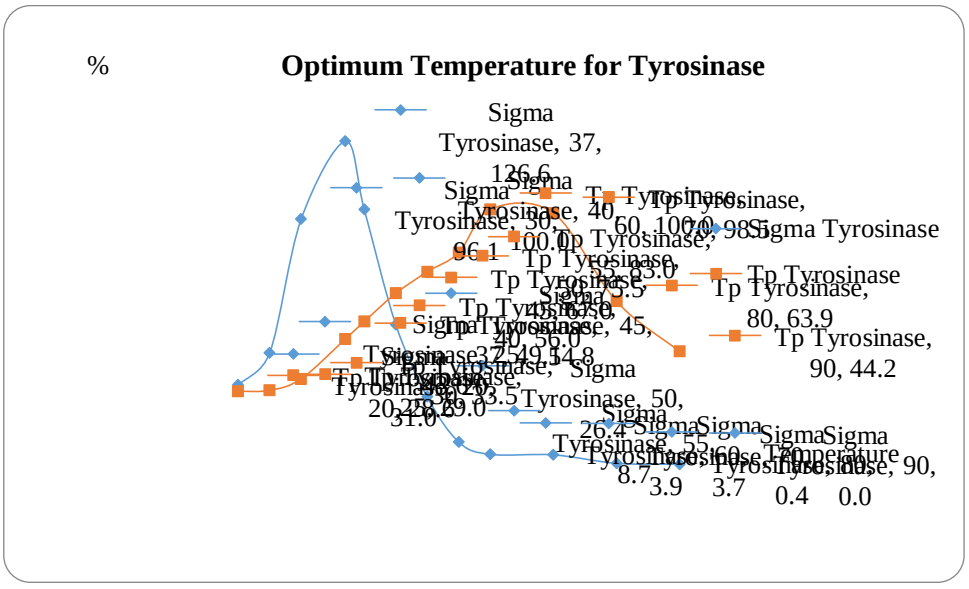

Figure 2. Effect of temperature on tyrosinase activity of *Thaeogyropous porentosus* and Sigma Tyrosinase

CONCLUSIONS

Thaeogyropous porentosus was used as the raw material for tyrosinase extract. *Thaeogyropous porentosus* is provided the highest total tyrosinase, SA, PF, and %Yield. For the extraction process, the extractants played an important role in maintaining tyrosinase from *Thaeogyropous porentosus*. Phosphate buffer solution pH 6.8 showed to be the most efficient in tyrosinase extraction from three cultivars mushroom by provide the highest activity. ATPS can be used as a primary purification step in the recovery of tyrosinase from three edible fungi. In the system of PEG - Salt, tyrosinase partitioning in greatly affected by PEG 6000 and Magnesium sulfate. Protein pattern of each edible fungi was varying due to the source of protein. Comparing with the protein pattern crude, top and bottom phase indicate that partitioning tyrosinase by using ATPS is sufficient to isolate desired enzyme into top phase and other contaminant protein partition to bottom phase.

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REFERENCES

1. Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.
2. Findrik Z, Geueke B, Hummel W, Vasic-Rack D. 2006. Modelling of L-DOPA enzymatic oxidation catalyzed by l-amino acid oxidase from *Crotalus adamanteus* and *Rhodococcus opacus*. Biochem. Eng. J., 27, 275-286
3. Hatti-Kaul R. 2001. Aqueous two-phase system: A general overview. Mol. Biotechnol.19, 269-277.
4. Ikehata K, Nicell JA. 2000. Characterization of tyrosinase for the treatment of aqueous phenol. Bioresource Technol. 74, 191-199.
5. Klomklao S, Benjakul S, Visessanguan W, Kishimaru H, Simpson BK. 2005. Partitioning and recovery of proteinase from spleen by aqueous two-phase system. Process Biochem. 3061-3067
6. Nalinnon S, Visessanguan W, Kishimura H. 2009. Partitioning of protease from stomach of albacore tuna (Thunnus alalunga) by aqueous two-phase system. Process Biochem. 44, 471-476.
7. Nisawang S, Hatti-Kaul R, Kanasawuda P. 2006. Purification of papain from Carica papaya latex: Aqueous two-phase extraction versus two-step salt precipitation. Enzyme Microb. Technol. 39, 1103-1107.
8. Pialis P, Saville BA. 1998. Production of L-DOPA from tyrosinase immobilized on nylon 6,6: enzyme stability and scale up. Enzyme Microb. Technol. 22,261-268.
9. Vissessanguan W, Benjakul S, Tanaka M. 2003. Effect of microbial transglutaminase on rheological properties of oxidized and non-oxidize natural actomyosin from two species of bigeye snapper. J. Sci. Food Agr. 83, 105-112.
10. Yamaguchi M, Hwang EM, Campbell JD. 1970. Latent o-diphenol oxidase in mushrooms (Agaricus bisporus). Can. J. Biochem. 48, 198.
11. Zhang X, William H. 1999. Purification and partial characteriszation of tyrosinase isoforms from cap flesh of Portabella mushrooms. Food Biochem. 23, 95-108.

Efficacy of Natural Products (ENP)
Full Paper

Potential antioxidant activities of extracts from proanthocyanidin rich fruits for food supplement and cosmetic

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ABSTRACT

Mulberry (*Morus alba* Linn.), Ma mao (*Antidesma thwaitesianum* Muell.) and Ma kiang (*Cleistocalyx nervosum* var. *paniala* Roxb.) are wild plant found in the northern and north-eastern area of Thailand. High contents of polyphenols and flavonoids in these fruits are known to possess strong antioxidant activity. The purposes of this study are to extract and analyze the proanthocyanidins from these fruits and verify their potential *in vitro* and *in vivo* antioxidant activities. The results showed that the supercritical fluid extract (SFE) contained significantly higher yield, total phenolic, flavonoid, and proanthocyanidin contents than those obtained from ethanol and water extract. The SFE had much higher antioxidant activities as assessed by ABTS radical cation decolorization, DPPH radical scavenging and ferric reducing antioxidant power (FRAP) assays. Further analysis using high-performance liquid chromatography with diode array and mass spectrometry detectors (HPLC-DAD/MSD) revealed the major presence of catechin. The experiment was successfully provided information of the antioxidant activities that demonstrated to reverse the cadmium effects on renal function, histological alterations, and improve several markers of oxidative stress in rats. The potential antioxidant activities of the SFE extract from these fruits can be further developed as food supplement and cosmetic.

Keywords: Fruits, Proanthocyanidins, Catechin, Qxidative stress, Cadmium

INTRODUCTION

Berries are popular in Northern Thailand for eating as a fresh fruit or as an active ingredient in the dietary supplement, cosmetic and healthy drinks [1]. Some of the Asian markets are also showing expanded interest for organically produced berry products. These fruits make good health and prevent diseases. Proanthocyanidin and flavonoid compounds are found in berries or these superfruits [2]. Proanthocyanidins are a class of polyphenols found in a variety of berries [3]. Chemically, they are oligomeric flavonoids of catechin, epicatechin and their gallic acid esters. Recently, some researchers have showed that these berries were proanthocyanidin rich fruits and possess antioxidant properties, but the photochemical contributed to their health benefits have not yet been clarified [4-6]. This study was designed to analyze the proanthocyanidin from proanthocyanin-rich fruits using HPLC-DAD/MSD, to investigate the potential of *in vitro* by ABTS, DPPH and FRAP assays and to demonstrate *in vivo* antioxidant benefit of fruit extract in healthy animal as well as in oxidative stress-induced injury animal model. Three selected plants are including *Antidesma thwaitesianum* Muell. (Ma mao; MM), *Morus alba* Linn. (Mulberry or Mon; MO) and *Cleistocalyx nervosum* var. *paniala* Roxb (Ma kiang; MK), which are tropical “berry-type” fruits cultivated and commonly consumed in fresh and widely found in Northern Thailand.

MATERIALS AND METHODS

Chemicals and Reagents

All the chemicals were analytical grade and reagents used in this study were chromatographic grade for HPLC analysis.

Plant Materials and Sample Preparation

Ripe berries fruits were obtained from Doi Kham Food Products Co., Ltd. (Royal Project, Chiang Mai, Thailand). The whole fruit was cleaned and rapidly stored at −20 °C. The pulp was subsequently isolated, weighed, Freeze-dried or lyophilized, and ground into powder. The powdered sample was kept at 4°C in an air tight container protected from light until extracted.

Supercritical CO_2 Extraction

Extraction was conducted using a laboratory scale supercritical fluid extractor unit (model SFE100, Thar Technologies Inc., PA, USA). The extraction protocol was set up according to the study of Yilmaz et al [7]. Freeze-dried fruits (100 g) were extracted using conventional solvent extractions (water and ethanol) and Supercritical Fluid Extraction (SFE) using CO_2 and ethanol as a co-solvent.

Identification and Quantification

All these extracts were examined for total phenolic content [8], total flavonoids as described by Lin and Tang [9], total proanthocyanidin contents [10], and antioxidative activities such as; ABTS Assay, DPPH Assay and FRAP Assay [11]. The modified method of Prasain et al. [12] of HPLC-DAD/MSD (Agilent Technologies, USA) was used to identify and quantify the proanthocyanidin contents. All the assays were performed in triplicate and the results were expressed as mean±SD from the three sets of observations. Comparisons were performed by one way ANOVA followed by Fisher's least significant difference (LSD) test using the SPSS 16.0 software (SPSS Inc., Chicago, IL, USA).

Cd-induced oxidative stress

Since kidney is the main target organ of Cd toxicity, the potential of *in vivo* antioxidant benefit of fruit extract in healthy animal as well as in oxidative stress-induced injury animal model [13]. This study was conducted to evaluate the possible beneficial role of Ma kiang or MK in chronic Cd exposure using a rat model of Cd-induced nephrotoxicity. Seven groups of male Wistar rats were studied: control, MK (MK fruit extract 2 g/kg, orally), Cd ($CdCl_2$ 0.2 mg/kg, i.p.), CdMK (combination of Cd and MK at 0.5, 1, 2 g/kg, respectively) and CdCT (combination of Cd and catechin 100 mg/kg). The renal function was calculated and oxidative stress parameters were composed of SOD, MDA, NO and Thiols group. Moreover, renal histology was confirmed in renal tissue.

RESULTS

The results showed the effect of extraction methods with solvent (water and ethanol) extraction and supercritical carbon dioxide extraction (SC-CO_2). The highest percent yield was found in SC-CO_2 of *C. nervosum* and the lowest was found in water extraction of *M. alba* as shown in Table 1. The percent yield of *C. nervosum* from SC-CO_2, ethanol and water extraction were 19.50 ± 0.85, 14.47 ± 0.85 and 8.60 ± 0.92 % dry wt., respectively. The percent yield of *A. thwaitesianum* from SC-CO_2, ethanol and water extraction were 17.87 ± 0.55, 11.50 ± 1.08 and 7.67 ± 0.86 % dry wt., respectively. While the percent yield of *M. alba* from SC-CO_2, ethanol and water extraction were 19.33 ± 0.67, 11.53 ± 0.91 and 7.07 ± 0.85 % dry wt., respectively. A modifier used in this study was ethanol which increased the solubility of polar solutes, induced changes in the structure of cellular matrix *via* intra-crystalline and break analyze matrix binding by competing with polar interaction between matrix and extracted compounds. Other factors were affected on optimize extraction method for amount of crude extracted such as, pressure, time and % modifiers [14].

Table 1. The percent yield of of *C.nervosum* (MK), *A.thwaitesianum* (MM), and *M.alba* (MO), from SC-CO_2, ethanol and water extraction, respectively.

Yield (%)	SC-CO_2	Ethanol	Water
C.nervosum (MK)	19.50 ± 0.85[a]	14.47 ± 0.85[b]	8.60 ± 0.92[c]
A.thwaitesianum (MM)	17.87 ± 0.55[a]	11.50 ± 1.08[b]	7.67 ± 0.86[c]
M.alba (MO)	19.33 ± 0.67[a]	11.53 ± 0.91[b]	7.07 ± 0.85[c]

Values are mean ± SD of three replicates from three independent experiments in a row with different letters indicate significant differences (p<0.05).

The results on phytochemical constituents and antioxidant abilities of SC-CO_2 extract of *C.nervosum* (MK), *A.thwaitesianum* (MM), and *M.alba* (MO) demonstrated that the percent yield, total flavonoid content and total proanthocyanidin content from SC-CO_2 were significantly higher than that of the common solvent extraction (Table 2). From all fruits, especially Ma kiang or MK extract showed significantly highest value than other fruits. SC-CO_2 extract also exhibited the greatest antioxidant properties as assessed by ABTS, DPPH and FRAP assay. The highest final yield was found in SFE of MK (0.20 g/100g dry wt). SFE of MK contained significantly higher total flavonoids (10.65 mg/100g dry wt) and proanthocyanidin (216.08 mg/100g dry wt) contents. SFE of MK also exhibited the greatest antioxidant properties as assessed by ABTS 0.60 mM trolox equivalent/g, DPPH in term of % inhibition (93.85%), and FRAP (63.02 mM trolox equivalent/g). The SC-CO_2 method was the most suitable for extraction of Ma kiang, Ma mao and Mon. it is suggested that supercritical CO_2 extraction is a promising technology for extraction and purification of antioxidant compounds from plant materials [15].

Table 2. Phytochemical constituents and antioxidant abilities of SC-CO_2 extract of *C.nervosum* (MK), *A.thwaitesianum* (MM), and *M.alba* (MO), respectively.

	MK	MM	MO
Phytochemical constituents			
Yield (%)	19.50 ± 0.85[a]	17.87 ± 0.55[a]	19.33 ± 0.67[a]
Total phenolic (g GAE/100 gDW)	14.47 ± 0.20[a]	7.05 ± 0.17[a]	3.52 ± 0.15[a]
Total flavonoid (g QE/100 gDW)	10.65 ± 0.18[a]	4.04 ± 0.18[a]	7.70 ± 0.13[a]
Total proanthocyanidin (g CE/100 gDW)	2.16 ± 0.04[a]	0.87 ± 0.01[a]	0.45 ± 0.03[a]
Catechin (g CE/100 gDW)	1.42 ± 0.01[a]	0.63 ± 0.01[a]	0.09 ± 0.01[a]
Antioxidant abilities			
ABTS (mg TE/g DW)	0.60 ± 0.01[a]	0.45 ± 0.01[a]	0.31 ± 0.01[a]
DPPH (mg TE/g DW)	9.32 ± 0.10[a]	8.22 ± 0.08[a]	5.32 ± 0.13[a]
FRAP (mg TE/g DW)	63.02 ± 1.26[a]	48.01 ± 2.14[a]	27.08 ± 1.11[a]

Values are mean ± SD of three replicates from three independent experiments in a row with different letters indicate significant differences (p<0.05).
GAE: gallic acid equivalents; QE: quercetin equivalents; CE: catechin equivalents; TE: trolox equivalents; dry wt: dry weight of the extract.

HPLC chromatographic fingerprint of polyphenol standards such as gallic acid, catechin, rutin, i-quercetin, eriodictyol, hydroquinin, quercetin, apigenin and keampferol by HPLC-DAD/MSD at wavelength 270 nm was shown in Figure 1. The results of identification and quantification were found that catechin content was highest in *A.thwaitesianum* (MM) from SC-CO_2 extraction (Figure 2). However, other substances those enhance the activity of antioxidant as catechin such as, quercetin, rutin, gallic acid and kaempferol, which might be combination reactions of catechin derivatives such as epicatechin or own catechin and change into proanthocyanidin, which have more effect of antioxidants properties [16-18].

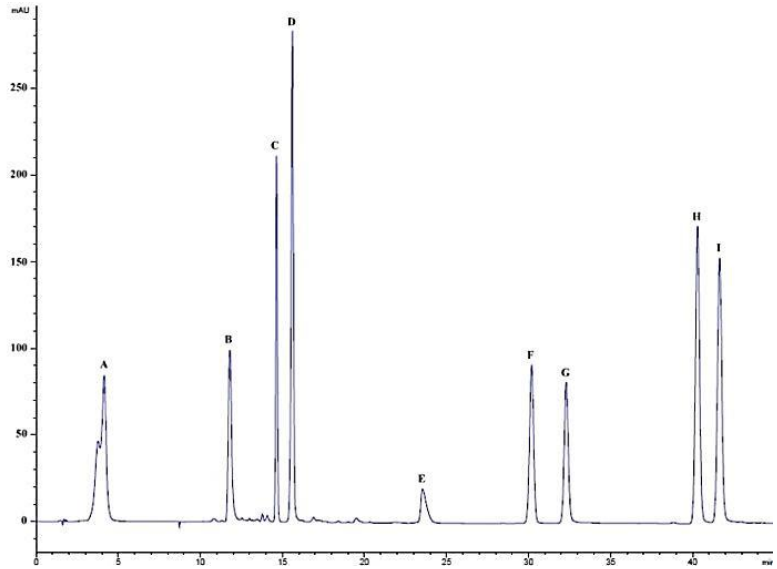

Figure 1. HPLC chromatographic fingerprint of polyphenol standards; (A) Gallic acid (B) Catechin (C) Rutin (D) i-Quercetin (E) Eriodictyol, (F) Hydroquinin (G) Quercetin (H) Apigenin (I) Keampferol at wavelength 270 nm.

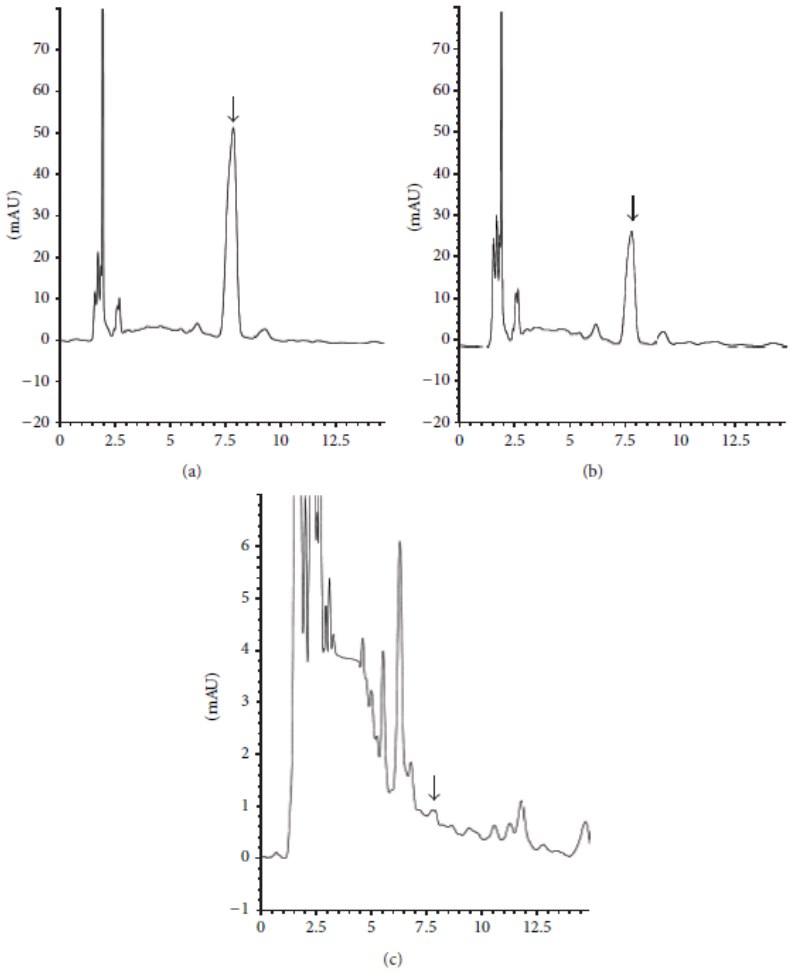

Figure 2. Chromatogram at 270 nm of the extract from *A.thwaitesianum* (MM), by (a) supercritical carbon dioxide, (b) ethanol, and (c) water extraction. Arrow indicates catechin peak at retention time 7.84 min.

In the next part of study, Ma kiang or MK was selected to further investigation for its antioxidative properties against oxidative stress in animal model with cadmium-induced nephrotoxicity. Ma kiang or MK had the ability to protect against the oxidative stress in the kidney of cadmium-induced toxicity *via* its antioxidant properties. The results were confirmed from the *in vivo* study by blood sample analysis data and kidney sample analysis data, together with histopotological alterations. The evident data were showed in animal group with received both cadmium and Ma kiang extract. Co-treatment with MK, particularly at the doses of 1 and 2 g/kg, significantly reduced these indexes of renal damage and restored all the changes of oxidative indexes (nitric oxide, malondialdehyde, thiols, superoxide dismutase, and catalase) resulting from toxic effect of cadmium. After 4 wk treatment, increased blood urea nitrogen and serum creatinine, decreased creatinine clearance, together with histopathological alterations were evident in Cd-intoxicated rats (as shown Table 3, 4). No significant changes of these parameters from the baseline controls were observed in rats received MK alone. The outcomes also provide essential information regarding health benefits of this naturally occurring antioxidant to combat other conditions elicited by redox alteration [19].

Table 3. Blood sample analysis data (BUN, serum creatinine and creatinine clearance of rat in different treatments.

Groups/Data	BUN (mg/dl)	SCr (mg/dl)	CCr (ml/min/100 g BW)
Control	19.33 ± 1.17†	0.33 ± 0.01†	0.70 ± 0.03†
MK	19.33 ± 1.50†	0.34 ± 0.04†	0.63 ± 0.06†
Cd	26.83 ± 0.95*	0.51 ± 0.03*	0.24 ± 0.02*
Cd+MK 0.5	22.17 ± 1.11$^{\dagger\#}$	0.42 ± 0.02$^{\dagger*\#}$	0.44 ± 0.15$^{*\#}$
Cd+MK 1	20.17 ± 0.70†	0.35 ± 0.03†	0.59 ± 0.06†
Cd+MK 2	18.50 ± 0.56$^{\dagger\#}$	0.33 ± 0.03$^{\dagger\#}$	0.71 ± 0.08$^{\dagger\#}$
Cd+CT	18.33 ± 0.67†	0.35 ± 0.02†	0.69 ± 0.04†

Means ± SE (n=6) in a column with symbol (†) are not significantly different with control group, ($P < 0.05$).
Means ± SE (n=6) in a column with symbol (*) are not significantly different with cadmium group, ($P < 0.05$).
Means ± SE (n=6) in a column with symbol (#) are not significantly different between Cd+MK group, ($P < 0.05$).

Table 4. Kidney samples analysis data (SOD, CAT, NO and MDA) of rat with different treatments.

Groups/Data	SOD (Unit/g KW)	CAT (Unit/g KW)	NO (μM/g KW)	MDA (μg/g KW)
Control	9.00 ± 0.35†	84.52 ± 2.24†	63.30 ± 1.97†	47.11 ± 2.47†
MK	9.05 ± 0.43†	81.16 ± 2.25†	67.51 ± 1.43†	47.57 ± 1.82†
Cd	5.13 ± 0.18*	26.14 ± 1.75*	79.58 ± 2.05*	67.04 ± 7.08*
Cd+MK 1	9.13 ± 0.78†	81.48 ± 2.37†	67.89 ± 3.55†	53.99 ± 2.46†
Cd+MK 2	9.00 ± 0.54†	80.49 ± 2.54†	63.68 ± 0.97†	50.91 ± 1.75†
Cd+CT	9.05 ± 0.49†	82.76 ± 0.68†	60.33 ± 2.61†	48.26 ± 1.60†

Means ± SE (n=6) in a column with symbol (†) are not significantly different with control group, ($P < 0.05$).
Means ± SE (n=6) in a column with symbol (*) are not significantly different with cadmium group, ($P < 0.05$).

The future for healthy nutrition and functional foods will be dominated by beverage concepts. Consumers are increasingly looking to beverages of all kinds to deliver health benefits – berries are in a good position to exploit this demand. Competition in the berry industry in value added of suitable extracts for dietary supplements. The cosmetic market is very attractive for high performance natural ingredients that are proven to be effective and safe. Berries are used as ingredients in cosmetics as an active antioxidant or color. Proanthocyanidin contents contribute to overall skin health and scavenging free radicals [20-21].

CONCLUSIONS

The results showed that the SC-CO2 extract contained significantly higher yield, total phenolic, flavonoid, and proanthocyanidin contents than those obtained from ethanol and water. It also demonstrated the greatest antioxidant activities as assessed by ABTS radical cation decolorization, DPPH radical scavenging, and ferric reducing antioxidant power (FRAP) assays. Further analysis using high-performance liquid chromatography with diode array and mass spectrometry detectors (HPLC-DAD/MSD) revealed the presence of catechin as a major phenolic compound of *Antidesma thwaitesianum* (Phyllanthaceae), with the maximum amount detected in the SC-CO2 extract. Ma kiang extract has benefit effect to prevent against Cd-induced oxidative damage in renal tissue. Meanwhile, chemical compositions of Ma kiang extract demonstrated some information of the antioxidant activities. However, the optimum dose to prevent the oxidative damage from Cd-induce remains unclear and requires further studies. Overall, the study provides evidence of the protective effect of Ma kiang extract on Cd-induce renal damage and suggests that it may be a possible therapeutic material for the treatment of Cd toxication.

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REFERENCES

1. Jansom C, Bhamarapravati B, Itharat A. 2008. Major anthocyanin from ripe berries of *Cleistocalyx nervosum* var. paniala. Thammasat Med. J. 8 (3), 364-370.
2. Hosseinian FS, Li W, Hydamaka AW, Tsopmo A, Lowry L, Friel J, Beta T. 2007. Proanthocyanidin profile and ORAC values of Manitoba berries, chokecherries, and seabuckthorn. J Agric Food Chem. 55(17), 6970–6976.
3. Szajdek A, Borowska E. 2008. Bioactive compounds and health-promoting properties of berry fruits: A review. Plant Foods. Hum. Nutr. 63,147-156.
4. Skerget M, Kotnik P, Hadolin M, Hraˇs AR, Simoniˇc M, Knezˇ Z. 2005. Phenols, proanthocyanidins, flavones and flavonols in some plant materials and their antioxidant activities. Food Chem. 89(2), 191–198.
5. Lee YA, et al. 2007, Ameliorative effects of proanthocyanidin on oxidative stress and inflammation in streptozotocin-induced diabetic rats. J. Agri. Food Chem. 55(23), 9395-9400.
6. Taheri R, Connolly BA, Brand MH, Bolling BW. 2013. Underutilized Chokeberry (*Aronia melanocarpa, Aronia arbutifolia, Aronia prunifolia*) Accessions Are Rich Sources of Anthocyanins, Flavonoids, Hydroxycinnamic Acids, and Proanthocyanidins. J. Agri. Food Chem. 61(36), 8581–8588.
7. Yilmaz EE, Ozvural EB, Vural H. 2011. Extraction and identification of proanthocyanidins from grape seed (*Vitis Vinifera*) using supercritical carbon dioxide. J. Supercrit. Fluids. 55(3), 924–928.
8. Maisuthisakul P, Suttajit M, Pongsawatmanit R. 2006. Assessment of phenolic content and free radical-scavenging capacity of some Thai indigenous plants. Food Chem. 100,1409-1418.
9. Lin JY, Tang CY. 2006. Determination of total phenolic and flavonoid contents in selected fruits and vegetables, as well as their stimulatory effects on mouse splenocyte proliferation. Food Chem. 101(1), 140–147.
10. Denev P, Yordanov A. 2013. Total polyphenol, proanthocyanidin and flavonoid content, carbohydrate composition and antioxidant activity of persimmon (*Diospyros kaki* L.) fruit in relation to cultivar and maturity stage. Bulg. J. Agric. Sci. 19, 981-988.
11. Thaipong K, Boonprakob U, Crosby K, Zevallos LC, Byrne DH. 2006. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. J. Food Compost. Anal. 19(6-7), 669-675.
12. Prasain JK, Peng N, Dai Y, et al. 2009. Liquid chromatography tandem mass spectrometry identification of proanthocyanidins in rat plasma after oral administration of grape seed extract. Phytomedicine. 16(2-3), 233–243.
13. Renugadevi J, Prabu SM. 2010. Quercetin protects against oxidative stress-related renal dyfunction by cadmium in rats. Exp. Toxicol. Patho. 62(5), 471-481.
14. Bergeron C, et al. 2005. Comparison of the Chemical Composition of Extracts from *Scutellaria lateriflora* Using Accelerated Solvent Extraction and Supercritical Fluid Extraction versus Standard Hot Water or 70% Ethanol Extraction. J. Agri. Food Chem. 53, 3076-3080.
15. ofAntidesma thwaitesianum by SupercPoontawee W, Natakankitkul S, Wongmekiat O . 2015. Enhancing Phenolic Contents and Antioxidant Potentials ritical Carbon Dioxide Extraction. J. Anal. Methods Chem. 2015, 1-7.

16. Bagchi D, Garg A, Krohn RL, et al. 1998. Protective effects of grape seed proanthocyanidins and selected antioxidants against TPA-induced hepatic and brain lipid peroxidation and DNA fragmentation, and peritoneal macrophage activation in mice. Gen. Pharmacol. 30(5), 771–776.
17. *Engström MT, Pälijärvi M, Fryganas C, Grabber JH, Mueller-Harvey I, Salminen JP. 2014. Rapid Qualitative and Quantitative Analyses of Proanthocyanidin Oligomers and Polymers by UPLC-MS/MS. J. Agri. Food Chem. 62(15), 3390–3399.*
18. Nuengchamnong N, Ingkaninan K. 2010. On-line HPLC-MS-DPPH assay for the analysis of phenolic antioxidant compounds in fruit wine: *Antidesma thwitesianum* Muell. Food Chem., 118(1), 147-152.
19. Wei R, et al. 2012. Grape seed proanthocyanidin extract reduces renal ischemia/reperfusion injuries in rats. Am J. Med. Sci. 343(6), 452-457.
20. Wang W, Zu Y, Fu Y. 2012. In vitro antioxidant and antimicrobial activity of extracts from *Morus alba* L. leaves, stems and fruits. Am. J. Chin. Med. 40(2), 349-356.
21. Krenn L, et al. 2007. Anthocyanin-and proanthocyanidin-rich extracts of berries in food supplements--analysis with problems. Pharmazie. 62(11), 803-812.

Anti-angiogenesis activity of Hoan-Ngoc *(Pseuderanthemum palatiferum* (Nees) Radlk.) leave extract on CAM model

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ABSTRACT

Pseuderanthemum palatiferum (Nees) Radlk. (*P. palatiferum*) known as Hoan-Ngoc or Payawanon is one of the most frequently used medical plants in Thailand for treating a variety of inflammatory diseases including cancer. Angiogenesis is a key process in human cancer growth, progression, and metastasis. There are several families of growth factors with angiogenic activities that have been identified such as fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF).This study aimed to investigate the effect of the water extract of *P. palatiferum* (WEP), fractionated from 95% ethanol extract of fresh leaves, on angiogenesis occurring naturally or induced by tumor melanoma B16F10 using the *in ovo* chick chorioallantoic membrane (CAM) model. The results showed that B16F10 induced a stronger angiogenic response than that of 100 ng/ml of angiogenic cytokine basic fibroblast growth factor (bFGF) ($p < 0.05$). No lethality of chick embryos was observed post exposure to WEP at 100-1,500 µg/ml for 24 and 48 hours. WEP at 100-300 µg/ml selectively and significantly suppressed the B16F10-induced angiogenesis by about 21 to 91% at 24 and 48 hr. ($p < 0.05$) and did not inhibit the normal neovascularization on the CAM. Overall, the data suggested that WEP might exhibit anti-cancer B16F10 cells, at least in part, through its anti-angiogenesis activity.

Keywords: anti-angiogenesis, *Pseuderanthemum palatiferum* (Nees) Radlk., B16F10 cell, CAM model

INTRODUCTION

Pseuderanthemum palatiferum (Nees) Radlk (*P. palatiferum*) is a new medicinal plant belonging to the Acanthaceae family. Its vernacular names are “Hoan-Ngoc”, “Wan ling” or “Payawanorn”. Hoan-Ngoc leaves have been reported to possess many pharmacological properties including high efficiency against cancer through apoptosis induction [1]. The leaf extract of Hoan-Ngoc showed high antioxidant activity against hydrogen peroxide radicals in the human blood. The ethyl acetate extract of the leaves showed strong antibacterial and antifungal activities [2]. *P. palatiferum* is used widely as a medicinal plant in Thailand to treat various diseases such as hypertension, diabetes, and tumor [3]. Previous work from this laboratory has revealed anti-inflammatory activity of the crude water leave extract of Hoan-Ngoc as evidenced by decreased nitric oxide production and suppression of inducible nitric oxide synthase and cyclooxygenase-2 in lipopolysaccharide and interferon gamma-activated RAW 264.7 cells [4]. As angiogenesis is essential in almost all tumor growth, progression, and metastasis, and it is related and is promoted by inflammation, Hoan-Ngoc which possesses anti-inflammatory property may exert its activity against various cancers through targeting the angiogenesis pathway. Therefore, this study aimed to investigate anti-angiogenic activity of the crude water leave extract of Hoan-Ngoc.

MATERIALS AND METHODS

Preparation of the water leaves extract of *Pseuderanthemum palatiferum*

Fresh leaves of Hoan-ngoc were blended in 95% ethanol and filtered through gauze. The extract was centrifuged at 3,500 x g for 10 minutes and then the supernatant was filtered through a Whatman No.1 filter paper. After that, the ethanolic filtrate was concentrated using a vacuum rotary evaporator and lyophilized into powder of ethanol crude extract (EEP). The EEP was partitioned between hexane and water (1:1, v/v). The water fraction was collected, centrifuged at 14,000 x *g* for 10 min at 4 ^{o}C, and then the supernatant was filtered through a Whatman No.1 filter paper. After that, the water fraction was evaporated and lyophilized into powder of water fraction of 95% ethanol crude extract (WEP).

Cell culture

B16-F10 (mouse melanoma cell line) was purchased from American Type Culture Collection (ATCC, USA). B16-F10 cells were cultured in Dulbecco's Modified Eagle's medium (DMEM) with high glucose supplemented with 10% FBS and 100 U/ml penicillin and 100 µg/ml streptomycin without HEPES. The cells were maintained at 37 ºC in 5% CO_2 and 95% humidity.

B16F10 and bFGF- induced angiogenesis in chick CAM model

Five-day-old chick embryos were purchased from Suranaree University of Technology Farm. An approximately 2 cm^2 window was cut in the shell over the false air sac, allowing access to the CAM. Angiogenesis in the chick CAM was induced by adding 10 µl of 100 ng/ml basic-fibroblast growth factor (bFGF) or 10 µl DMEM (uninduced control) onto a sterile filter paper disc placed onto the CAM. For tumor induction, pellets of 1×10^6, 3×10^6 or 6×10^6 B16F10 cells were inoculated directly onto the CAM. Then, 30 µl 100 U/ml penicillin was immediately added to the placed disc or to the tumor pellet in the CAM prior covering tumor pellet with a paper disc (1 disc/CAM). The exposed hole in the shell of each egg was closed with tape and further incubated at 37 ºC in 5% CO_2 and 95% humidity for 24 hours and 48 hours. Before and after incubation, the images of each treated CAM were captured and the number of blood vessels in contact with the paper disc within the focal plane of the CAM were quantified when viewed under a stereomicroscope.

Cytotoxic effect of WEP against B16F10

To rule out the direct cytotoxicity of WEP on B16F10, the effect of WEP on cell viability of B16F10 was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [5].

The effect of WEP on chick chorioallantoic membrane (CAM)

To find out the concentration of WEP that was not toxic in the CAM model, the effects of WEP on natural angiogenesis in the CAM was evaluated at 24 and 48 hours post exposure. A filter disc in the presence or absence of different concentrations (100-1,500 µg/ml) of WEP was placed upon the CAM and 30 µl 100 U/ml penicillin was immediately added. The exposed hole in each egg was closed and incubated for 24 hours and 48 hours. Angiogenesis at each time point was quantified as described above.

The effect of WEP on B16F10-induced agiogenesis in CAM

A Pellet of 3×10^6 B16F10 cells were placed directly onto the CAM, then a filter disc in the presence or absence of different concentrations (100-300 µg/ml) WEP was placed upon the CAM prior adding 30 µl 100 U/ml penicillin. The exposed hole in each egg was closed with tape and further incubated for 24 hours and 48 hours. Angiogenesis at each time point was quantified as described above.

All experiments were performed at least twice with four replicates per treatment, unless stated otherwise. Data shown are representative of at least two independent experiments with similar results.

RESULTS

When compared to the uninduced control, the neovascular vessels were increased 12 and 4 folds upon exposure to the angiogenic control 100 ng/ml of bFGF for 24 and 48 hours, respectively (Figure 1). B16F10 also increased the number of neovascular vessels in both dose- and time-dependent manners ($p < 0.05$). Notably, the increased number of neovascularization induced by B16F10 was significantly greater ($p < 0.05$) than that of 100 ng/ml of bFGF in both time points. Furthermore, the angiogenesis induction by both B16F10 and bFGF were significantly higher at 48 hours than 24

hours (Figure 1). As doubling the cell number of B16F10 from 3×10^6 to 6×10^6 cells only increased the number of neovascular vessels by about 16%, 3×10^6 cells of B16F10 was selected for further study of anti-angiogenesis activity of WEP on the CAM.

The result of cytotoxicity of WEP towards B16F10 in Figure 2A showed that WEP at the concentration of 50 and 100 µg/ml had no toxicity towards B16F10 cells ($p < 0.05$), whereas 300 µg/ml of WEP decreased the viability of B16F10 by 8.97%. WEP at 600-1500 µg/ml showed the cytotoxicity towards B16F10 by 30-70%. Therefore, the concentration from nontoxic till maximum tolerated concentration of WEP (50-300 µg/ml) was selected for further study in the B16F10-induced angiogenesis CAM model. The results in Figure 2B showed that exposure to WEP up to 300 µg/ml for 24 and 48 hour did not decrease the natural neovascular formation in the CAM. In contrast, exposure to higher concentration of WEP (900 and 1,500 µg/ml for 24 hr., and 600-1,500 µg/ml for 48 hr.) could suppress the natural neovascularization. In spite of some toxicity on neovascular formation of WEP at high concentrations, there was still no lethality of the chick embryo was observed in any treatment groups. Consequently, the concentration up to 300 µg/ml of WEP which had no suppression on natural angiogenesis generation was chosen for subsequent anti-angiogenesis study.

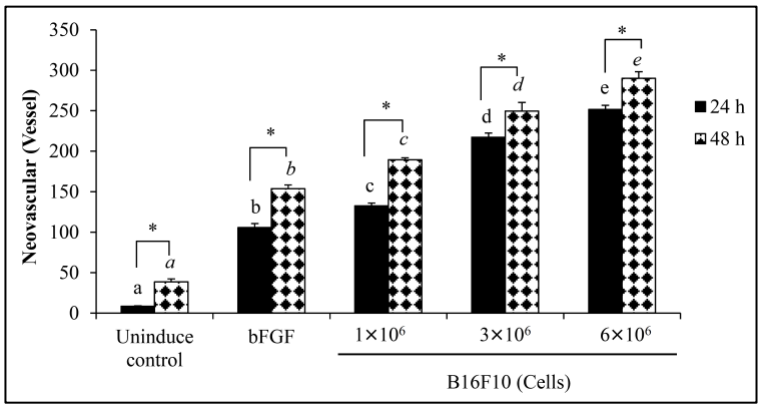

Figure 1. Comparison of angiogenesis induction by bFGF and B16F10 in the CAM model at 24 and 48 hours. Values are mean ± SD. Bars marked with different letters (24 h) or italic letters (48 h) are significantly different within the same time point at p< 0.05, as performed by one-way ANOVA. The asterisk indicates the different of mean values between 24 and 48 hr. ($p < 0.05$) using the Student's *t*-test.

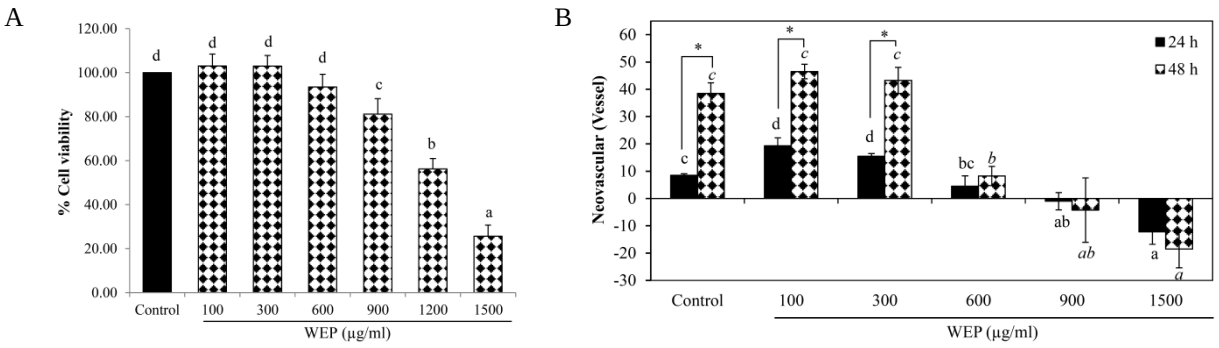

Figure 2. (A) Cytotoxic effect of WEP against B16F10 cells. The cells were exposed to various concentrations of WEP for 24 hour. Values are mean ± SD. Means with different letters are significantly different at p <0.05 as determined by one-way ANOVA. (B) The effect of WEP on normal angiogenesis at 24 and 48 hours. Values are means ± SD. Values marked with different letters (24 h) or italic letters (48 h) are significantly different at p< 0.05 within the same time point, as performed by one-way ANOVA. The asterisk indicates the different of mean values between 24 and 48 hr. ($p < 0.05$) using the Student's *t*-test.

Anti-angiogenesis activities of WEP on B16F10-induced angiogenesis in the CAM are clearly demonstrated in Figure 3A and 3B. WEP produced a dose-dependent suppression of B16F10-induced angiogenesis in the CAM ($p < 0.05$) at both 24 and 48 hr. Concomitantly exposure of B16F10 with WEP on the soaked paper disc for 24 hours caused the reduction of tumor-induced neovascularization by 20.48%, 50.90% and 87.65% at 30, 100 and 300 µg/ml of WEP, respectively. Likewise, After 48 hours, the number of neovascularization induced by of B16F10 was also reduced by 14.7%, 49.8% and 90.8% upon exposure to WEP at 30, 100 and 300 µg/ml, respectively. Notably, the inhibition of B16F10-induced neovascularization by WEP was more pronounced at 300 µg/ml which was barely non-toxic towards tumor cells. Therefore, the strong suppression of neovascularization by WEP was not solely due to direct cytotoxicity towards B16F10 tumor cells (Figure 2A and 3A).

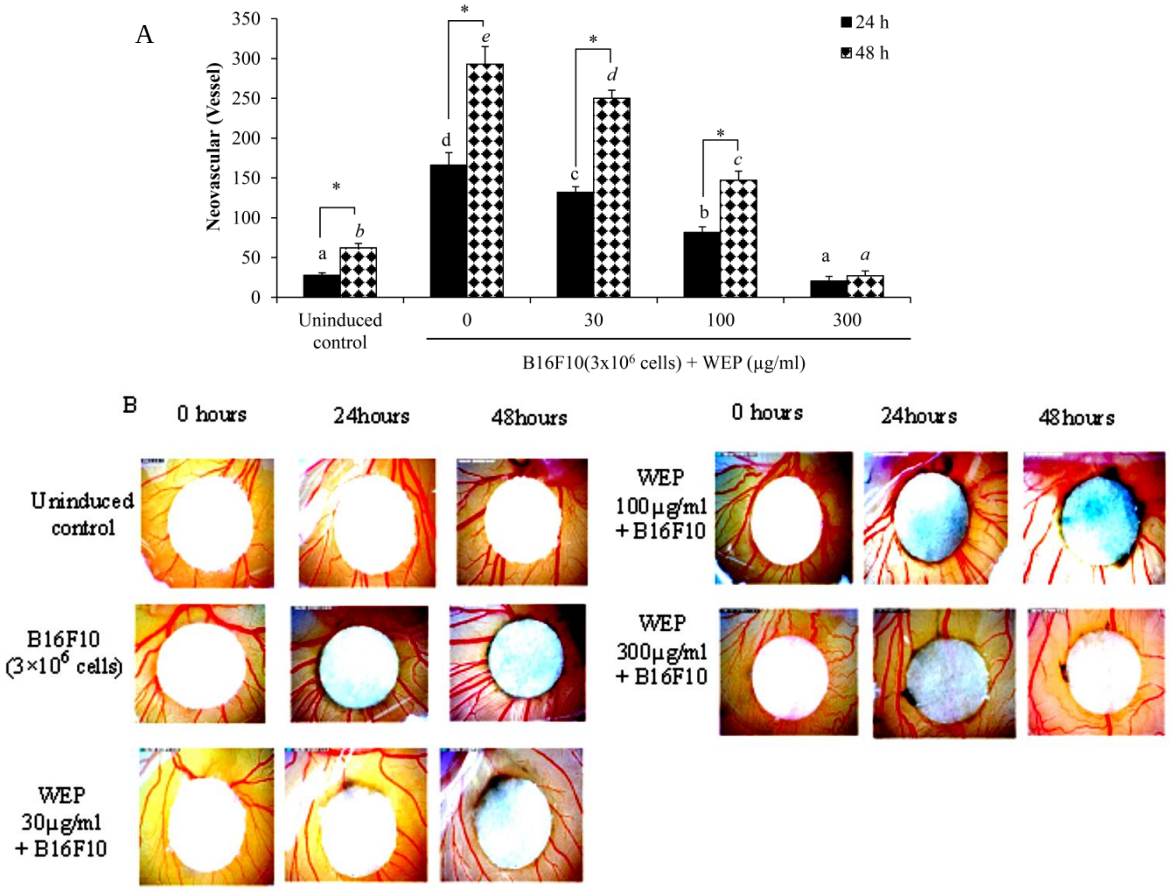

Figure 3. (A) Anti-angiogenic activity of WEP on B16F10-induced angiogenesis in CAM at 24 and 48 hours. Values are expressed as means ± SD (n = 6). Bars marked with different letters (24 h) or italic letters (48 h) are significantly different at p< 0.05 as performed by one-way ANOVA. (B) Photograph of neovascular vessels formation in CAM concomitantly exposure to B16F10 and WEP at 24 hr. and 48 hr. time points (6.7x magnification).

CONCLUSIONS

The water fresh leaf crude extract of *P. palatiferum* at 100-300 µg/ml produced a dose-dependent suppression of B16F10-induced angiogenesis in chick CAM model. The activity of WEP was highly specific as the extract only selectively inhibited the tumor-induced angiogenesis without targeting the normal or natural neovascularization on the CAM.

ACKNOWLEDGEMENTS

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REFERENCES

1. Chayarop K, Peungvicha P, Wongkrajang Y, Chuakul W, Amnuoypol S, Temsiririrkkul R. 2011. Pharmacognostic and phytochemical investigations of *Pseuderanthemumpalatiferum* (Nees) Radlk. ex Lindauleaves. Pharmacognosy J. 3(23), 18-23.
2. Padee P, Nualkaew S, Talubmook C, Sakuljaitrong S. 2010. Hypoglycemic effect of a leaf extract of *Pseuderanthemumpalatiferum* (Nees) Radlk. in normal and streptozotocin-induced diabetic rats. J. Ethnopharmacol. 132(2), 491-496.
3. Pan R, Dai Y, Gao X, Lu D, Xia Y. 2010. Inhibition of vascular endothelial growth factor-induced angiogenesis by scopoletin through interrupting the autophosphorylation of VEGF receptor 2 and its downstream signaling pathways.Vascular Pharmacology. 54(1-2), 18-28.
4. Huynh Kim D, 2008. KHẢO SÁT THÀNH PHẦN HÓA HỌC CỦA LÁ XUÂN HOA (*Pseuderanthemum palatiferum*), Tạp chí Khoa học. 9, 232-240.
5. Sashwati R, Savita K, Helaine A, Jelena V, Debasis B, Manashi B, Chandan K. 2002. Anti-angiogenic property of Edible Berries. Free Radical Res. 36 (9), 1023–1031.

Antioxidant activity of bioactive compounds from *Camellia oleifera* seed cake

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ABSTRACT

Camellia oleifera seed cake is by-product of oil production, which is normally discarded as waste about one million tons every year. However, this waste contains many active substances including saponins, flavonoids, and polysaccharides which possess many benefits to health. Therefore, this study aimed to focus on the chemical compounds and antioxidant activity investigations of crude extracts and pure compounds from *C. oleifera* seed cake. The tea seed cake of *C. oleifera* Abel was obtained from Chaipattana Foundation in Thailand. The plant materials were extracted by maceration with distilled hexane, ethyl acetate, and methanol, respectively. The preliminary chemical constituents of tea seed cake crude extracts were analyzed by TLC using silica gel F_{254} and screened for scavenging effect on DPPH radical by TLC-DPPH assay. The active compound was successively isolated from active extract by chromatographic techniques. Initial separation of the ethyl acetate extract was started to partition with hexane and methanol. Then, the methanol part of ethyl acetate extract was separated by quick column chromatography using silica gel, as adsorbent and gradient eluted with hexane and gradually increased polarities by ethyl acetate and methanol. Fractions with similar chromatographic pattern were combined and determined the antioxidant activity by DPPH, Photochemiluminescence (PCL), and Ferric reducing antioxidant power (FRAP) assay. The potential fraction was successively chromatographed on a Sephadex LH-20 column and eluted with 100% methanol. The chemical structure of the isolated compound was elucidated on the basis of spectroscopic techniques such as UV-Vis spectroscopy, infrared spectroscopy (IR), mass spectrometry (MS), and nuclear magnetic resonance spectroscopy (^{1}H-NMR and ^{13}C-NMR). Moreover, isolated compound was evaluated for the antioxidant activity and cytotoxicity. The cytotoxicity of osteoblast MC3T3-E1 cells were analyzed using a colorimetric assay of the tetrazolium salt (WST-1) conversion by mitochondrial dehydrogenases. Results from TLC chromatograms indicated that ethyl acetate extract contained remarkable result with many active spots and was further investigated for active components by using chromatographic techniques. Activity-guided separation of the ethyl acetate crude extract led to the isolation of 9 fractions (Q1-Q9). Among the isolated fractions, Q9 demonstrated the strongest antioxidant activity. This fraction showed value of activities with IC_{50} of 75.11 ± 0.19 µg/ml on DPPH assay, 0.42 ± 0.16 nmol Trolox equivalent/µg extract on PCL assay, and FRAP value of 80.88 ± 0.83 µg/mg extract. Therefore, active fraction was collected to further separation by flash column, yielding 6 fractions (F1-F6). Fraction F5 appeared as yellow crystalline solid, which later identified as kaempferol. The completed structure of this compound was characterized based on spectral data and comparison with previous report. Kaempferol demonstrated moderate antioxidant activity with IC_{50} of 3.29 ± 0.14 (µg/ml), 18.44 ± 7.74 nmol Trolox equivalent/µg extract, and FRAP value of 42.04 ± 0.06 µg/mg extract, using DPPH, PCL, and FRAP assay, respectively. Moreover, kaempferol was treated with osteoblast MC3T3-E1 cells. The results suggested that kaempferol had no toxic effects in MC3T3-E1 cells at 0.01 mM concentration.

KEYWORDS: *C. oleifera*, seed cake, by-product, natural products, antioxidant

INTRODUCTION

Camellia oleifera Abel belongs to the family of *Theaceae*, the same as tea, *C. sinensis* [1]. It is commonly known as the oil seed Camellia, tea seed oil or tea oil Camellia. In Thailand, this plant was cultivated and developed under the Chaipattana Foundation in the name of Tea Oil and Plant Oils Development Center.

Leaves of *C. oleifera* cannot be used as beverage, but its seeds contain 30% oil with good quality as the same as olive oil [2]. Camellia oil is an important source of edible oil in China and is used in cooking and as a medicine for the treatment of intestinal disorders and burn injuries [3]. The remainder of defatted seeds after oil extraction is called *C. oleifera* cake or tea seeds cake. These by-products are always discarded as large amount that leads to a lot of wasted bioactive compounds. In the past, the defatted seeds were used as washing hair material for women, eliminating unwanted fish in prawn ponds, and found with the function of stopping itching and pain [4].

Recent researches disclose that the cake of *C. oleifera* containing many bioactive compounds, especially polysaccharides, saponins, and flavonoids [5, 6]. For several years, tea seed cake has been widely studied for their chemical properties and biological activities, including antibacterial, antifungal, cytoprotective, antitumor, and antioxidant effects [7].

Although the prior reports have indicated the presence of phytochemical compounds, however, they have not been specifically isolated active compounds and determined for antioxidant activity. Therefore, this study aimed to focus on the chemical and biological investigations of the *C. oleifera* seed cake. The crude extracts were separated by chromatographic techniques and the isolated compounds were elucidated by spectroscopic methods. For evaluation of the biological activity, the antioxidant activity of crude extracts and pure compounds from tea seed cake was determined.

MATERIALS AND METHODS

Plant material

The tea seed cake of *C. oleifera* was kindly provided by the Chaipattana Foundation in Thailand.

Extraction and isolation

The tea seed cake of *C. oleifera* was successively extracted by maceration with distilled hexane, ethyl acetate, and methanol. The solvent was removed under reduced pressure by rotary evaporator and freeze dryer to give hexane, ethyl acetate, and methanol crude extracts, respectively. The separation of bioactive crude extract was performed by antioxidant activity-guided separation using chromatographic methods. The active compound was successively isolated from active extract by chromatographic techniques. Initial separation of the ethyl acetate extract was started to partition with hexane and methanol. Then, the methanol part of ethyl acetate extract was separated by quick column chromatography using silica gel, as adsorbent and gradient eluted with hexane and gradually increased polarities by ethyl acetate and methanol. Fractions with similar chromatographic pattern were combined and determined the antioxidant activity. The potential fraction was successively chromatographed on a Sephadex LH-20 column and eluted with 100% methanol. The chemical structure of the isolated compound was elucidated on the basis of spectroscopic techniques such as UV-Vis spectroscopy, infrared spectroscopy (IR), mass spectrometry (MS), and nuclear magnetic resonance spectroscopy (^{1}H-NMR and ^{13}C-NMR).

Antioxidant activity testing

The preliminary chemical constituents of tea seed cake crude extracts were analyzed by TLC using silica gel F_{254} and screened for scavenging effect on DPPH radical by TLC-DPPH assay. Briefly, the solution of test sample was prepared in suitable solvent and applied similarly to two TLC plates. After that, both plates were developed with the same solvent system. Subsequently, the first plate was spray with 10% sulfuric acid spray reagent and another plate was spray with a 0.2% DPPH reagent in methanol. Moreover, crude extracts and isolated compound were tasted for antioxidant activities using 1, 1-diphenyl-2-picryl hydrazyl (DPPH), Photochemiluminescence (PCL), and Ferric reducing antioxidant power (FRAP) assay.

The first method determined the free radical-scavenging activity using DPPH• radical.[8] DPPH is commercially available organic nitrogen radicals and has a UV-Vis absorption maximum at 520 nm. DPPH free radical scavenging activity is measured by reducing of absorbance at 520 nm, which resulted from reduction of DPPH free radicals (DPPH•) to the hydrazine form (DPPH-H). Sample was prepared in methanol at least five concentrations. In 96-well microtiter plate, reaction mixtures containing 100 µL of various concentrations of the test sample were thoroughly mixed with 100 µL of freshly prepared DPPH solution. After incubation at room temperature for 30 min, the absorbance was read at 520 nm on a microplate reader.

FRAP is another method of wide suitability for assay of antioxidants in vitro as well as in organisms. Fe (III) tripyridyltriazine complex is reduced to the ferrous form, with an increase in absorbance at 700 nm. Absorbance is proportional to the combined ferric reducing antioxidant power (FRAP value) of the antioxidants in the samples.[9] Shortly, various concentrations of sample were mixed with 100 µL of sodium phosphate buffer at pH 6.6 and 100 µL of 1%

potassium ferricyanide. The mixture was incubated at 60 °C for 20 min. After 100 µL of 10% trichloroacetic acid (w/v) were added, the mixture was transferred to 96-well microtiter plate. Then, mixture was mixed with 100 µL of deionized water and 20 µL of 0.1% of ferric chloride, and the absorbance was measured at 700 nm. Additional dilution was needed if the FRAP value measured was over the linear range of the standard curve.For PCL assay, superoxide anion radical ($O_2^{-\bullet}$) are produced by optical excitation of a photosensitizer substance. The antioxidant capacities of samples are measured by their inhibitory effects on luminescence generation, compared with the standard antioxidant. Samples were measured in the Photochem® with the antioxidative capacity of lipid soluble substances (ACL) kit. A 2.30 mL of methanol, 0.2 mL of buffer solution, 25 µL of photosensitizer, and 10 µL of standard or sample solution were mixed and measured. The antioxidant capacities of the samples were quantified by comparison with the TROLOX® standard and were given in equivalent units of standard. All determinations were performed in triplicate.

Moreover, isolated compound was evaluated for the cytotoxicity. The cytotoxicity of osteoblast MC3T3-E1 cells were analyzed using a colorimetric assay of the tetrazolium salt (WST-1) conversion by mitochondrial dehydrogenases. WST-1 cell cytotoxicity assay is a sensitive and accurate assay for cell cytotoxicity and proliferation. The MC3T3-E1 at density of $1x10^5$ cells/ml was seeded in 96-well microtiter plates. After 24 hours incubation, the cells were treated with isolated compound at concentrations of 0.01 mM and incubated at 37 ºC for 48 hours. Finally, 100 µL of WST-1 solution was added and the sample was left to incubate for 30 min before measuring absorbance at 450 nm.

RESULTS

The powder of *C. oleifera* seed cake (1 kg) was successively extracted with hexane, ethyl acetate, and methanol. The yields of hexane, ethyl acetate, and methanol extracts were 7.65, 2.23, and 9.69 % w/w, respectively. The crude extracts were preliminary determined for antioxidant activity using TLC-DPPH method. The TLC patterns of the crude extracts were shown in **Figure 1**. Results from TLC chromatograms indicated that ethyl acetate extract contained remarkable result with many active spots and was further investigated for active components by using chromatographic techniques. Activity-guided separation of the ethyl acetate crude extract led to the partition with hexane and methanol. Then, the methanol part of ethyl acetate extract was separated by quick column chromatography, to give 9 fractions (Q1-Q9).

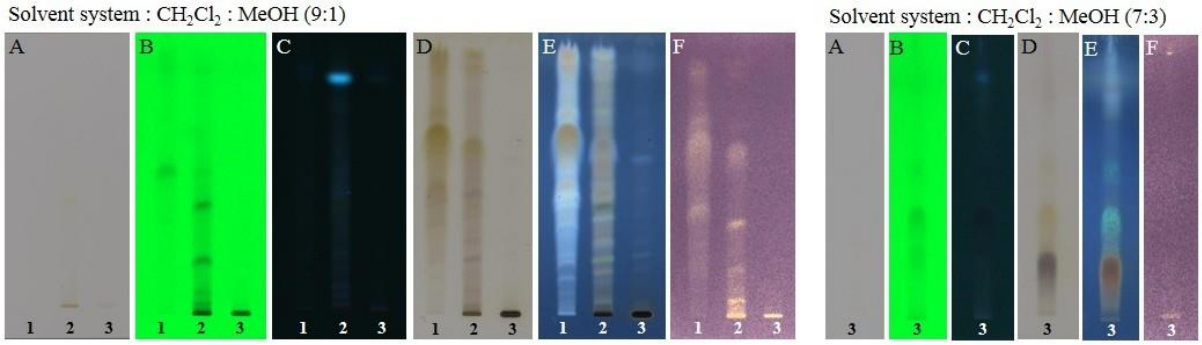

Figure 1. TLC chromatograms of the crude extracts from *C. oleifera* seed cake
1 = Hexane extract, **2** = Ethyl acetate extract, and **3** = Methanol extract
Stationary phase: Silica gel F_{254}
Detector: **(A)** white light; **(B)** UV 254 nm; **(C)** UV 366 nm;
(D) 10% H_2SO_4, white light; **(E)** 10% H_2SO_4, UV366 nm; and
(F) DPPH

The separated extracts and all fractions were determined for scavenging and reducing properties using DPPH, PCL, and FRAP assay, respectively. The antioxidant in the term of free radical scavenging and reducing properties of the crude extracts were shown in **Table 1**, indicating that the methanol part of ethyl acetate extract exhibited good activity. Among the isolated fractions, Q9 demonstrated the strongest antioxidant activity.

Table 1. Antioxidant activity of crude extracts and fractions Q1-Q9

Sample	DPPH[a]	PCL[b]	FRAP[c]
Ethyl acetate – Hexane	ND*	ND*	ND*
Ethyl acetate – MeOH	361.76 ± 0.89	0.39 ± 0.04	57.87 ± 0.61
Q1	ND*	ND*	ND*
Q2	ND*	ND*	ND*
Q3	ND*	ND*	ND*
Q4	ND*	ND*	ND*
Q5	325.45 ± 1.26	0.25 ± 0.03	39.58 ± 0.43
Q6	253.71 ± 0.69	0.30 ± 0.27	43.42 ± 0.22
Q7	191.39 ± 2.11	0.30 ± 0.07	54.01 ± 0.18
Q8	103.05 ± 1.21	0.41 ± 0.30	84.45 ± 0.45
Q9	75.11 ± 0.19	0.42 ± 0.16	80.88 ± 0.83

[a] Values expressed as IC_{50} [µg/mL]
[b] Values expressed as nmol Trolox equivalent/µg extract
[c] Values expressed as µg $FeSO_4$ equivalent/mg extract
ND*, not determined

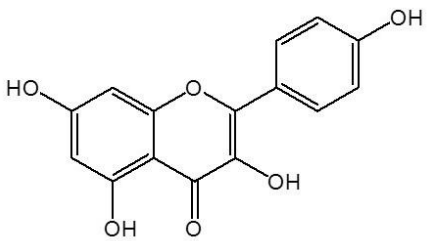

Figure 2. The structure of kaempferol

Therefore, active fraction was collected to further separation by flash column, yielding 6 fractions (F1-F6). Fraction F5 appeared as yellow crystalline solid, which later identified as kaempferol **(Figure 2)**. The completed structure of this compound was characterized based on spectral data and comparison with previous report.[10] Kaempferol demonstrated moderate antioxidant activity with IC_{50} of 3.29 ± 0.14 (µg/ml), 18.44 ± 7.74 nmol Trolox equivalent/µg extract, and FRAP value of 42.04 ± 0.06 µg/mg extract, using DPPH, PCL, and FRAP assay, respectively **(Table 2)**. Moreover, kaempferol was treated with osteoblast MC3T3-E1 cells. The results suggested that kaempferol had no toxic effects in MC3T3-E1 cells at 0.01 mM concentration.

Table 2. Antioxidant activity of kaempferol

Sample	DPPH[a]	PCL[b]	FRAP[c]
Kaempferol	3.29 ± 0.14	18.44 ± 7.74	42.04 ± 0.06
Ascorbic acid	1.97 ± 0.03	ND*	87.81 ± 0.46

[a] Values expressed as IC_{50} [µg/mL]
[b] Values expressed as nmol Trolox equivalent/µg extract
[c] Values expressed as µg $FeSO_4$ equivalent/mg extract
ND*, not determined

CONCLUSIONS

As part of our continuing investigation on bioactive substances from by-product of *C. oleifera*, the ethyl acetate extract of tea seed cake showed interesting antioxidant activity. The activity-guided fractionation along with chromatographic techniques led to isolation of known active compound, kaempferol.

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REFERENCES

1. Zhang Y, Chen Q, Luo X, Dai T, Lu B, Shen J. 2011. Mutagenicity and safety evaluation of the water extract of *Camellia oleifera* Abel. J. Food Sci. 76(3), 84-89.
2. Jin X. 2012. Bioactivities of water-soluble polysaccharides from fruit shell of *Camellia oleifera* Abel: Antitumor and antioxidant activities. Carbohydr. Polym. 87(3), 2198-2201.
3. Chaicharoenpong C, Petsom A. 2009. Quantitative thin layer chromatographic analysis of the saponins in tea seed meal. Phytochem. Anal. 20(3), 253-255.
4. Liu X, Jia L, Gao Y, Li B, Tu Y. 2014. Anti-inflammatory activity of total flavonoids from seeds of *Camellia oleifera* Abel. Acta Bioch. Bioph. Sin. 46(10), 920-922.
5. Jin X, Ning Y. 2012. Antioxidant and antitumor activities of the polysaccharide from seed cake of *Camellia oleifera* Abel. Int. J. Biol. Macromol. 51(4), 364-368.
6. Ye Y, Fang F, Li Y. 2014. Isolation of the sapogenin from defatted seeds of *Camellia oleifera* and its neuroprotective effects on dopaminergic neurons. J. Agri. Food Chem. 62(26), 6175-6182.
7. Gulcin I. 2012. Antioxidant activity of food constituents: an overview. Arch. Toxicol. 86(3), 345-391.
8. Shahmohamadi R, Sariri R, Rasa M, Aghamali M. 2014. Antioxidant Activity of Gilan *Mentha pulegium* During Growth. Pak. J. Biol. Sci. 17(3), 380-387.
9. Ferreira ICFR, Baptista P, Vilas-Boas M, Barros L. 2007. Free-radical scavenging capacity and reducing power of wild edible mushrooms from northeast Portugal: Individual cap and stipe activity. Food Chem. 100(4), 1511-1516.
10. Gangwal A, Parmar S, Sheth N. 2010. Triterpenoid, flavonoids and sterols from *Lagenaria siceraria* fruits. Der Pharmacia Lettre. 2(1), 307-317.

Assessment of synergistic effects on antimicrobial activity in vapor- and liquid-phase of cinnamon and oregano oils against *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus is an important human pathogen in medical treatment and foodborne illness prevention. The applications of antibiotics in treatment of *S. aureus* become ineffective due to an increase in antibiotic resistance and the applications of chemical preservatives in foods because negative effects on human health. Therefore, natural antibacterial compounds are considered as a promising alternative approach. In this study, bacterial inhibition efficacy of cinnamon and oregano oils was evaluated by using disc volatilization assay and microdilution assay. Synergistic effects of their combinations were also indicated by a mixture of cinnamon : oregano oils at various ratios including 0:10, 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1 and 10:0. Inhibition zones in vapor-phase at the highest test concentration of EOs (160 µL L^{-1} air) for 11 combinations varied from 31.67±0.58 mm to 44.67±0.58 mm. Minimum inhibitory concentration (MIC) in volatilization assay was indicated at 5 µL L^{-1} air when the ratios of cinnamon and oregano oils were 8:2 and 9:1. *S. aureus* was more sensitive to essential oils (EOs) in vapor-phase than in liquid-phase due to greatly higher MIC values in liquid-phase. Moreover, the MICs of EO vapor also showed bactericidal activity. The results of synergistic integration indicated that there was only the combination 9:1 in liquid-phase showing synergistic effects while all combinations in vapor-phase exhibited this synergy. In vapor-phase, strongly synergistic activities were observed at the combined ratios 8:2 and 9:1 (FIC=0.25). The optimal combinations of the EOs would be potential to the application in medical development or food preservation.

Keywords: cinnamon oil, isobologram, MIC, oregano oil, synergistic effects

INTRODUCTION

Staphylococcus aureus is a bacterial pathogen causing varieties of health problems from local infections such as skin infections, cellulitis, myositis, and pneumonia to systemic infections such as bacteremia and sepsis [1]. In terms of foodborne illness, *S. aureus* is a leading cause of gastroenteritis resulting from the consumption of contaminated food. Staphylococcal food poisoning is due to the absorption of staphylococcal enterotoxins performed in food [2]. Antibiotic resistant strains, specifically methicillin resistant, make medical treatment ineffective and cause problems for medicinal therapy [3]. In terms of food safety, chemical preservatives have been employed to prevent the spoilage and foodborne pathogens. In recent years, however, a consideration of using synthetic compounds as preservatives in foods has been expressed by consumers. Therefore, natural antimicrobials, e.g. medicinal plant extracts and essential oils, have recently gained a great scientific interest. Essential oil of cinnamon contains 66.28–81.97% of trans-cinnamaldehyde [4] contributing to antimicrobial activity of cinnamon oil against a wide range of microorganisms [5, 6, 7]. Cavacrol (26.7%), p-Cimene (15.2%) and γ-Terpinene (15.1%) are main components of *Origanum vulgare* essential oil [8]. Cinnamon oil has strong inhibition effect on *Haemophilus influenze*, *Streptococcus pyogenes*, *Streptococcus pneumonia*, *Escherichia coli*, and *Staphylococcus aureus* at minimum inhibitory dose 3.13, 6.25, 3.13, 12.5 and 6.25 mg L^{-1} air, respectively [9]. By

studying on the antibacterial effect of lavender and oregano EOs, Martucci et al. [8] found that *S. aureus* was more sensitive to the selected essential oils than *Escherichia coli*. Cinnamon and oregano have been applied in food industry to prevent contaminated spoilages and pathogens [10]. The combinations of certain essential oils or their major constituents are reported to perform synergistic effects on microbial inhibition [11, 12, 13]. It is very vital to evaluate a synergistic effect of EOs since the applied concentration could be reduced significantly by using an optimal combination. The objectives of this study were to investigate the liquid- and vapor-phase antimicrobial activity of cinnamon and oregano oil mixtures against *S. aureus* and their synergistic effects to demonstrate an alternative approach on inhibition of the pathogen using natural antimicrobials.

MATERIALS AND METHODS

Chemicals

Cinnamon essential oil (*Cinnamomum zeylanicum*) and oregano essential oil (*Origanum vulgare*) were obtained from Botanic Essence Co.,Ltd. (Bangkok, Thailand). Mueller Hinton agar, Mueller Hinton broth (HiMedia), resazurin (BDH), Whatman filter paper No.1 were used in antimicrobial assays.

Bacterial strains

Staphylococcus aureus DMST 8840 (ATCC 25923) was obtained from The Department of Medical Science, National Institute of Health, Ministry of Public Health, Thailand.

Inoculum preparation

S. aureus was subcultured twice in Mueller Hinton broth (HiMedia) at 37°C for 24 hours. Cells were harvested by centrifuging at 6000× g for 2 min and washed with sterile NaCl solution (0.85 %). Sodium phosphate buffer (0.1 M) was used to dilute cell suspension to obtain cell concentration at 10^8 CFU mL^{-1}.

Antimicrobial assay

Disc volatilization assay

Antimicrobial activity of essential oils in vapor-phase was determined by employing modified disc volatilization assay [14]. Mueller Hinton agar (MHA) plates were prepared by using 90-mm plastic Petri dishes containing 20 ml of MHA medium. Bacterial suspension (10^8 CFU mL^{-1}) was inoculated onto agar surface by using a sterile cotton swab. Eleven combinations of cinnamon : oregano EOs including 0:10, 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1 and 10:0 [12] were diluted in ethyl acetate to obtain two-fold serial dilutions. Ten µL of each dilution was completely absorbed by a 10-mm diameter sterile blank filter disc (Whatman Grade 1) which was then placed in the center of internal surface of the lid of Petri dishes. EO concentrations in the headspace varied from 5 µL L^{-1} to 160 µL L-1. A filter disc containing 10 µL of ethyl acetate was placed in the control dish. After the discs were dried in aseptic condition for 1 min, the plates were sealed with sterile adhesive tape and incubated at 37°C for 24 h. Minimum inhibitory concentrations (MICs) were indicated after incubation, showed as the amount of EOs per a litter of the headspace atmosphere above agar surface that could inhibit *S. aureus* and create an apparent inhibition zone. The diameter of inhibition zones was also recorded.

Following the period of incubation, the antimicrobial atmosphere was removed by preplacing a lid of plastic dish with a blank one to check whether the effects are bacteriostatic or bactericidal. After incubation at 37°C for 14 days, if bacterium starts to grow from the clear zone, the effect was bacteriostatic, whereas if no growth was observed, the effect was bactericidal (minimum bactericidal concentrations were recorded). The test was performed in three replicates.

Broth microdilution assay

Broth microdilution method modified from Sarrazin et al. [15] was employed to test the antimicrobial effects of essential oils by using 96-well plates. Eleven combinations of cinnamon and oregano EO including 0:10, 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1 and 10:0 [12] (from row 1 to row 11 in Figure 1, respectively) were diluted in 1% Tween80 to obtain two-fold serial dilutions. Final concentrations of EO in the wells were 8, 4, 2, 1, 0.5, and 0.25 µL mL^{-1} (from column C to column H in Figure 1, respectively). Each well contained 100 µl of essential oils at specific concentration and 100 µL MHB inoculated with *S. aureus* (final concentration approximately 5×10^4 CFU mL^{-1}). The sterility control wells contained 200 µL of sterile MHB, no microorganism (column A in Figure 1). The positive solvent control wells were filled with 100 µL of 1% Tween 80 instead of essential oil solution (column B in Figure 1). The 96-well plates were incubated at 37°C for 24 h. After incubation, bacterial growth was indicated by adding 20 µL of resazurin solution and incubation for 3 h. A change of solution color from blue to pink showed the presence of *S. aureus*. MIC was the lowest concentration of EO that could prevent the change of resazurin color from blue to pink.

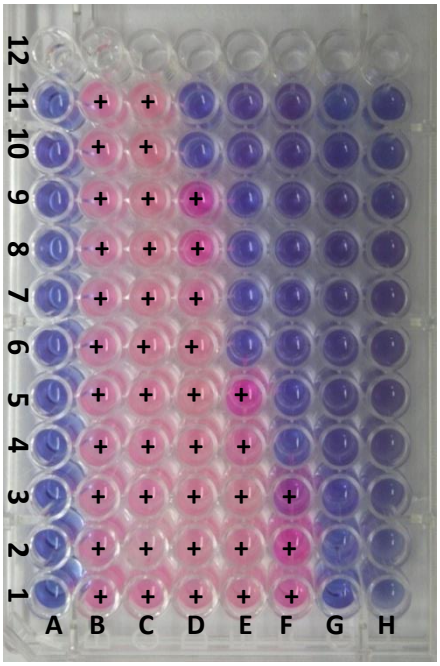

Figure 1. Plates after 24 h of incubation in broth microdilution assay. Pink color (+) indicated the growth of *S. aureus*; blue showed the inhibition of the growth.

Minimum bactericidal concentrations (MBC) of EOs were determined by confirming onto MHA. Total suspension (220 µL) from the well remaining blue color was centrifuged at 6000× g for 2 min. The supernatant was discarded and the bacterial cells were suspended in NaCl solution (0.85 %). This step was carried out to remove the accumulation of essential oils. The suspension (5 µL) was then spotted onto MHA plates at three different points and incubated at 37°C for 24 h. The bactericidal concentration was the lowest concentration of EOs that could cause the microorganism failed to grow on MHA. The test was conducted with three replicates.

Synergistic analysis

In order to determine the synergistic effect of combination between cinnamon oil (A) and oregano oil (B), a fractional inhibition concentration index (FIC) will be estimated. FIC for each combination was calculated by the following equation [16].

$$\mathbf{FIC_A} = \frac{\text{MIC of A in presence of B}}{\text{MIC of A}}$$

$$\mathbf{FIC_B} = \frac{\text{MIC of B in presence of A}}{\text{MIC of B}}$$

$$\mathbf{FIC_{index}} = \mathbf{FIC_A} + \mathbf{FIC_B}$$

FIC < 1: synergistic effect.
FIC = 1: additive effect.
FIC > 1: antagonistic effect.

The effects of all combinations were graphically described by plotting on an isobologram using the mean FIC values from triplicated experiments. FIC_A values were plotted (on the vertical axis) versus FIC_B (on the horizontal axis). In the isolobogram, if the points of combination falling on the straight line show additive effect, the points above show antagonistic, and those below indicate synergistic [17].

Statistical analyses

The analyses were carried out in triplicate and results were expressed as mean ± standard deviation. Analyses of variance (ANOVA) were conducted and differences among samples means were analyzed by Duncan's multiple range test (p<0.05) by using SPSS (IBM SPSS Statistics 19.0, SPSS Inc., USA).

RESULTS AND DISCUSSION

Inhibition zones

In vapor-phase, the inhibition effects of different EO combinations against *S. aureus* are presented in Table 1. All combinations showed antimicrobial activity against the pathogen as they were able to cause apparent halos on the surface of MHA. Generally, the diameters of inhibition zones increased with the increasing of EO concentrations. The values for the diameter of inhibition zones ranged from 31.67±0.58 mm to 44.67±0.58 mm at 160 µL L^{-1} of EO concentration and were significantly different from other concentrations of EOs except the combination ratios 7:3, 8:2, and 9:1.

Table 1. Diameter of inhibition zones (mm) evaluated by using disc volatilization assay

Ratios (v/v)		Concentrations (µL L^{-1} air)					
Cin	**Ore**	**5**	**10**	**20**	**40**	**80**	**160**
0	10	0^{e}	0^{e}	$12.76±0.58^{d}$	$34.67±0.58^{c}$	$36.67±1.53^{b}$	$44.67±0.58^{a}$
1	9	0^{e}	0^{e}	$10.67±1.16^{d}$	$30.00±0.00^{c}$	$32.00±0.00^{b}$	$35.00±0.00^{a}$
2	8	0^{e}	0^{e}	$10.00±0.00^{d}$	$25.33±0.58^{c}$	$30.00±0.00^{b}$	$31.67±0.58^{a}$
3	7	0^{f}	$10.00±0.00^{e}$	$24.67±0.58^{d}$	$30.00±0.00^{c}$	$33.67±0.58^{b}$	$35.00±0.00^{a}$
4	6	0^{f}	$10.67±0.58^{e}$	$24.33±1.16^{d}$	$28.00±0.00^{c}$	$30.00±0.00^{b}$	$35.33±1.16^{a}$
5	5	0^{e}	$17.00±0.00^{d}$	$27.33±2.89^{c}$	$32.00±0.00^{b}$	$32.67±0.58^{b}$	$34.67±0.58^{a}$
6	4	0^{f}	$17.00±0.00^{e}$	$29.33±1.16^{d}$	$34.67±0.58^{c}$	$36.00±0.00^{b}$	$37.00±0.00^{a}$
7	3	0^{e}	$22.67±2.31^{d}$	$28.00±0.00^{c}$	$34.33±0.58^{b}$	$35.00±0.00^{ab}$	$36.00±0.00^{a}$
8	2	$17.00±1.73^{e}$	$24.67±0.58^{d}$	$29.33±1.16^{c}$	$33.33±1.16^{b}$	$34.67±0.58^{ab}$	$35.33±0.58^{a}$
9	1	$19.00±1.73^{d}$	$24.00±1.73^{c}$	$27.00±1.73^{b}$	$34.00±1.73^{a}$	$34.33±1.16^{a}$	$35.67±0.58^{a}$
10	0	0^{e}	0^{e}	$20.67±1.16^{d}$	$30.00±0.00^{c}$	$36.67±0.58^{b}$	$38.00±0.00^{a}$

Means followed by a different superscript letter within a row are significantly different (p < 0.05).
Cin = cinnamon; Ore = oregano

Combinations of essential oils exhibited higher antimicrobial potential than individual essential oils. Individual cinnamon and oregano oils caused inhibition zone at the concentration 20 µL L^{-1}, the similar effects were observed in two combination ratios (1:9 and 2:8). An increase in cinnamon oil percentage in the other combinations show a stronger action against *S. aureus* since the pathogen was inhibited at lower concentration (10 µL L^{-1}). In particular, when cinnamon and oregano were employed at the ratios 8:2 and 9:1, these combinations could render a halo on the surface of agar inoculated with *S. aureus* at lower concentration 5 µL L^{-1}. Figure 2 shows apparent inhibition zones caused by these combinations of EOs. Therefore, the combinations of these EOs might encourage their antimicrobial activity which was concentration-dependent.

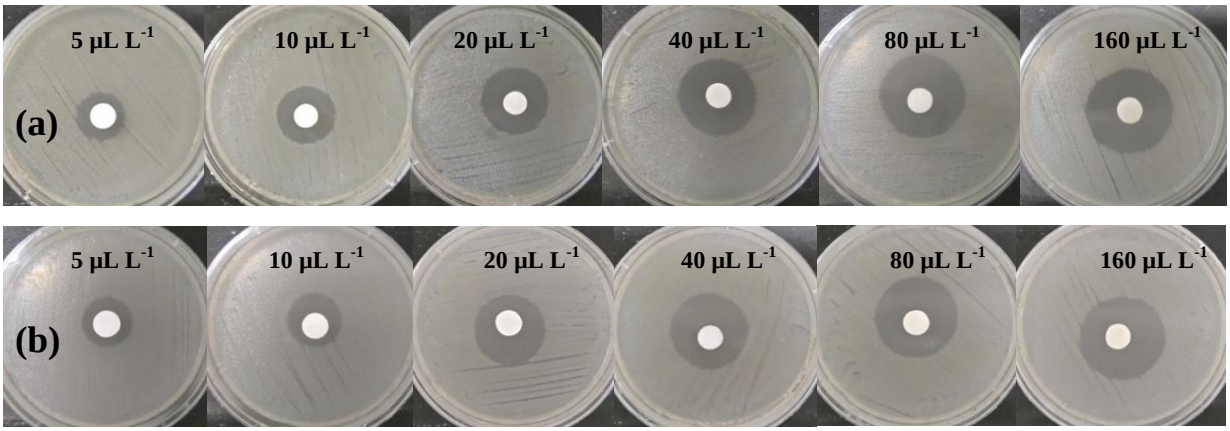

Figure 2. Plastic Petri dishes with filter paper discs containing EOs of cinnamon and oregano at ratio (a) 8:2 and (b) 9:1, showing the inhibition zones against *S. aureus*.

Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs)

Table 2 shows the minimum inhibitory concentration (MIC), minimum bactericidal concentration (MBC), and fractional inhibition concentration (FIC) index of different combinations of EOs against *S. aureus* in vapor and liquid-phase. In general, *S. aureus* was more sensitive to the vapor of the EOs than aqueous EOs. The MICs in vapor-phase were greatly lower than those in liquid-phase although the definition and the unit of MIC in two methods are different. MICs in vapor contact assay varied from 5 to 20 µL L^{-1} air while those values in microdilution assay ranged from 1 to 4 µL mL^{-1} solution. The EOs in both phase shared the same trend of antimicrobial inhibition on *S. aureus*, the microorganism seemed to be more sensitive to the EOs when increasing the ratios of cinnamon oil. However, the lowest MIC in vapor-phase was obtained at the combination 8:2 and 9:1 (MIC=5 µL L^{-1}) and the ones in liquid-phase were observed at the combinations 9:1 and 10:0 (MIC=0.5 µL mL^{-1}). MBCs in volatilization assay were equal to MICs because the inhibition effects maintained for at least 14 days after removing the antimicrobial atmosphere. These finding are in agreement with other studies reporting that some certain essential oils including oregano and cinnamon oils in vapor-phase had a lethal effect on *Staphylococcus aureus and Pseudomonas aeruginosa,* even in small amount [18]. Besides, López et al. [14] reported that their selected essential oils had bactericidal effects on test microorganisms in disc volatilization assay. For microdilution assay, MBCs were higher than MICs in almost combinations, except the ratios 0:10, 1:9, 2:8 and 8:2. Hence, it could be inferred that the antimicrobial activity of the selected EOs in vapor-phase could be achieved at lesser amount than that in liquid-phase. The results are consistent with the results of other study, which compare the inhibition of lemon grass oil against *Escherichia coli* in vapor- and liquid-phase [19]. Carvacrol, a main constituent in oregano oil, was found by Ultee et al. [20] to interact with the phospholipid bilayer and change the structure of *Bacillus cereus* membrane as well as increase bacterial cell membrane permeability [21]. Besides, cinnamaldehyde can inhibit the activity of enzymes related to cytokine interaction or less functional responsibility in cells when it is used at low concentration. It can also inhibit the activity of ATPase at higher concentration. At a lethal level, it is able to perturb the cell membrane [22]. In direct contact approaches, the effectiveness of EOs might decrease due to the binding of active compound to other components and affinity with water [23].

Table 2. Minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs) and fractional inhibition concentrations (FICs) index indicated by disc volatilization and broth microdilution assay

Ratio (v/v)		MIC		MBC		FIC	
Cin	Ore	Vapor-phase ($\mu L\ L^{-1}$)	Liquid-phase ($\mu L\ mL^{-1}$)	Vapor-phase ($\mu L\ L^{-1}$)	Liquid-phase ($\mu L\ mL^{-1}$)	Vapor-phase	Liquid-phase
0	10	20	4	20	4	1	1
1	9	20	4	20	4	1	1.7
2	8	20	4	20	4	1	2.4
3	7	10	2	10	4	0.5	1.55
4	6	10	2	10	4	0.5	1.9
5	5	10	1	10	2	0.5	1.13
6	4	10	1	10	2	0.5	1.3
7	3	10	1	10	2	0.5	1.48
8	2	5	1	5	1	0.25	1.65
9	1	5	0.5	5	1	0.25	0.91
10	0	20	0.5	20	1	1	1

Cin = cinnamon; Ore = oregano

Synergistic effects

Davidson and Parish [19] classified and described the synergistic effect of EOs or their components when they are combined. Synergistic effect is showed when the effect of combination is higher than the sum effects of individuals. If these values are equal, the combination shows an additive effect. Finally, antagonistic effect is observed when the combination of EOs shows a lower antimicrobial effect in comparison with that of individual EO.

Fraction inhibitory concentration index could be estimated in different approaches. In this study, FIC values of cinnamon and oregano oils were calculated basing on the MICs and these were plotted in isobolograms (Figure 3). Table 2 also shows FIC index of the combinations of EO. A synergistic effect was apparently observed for the inhibition of *S. aureus* in vapor-phase of EOs. Regardless to individual EO treatments, there were two combinations showing additive effect (FIC index was equal to 1) including 1:9 and 2:8 while the rest of them significantly contributed to improve the inhibition effects. Particularly, strong synergistic activity was found in the combination 8:2 and 9:1 (FIC=0.25). Conversely, the combination of cinnamon and oregano oil in liquid-phase indicated antagonistic, there was only the combination 9:1 giving the slightly synergistic inhibition (FIC=0.91). Cinnamon oil showed higher contribution to the antimicrobial activity of the mixture of EOs in both methods since an increase in cinnamon ratios provided more synergistic effect. In another study, Goñi et al. [11] found that the components of combined mixture in vapor-phase were more similar to those of cinnamon vapor when mixing cinnamon and clove oil. Besides, vapor-phase was more effective approach to combine these oils for inhibition of *S. aureus*. This might be due to a difference in mode of action and composition of antimicrobial agents in both assays. In solution, the antimicrobial activity is obtained by more hydrophilic and less volatile substances. In vapor-phase, it depends on the volatility of compounds and high volatility property of EO compounds contributes to their antimicrobial effect [19].

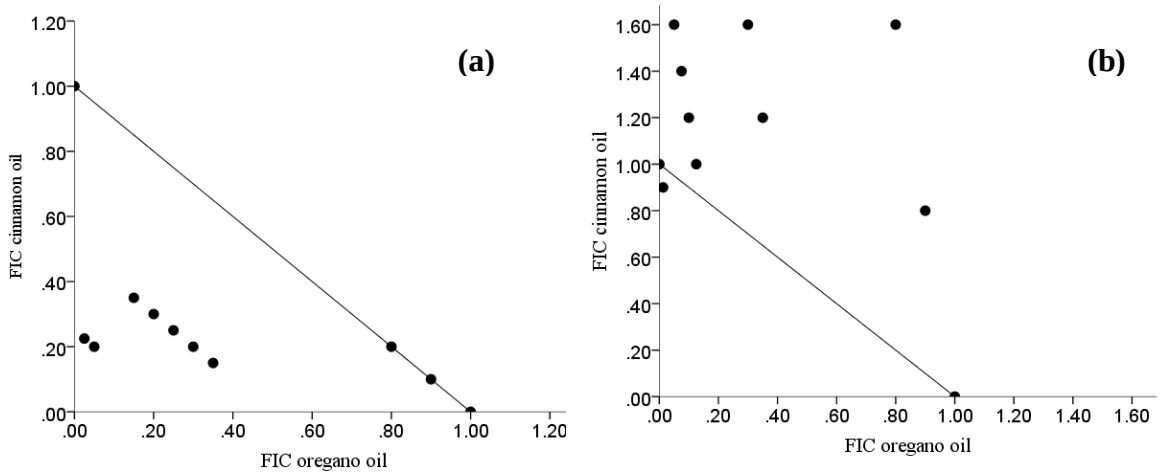

Figure 3. Isobolograms of cinnamon and oregano oils against *Staphylococcus aureus* in vapor (a) and liquid (b) phase.

CONCLUSIONS

This work evaluated and compared the antimicrobial activity of the combination of cinnamon and oregano essential oils at different ratios against *S. aureus* in vapor and liquid-phase. Synergistic effects were also integrated to indicate the optimal combination of EOs for inhibition of the pathogen. In general, vapor-phase was the suitable approach to apply the combination of EOs since less concentration was required. In fact, MICs of the combination ratios 8:2 and 9:1 were very low at 5 µL L^{-1} air. Moreover, these combinations showed high synergistic effect with FIC=0.25. The results provide a powerful approach of using natural compounds for developing medical therapies or active packaging against foodborne spoilages and pathogens.

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REFERENCES

1. Kurlenda J, Grinholc M. 2012. Alternative therapies in *Staphylococcus aureus* diseases. Acta Biochim. Pol. 59, 171-184.
2. Loir YL, Baron F, Gautier M. 2003. *Staphylococcus aureus* and food poisoning. Genet. Mol. Res. 2, 63-76.
3. Ito T, Hiramatsu K .2003. MRSA (methicillin-resistant *Staphylococcus aureus*). Nippon Rinsho. 61, 164-170.
4. Li Y, De-xin K, Hong W. 2013. Analysis and evaluation of essential oil components of cinnamon barks using GC–MS and FTIR spectroscopy. Ind. Crops Prod. 41, 269-278.
5. Gill AO, Richard AH. Mechanisms of bactericidal action of cinnamaldehyde against *Listeria monocytogenes* and of eugenol against *L. monocytogenes* and *Lactobacillus sakei*. Appl. Environ. Microbiol. 70, 5750-5755.
6. Sanla-Ead N, Jangchud A, Chonhenchob V, Suppakul P. 2006. Antimicrobial activity of cinnamon, clove and galangal essential oils and their principal constituents, and possible application in active packaging. The Proceedings of 15th IAPRI World Conference on Packaging. 214-218.
7. Ooi LS, Li Y, Kam SL, Wang H, Wong EY, Ooi VE. 2006. Antimicrobial activities of cinnamon oil and cinnamaldehyde from the Chinese medicinal herb *Cinnamomum cassia* Blume. Am. J. Chinese Med. 34, 511–22.
8. Martucci JF, Gende LB, Neira LM, and Ruseckaite RA. 2015. Oregano and lavender essential oils as antioxidant and antimicrobial additives of biogenic gelatin films. Ind. Crops Prod. 71, 205-213.

9. Inouye S, Toshio T, and Hideyo Y. 2001. Antibacterial activity of essential oils and their major constituents against respiratory tract pathogens by gaseous contact. J. Antimicrob. Chemother. 47:565-573.
10. Burt S. 2004. Essential oils: Their antibacterial properties and potential applications in foods – A review. Int. J. Food Microbiol. 94, 223-253.
11. Goñi P, López P, Sánchez C, Gómez-Lus R, Becerril R, and Nerín C. 2009. Antimicrobial activity in the vapour phase of a combination of cinnamon and clove essential oils. Food Chem. 116, 982-989.
12. Sukatta U, Haruthaithanasan V, Chantarapanont W, Dilokkunanant W and Suppakul P. 2008. In vitro evidence of antifungal synergy between clove and cinnamon oil and possible application in active packaging for controlling postharvest decay of table grape. The Proceedings of 16th IAPRI World Conference on Packaging. Bangkok, Thailand.
13 Pei R, Feng Z, Bao-Ping J, and Jing X. 2009. Evaluation of combined antibacterial effects of eugenol, cinnamaldehyde, thymol, and carvacrol against *E. Coli* with an improved method. J. Food Sci. 74, M379-M383.
14. López P, Sánchez C, Batlle R, Nerín C. 2005. Solid- and vapor-phase antimicrobial activities of six essential oils: susceptibility of selected foodborne bacterial and fungal strains. J. Agr. Food Chem. 53, 6939-6946.
15. Sarrazin SLF, Ricardo BO, Lauro ESB, Rosa HVM. 2012. Chemical composition and antimicrobial activity of the essential oil of *Lippia grandis* Schauer (Verbenaceae) from the Western Amazon. Food Chem. 134, 1474-1478.
16. Davidson PM. and Parish ME. 1989. Methods for testing the efficacy of food antimicrobials. Food Technol. 43, 148-155.
17. Ocana A, Amir E, Yeung C, Seruga B, Tannock IF. 2012. How valid are claims for synergy in published clinical studies? Annal. Oncol. 23, 2161–2166.
18. Pibiri MC. 2006. Assainissement microbiologique de l'air et des systèmes de ventilation au moyen d'huiles essentielles PhD thesis, Federal Polytechnic Institute., Lausanne, France.
19. Tyagi AK, Anushree M. 2012. Bactericidal action of lemon grass oil vapors and negative air ions. Innov. Food Sci. Emerg. Technol. 13, 169-77.
20. Ultee A, Kets EPW, Alberda M, Hoekstra FA, Smid EJ. 2000. Adaptation of the food-borne pathogen *Bacillus cereus* to carvacrol. Arch. Microbiol. 174, 233- 238.
21. Lambert RJW, Skandamis PN, Coote PJ, Nychas GJE. 2001. A study of the minimum inhibitory concentration and mode of action of Oregano essential oil, thymol and carvacrol. J. Appl. Microbiol. 91, 453-462.
22. Nazzaro F, Florinda F, Laura DM, Raffaele C, Vincenzo DF. 2013. Effect of essential oils on pathogenic bacteria. Pharmaceuticals. 6, 1451-1474.
23. Avila-Sosa R, Enrique P, María TJM, Guadalupe VN-M, Addí RNC, Aurelio L-M. 2012. Antifungal activity by vapor contact of essential oils added to amaranth, chitosan, or starch edible films. Int. J. Food Microbiol. 153, 66-72.

Comparative evaluation of different concentration methods for the assays of total phenolic content and antioxidant activity of Thai pomegranate juice

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ABSTRACT

Pomegranate juice is considered to be a rich source of antioxidants and total polyphenol content. This study aimed to determine the best concentration method for the assay of total phenolic content and antioxidant activity of Thai pomegranate juice (TPJ). Fresh TPJ (14-15 °Brix) was concentrated to 60±0.5 °Brix by rotary vacuum evaporation, and microwave evaporation which were achieved within 21 and 11 minutes, respectively. Fresh TPJ and concentrated TPJs were investigated in triplicate for total phenolic content using Folin-Ciocalteau's reagent and radical scavenging potential using DPPH and ABTS assays. Total phenolic contents of concentrated TPJs by rotary vacuum evaporator and by microwave evaporator were found to be similar (6.61±0.75 and 6.68±0.60 mg GAE/L juice, respectively) which were significantly higher than that of fresh TPJ (1.94±0.36 mg GAE/L juice, $P<0.001$). Concentrated TPJs by rotary vacuum evaporator and by microwave evaporator exhibited similar radical scavenging activity on DPPH and ABTS assays, which were significantly lower than that of fresh TPJ ($P<0.001$). Concentrated TPJs (by rotary vacuum evaporator and by microwave evaporator) and fresh TPJ showed antiradical activity against DPPH radicals (IC_{50} values 0.22±0.01, 0.24±0.01, and 0.93±0.07 µl/ml) and against ABTS radicals (IC_{50} values 0.16±0.05, 0.17±0.05, and 0.65±0.13 µl/ml). The results indicate that both concentration methods can be used for quantitative analysis of total phenolic content and antioxidant activity of TPJ. However, microwave evaporation may be the best choice since TPJ can be concentrated in a short period of time. Microwave evaporation has the advantage of heating the TPJ rapidly and uniformly.

Keywords: Thai pomegranate juice, Total phenolic content, Antioxidant activity, DPPH, ABTS

INTRODUCTION

Pomegranate (*Punica granatum* L.) is one of the most popular nutritious and healthy fruits. It is an antioxidant-rich food that contains three types of antioxidant polyphenols, including tannins, anthocyanins, and ellagic acid [1]. Pomegranate juice is considered to be a rich source of antioxidants and total polyphenol content. Pomegranate juice contains many components such as sugar content, the major acids (citric and malic), phenolic, tannins, ellagic acid, gallic acid, protocatechuic acid, chlorogenic acid, caffeic acid, ferulic acid, p-coumaric acid, catechin, phloridzin, quercetin, anthocyanins, punicalagin, phytoestrogenic, flavonoids and tannins [2-6]. Pomegranate juice has been reported to possess various properties, including antiproliferative, anticarcinogenic, antimicrobial, antiviral, and anti-atherosclerotic properties. Moreover, pomegranate juice consumption has been found to have several medical benefits to prevent and treat a wide variety of diseases such as cancer [7], cardiovascular disease [8], diabetes [9], Alzheimer's [10] and Parkinson's disease [11]. These health benefits are attributed to its antioxidant activity and its high total polyphenol content.

Pomegranate juice contains high amount of water. The majority of water can be separated by concentration methods such as conventional heating (boiling), microwave heating, vacuum evaporation, membrane concentration, ultrafiltration, nanofiltration, and reverse osmosis. This study aimed to determine the best concentration method

(between rotary vacuum evaporation and microwave evaporation) for the assay of total phenolic content and antioxidant activity of Thai pomegranate juice (TPJ).

MATERIALS AND METHODS

Pomegranate juice preparation

Thai pomegranate fruits were collected from pomegranate garden in Pak Chong, Nakhon Ratchasima Province, Thailand. The arils of the fruits were manually separated from the peels, and squeezed to produce the juice. The juice was then filtered through layers of cotton wool laid out over a sieve, and centrifuged at 4000 RCF for 15 min at 4 °C. Soluble solids or degree Brix measured by refractometer of fresh TPJ was 14-15 °Brix. TPJ was then concentrated by 2 methods: rotary vacuum evaporation, and microwave evaporation. Fresh TPJ with an initial total solids content of 14-15 °Brix was concentrated to a final concentration of 60 °Brix. Concentrations were achieved within 21 minutes by using a rotary vacuum evaporator at 65 °C, and 11 minutes by using microwave evaporator with maximum output of 450 W.

Determination of total phenolic content

Total phenolic content was determined by using Folin-Ciocalteu (FC) method that was modified from the method described by Minussi and colleagues (2003) [12]. Briefly, 100 µl of diluted TPJ in the ratio of 1:11 with 10% ethanol was mixed with 2000 µl of 2% sodium carbonate and 100 µl of Folin-Ciocalteu reagent. All solutions were freshly prepared and protected from light. The mixture was allowed to stand for 30 minutes before measuring the absorbance at 750 nm by using a spectrophotometer (CECIL 1011, England). Results were expressed as mg of gallic acid equivalents (GAE)/L juice. All determinations were performed in triplicate.

Antioxidant activity

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging method.

In this method, the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was used to measure the antioxidant activity of juices as described by Villano and colleagues (2006) [13] with some modification. Briefly, 50 µl of diluted samples and vitamin C (positive control, 0.001g/ml) was mixed with 1950 µl of methanol solution (25 mg/L) of the radical DPPH. All solutions were freshly prepared and protected from light. The mixture was incubated at room temperature for 30 min in the dark. The absorbance was then measured at 515 nm by using microplate reader (BioRad Benchmark Plus microplate reader). The distilled water mixed with DPPH working solution was used as the control and methanol solution was used as sample blank. Radical scavenging activity was defined as the inhibition percentage. All determinations were performed in triplicate.

2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS) radical scavenging method.

In this method, the 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid or ABTS radical was used to measure the antioxidant activity of juices as described by Re and colleagues (1999) [14] with some modification. Briefly, 25 µl of diluted samples and vitamin C (positive control, 0.001g/ml) was mixed with 2000 µl ABTS working solution (5 ml of 7 mmol/L ABTS solution reacts with 88 µl of 140 mmol/L potassium persulfate solution and then the reaction solution was diluted with ethanol to the absorbance of 0.700 ± 0.005 at 734 nm). All solutions were freshly prepared and protected from light. The mixture was incubated at room temperature for 10 min in the dark. The absorbance was then measured at 734 nm by using microplate reader (BioRad Benchmark Plus microplate reader). The distilled water mixed ABTS working solution was used as the control and ethanol solution was used as sample blank. Radical scavenging activity was defined as the inhibition percentage. All determinations were performed in triplicate.

The scavenging capacity (SC) for both DPPH and ABTS assays was calculated by using the following equation

$$\% (SC) = [(Abs_{control} - Abs_{sample}) / Abs_{control}]*100$$

The IC_{50} values of DPPH and ABTS assay calculated by using % scavenging capacity. The IC_{50} graph used GraphPad Prism software (version 6, GraphPad Software Inc, California, USA).

Statistical analysis

Data were presented as means of triplicates±SD. Data were analyzed by one-way analysis of variance (ANOVA) followed by post-hoc testing analysis using Sigma Stat 3.5 software (San Diego, USA). The P values less than 0.05 were considered statistically significant.

RESULTS

The percentage yields of concentrated TPJ for both rotary vacuum evaporation, and microwave evaporation methods were 16%. The data revealed that TPJ contained high concentrations of total phenolic content and strong antioxidant activity by means of radical scavenging potential using DPPH and ABTS assays. Total phenolic contents of concentrated TPJ by rotary vacuum evaporator (6.61±0.75 mg GAE/L juice) and concentrated TPJ by microwave evaporator (6.68±0.60mg GAE/L juice) were significantly higher than that of fresh TPJ (1.94±0.36 mg GAE/L juice, *P*<0.001). There was no significant difference in total phenolic content between both concentrated TPJs (Figure 1). The data demonstrated the presence of strong antioxidant properties (radical scavenging potential using DPPH and ABTS assays) of both concentrated TPJs. Both concentrated TPJs showed higher antioxidant activity than fresh TPJ. The IC_{50} of DPPH radical scavenging activity of concentrated TPJ by rotary vacuum evaporator, and concentrated TPJ by microwave evaporator were 0.22±0.01 and 0.24±0.01 µl/ml, respectively. These IC_{50} values were significantly lower than that of fresh TPJ IC_{50} (0.93±0.07 µl/ml, *P*<0.001). There was no significant difference in antioxidant activity by DPPH assays between concentrated TPJ by rotary vacuum evaporator and concentrated TPJ by microwave evaporator (Figure 2A). The IC_{50} of ABTS radical scavenging activity of concentrated TPJ by rotary vacuum evaporator and concentrated TPJ by microwave evaporator were 0.16±0.05 and 0.17±0.05 µl/ml, respectively. The IC_{50} of ABTS radical scavenging activity of concentrated TPJ by rotary vacuum evaporator and microwave evaporator were statistically significantly lower than that of fresh TPJ IC_{50} (0.65±0.13 µl/ml, *P*<0.001). No significant difference in antioxidant activity by ABTS assays between concentrated TPJ by rotary vacuum evaporator and concentrated TPJ by microwave evaporator was found (Figure 2B). Vitamin C was used as positive control in DPPH (IC_{50}= 0.00±0.01 mg/ml) and ABTS (IC_{50}= 0.01±0.00 mg/ml) radical scavenging activities, which were significantly lower than that of fresh TPJ, and both concentrated TPJs (*P*<0.001)**.** Both concentrated TPJs and fresh TPJ showed lower antioxidant activity than Vitamin C.

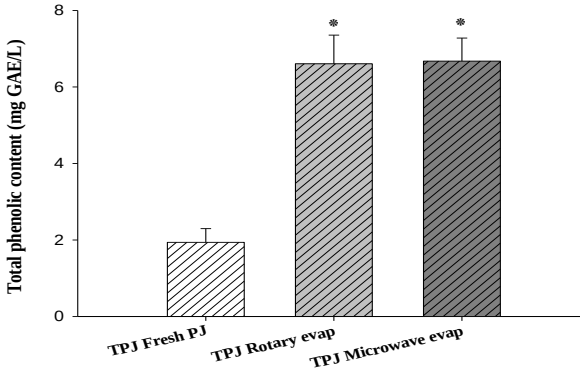

Figure 1. Total phenolic contents of fresh TPJ, concentrated TPJ by rotary vacuum evaporator and concentrated TPJ by microwave evaporator. Values are expressed as means± SD, n=3 per group. *, P <0.001 compared with fresh TPJ.

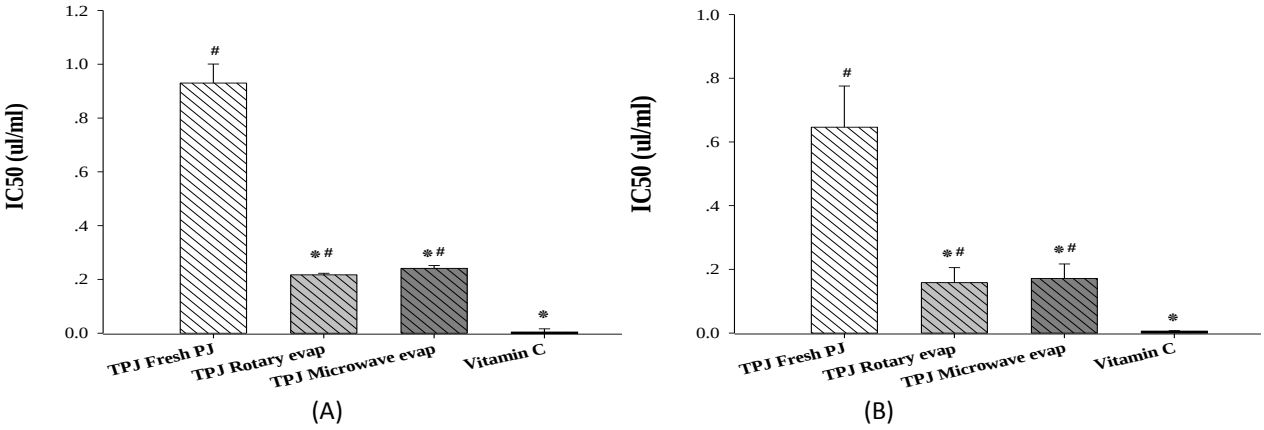

Figure 2. The IC_{50} values of DPPH (A) and ABTS (B) radical scavenging activities of fresh TPJ, concentrated TPJ by rotary vacuum evaporator, concentrated TPJ by microwave evaporator, and vitamin C. Values are expressed as means**±**SD, n=3 per group. *, P<0.001 compared with fresh TPJ. #, P<0.001 compared with vitamin C.

CONCLUSIONS

Similar to other pomegranate cultivars, TPJ contains high concentration of total phenolic content and possesses strong antioxidant activity. Antioxidant capacities measured by DPPH and ABTS assays of both concentrated TPJs were strongly correlated with their total phenolic contents, suggesting that phenolic compounds may be a contributor to antioxidant capacity of TPJ. The present results indicate that both concentration methods can be used for quantitative analysis of total phenolic content and antioxidant activity of TPJ. However, microwave evaporation may be the best choice since TPJ can be concentrated in a short period of time. Microwave evaporation method present the advantages of heating the TPJ simply, rapidly, reproducible, and uniformly.

REFERENCES

1. Qu W, Breksa Iii AP, Pan Z, and Ma H. 2012. Quantitative determination of major polyphenol constituents in pomegranate products. Food Chem. 132, 1585-1591.
2. Tezcan F, Gültekin-Özgüven M, Diken T, Özçelik B, and Erim F.B. 2009. Antioxidant activity and total phenolic, organic acid and sugar content in commercial pomegranate juices. Food Chem. 115, 873-877.
3. Mousavinejad G, Emam-Djomeh Z, Rezaei K, and Khodaparast MHH. 2009. Identification and quantification of phenolic compounds and their effects on antioxidant activity in pomegranate juices of eight Iranian cultivars. Food Chem. 115, 1274-1278.
4. Poyrazoğlu E, Gökmen V, and Artık N. 2002. Organic Acids and Phenolic Compounds in Pomegranates (*Punica granatum* L.) Grown in Turkey. J. Food Compos Anal. 15, 567-575.
5. Gil MI, Tomás-Barberán FA, Hess-Pierce B, Holcroft DM and Kader AA. 2000. Antioxidant activity of pomegranate juice and its relationship with phenolic composition and processing. J. Agr. Food Chem. 48, 4581-4589.
6. Vegara S, Marti N, Lorente J, Coll L, Streitenberger S, Valero M, and Saura D. (2014). Chemical guide parameters for Punica granatum cv. 'Mollar' fruit juices processed at industrial scale. Food Chem. 147, 203-208.
7. Wang L, Ho J, Glackin C, and Martins-Green M. 2012. Specific Pomegranate Juice Components as Potential Inhibitors of Prostate Cancer Metastasis. Transl. Oncol. 5, 344-IN345.
8. Fuhrman B, Volkova N, and Aviram M. 2005. Pomegranate juice inhibits oxidized LDL uptake and cholesterol biosynthesis in macrophages. J. Nutr. Biochem. 16, 570-576.
9. Rosenblat M, Hayek T, and Aviram M. 2006. Anti-oxidative effects of pomegranate juice (PJ) consumption by diabetic patients on serum and on macrophages. Atherosclerosis. 187, 363-371.
10. Hartman RE, Shah A, Fagan AM, Schwetye KE, Parsadanian M, Schulman RN, Finn MB, and Holtzman DM. 2006. Pomegranate juice decreases amyloid load and improves behavior in a mouse model of Alzheimer's disease. Neurobiol. Dis. 24, 506-515.
11. Tapias V, Cannon JR, and Greenamyre JT. 2014. Pomegranate juice exacerbates oxidative stress and nigrostriatal degeneration in Parkinson's disease. Neurobiol. Aging. 35, 1162-1176.
12. Minussi RC, Rossi M, Bologna L, Cordi L v, Rotilio D, Pastore GM and Durán N. 2003. Phenolic compounds and total antioxidant potential of commercial wines. Food Chem. 82, 409-416.
13. Villano D, Fernandez-Pachon MS, Moya M.L, Troncoso AM and Garcia-Parrilla MC. 2007. Radical scavenging ability of polyphenolic compounds towards DPPH free radical. Talanta. 71, 230-235.
14. Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, and Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Bio. Med. 26, 1231-1237.

In vitro mutagenic and antimutagenic effects of *Diospyros castanea* branch and leaf extracts

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ABSTRACT

Diospyros castanea is a plant in Ebenaceae family and has been used as a folk medicine in Thailand. The objectives of this study are to determine the mutagenicity and antimutagenic effects of the ethanolic extracts of *D. castanea* branch and leaf by using bacterial model based on Ames' test. In mutagenicity test, the extracts were pre-incubated with *Salmonella typhimurium* TA98 and TA100 and then cultivated in the presence and absence of S9-mix, a metabolic activation system containing rat-liver microsome fraction plus cofactors. The result showed that both extracts, at the concentration of 1 to 10 mg/plate, had no mutagenic effect. In the absence of S9-mix, the extracts were pre-incubated with the bacterial cultures containing standard mutagens (AF2 and 4NQO). The result showed that the extracts could slightly reduce the number of revertant colonies. In the presence of S9-mix, the extracts were pre-incubated with the bacterial cultures (TA98 and TA100) contained standard mutagens, 2AA. Interestingly, it was found that the branch extract showed strongly antimutagenicity effect with IC_{50} of 0.76 and 0.70 mg/plate, respectively, and the leaf extract showed the similar effect with IC_{50} of 0.80 and 0.70 mg/plate, respectively. These results indicate that the ethanolic extracts from *D. castanea* branch and leaf can decrease the mutagenic effect of the standard mutagens in this study and have no mutagenic effect against this bacterial model.

Keywords: Mutagenicity, Antimutagenicity, *Diospyros castanea*

INTRODUCTION

Diospyros castanea Fletcher or Dak Dam in Thai is a local plant found in northern and northeastern parts of Thailand. It belongs to Ebenaceae family. It has been used as traditional medicine for the treatment of diarrhea and vomit. In addition, the hot water extract from its branch has been used as a traditional tonic. Sripanidkulchai and colleagues [1] reported the screening of its chemical composition and some biological activity. The ethanolic extract of *D. castanea* contained tannins, flavonoids, alkaloids, cardiac glycosides and has antioxidative activity [1]. This plant extract showed antibacterial activity against *Staphylococcus aureus*, *Salmonella* sp., *Shigella* sp. and *Vibrio cholera* and antiviral activity against Herpes simplex virus type I [2]. However, it was found that these *D. castanea* extracts had no immunomodulatory effect against murine splenocytes in *in vitro* [3].

The point mutations are the cause of several human genetic diseases and involve in tumor formation in humans and experimental animals. The U.S. Food and Drug Administration announces a guideline or an alternative approach for the safety assessment of food and drug including the natural products by using the bacterial reverse mutation test or Ames' test [4, 5]. Mutant strains of *Salmonella typhimurium* and *Escherichia coli* are used in the detection of the chemicals that induce mutations by reverting of the mutation in the bacterial strains resulting in restoration of the essential amino acid synthesis function [5]. Therefore, the bacterial reverse mutation test is useful method in the test of mutagenic and antimutagenic potentials of natural or synthetic chemicals. This bacterial reverse mutation test is not only sensitive method but it is also rapid, inexpensive and easy method for detection of mutation. The objectives in this study are to investigate the mutagenic and antimutagenic effects of *D. castanea* branch and leaf extracts by using modified bacterial reverse mutation test.

MATERIALS AND METHODS

Plant extract preparation

The *D. castanea* branch or leaf was dried and then milled to a powder. The powder was macerated in 50% ethanol at a ratio of 1:5 for 7 days then filtered through Whatman No.1 paper. The solution was evaporated under rotary evaporator and then dried by using freeze dryer. The yields of branch and leaf extracts were 8.74% and 7.32%, respectively. For extract stock solution, the extracts were dissolved in dimethyl sulfoxide (DMSO) to 100 mg/ml and filtered by using 0.4 µm syringe filter.

Bacterial strains and media

The bacterial model in this study was *Salmonella typhimurium* strain TA98 and TA100. The bacteria were maintained in 1 mM histidine- and 1 mM biotin-supplemented Vogel–Bonner (VB) agar. A loopful of bacteria was inoculated into 20 ml of Nutrient broth and then incubated at 35°C in water bath shaker (100 rpm) for 18 h before performing the experiments.

Determination of mutagenic activities

The pre-incubation method which modified from bacterial reverse mutation test or Ames' test [6] was used to determine the mutagenic activity of the extract. Briefly, the bacteria were separated into two conditions, the presence and absence of S9-mix. S9 mix was consisted of 10% of male Sprague-Dawley rat microsomal liver enzyme, 4 mM NADPH, 4 mM NADH, 5 mM glucose-6-phosphate, 8 mM $MgCl_2$, 33 mM KCl, 100 M sodium phosphate buffer (pH 7.4) [7]. The overnight bacterial culture (100 µl) was mixed with the various concentrations of the extract (50 µl). For the presence of S9-mix, the culture was added with 0.5 ml S9-mix. For the absence of S9-mix, the culture was added with 0.5 ml of 0.2 M phosphate buffer (pH 7.4). The DMSO alone (50µl) was mixed with bacterial culture (100 µl) in the presence or absence of S9-mix as a negative control or background. The bacterial mixture was pre-incubated at 35°C and shaken (100 rpm) for 30 min. Two milliliter of melting 0.1 mM histidine – 0.1 mM biotin supplemented top agar (sterile mixture of 0.6 % w/w agar and 0.5 % w/w sodium chloride) was then added to the bacterial mixture. After mixing well, the whole mixture was then overlaid on the surface of VB agar. After the agar solidity, the plates were then incubated at 37°C for 48 h. The bacterial colonies from the duplicate experiments were counted and expressed as number of revertants/plate (mean ± SD).

Determination of antimutagenic activities

Antimutagenic activity of the extract was also determined by using pre-incubation method which modified from bacterial reverse mutation test or Ames' test [6]. Briefly, in the presence of S9-mix, the bacterial culture (100 µl) was added with the various concentration of the extract (50 µl), 50 µl of 10 µg/ml standard mutagen (2-aminoanthracene or 2-AA) for both strains and 0.5 ml of S9-mix. In the absence of S9-mix, the bacterial culture (100 µl) was added with the extract (50 µl), 0.5 ml of 0.2 M phosphate buffer and 50 µl of standard mutagens. Strain TA98 was treated with standard mutagens included 2 µg/ml of AF2 (2(2-furyl)-3(5-nitro-2-fury)acryl amide) and 4 µg/ml of 4NQO (4-nitroquinoline 1-oxide). Strain TA100 was treated with 0.2 µg/ml of AF2 and 1 µg/ml of 4NQO. The DMSO alone (50µl) was mixed with bacterial culture (100 µl) and standard mutagens (50 µl) in the presence or absence of S9-mix as a positive control. Then the mixture was pre-incubated at 35°C and shaken (100 rpm) for 30 min. The melting histidine - biotin supplemented top agar (2 ml) was then added as described above. After the agar solidity, the plates were incubated at 37°C for 48 h. The bacterial colonies from the duplicate experiments were counted and expressed as number of revertants/plate (mean ± SD) and calculated for 50% inhibitory concentration (IC_{50}).

RESULTS

The criteria for determining the positive result in mutagenicity test include 1) a concentration-related increase of revertant colonies over the range tested, 2) a reproducible increase at one or more concentrations in the number of revertant colonies per plate in at least one strain with or without metabolic activation system and 3) at least three times higher in the number of revertant colonies of positive results than those of spontaneous colonies or background [4, 8]. Both *D. castanea* extracts were tested at the concentration of 1 to 10 mg/plate. In the presence and absence of S9-mix, the extracts did not induce the increasing of revertant colonies in both strains compared with control or spontaneous colonies and there was no concentration-related increasing of revertant colonies suggesting that the *D. castanea* branch and leaf extracts had no mutagenic effect (Table 1).

For antimutagenic effect, the bacteria were pre-incubated with 2AA in the presence of S9-mix resulted in the highly increasing of revertant colonies compared with number of spontaneous colonies. But in the addition of the *D. castanea* branch and leaf extracts, the number of 2AA induced revertant colonies was highly deceased in dose dependent manners (Figure 1) with IC_{50} of 0.76 and 0.80 mg/plate for TA98 and 0.70 and 0.70 mg/plate for TA100, respectively (Table 2). On the other hand, the extracts showed lower antimutagenic activity in the absence of S9-mix than those in the presence of S9-mix (Figure 1 and Table 2). These results indicate that the extracts can exhibit stronger antimutagenic effect in the liver enzymatic activation or in metabolic forms than the direct contact activation, however, both branch and leaf extracts showed the similar trend of this effect.

Table 1. The mutagenic effects of *D. castanea* extracts

Extract conc. (mg/plate)	Number of revertants/plate							
	D. castanea branch extract				*D. castanea* leaf extract			
	-S9-mix		+ S9-mix		-S9-mix		+ S9-mix	
	TA98	TA100	TA98	TA100	TA98	TA100	TA98	TA100
0	22 ± 6	127 ± 5	38 ± 3	138 ± 7	29 ± 7	117 ± 6	33 ± 17	135 ± 13
1	20 ± 0	127 ± 4	36 ± 2	133 ± 3	39 ± 1	150 ± 1	32 ± 2	130 ± 35
2.5	21 ± 2	119 ± 2	38 ± 6	130 ± 20	43 ± 2	118 ± 1	39 ± 1	125 ± 1
5	19 ± 4	119 ± 11	25 ± 3	108 ± 8	19 ± 4	111 ± 3	34 ± 8	106 ± 1
10	12 ± 2	137 ± 1	16 ± 3	102 ± 18	18 ± 4	130 ± 13	31 ± 13	103 ± 19

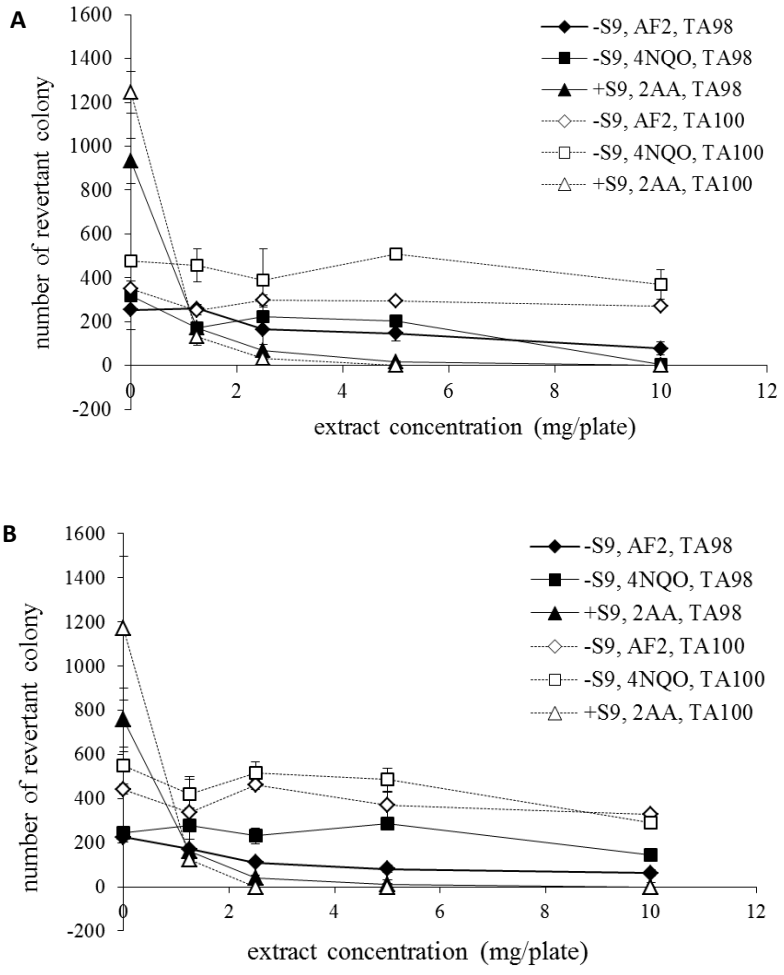

Figure 1. Antimutagenic activities of *D. castanea* branch (A) and leaf (B) extracts

CONCLUSIONS

This present study demonstrates that the ethanolic extracts from *D. castanea* branch and leaf do not induce the genetic reversion of *S. typhimurium* TA98 and TA100 in this study. It confirms that the consumption of these extracts do not create a mutation which may lead to the carcinogenesis. Moreover, the extracts in the enzymatic activation system can strongly decrease the mutagenic effect of the standard mutagens in this study suggesting that the consumption of the *D. castanea* extracts may help to prevent the carcinogenesis. In conclusion, the extracts from *D. castanea* branch and leaf in the present study have efficacy and potential for further natural product development.

Table 2. The IC_{50} of the antimutagenic effects of *D. castanea* branch and leaf extracts

Plant extracts	IC_{50} (mg/plate)					
	-S9-mix				+ S9-mix	
	AF2		4NQO		2AA	
	TA98	TA100	TA98	TA100	TA98	TA100
D. castanea branch	6.61	5.32	>10	>10	0.76	0.70
D. castanea leaf	2.94	>10	>10	>10	0.80	0.70

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REFERENCES

4. Sripanidkul B, Junlatat J, Thitimatharoch T, Saoha J. 2010. Research report: Standards and chemical constituents of Plants in Plant Genetics Conservation Project as The Royal Initiative of Her Royal Highness Princess Maha Chakri Sirindhorn of Khon Kaen University at Ubonrat Dam, Khon Kaen.
5. Tattawasart U. 2010. Research report: Investigation on biological activities of herbal plants from Amphur Nampong for use in infection.
6. Sripanidkul B. 2010. Research report: Study on anti-mutagenicity and immunomodulatory effect of Indigenous Plants Conservation Project as The Royal Initiation of Her Royal Highness Princess Maha Chakri Sirindhorn of Khon Kaen University at Ubonrat Dam, Khon Kaen.
7. U.S. Food and Drug Administration. 2000. Toxicological Principles for the Safety Assessment of Food Ingredients Redbook 2000. In: Chapter IV.C.1.a. Bacterial Reverse Mutation Test.
8. Ames BN, McCann J, Yamasaki E. 1975. Methods for detecting carcinogens and mutagens with the *Salmonella*/Mammalian-microsome mutagenicity test. Mutat. Res. 31, 347-364.
9. Sripanidkulchai B, Tattawasart U, Laupatarakasem P, Vinitketkumneun U, Sripanidkulchai K, Furihata C, Matsushima T. 2002. Antimutagenic and anticarcinogenic effects of *Phyllanthus amarus*. Phytomedicine. 9, 26-32.
10. Matsushima T, Sawamura M, Hara K, Sugimra T. 1976. A safe substitute for polychlorinated biphenyls as an inducer of metabolic activation system. In: *in vitro* Metabolic Activation in Mutagenesis Testing. Elsevier/North-Holland, Amsterdam, USA, 85–88.
11. Claxton LD, Allen J, Auletta A, Mortelmans K, Nestmann E, Zeiger E. 1987. Guide for the *Salmonella typhimurium*/Mammalian microsome tests for bacterial mutagenicity. Mutat. Res. 189, 83-91.

The interactive effect of the combinations of *Kaempferia parviflora* extract with antibiotic agents against methicillin resistant *Staphylococcus aureus*

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ABSTRACT

The extracts from rhizome of *Kaempferia parviflora* have been found to possess antimicrobial activities such as antifungal and antibacterial activities. The study was aimed to investigate the antibacterial activities of the methanol extract and antibiotic agents against various MRSA strains. The antibacterial activities of methanol extract and antibiotic agents were assessed by agar dilution method. The interactive effect of the combinations of methanol extract with antibiotic agents: penicillin and vancomycin, were assessed by checkerboard dilution method in 20 isolates of MRSA. The data were analyzed for minimum inhibitory concentration (MIC) and Fractional inhibitory concentration index (FICI). The results showed that methanol extract exhibited the inhibition on 8 isolates of MRSA at the MICs ranged from 1000 – 2000 μg/ml. The MICs of penicillin against 20 isolates of MRSA ranged from 0.125 – >256 μg/ml are implied that all of isolates are resistant to penicillin. The MICs of vancomycin against 19 isolates of MRSA ranged from 0.25 – 2 μg/ml are implied that most isolates are sensitive to vancomycin, except one isolate showing resistance to vancomycin with MIC of 4 μg/ml. The interactive effect of combinations between methanol extract and vancomycin exhibited synergistic effect against MRSA 5 isolates (FICI ≤ 0.5) and neutral effect on MRSA 3 isolate (FICI = 1). The interactive effect of methanol extract and penicillin showed to be synergistic for all 8 isolates of MRSA (FICI ≤ 0.5). In conclusion, *Kaempferia parviflora* methanol extract demonstrates a possible enhanced antibacterial activity in the treatment of MRSA infection.

Keywords: *Kaempferia parviflora* extract, methicillin resistant *Staphylococcus aureus*, antibiotic agents

INTRODUCTION

Evidence from clinical studies have demonstrated that *Staphylococcus aureus* is a human pathogen that is highly resistant to various antibiotic drugs. Methicillin resistant *S. aureus* (MRSA) strains is a major human pathogen that causes an increasing number of infections in hospital setting as well as in the community [1].

Kaempferia parviflora belong to Zingiberaceae family and locally known in Thai as Kra-Chai-dum [2]. The rhizome of *Kaempferia parviflora* has been found to possess antimicrobial activities such as antifungal and antibacterial activities [2]. The present study was aimed to investigate the antibacterial activity of the *K. parviflora* extract and its interactive effect on the combination of *K. parviflora* extract with antibiotic agents against various MRSA strains.

MATERIALS AND METHODS

Plant collection and crude extract

Fresh rhizomes of *K. parviflora* were collected from Dan Sai district, Loei province, Thailand during December 2012 – January 2013. Crude extract were prepared as previously described [3]. Briefly, dried rhizomes of *K. parviflora* (1.63 kg) were ground and macerated twice with methanol (8 L) at room temperature for seven days and the solvent was removed in vacuo at 65°C (rotary evaporator, Buchi rotavapor R-124, German) to yield *K. parviflora* crude extract was approximately 1.4% w/w (22.72 g).

Microorganism

Twenty methicillin-resistant *Staphylococcus aureus* [MRSA] strains were obtained from Clinical Microbiology Unit of Srinagarind Hospital, Faculty of Medicine, Khon Kaen University.

Antibacterial assay

The antibacterial activities of methanol extract and antibiotic agents were evaluated by agar dilution method [4, 5] using Mueller Hinton agar (Himedia, India) for the assay. The microorganism were grown on Nutrient agar (NM018, India) at 37°C for 24 h. and activated by inoculating 3 – 4 loopful of the strain in the 0.85% NaCl (inoculum size was 10^8 cells/ml as per 0.5 McFarland standard) [6]. Then 0.01 ml of inoculum was spotted into the Mueller Hinton agar (Himedia, India) supplemented with an extract or antibiotic at concentrations ranging from 15.62-2000 µg/ml [2] for crude extract, 0.125-256 µg/ml [7] for penicillin (PEN) (M&H manufacturing, Thailand) and vancomycin (VAN) (Edicin, Slovenia) and all the plates were incubated at 37°C for 24 h. The experiment was performed 3 times. The MIC (minimum inhibitory concentration) was defined as the lowest concentration at which no visible growth was observed.

Determination of the interactive effect of combinations of antimicrobial agents

The antibacterial activities of different combinations of 2 antibacterial agents were assessed using the checkerboard dilution method [5, 8, 9]. The interactive effect of the combinations of methanol extract with antibiotic agents: penicillin and vancomycin, were tested at the MIC. Then 0.1 ml of serial dilutions (1x MIC to 8x MIC) of antibiotics with 0.1 ml of serial dilution (1x MIC to 8x MIC) of methanol extract into the Mueller Hinton broth tubes (Himedia, India). The microorganism were grown on Nutrient agar (NM018, India) at 37°C for 24 h. and activated by inoculating 3 – 4 loopful of the strain in the 0.85% NaCl (inoculum size was 10^8 cells/ml as per 0.5 McFarland standard) [6]. Then 0.1 ml of inoculum was inoculated into the Mueller Hinton broth tubes (Himedia, India). All tubes were incubated at 37°C for 24 h. The fractional inhibitory concentration index (FICI) [8] was calculated by adding the FICs (MIC of drug A in combination with drug B/MIC of drug A alone) of methanol extract and antibiotic agents. An FICI of ≤0.5 was defined as synergy, an FICI of >0.5 to 4.0 was defined as indifferent, and an FICI of >4.0 was defined as antagonism. Checkerboard test results represented the average of triplicate tests.

RESULTS

The MICs of methanol extract and antibiotic agents

Antibacterial activities of methanol extract and antibiotic agents against 20 isolates of MRSA were performed using the agar dilution method. The plant methanol extract showed the inhibition on 8 isolates of MRSA at the MICs ranged from 1,000 – >2,000 µg/ml (Table 1). Penicillin showed the inhibition on 20 isolates of MRSA at the MICs ranged from 0.125 – >256 µg/ml are implied that all of isolates are resistant to penicillin (Table 1). Vancomycin showed the inhibition on 19 isolates of MRSA at the MICs ranged from 0.25 – 2 µg/ml are implied that most isolates are sensitive to vancomycin, except one isolate showing resistance to vancomycin (MRSA 5-333) with MIC of 4 µg/ml (Table 1). Our results are in line with previous study that *K. parviflora* extract showed the inhibition on *S. aureus* [11].

Table 1. Antibacterial activities of methanol extract and antibiotic agents against 20 isolates of MRSA.

MRSA isolates	MIC (µg/ml)		
	Methanol extract	Penicillin*	Vancomycin**
5-456	2,000	0.25	0.25
5-183	1,000	16	1
5-163	>2,000	16	0.25
5-184	2,000	0.25	0.5
5-254	>2,000	0.25	0.25
5-30	>2,000	0.25	0.5
5-490	>2,000	>256	0.5
5-85	>2,000	4	0.5
5-104	1,000	0.25	0.25
5-272	>2,000	0.25	0.5
5-260	>2,000	>256	0.25
5-283	>2,000	0.25	0.25
5-151	2,000	0.25	0.25
5-253	2,000	>256	0.5
5-333	>2,000	>256	4
5-549	2,000	0.5	0.5
5-328	>2,000	0.5	0.5
5-522	2,000	4	2
5-489	>2,000	128	2
5-129	>2,000	>256	1

* EUCAST [10] define susceptibility as ≤0.12 µg/ml
** EUCAST [10] define susceptibility as ≤2 µg/ml

Evaluation of interactive effects of combinations of antibiotic agents

The interactive effect of combinations between methanol extract and antibiotic agents against 8 isolates of MRSA were performed using the checkerboard dilution method. The interactive effect of combinations between methanol extract and vancomycin showed the synergistic effect against on MRSA 5 isolates (FICI≤0.5) and indifferent effect or neutral effect from single agent on MRSA 3 isolates (FICI=1) (Table 2). The interactive effect of methanol extract and penicillin showed to be synergistic for all MRSA isolates (FICI≤0.5) (Table 3). Our results are in line with the previous studies that Zingiberaceae family showed antibacterial activity and synergistic effect when combined with the antibiotics [1, 12].

Table 2. Susceptibility of MRSA isolates to combination *K. parviflora* extract and vancomycin.

MRSA strains	Agents	MIC (μg/ml)		FICI	Outcome
		alone	Methanol extract + VAN		
5-456	Methanol extract	2,000	1,000	1	Indifference
	Vancomycin	0.25	0.125		
5-183	Methanol extract	1,000	500	1	Indifference
	Vancomycin	1	0.5		
5-184	Methanol extract	2,000	1,000	1	Indifference
	Vancomycin	0.5	0.25		
5-104	Methanol extract	1,000	250	0.5	Synergy
	Vancomycin	0.25	0.0625		
5-151	Methanol extract	2,000	500	0.5	Synergy
	Vancomycin	0.25	0.0625		
5-253	Methanol extract	2,000	250	0.25	Synergy
	Vancomycin	0.5	0.0625		
5-549	Methanol extract	2,000	500	0.5	Synergy
	Vancomycin	0.5	0.125		
5-522	Methanol extract	2,000	500	0.5	Synergy
	Vancomycin	2	0.5		

FICI≤0.5, synergy; FICI >0.5 to 4.0, indifference

Table 3. Susceptibility of MRSA isolates to combination *K. parviflora* extract and penicillin.

MRSA strains	Agents	MIC (μg/ml)		FICI	Outcome
		alone	Methanol extract + PEN		
5-456	Methanol extract	2,000	62.50	0.062	Synergy
	Penicillin	0.25	0.0078		
5-183	Methanol extract	1,000	<7.8	<0.016	Synergy
	Penicillin	16	<0.125		
5-184	Methanol extract	2,000	62.50	0.062	Synergy
	Penicillin	0.25	0.0078		
5-104	Methanol extract	1,000	<7.8	<0.016	Synergy
	Penicillin	0.25	<0.00195		
5-151	Methanol extract	2,000	31.25	0.031	Synergy
	Penicillin	0.25	0.0039		
5-253	Methanol extract	2,000	31.25	0.047	Synergy
	Penicillin	128	4		
5-549	Methanol extract	2,000	500	0.5	Synergy
	Penicillin	0.5	0.125		
5-522	Methanol extract	2,000	<15.6	<0.023	Synergy
	Penicillin	2	<0.031		

FICI≤0.5, synergy; FICI >0.5 to 4.0, indifference

CONCLUSIONS

K. parviflora extract showed antibacterial activity against on 8 isolates of MRSA and interactive effect of combinations between vancomycin showed synergist effects against 5 isolates of MRSA. The interactive effect of combinations between penicillin exhibited synergist effect against all 8 isolates of MRSA. The study provides a strategy in utilizing compounds from natural source in combination with modern antibiotic agents to enhance antibacterial activity in the treatment of MRSA infection.

ACKNOWLEDGEMENTS

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REFFERENCES

1. Mun SH, Joung DK, Kim YS, Kang OH, Kim SB, Seo YS, et al. (2013). Synergistic antibacterial effect of curcumin against methicillin-resistant *Staphylococcus aureus*. Phytomedicine. 20, 714 - 718.
2. Kummee S, Tewtrakul S, Subhadhirasakul S. 2008. Antimicrobial activity of the ethanol extract and compounds from the rhizomes of *Kaempferia parviflora*. Songklanakarin J. Sci. Technol. 30, 463-466.
3. Dowrote N. 2010. The effects of *Kaempferia parviflora* and *Hibiscus sabdariffa* extracts on arterial blood pressure and renal functions in rats. Thesis for the Degree of Master of Science in Physiology, Prince of Songkla University.
4. Hausler WJ, Herrmann KL, and Isenberg HD. 1991. Manual of clinical microbiology. 10th ed. Washington, DC: American Society for Microbiology.
5. Parpekh J, Jadeja D, and Chanda S. 2005. Efficacy of aqueous and methanol extracts of some medicinal plants for potential antibacterial activity. Turk. J. Biol. 29, 203-210.
6. Medical Technology Laboratory, Thammasat University Hospital. 2551. Direct colony suspension method (DCS). 4.
7. Clinical and Laboratory Standards Institute. 2009. Performance Standards for Antimicrobial Disk Susceptibility Tests, 10th ed. Approved standard M2-A10. Clinical and Laboratory Standards Institute, Wayne, PA.
8. Climo MW, Patron RL, and Archer GL. 1999. Combinations of vancomycin and beta-lactams are synergistic against staphylococci with reduced susceptibilities to vancomycin. Antimicrob. Agents Ch. 43, 1747-1753.
9. Spoorthi NJ, Vishwanatha T, Reena V, Divyashree BC, Aishwarya S, Siddhalingeshwara KG, et al. 2011. Antibiotic synergy test: checkerboard method on multidrug resistant Pseudomonas aeruginosa. Int. Res. J. Pharm. 2(12), 196-198.
10. The European Committee on Antimicrobial Susceptibility Testing. 2015. Routine and extended internal quality control as recommended by EUCAST. Version 5.0. http://www.eucast.org
11. Sirikhansaeng P, Vichitphan K, and Vichitphan S. 2008. The flavonoid content and antibacterial activity from *Kaempferia parvifora* Wall. ex Baker in Krachai-Dum herbal wine (abstract). J. Biotechnol. 136s, s746-s747.
12. Eumkeb G, Siriwong S, Phitaktim S, Rojtinnakorn N, and Sakdarat S. 2011. Synergistic activity and mode of action of flavonoids isolated from smaller galangal and amoxicillin combinations against amoxicillin-resistant *Escherichia coli*. Journal of Applied Microbiology. 112, 55-64.

Phyllantus niruri L. extract instant granules as an antithrombocytopenia

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ABSTRACT

Meniran (*Phyllantus niruri L.*) has been proven as antithrombocytopenia in mice. So a well made preparation is necessary in order to have easy consumption. Instant granules form were chosen by making three formulations with various of sucrose and citric acid. The study was conducted through the stages of extraction, phytochemical screening, determination of standardized extract, formulation, granulation, physical evaluation and observation of physical appearance. Phytochemical screening results showed that the *Phyllantus niruri* extract contained polyphenols, tannins, saponins, flavonoids, monoterpenes and sesquiterpenes, steroids, triterpenoids, and quinones. The results showed that the *Phyllantus niruri* extract contained 7.45% water content, 41.63% ethanol soluble content, 62.24% water soluble content, and 2.51% ash content. Then it was formulated into granules and then evaluated for various parameters. The evaluation of all the instant granules showed a good result of quality with 11.65° of angle of repose, 2.43% of water levels, 13.05 g/s of flow rates and 27.33% compressibility. Based on the hedonic test performed on the third formula and it can be concluded that formula was the most preferred formulation. Based on thin layer chromatography, it can be concluded that the active compound is still remained after formulated.

Keywords: formulation, *Phyllantus niruri* extract, instant granules

INTRODUCTION

1.

Indonesia is a tropical country that is becoming endemic regions diverse tropical diseases such as Dengue Hemorrhagic Fever [1]. There is more than 50 millions people in Indonesia infected by dengue virus and 2.5 millions of them are dead. Based on WHO, there was an increased incidence rate of 0.005% per 100,000 populations in 1968 to be 6-27 per 100,000 populations in 2009 in Indonesia [2]. The current treatment is merely supportive therapy. Meniran exhibits significant antithrombocytopenia activity as well as immunodulator activity [3]. *Phyllanthus niruri* extract administrated oral to rats showed increased the number of plateles [4]. In this communication, formulation of *Phyllantus niruri* extract needed in order to easier to use.

MATERIALS AND METHODS

Collecting of Materials and Determination Plant

The plant meniran (*Phyllantus niruri L*) obtained from the National Agency for Medicinal Plant Research, Research Installation Manoko, Bandung. The *Phyllantus niruri* sorted and carried downsizing. Determination plants conducted at the Laboratory of Plant Taxonomy Department of Biological Science Faculty.

Extraction of *Phyllantus niruri*

The dried powder of the plant was extracted by maceration method for 96 hours with 70% ethanol at 25°C. The extract thus obtained was concentrated using evaporator and subjected to preliminary chemical screening to indentify the phyto-consituents like flavonoids, saponins, alkaloids, phenolic compounds. The standard of *Phyllantus niruri* extract was measured and compared it with herbal pharmacopoeia.

Formulation of Herbal Instant Granules

Herbal instant granules were prepared by wet granulation method with three different formulation which contained *Phyllantus niruri L* extract. Three formula instant granules were made with different levels of sucrose and citric acid (Table 1). Each formula contained the extract powder meniran 46.5% or 9.3 grams of 20 grams of the preparation of instant granules.

Table 1. Composition of formulation of *Phyllantus niruri* granules

Ingredients	Formulation 1	Formulation 2	Formulation 3
***Phyllantus** niruri L* **extract**	9.3 g	9.3 g	9.3 g
Sucrose	10 g	8 g	6 g
Sodium Cyclamate	1 g	1 g	1 g
Citric acid	0.3 g	0.4 g	0.5 g
Sodium Chloride	0.2 g	0.2 g	0.2 g
Starch Pregelatinized	5 g	5 g	5 g
Maltodextrin	8 g	8 g	8 g
Citrus flavor	0.4 g	0.4 g	0.4 g

The extract was dried at 60°C to constant weight and triturated in a mortar and pestle to make powder then mixed with calculated amount of the other components. The binder was added and formed into a paste and granulated using sieve No. 40. This mass was passed through sieve No. 20 to get granules and these granules were dried at 40°C.

Evaluation of formulated Herbal Instant Granules

Angle of repose

A funnel was secured with its tip at a given height, above a graph paper that was placed on a flat horizontal surface. The blend was carefully pored through the funnel until the apex of the conical pile just touches the tip of the funnel. The radius of the base of the conical pile was measured. The angle of repose was calculated using the following formula:

$$\mathbf{Tan\ \theta = h/r} \qquad \mathbf{(1)}$$

θ = Angle of repose, h = Height of the cone, r = Radius of the cone base.

Values for angle of repose ≤ 30° usually indicate a free flowing material and angles ≥ 40° suggest a poorly flowing material, 25°- 30° show excellent flow properties, 31°-35° show good flow properties, 36°-40° show fair flow properties and 41°-45° showing passable flow properties.

Bulk Density

The powder blend (15 g) was introduced into a dry 100 mL-cylinder, without compacting. The powder was carefully leveled without compacting and the unsettled apparent volume was read. The bulk density was calculated using the following formula:

$$\mathbf{\rho_b = M / V_o} \qquad \mathbf{(2)}$$

ρ_b = Apparent bulk density, M = Weight of sample, V_o = Apparent volume of powder

Tapped Density

After carrying out the procedure as given in the measurement of bulk density. The cylinder containing the sample was tapped 500 times initially followed by an additional taps of 750 times until difference between succeeding measurement was less than 2% and then tapped volume, tapped volume of powder was measured, to the nearest graduated unit. The tapped density was calculated, in gram per milliliter, using the following formula:

$$\mathbf{\rho_{tap} = M / V_f} \qquad \mathbf{(3)}$$

ρ_{tap} = Tapped density, M = Weight of sample, V_f = Tapped volume of powder

Compressibility index

The Compressibility index (Carr's index) is a measure of the propensity of a powder to be compressed. It was determined from the bulk density and tapped density. As such, it is a measurement of the relative importance of inter-particulate interactions. In a free flowing powder, such interactions were generally less significant, and the bulk and tapped densities will be closer in value. For poorer flowing materials, there were frequently greater inter-particulate interactions, and a greater difference between the bulk and tapped densities will be observed. These differences were reflected in the Carr's Index which is calculated using the following formulas:

$$\mathbf{Compressibility\ index = [(\rho_{tap} - \rho_b) / \rho_{tap}] \times 100} \qquad \mathbf{(4)}$$

ρ_b = Apparent bulk density, ρ_{tap} = Tapped density

Hausner's Ratio

Hausner's ratio was an indirect index of ease of powder flow. It was calculated by the following formula:

$$\text{Hausner's Ratio} = \rho_{tap} / \rho_b \qquad (5)$$

ρ_b = Apparent bulk density, ρ_{tap} = Tapped density

Hausner's ratio less than 1.25 indicates better flow properties than higher one, between 1.25 to 1.5 indicates moderate flow properties and more than 1.5 indicates poor flowability.

Thin-layer Chromatography (TLC) test of *Phyllantus niruri L* Granules

The extract and the instant granules were spotted on TLC plate. First, the chamber was prepared, then put the n-hexane : ethyl acetate (9:2) as a mobile phase into the chamber. The spot was observed and marked, then sprayed the plate with 10% sulfuric acid. The appeared spots were observed and compared between the extract and the formula.

Hedonic Test

Hedonic test was tested on 30 volunteers who were given the instant granules that had been dissolved with water. For each test formula, the volunteer was given the lag time to neutralize the sense of taste. Then the volunteer fill out questionnaires about the instant granules.

RESULTS

The alcoholic extract of *Phyllantus niruri L.* gave yield of 12.37% w/w. When subjected to preliminary chemical screening showed the presence of phenolic compounds, tannins, saponins, flavonoids, steroids, quinons, monoterpenes &sesquiterpenes, steroids, triterpenoids (Table 2).

Table 2. Phytoconstituents of *Phyllanthus niruri* extract

Phytoconstituents	test
Alkaloids	-
Phenolic compounds	+
Tannins	+
Saponins	+
Flavanoids	+
Monoterpenes & Sesquiterpenes	+
Steroids	+
Triterpenoids	+
Quinons	+

(-) absent (+) detected

Parameter determination of Standard Extract according Pharmacope Herbal indoneisa

Nonspecific and Specific parameters

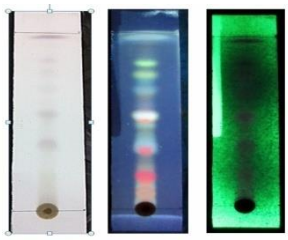
Testing of nonspecific parameters such as density determination was done by using pycnometer to impose limits the amount of mass per unit volume and the result showed 1.01 g/mL. Measurement of water content was performed using toluene and the result showed that water content was 7.45%. Ash content testing was a way of testing the mineral content, salts content and oxides in the extract. The result showed that the content of ash was 2.51%. The results showed that levels of drying loss amounted to 8.61% (Table 3)

Testing of spesific parameters such as organoleptic, the contents of soluble ethanol and testing of water content soluble. The results showed organoleptic was condensed form, blakish green color, sweet scent and had bitter taste. The results showed contents of soluble ethanol was 41.63% and 62.24% contents of soluble water.

Table 3. Evaluation of extarct

Parameter	Value	Requirements
Density	1.01 g/mL	-
Water content	7.45 %	< 17%
Contents of ash	2.51%	≤ 7.2 %
Loss of drying	8.61 %	≤ 14 %

TLC test of *Phyllantus niruri L* Extract



a b c

Figure 1. Result of TLC test of *Phyllantus niruri* extract under (a) visible light, (b) UV 254 nm after using H_2SO_4 in 10% ethanol, and (c) UV 366 nm after using H_2SO_4 in 10% ethanol

The yellow-orange spot was suspected as flavonoids spot. Quersetin is one of flavonoids group has a funtion as antithrombocytopenia [6].

Granules Evaluation

Loss of drying

Those formulation showed loss on drying below 10% (Table 4) according to herbal pharmacopoeia.

Table 4. Evaluation of loss drying granules instant

Formulation	Loss of drying (%)
I	2.09
II	2.53
III	2.67

Flow rate

The flow rate of three formulations showed excellent flow rate according to herbal pharmacopoeia (Table 5).

Table 5. Evaluation of flow rate granules instant

Formulation	Flow rate (g/s)	Flow rate index	Properties
I	13.33	>10	Excellent
II	13.33	4-10	Good
III	12.5	1.6-4	Poor
		<1.6	Very poor
Mean	13.05		Excellent

Compressibility

Those formulations showed the formulation I had fair compressibility, formulation II had very poor compressibility, formulation III had poor compressibility and the mean of three formulations showed that those formulations could not continued for tablet formulation becasue they had poor compressibility (Table 6).

Table 6. Evaluation of compressibility granules instant

Formulation	Compressibility	Compressibility index	Properties
I	19.92	≤10	Excellent
II	33.25	11-15	Good
III	28.47	16-20	Fair
		21-25	Passable
		26-31	Poor
Mean	27.33		Poor

Angle of repose

The angle of repose is the elevation angle was formed between the pile of particles in the horizontal plane. The angle of repose is a characteristic fluidity that is closely linked with the cohesion each particle. Angle of repose will be greater if the particle size gets smaller [7]. From the obtained results, the resulting granules had angle of repose of 11.65° (Table 7).

Table 7. Evaluation of angle of repose granules instant

Formulation	Angle of repose (°)	Angle of repose index	properties
I	11.30°	≤25	Excellent
II	12.36°	25-30	Good
III	11.30°	30-40	Fair
		>40	Poor
Mean	11.65°		Excellent

Density

The granule density can affect the solubility, the bigger density of the granules then the solubility will be slower. The result showed that formulation I was the highest density which means it had the slowest solubility (Table 8).

Table 8. Evaluation of angle of density granules instant

Formulation	Density (g/mL)
I	1.305
II	1.152
III	0.995

TLC test of *Phyllantus niruri L* Granules

The result showed that the spot of the extract and the formula did not change, which means the formulation did not change the existing content in the extract (Figure 2).

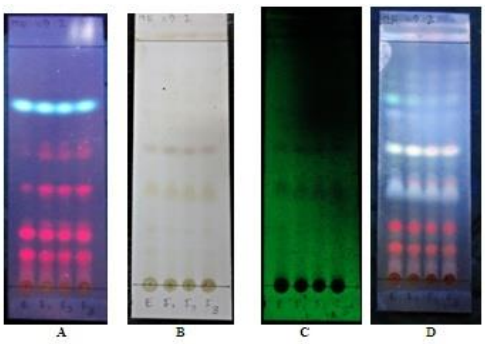


Figure 2. Result of TLC test of *Phyllantus niruri* granules under (a) UV 254 nm before using H_2SO_4 in 10% ethanol, (b); visible light, (c) UV 254 nm after using H_2SO_4 in 10% ethanol, (d) UV 366 after using H_2SO_4 in 10% ethanol

There were 4 spots which were extract (E), Formulation 1(F1), Formulation 2(F2), Formulation 3 (F3). We saw that there was not significantly different spots between the extract and formulation means allegedly efficacious compounds were not lose by formulation process.

Hedonic Test

Hedonic test was tested to 30 velunterrs to test the taste of the formulation. The results obtained from the analysis of hedonic test on 30 volunteers are formula I was preferred over formula II and III

Evaluation granules Instant Physical Appearance

The granules was kept at three conditions which are at 25°C, 40°C and fresh air. The observation for 30 days showed that there was no significant changes to physical appearance. The granules was gained weight during the observation, may be it caused by particle contamination.

CONCLUSIONS

From the research that has been done can be concluded that the herbal extract *Phyllantus niruri L*. can qualify standardization standardized extracts because it meets the standard parameters based extracts Herbal Pharmacopoeia (2008). Instant granules formulation of extract *Phyllantus niruri L*. can be created and meet the requirements of a good instant granules where in formula I is the most preferred.

REFERENCES

1. Indonesian Health Ministry. 2010. *Demam Berdarah Dengue.* Pusat Data dan Surveilans Epidemiologi.
2. World Health Organization. 2009. Dengue and Dengue Haemorrhagic Fever. at: http://www.who.int/mediacentre/factsheets/fs117/4.
3. Jose J, Sudhakaran S, Kumar S, Jayaraman S, Variyar J. 2014. Study of *In vitro* immunomodulatory effect of flavonoid isolated from *Phyllanthus niruri* on human blood lymphocytes and evaluation of its antioxidant potential. Int. J. Pharmacognosy and Phytochem. Res. 6(2), 284-289.
4. Dhani Adriati Koswara. Y. Susilawati. 2005. Aktivitas Ekstraks Etanol Meniran (*Phyllantus niruri* L.), Daun Pepaya (*Carica Papaya L*.) Dan Daun Jambu Biji Merah (*Psidium guajava L*.) Terhadap Jumlah Trombosit, Eritrosit, dan Kadar Hematokrit Pada Tikus Putih (*Rattus novergicus*. thesis. Padjadjaran University Press. Bandung. Indonesia.
5. Srinath KR, Chowdary P, Palaniswamy P, Vamsy Krishna A, Aparna S, Ali SS et al. 2011. Formulation and evaluation of effervescent tablets of Paracetamol. IJPRD. 3(3), 76-104.
6. Siswono. 2004. *Ekstrak Daun Jambu Biji Naikkan Trombosit Penderita DB*.at: http://www.MediaIndonesia.co.id
7. Parrott L. 1971. Pharmaceutical Technology Fundamental Pharmaceutica. Burger Publishing Company. 158, 171, 389. Mineapolis. 3rd edition.

Antioxidative and anticancer activities of selected microalgae extracts isolated from East coast of Thailand

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ABSTRACT

Microalgae are a potential bioresource containing nutritional and medical values. In this study, three microalgae were isolated from East coast of Thailand including *Amphora* sp., *Cheatoceros* sp. and *Spirulina* sp. The methanolic extracts of these microalgae were analyzed their antioxidative activity and total phenolic content. In addition, the extracts were investigated the cytotoxic activity against hepatocarcinoma (HepG2) and breast cancer (MDA-MB-231) cells in *in vitro*. The result showed that *Amphora* sp. significantly exhibited higher antioxidative activity than *Spirulina* sp. and *Cheatoceros* sp. with IC_{50} of 285.22 ± 3.59, 452.33 ± 18.44 and 2,406.86 ± 201.39 µg/ml, respectively. The *Amphora* sp.contained higher total phenolic content than *Cheatoceros* sp. (17.23 ± 0.07 and 8.68 ± 0.77 mg tannic acid equivalent/g crude extract, respectively) at $P<0.05$. The total phenolic contents in *Amphora* sp. and *Spirulina* sp. were not significant difference (17.23 ± 0.07 and 24.20 ± 3.50 mg tannic acid equivalent/g crude extract, respectively) at $P>0.05$. Nevertheless, *Cheatoceros* sp. showed stronger cytotoxicity against HepG2 and MDA-MB-231 cells (IC_{50} of 538.79 ± 8.43 and 413.91 ± 20.16 µg/ml, respectively) than those of *Amphora* sp. and *Spirulina* sp., respectively. These results indicated that isolated microalgae in this study can be the good antioxidative and anticancer sources.

Keywords: Microalgae, *Amphora* sp., *Cheatoceros* sp., *Spirulina* sp., antioxidants, cytotoxicity

INTRODUCTION

Microalgae which widely found in East coast of Thailand include green algae, chlorophycae, and the intermediated species between plants and bacteria. There are many components in microalgae such as carotenoids, polyunsaturated fatty acid, tocopherols, sterols, vitamins, minerals [1], omega-3, and sulfated polysaccharide [2]. Carotenoids possess antioxidant activity, antitumoural activity, and reduce incidence of chronic diseases [1]. The sulfated polysaccharides showed anti-herpes simplex virus type 1 and type 2 as well as anti-influenza A virus [2]. However, there is no data of pharmacological activities of microalgae in the East coast of Thailand especially *Amphora* sp., *Cheatoceros* sp., and *Spirulina* sp. Therefore, the aim of this study was determination of their antioxidative activity, cytotoxic activity against hepatocarcinoma (HepG2) and breast cancer (MDA-MB-231) cells in *in vitro* and total phenolic content.

MATERIALS AND METHODS

Microalgae extract preparation

The microalgae in this study (Figure 1) was isolated from East coast of Thailand, identified and cultured by Dr. Maliwan Kutako from Faculty of Marine Technology, Burapha University Chanthaburi Campus, Thailand. The microalgae was harvested and extracted by Dr. Sasipawan Machana from Faculty of Pharmaceutical Sciences, Burapha University, Thailand. Briefly, microalgae were centrifuged (6000 ×g for 10 min) to separate the cells from the culture media. The cell pellet was extracted by using methanol for 24 h. The extract solution was filtered and then dried by using rotary evaporator. The yields of the extracts were calculated. The extract stock solution was prepared by dilution the extract with dimethyl sulfoxide (DMSO) to the concentration of 100 mg/ml and then filtrated by using 0.4 µm syringe filter.

Determination of antioxidative activity

The *in vitro* antioxidant activity was analyzed using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay following Brand-Williams et al. (1995) [3]. Briefly, the reaction mixture contained the various concentrations of extract (0-8,000 µg/mL), 1 mM DPPH (200 µL), and methanol. After incubation for 15 min, the absorbance was measured at 515 nm using UV-Vis spectrophotometer (Shimadzu. Australia).Vitamin C and vitamin E were used as the positive control. The 50% of inhibition (IC_{50}) was determined from curves of % inhibition and concentration of the extract. The % inhibition was calculated by

$$\% \text{ inhibition} = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100$$

When; $Abs_{control}$: absorbance of control (not contain the extract)
Abs_{sample} : absorbance of the microalgae extract.

Total phenolic contents

Total phenolic contents in the selected microalgae extract were determined by the Folin-Ciocalteau method using tannic acid as standard [4]. The extract (0.1 mg/L) was added to the mixture of Folin-Ciocalteau's phenol reagent. After that, the mixture was added to 1.2 mL of sodium carbonate (0.2 mg/mL). Then, the mixture solution was measured for absorbance at 725 nm using UV-Vis spectrophotometer (Shimadzu. Australia).

Determination of cytotoxicity

The cancer cell lines in this study included hepatocarcinoma (HepG2) and breast cancer (MDA-MB-231) were cultured by using culture media Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and incubated in 37°C and 5% CO_2 atmosphere. The cells were seeded into 96 well-plate (1×10^4 cells/well) and then incubated for 24 h. The extracts were diluted with culture media to various concentrations and then added into the cells. After 2 days incubation, cell viability was determined by using MTT assay [5]. Briefly, the cells were added with 50 µl of 1 mg/ml MTT solution and then incubated at 37°C with 5% CO_2 atmosphere for 3 h. The culture media was removed and then added with DMSO. The solution absorbance was measured at 570 nm. The cytotoxicity of the extracts was calculated and expressed as 50% inhibitory concentration (IC_{50}).

Statistical analysis

Data were reported as the mean ± SD. Statistical significances among groups were evaluated using ANOVA followed by post hoc test (least square mean) by SPSS program version 16.0. Values of $P<0.05$ were considered to be statistically significant.

RESULTS

The yields of the *Amphora* sp., *Cheatoceros* sp. and *Spirulina* sp. extracts were 3.89, 2.77 and 4.72%, respectively. The morphologies of three microalgae were shown in Figure 1.

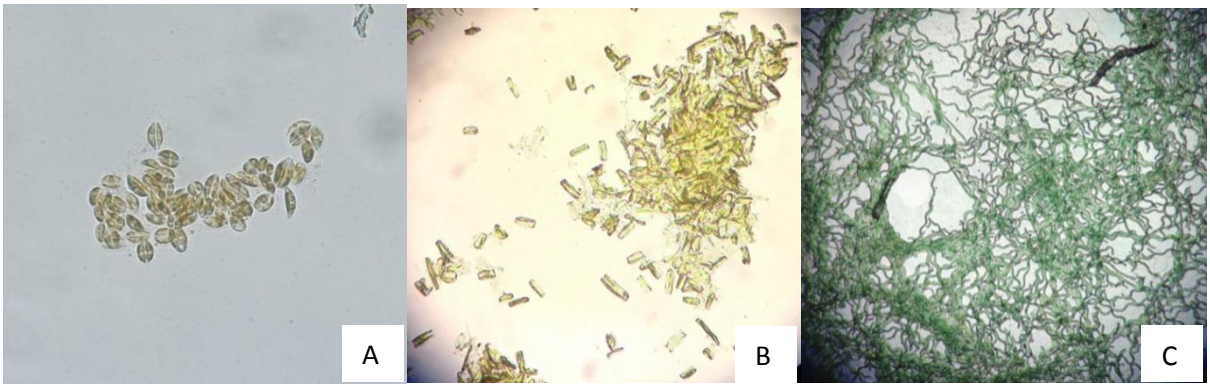

Figure 1. Morphologies of microalgae in this study which kindly received from Dr. Maliwan Kutako. A) *Amphora* sp., B) *Cheatoceros* sp. and C) *Spirulina* sp.

Antioxidative activities of three microalgae by using DPPH assay and their total phenolic contents were shown in Table 1. The result showed that *Amphora* sp. significantly showed higher antioxidative activity than *Spirulina* sp. and *Cheatoceros* sp. with IC_{50} of 285.22 ± 3.59, 452.33 ± 18.44 and 2,406.86 ± 201.39 µg/ml, respectively. Accordingly, the total phenolic content of *Amphora* sp. which showed the higher value than that of *Cheatoceros* sp. (17.23 ± 0.07 and 8.68 ± 0.77 mg tannic acid equivalent/g crude extract, respectively) at $P<0.05$. The total phenolic contents in *Amphora* sp. and *Spirulina* sp. were not significant difference (17.23 ± 0.07 and 24.20 ± 3.50 mg tannic acid equivalent/g crude extract, respectively) at $P>0.05$. However, the antioxidative activities of the selected microalgae were lower than the positive controls including vitamin C and vitamin E.

Table 1. Antioxidative activities and total phenolic content of the microalgae extracts

Extracts	Antioxidative activities (IC_{50}, µg/ml)	Total phenolic content (mg tannic acid equivalent/g crude extract)
Amphora sp.	285.22 ± 3.59$^{*,\psi}$	17.23 ± 0.07
Cheatoceros sp.	2,406.86 ± 201.39$^{*,\psi}$	8.68 ± 0.77$^{\psi}$
Spirulina sp.	452.33 ± 18.44$^{*,\psi}$	24.20 ± 3.50
Vitamin C	2.68 ± 0.04	-
Vitamin E	7.75 ± 0.02	-

Data shows mean±SD. *: significant difference at $P<0.05$ compared to positive controls (vitamin C and vitamin E); ψ: significant difference at $P<0.05$ compared to among two the microalgae.

The microalgae extracts at the study concentrations suppressed the proliferation of both cancer cells with dose dependent manner (data not shown) especially *Cheatoceros* sp. that showed significantly stronger inhibitory effect on both HepG2 and MDA-MB-231 proliferations with IC_{50} of 538.79 ± 8.43 and 413.91 ± 20.16 µg/ml, respectively, than those of *Amphora* sp. and *Spirulina* sp. (Table 2 and Figure 2). This cytotoxic effect of *Cheatoceros* sp. was contrary with the antioxidative activity and the content of phenolics, therefore, it is possible that the *Cheatoceros* sp. inhibitory effect against these cancer cells did not from the antioxidant or phenolic content in the extract.

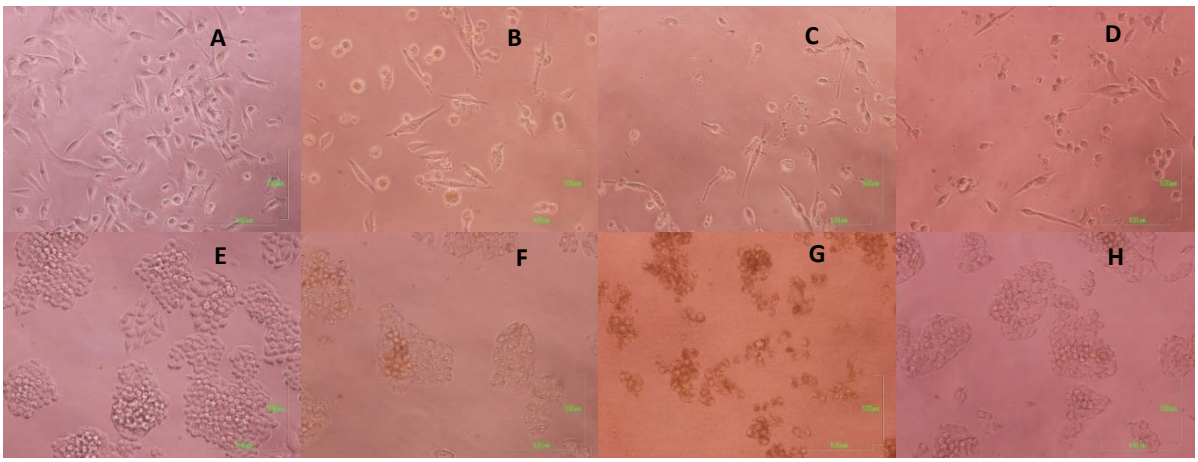

Figure 2. Cytotoxic effects of microalgae extracts on MDA-MB-231(upper) and HepG2 cells (lower). A) and E) untreated cells, B) and F) 800 µg/ml *Amphora* sp. extract, C) and G) 800 µg/ml *Cheatoceros* sp. extract, D) and H) 800 µg/ml *Spirulina* sp. extract.

Table 2. The cytotoxic effects of microalgae extracts against HepG2 and MDA-MB-231 cells

Extracts	IC_{50} (µg/ml)	
	HepG2 cells	MDA-MB-231 cells
Amphora sp.	>800	$576.04 \pm 39.54^{*}$
Cheatoceros sp.	538.79 ± 8.43	$413.91 \pm 20.16^{*}$
Spirulina sp.	>800	$700.28 \pm 47.96^{*}$

Data shows mean±SD. *: significant difference at P<0.05 compared to among two the microalgae.

CONCLUSIONS

In conclusion, the selected microalgae from East coast of Thailand were firstly reported their antioxidative and anticancer activities. *Amphora* sp. significantly exhibited highest antioxidative activity. Total phenolic content in *Amphora* sp. and *Spirulina* sp. contained higher than that of *Cheatoceros* sp. Moreover, *Cheatoceros* sp. showed stronger cytotoxicity against HepG2 and MDA-MB-231 cells than those of *Amphora* sp. and *Spirulina* sp. The results in this study revealed the potent of microalgae isolated from East coast of Thailand including *Amphora* sp., *Cheatoceros* sp., and *Spirulina* sp. as the antioxidant and anticancer biosources that may be useful for the further development of natural health products.

ACKNOWLEDGEMENTS

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REFERENCES

1. Christaki E, Bonas E, Giannenas I, Paneri P. 2013. Functional properties of carotenoids originating from algae. J. Sci. Food Agric. 93, 5-11.
2. Lee JB, Hayashi M, Kurosa E, Suzuki E, Kubo Y, Hayashi T. 2006. Antiviral sulfated polysaccharide from *Navicula directa*, a diatom collected from deep-sea water in Toyama bay. Biol. Pharm. Bull. 29, 2135-2139.
3. Brand-Williams W, Cuvelier ME, Berset C. 1995. Use of a free-radical method to evaluate antioxidant activity. Lebensm-Wiss Technol. 28(1), 25–30.
4. Makkar H, Blummel M, Borowy NK, Becker K. 1993. Gravimetric determination of tannins and their correlations with chemical and protein precipitation methods. J. Sci. Food Agric. 61, 161–165.
5. Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survivals: application to proliferation and cytotoxicity assays. J. Immunol. Methods. 65, 55–63.

The efficiency of crude leaf extracts from five plants species in moracae family as potentially active antibacterial agents

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ABSTRACT

The main purpose of this research is to study the efficiency of plants extracts for determining them as antibacterial agents. The effective compounds from five leafs species as mulberry (*Morus alba*), jackfruit (*Artocarpus heterophyllus*), Siamese rough bush (*Streblus aspera* Lour), weeping fig (*Ficus benjamina*) and common fig (*Ficus carica*) were extracted by cremation technique with two different polar solvents of hexane and methanol. The results indicated that the methanol extraction gave higher product yields of all five plants than that of the hexane extraction. Then the activity on *Staphylococcus aureus* of all crude extracts was subjected with the concentration of sample as 100 mg/ml. The result revealed that there are two effective hexane crude extracts showed the activity against bacteria. The crude extract from common fig displayed the width of clear zone as 14 mm that was slightly better than crude extract from weeping fig, presenting the length of clear zone as 7.3 mm. In addition, the further investigation was performed in methanol crude extracts. The result demonstrated that crude extracts from common fig and jackfruit showed the efficiency on *Staphylococcus aureus* with clear zone dimension as 7.6 mm and 10 mm, respectively. Nevertheless, the efficiency of effective crude extracts was lower than antibiotic references of tetracycline and ampicillin. More importantly, the effective crude extracts that showed activity against bacteria were chosen to determine their phytochemical compositions. The result revealed the interesting existence of anthraquinone and alkaloid from hexane crude extracts and the existence of coumarin, phenolic compound and tannin from methanol crude extracts.

Keywords: moracae, antibacterial activity, phytochemical compositions

INTRODUCTION

Nowadays, it is inevitable to note that herbal medicine is being one of the extensively interesting topics for traditional and modern pharmacology. The composition of phytochemical compounds inside several herbs presents an active ability in biological properties for curing many diseases such as antioxidant and antibacterial applications. However, the consumption of herbs for medicinal trials is declined due to the discovery of new synthesized agents as modern drugs. Unfortunately, the modern agents demonstrate side effects and toxicity to patients, including the high cost for purchasing. Consequently, to figure out this obstruct, the research in herb is one of the promising ideas for starting to achieve the new effective drugs. Like from the previous said, herbs present a lot of capabilities on pharmacology. They have been chosen for curing many infectious diseases in gastrointestinal system, lung, skin and diarrhea [1]. Especially, the infection in gastrointestinal system is commonly known as a serious cause of death and effects to a lot of people in poor hygiene countries. The major causes are bacteria such as *Escherichia coli, Staphylococcus aureus, Bacillus subtilis*. Consequently, it is a worthiness to conduct the research for solving this problem by studying in local Thai herbs that are expected to illustrate the hopeful competence on inhibition the growth of these bacteria.

Therefore, this research is done for investigating the proficiency of local Thai herbs in moracea family that is easily found in several parts of the country. The selected five plants of mulberry, jackfruit, Siamese rough bush, weeping fig and common fig were extracted by cremation technique with two different polar solvents of hexane and methanol. Then dried crude extracts were further studied to determine their medicinal activity on antibacterial against *Staphylococcus aureus*. More importantly, the further study was carried out to discover the phytochemical compounds in active crude extracts.

MATERIALS AND METHODS

Sample preparation and extraction

To prepare the extracts, five selected samples of mulberry, jackfruit, Siamese rough bush, weeping fig and common fig were collected by using the leaf as a target part for extraction. Samples were cut into small pieces and dried for removing contented humidity. Then the grinded leaf was transferred to extract by placing in hexane solution for 2 days. The solution and solid residue were obtained after the process completed. Then the solution was evaporated to remove hexane to obtain crude hexane extract. While the solid residue was extracted with methanol solution further for 2 days. Then crude methanol extract was obtained by removing methanol from mixed solution. In the final step, two crude extracts as hexane crude extract and methanol crude extract were gained for testing antibacterial activity and investigating phytochemical compounds.

Antibacterial assay

The antibacterial activity of all crude extracts was performed against gram positive bacteria of *Staphylococcus aureus* by agar well diffusion method. The culture was freshly prepared into NA broth. For the experimental work, 5 ml of incubated agar medium was transferred into dishes and the solid agar plates were swabbed with 50 µl of bacterial strain. After the adsorption of bacteria, wells of 6 mm diameter were made by the sterile metallic borer and each plate was filled with several concentrations of crude extracts in DMSO solution. Then all plates were incubated at 37 C for 24 h. The efficiency for killing bacteria of each crude extract was determined by measuring the zone of inhibition. The solvent of DMSO was used as a negative control while the positive controls are Ampicillin and Tetracyclin.

Phytochemical study

The preliminary screening for investigating the presence of phytochemical was done by treating active antibacterial crude extracts with chemical tests to identify the presence of alkaloids, anthraquinones, coumarins, phenolic compounds, tannins by using Thin Layer Chromatography (TLC) technique.

Test for alkaloids

Crude extracts were spotted on TLC plate, placed in mobile phase solution of chloroform: methanol:water (30:60:10). Then the dried TLC plate was sprayed with Dragendoff reagent and detected to verify the presence of alkaloids by notifying the reddish brown spot on the TLC plate.

Test for anthraquinones

The presence of anthraquinones in crude extract was performed by using KOH reagent for detecting the spots on TLC plate. The mixed active compounds were separated on TLC plate by using ethyl acetate:methanol:water (81:11:8) as a mobile phase solution. Change of colour to pink indicates the presence anthraquinones.

Test for coumarins

To determine the presence of coumarin in active crude extracts, the spots on TLC plate were separated by mixed mobile phase solutions of toluene:ethyl acetate (90:10). The plate was sprayed with KOH reagents to investigate the blue spot on TLC for confirming the presence of coumarin in crude extract.

Test for phenolic compounds

The solution of Ferric chloride ($FeCl_3$) was freshly prepared by dissolving in 10 mL of distilled water. The separation of crude extract was separated by toluene:ethyl acetate (93:7). The appearance of blue spot on the TLC plate indicates the presence of pholic compound in crude extract.

Test for tannins

The separated spot on TLC plate of extract by chloroform:methanol:water (75:15:10) was interacted with Ferric chloride ($FeCl_3$). The presence of tannin can be observed for a change of colour to bluish black under visible light.

RESULTS AND DISCUSSIONS

Table 1. The general properties of crude extracts and extraction method.

Samples	Solvents	Amount of sample (g)	Amount of Crude extract (g)	Percent yield	Physical property
Mulberry	**methanol**	**50**	**1.1**	**2.2**	**Viscous green**
	hexane	50	5.8	11.6	Viscous dark green
Jackfruit	**methanol**	**50**	**1.1**	**2.2**	**Viscous dark yellow**
	hexane	50	4.7	9.4	Viscous dark green
Weeping fig	**methanol**	**50**	**1.2**	**2.4**	**Viscous yellow**
	hexane	50	1.7	3.4	Viscous dark green
Siamese rough bush	**methanol**	**50**	**0.7**	**1.4**	**Viscous yellow**
	hexane	50	3.1	6.2	Viscous dark green
Common fig	**methanol**	**50**	**0.6**	**1.2**	**Viscous yellow**
	hexane	50	3.0	6.0	Viscous dark green

Effects of solvents on the extraction

Extraction of active compounds in plant sources uses various solvent systems. There are a lot of common solvents as nonpolar and polar solvents are used for extraction the bioactive compounds in plants. In this research, two solvents of methanol and hexane were used as shown in Table 1. Both of them presented a mild quantity from extraction. Hexane demonstrated higher yields than using methanol for all plant extracts. It might be that hexane can diffuse to the internal pores of plants then hexane can leach out more products than methanol [2]. Moreover, the other point is the physical property of hexane that has higher viscosity and vapor pressure than methanol [3]. However the ability of solvents on extraction was further studied on the efficiency of crude extracts on antibacterial activity and the investigation of *phytoconstituents compounds*.

Antibacterial activity

The antibacterial activity of all crude leaf extracts were assayed in vitro by agar disc diffusion method against the selected pathogenic Gram-positive bacteria of *S. aureus*. The result in figure 1 exhibited the zone of inhibition (in mm) values of crude extracts by hexane extraction. It revealed that the crude extract from common fig demonstrated a considerable activity against *S.aureus* at all of three prepared concentrations, while the crude extract from weeping fig presented its antibacterial activity at 100 mg/ml. Nevertheless, the activity levels of active crude extracts are still lower than reference antibiotic agents as Tetracycline and Ampicillin. On the other point, the activity of methanol crude extract was shown in Figure 2. Among them, the crude extract from jack fruit had the most significant activity with the presence of visible clear zone at all three concentrations. The crude extract from common fig also presented the activity to inhibit the growth of *S.aureus* at 10 and 100 mg/ml.

The overall results from the ability to inhibit the growth of bacteria of crude extracts could be due to the existence of active compounds in theirs structures. It can be noted that both solvents for using in this extraction did not show the activity on this test. Then, it is clear to mention that crude extract from common fig has the most diffusion activity than the others in an antibacterial test. Therefore, it is worth to identify the phytochemical constituents to specify them in therapeutic application.

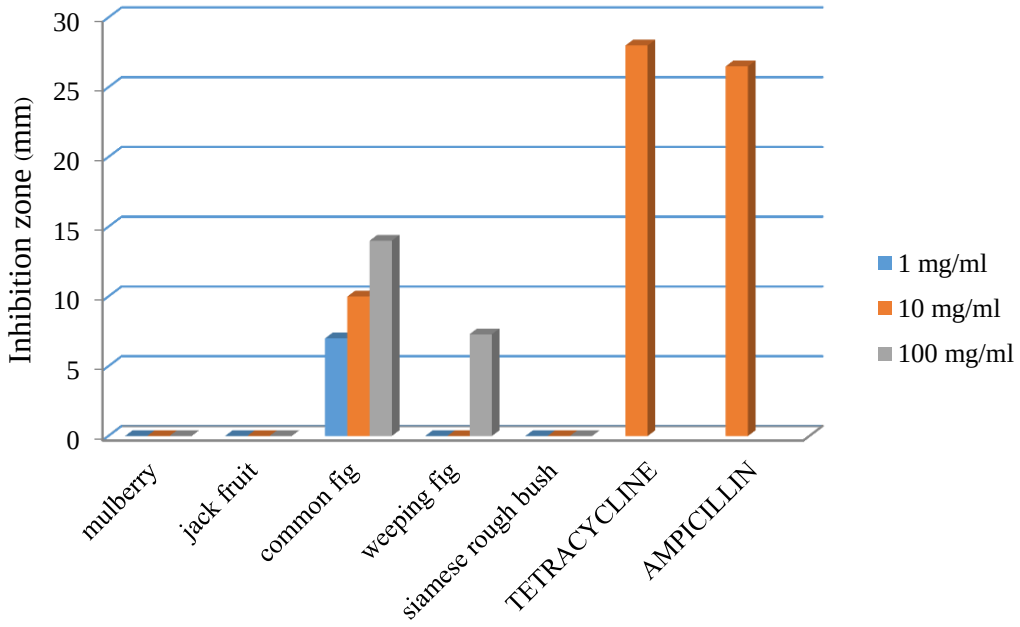

Figure 1. the effectiveness on antibacterial activity with the length of inhibition zone (mm) of hexane crude extracts at three concentrations as 1, 10 and 100 ng/ml.

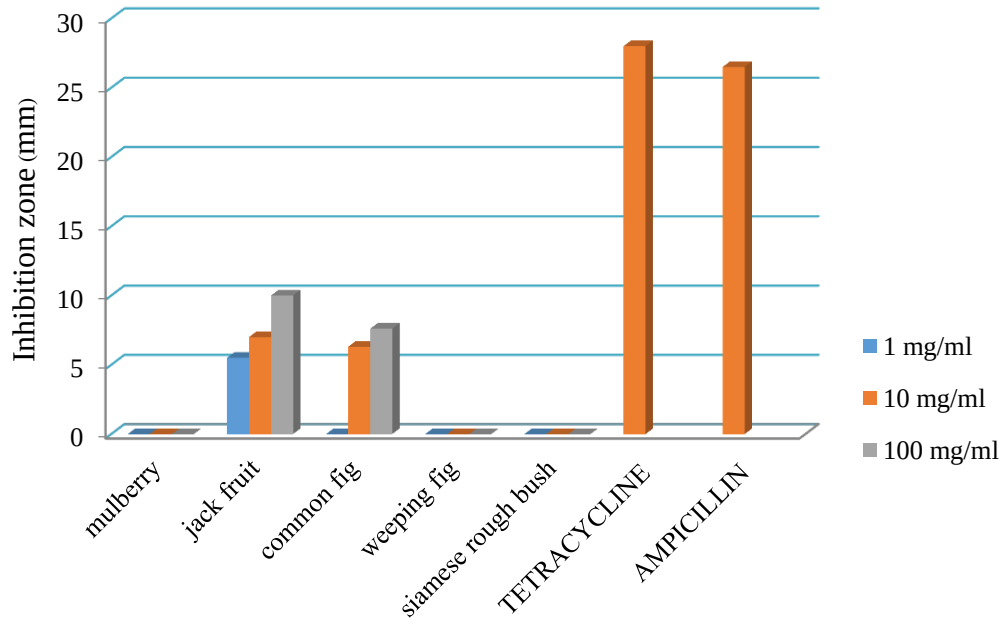

Figure 2. the effectiveness on antibacterial activity with the length of inhibition zone (mm) of methanol crude extracts at three concentrations as 1, 10 and 100 ng/ml.

Phytochemical study

The investigation on phytochemicals screening of active antibacterial crude extracts revealed the presence of alkaloids, anthraquinones, coumarins, phenolic compounds and tannins as shown in Table 2. These phytochemical compositions are related to the capability on medicinal treatment, resulting in the application in both research area and pharmaceutical companies for manufacturing the new promising antibacterial drugs. The hexane crude extract of common fig showed the presence of anthraquinones, whereas weeping fig presented the composition of alkaloids. The presence of alkaloids is one of a good sign for developing plants to use in drug companies. Alkaloids are the important phytochemicals in plants that had used in pain killer medications [4].

The methanol crude extracts of common fig and jack fruit demonstrated the presence of coumarins, phenolic compounds and tannins in both crude extracts. According to Shimida T. [6], tannins have an interesting activity to bound with protein that led to the inhibition the synthesis of protein in cell, resulting in the death of harm cell likes bacteria and cancer [5]. These observations from phytochemicals screening therefore support the use of moracae leaves as a potential source for antibacterial agents.

Table 2. The presence of phytochemical compounds in selected crude extracts.

Plants	Crude extracts			
	Common fig from hexane	Weeping fig from hexane	Common fig from methanol	Jack fruit from methanol
Alkaloids	-	✓	-	-
Anthraquinones	✓	-	-	-
Coumarins	-	-	✓	✓
Phenolic compounds	-	-	✓	✓
Tannins	-	-	✓	✓

CONCLUSIONS

In conclusion, this research studied the antibacterial activity and screening phytoconstituents from leaf extract of five local plants in Moracae family. The results revealed the effectiveness of common fig from both used extraction solvents, including weeping fig from hexane extraction and jack fruit from methanol extraction on inhibition the growth of *S. aureus*. Then the screening phytoconstituents demonstrated the presence of anthraquinones in common fig by using hexane extraction and alkaloids in weeping fig. In addition, the presence of coumarins, phenolic compounds and tannins were found in common fig and jack fruit by using methanol extraction. Therefore, it is appealing to apply these effective crude extracts to use in healthcare and personal care products for resisting the activity of *S.aureus*.

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REFERENCES

1. Zhao Q, Wan, TP, Yu SJ, Wang Q. 2008. Determination of antibacterial activities of Dicranostigma leptodum (Maxim.) Fedde alkaloids. Chinese Veterinary Science. 38(12), 1098–1101.
2. Ganesan P, Kumar CS, Bhaskar N. 2008. Antioxidant properties of methanol extract and its solvent fractions obtained from selected Indian red seaweeds. Bioresour. Technol. 99, 2717–2723.
3. Bhat R, Liong M-T, Abdorreza MN, Karim AA. 2012. Evaluation of free radical scavenging activity and antioxidant potential of a few popular green leafy vegetables of Malaysia. Int. J. Food Prop. 16, 1371–1379.
4. Ayyanar M, Ignacimuthu S. 2005. Traditional knowledge of Kani tribals in Kouthalai of Tirunelveli hills, Tamil Nadu, India. J. Ethnopharmacol. 102, 246–255.
5. Duraipandiyan V, Ignacimuthu S. 2009. Antibacterial and antifungal activity of Flindersine isolated from the traditional medicinal plant, Toddalia asiatica(L.) Lam. J. Ethnopharmacol. 123, 257–262.
6. Shimida T. 2006. Salivary proteins as a defense against dietary tannins. J. Chem. Ecol. 32(6), 1149-1163.

Effects of astaxanthin from *Lipopenaeus vannamei* on carrageenan-induced paw edema in mice

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ABSTRACT

Astaxanthin from *Haematococcus pluvialis* has been demonstrated its beneficial effect in antioxidant and inflammation. However, the anti-inflammatory effect of astaxanthin from white shrimp shell has not been elucidated. Thus, the present study aimed to investigate the anti-inflammatory activity of astaxanthin extracted from *Lipopenaeus vannamei* against carrageenan-induced paw edema in mice. The inflammation was induced by 2.5% carrageenan intraplantar injection in both hind paws. Astaxanthin was administered orally at dose of 10, 30, 50, 100 mg/kg for 21 days prior to carrageenan injection. Indomethacin (5 mg/kg) was used as a positive control. Paw thickness was measured using vernier caliper after 1, 3 and 6 h of carrageenan injection. We found that 100 mg/kg astaxanthin significantly inhibited both early and late phase of carrageenan-induced inflammation. Interestingly, this study demonstrated the potent effect of 100 mg/kg astaxanthin alleviated paw edema when compare to standard drug. Taken together, our results suggest that astaxanthin from *Lipopenaeus vannamei* possess anti-inflammatory activity, however the mechanisms of action should be further elucidated.

Keywords: astaxanthin, carrageenan, paw edema, mice

INTRODUCTION

Inflammation is a physiological process relative to the protective mechanism of the local microcirculation to tissue injury caused by physical trauma, noxious stimuli by chemical agents, heat, antigen-antibody interaction and infectious agents. It is known to be involved in the inflammatory reactions including the release of histamine, bradykinin, prostaglandins, fluid extravasations, cell migration, tissue breakdown and repair which targeted at host defense mechanism and usually activated in most disease condition. The mechanism of inflammation injury is attributed, in part, to release of reactive oxygen species from activated neutrophil and macrophages. This over production leads to tissue injury contributed to damage the macromolecules and lipid peroxidation of the membranes [1]. To date, natural products as a number of therapeutic drugs have been discovered from the active constituents obtained from biological sources.

Astaxanthin is one of the most common carotenoids and found in the red pigment of crustacean shell (crabs, shrimps, for example), salmon and the asteroidean [2]. Previous studies demonstrated wide range of biological activities of astaxanthin including antioxidant [3-5], antitumor [6-8] and anti-inflammation [9-11]. Therefore, the present study reported the anti-inflammatory activities of astaxanthin extracted from white shrimp shell mostly found in Thailand.

MATERIALS AND METHODS

Animals

Male ICR mice 8 weeks old, weighting 30-35 g, were acclimated to housing for at least 1 week prior to investigation. Mice were on a 12/12-h day/night cycle, a relative humidity of 50%, with food and water provided ad libitum. All administrations in this study were performed once daily between 8:00-9:00 a.m. Seven groups of ICR mice (n=4) were randomly assigned into naïve control, vehicle control (propylene glycol treated groups), positive control

(Indomethacin treated groups) and treatment groups (10, 30, 50 and 100 mg/kg of astaxanthin). Experimental procedures have been performed in accordance with the principles of animal care outlined by Faculty of Science, Prince of Songkla University. (Ethic no. MOE 0521.11/1045)

Model of inflammation

In this model, each mouse was injected with 50 µl of the inflammatory agent under brief isoflurane anesthesia (3%). To assess the effects of axtaxanthin from white shrimp shell on paw edema, 2.5% carrageenan suspension (w/v with 0.9% saline) was injected into the both hind paws.

Extraction of Astaxanthin from whites shrimp shell

Astaxanthin extraction was prepared according to the procedure described by Sachindra, Bhaskar, & Mahendrakar (2005) [12] with slight modification in that food grade ethanol was used as a solvent. Shrimp shell was blended with ethanol in the ratio of 1:2 (shrimp shell:ethanol) using a Waring laboratory blender (Waring Laboratory Science, Winsted, USA). Shrimp shell residues were vacuum filtered. The collected extract was then evaporated under vacuum at 40 ºC, 175 MPa using a Büchi R-124 rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland) to obtain astaxanthin.

Measurement of edema

Paw thickness was used as a measurement of inflammation-induced edema [14, 16-19]. Briefly, the dorsoventral thickness of each hind paw was measured using a caliper placed at the border of the phalanges and the metatarsals. The measurement was taken when each edge of the caliper was just touching the dorsal and ventral surface of the hind paw (i.e. the caliper was not squeezed onto the hind paw). Data are expressed as the mean paw thickness ± SD.

Experimental design

Astaxanthin were delivered as a solution with propylene glycol. Indomethacin (Wako Pure Chemical Industries, Ltd. Saitama, Japan), a potent non-steroidal anti-inflammatory agent, was suspended in a propylene glycol. Indomethacin (5 mg/kg) has been reported to reduce pain and inflammation associated with acute inflammation [13-15] and serves as a positive control in this study. Propylene glycol was used as a vehicle control.

Mice were randomly separated to each treatment group (n=4 mice per group). Mice received astaxanthin at various doses ranging from 10-100 mg/kg once daily for 3 weeks and received indomethacin at a daily dose of 5 mg/kg for 1 week before carrageenan-induced paw edema. At last dose of oral administration with astaxanthin (10-100 mg/kg), indomethacin (5 mg/kg), or propylene glycol, each animal received the intraplantar injection of 2.5% carrageenan (50µl) from Wako Pure Chemical Industries, Ltd. Saitama, Japan. The paw edema testing were performed 1, 3, 6 h after the carrageenan injection.

Statistical analyses

All data were analyzed using SPSS version 7.0. Paw thickness was analyzed using one-way ANOVA. A probability level < 0.05 was considered to be statistically significant.

RESULTS

The most widely used primary test to screen new anti-inflammatory agents in order to elucidate the ability of a compound to reduce local edema induced in the rat paw by injection of an irritant agent [20]. Carrageenan-induced edema has been commonly used as an experimental animal model for acute inflammation and is believed to be biphasic. The early phase (1-2 h) of the carrageenan model is mainly mediated by histamine, serotonin and increased synthesis of prostaglandins in the damaged tissue surroundings. The late phase is sustained by prostaglandin release and mediated by bradykinin, leukotrienes, polymorphonuclear cells and prostaglandins produced by tissue macrophages [21, 22].

Anti-inflammatory activity, as measured by the paw thickness of the carrageenan-induced edema in mouse paw, was compared with indomethacin (5mg/kg, p.o.). In carrageenan-induced paw edema, we found a significant reduction in paw edema in indomethacin group when compared to the vehicle control group. The anti-inflammatory effect similar to indomethacin which was observed only in the mice treated with 100 mg/kg astaxanthin which significantly decreased paw edema especially 6 h after carrageenan injection. However, low dose of astaxanthin (10-50 mg/kg) showed no significant difference when compare to vehicle control group. This possibly due to low dose of astaxanthin might not reach the therapeutic effect to protect inflammation (Figure 1).

Figure 2 demonstrated the % inhibition of carrageenan-induced paw edema at 1, 3 and 6 h in mice. Histogram was plotted between doses of standard drug indomethacin 5 mg/kg, astaxanthin 100 mg/kg in X axis and inhibition of paw edema in Y axis. Data showed the inhibition of paw edema by astaxanthin significantly increases from standard drug especially 6 h.

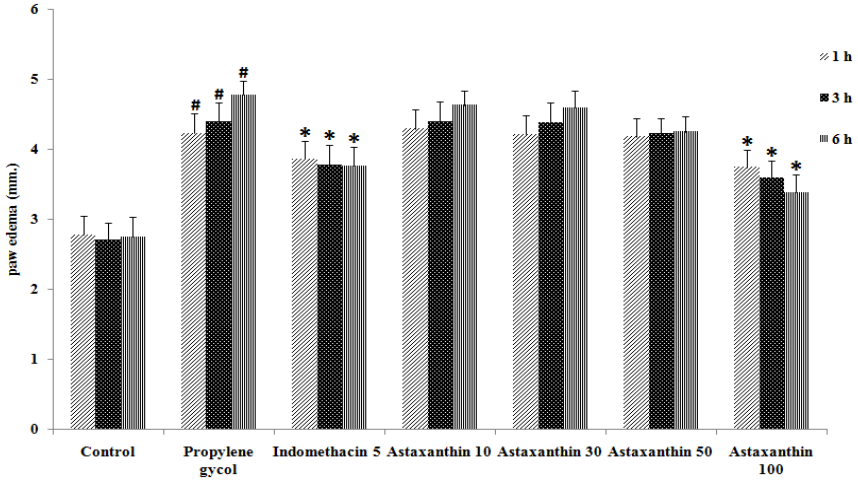

Figure 1. Effect of astaxanthin on inflammation induced by carrageenan-induced paw edema (mm). Data are presented as mean ± SD, n= 8 each group; # p <0.05 naïve control versus vehicle control, * p<0.05 astaxanthin and indomethacin versus vehicle control.

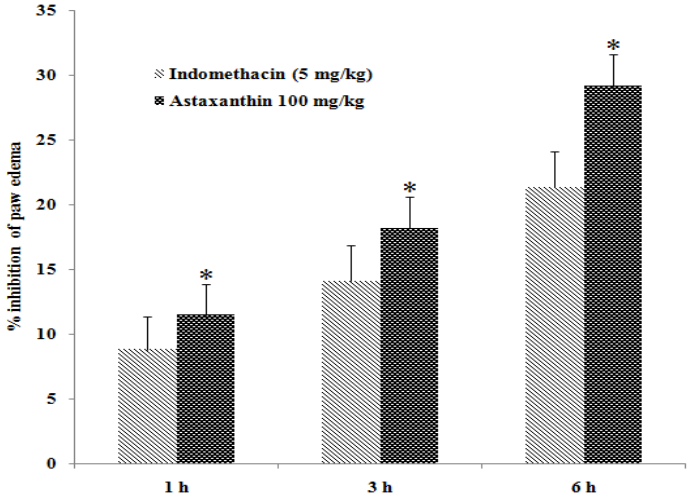

Figure 2. Effect of astaxanthin on the percentage inhibition of paw edema. Data are presented as mean ± SD, n= 8 each group; * p<0.05 astaxanthin 100 mg/kg versus indomethacin.

CONCLUSIONS

The present study showed that astaxanthin at dose 100 mg/kg BW and indomethacin administered via oral reduced experimentally induced inflammation, as measured as the paw thickness of the carrageenan-induced edema in mice.

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REFERENCES

1. Kumar V, Abbas AK, Fausto N, Aster JC. 2010. Robbins and Cotran Pathologic Basis of Disease. 8th ed. Elsevier Saunders. Philadelphia
2. Ambati RR, Phang SM, Ravi S. 2014. Astaxanthin: extraction, stability, biological activities and its commercial applications–a review. Mar. Drugs. 12, 128-152.
3. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J. 2007. Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell Biol. 39, 44-84.
4. Kim JH, Chang MJ, Choi HD, Youn YK, Kim JT, Oh JM. 2011. Protective effects of Heamatococcus astaxanthin on oxidative stress in healthy smokers. J. Med. Food. 14, 1469-1475.
5. Rodrigues E, Mariutti LR, Mercadante AZ. 2012. Scavenging capacity of marine carotenoids against reactive oxygen and nitrogen species in a membrane-mimicking system. Mar. Drugs. 10, 1784-1798.
6. Chew BP, Park JS, Wong MW, Wong TS. 1999. A comparison of the anticancer activities of dietary beta-carotene, canthaxanthin and astaxanthin in mice in vivo. Anticancer Res. May-Jun; 19 (3A), 1849-53.
7. Li Z, Wang Y, Mo B. 2002. The effects of carotenoids on the proliferation of human breast cancer cell and gene expression of bcl-2. Zhonghua Yu Fang Yi Xue Za Zhi. 36, 254-257.
8. Palozza P, Torelli C, Boninsegna A, et al. 2009. Growth-inhibitory effects of the astaxanthin-rich alga Haematococcus pluvialis in human colon cancer cells. Cancer Lett. 283, 108-117.
9. Choi SK, Park YS, Choi DK,Chang HI. 2008. Effects of astaxanthin on the production of NO and the expression of COX-2 and iNOS in LPS-stimulated BV2 microglial cells. J. Microbiol. Biotechnol. 18, 1990-1996.
10. Kishimoto Y, Tani M, Uto-Kondo H, Iizuka M, Saita E, Sone H. 2010. Astaxanthin suppresses scavenger receptor expression and matrix metalloproteinase activity in macrophages. Eur. J. Nutr. 49, 119- 126.
11. Yasui Y, Hosokawa M, Mikami N, Miyashita K, Tanaka T. 2011. Dietary astaxanthin inhibits colitis and colitis-associated colon carcinogenesis in mice via modulation of the inflammatory cytokines. Chem. Biol. Interact. 193, 79-87.
12. Sachindra NM, Bhaskar N, Mahendrakar NS. 2005. Carotenoids in crabs from marine and fresh waters of India. Lebensmittel-Wissenschaft und Technologie. 38, 221-225.
13. Conti S, Costa B, Colleoni M, Parolaro D, Giagnoni G. 2002. Antiinflammatory action of endocannabinoid palmitoylethanolamide and the synthetic cannabinoid nabilone in a model of acute inflammation in the rat. Br. J. Pharmacol. 135,181-187.
14. El Shenawy SM, Abdel-Salam OM, Baiuomy AR, El Batran S, Arbid MS. 2002. Studies on the anti-inflammatory and anti-nociceptive effects of melatonin in the rat. Pharmacol Res. 46, 235-243.
15. Lin CC, Chen MF, Chen CF. 1995. The anti-inflammatory effects of Chinese crude drug prescriptions on experimental arthritis. Am. J. Chin. Med. 23, 145-152.
16. Buritova J, Honore P, Besson JM. 1995. Indomethacin reduces both Krox-24 expression in the rat lumbar spinal cord and inflammatory signs following intraplantar carrageenan. Brain Res. 674, 211-220.
17. Doughty JR, Goldberg RL, Schenkelaars EJ, Singh HN, Peppard J, Haston W et al. 1991. Relationship of blood markers to disease severity and drug efficacy in rat adjuvant arthritis. Agents Actions. 34, 129-131.
18. Malmberg AB, Gilbert H, McCabe RT, Basbaum Al. 2003. Powerful antinociceptive effects of the cone snail venom-derived subtype-selective NMDA receptor antagonists conantokins G and T. Pain. 101, 109-116.
19. Wei F, Zou S, Young A, Dubner R, Ren K. 1999. Effects of four herbal extracts on adjuvant-induced inflammation and hyperalgesia in rats. J. Altern Complement Med. 5, 429-436.
20. Winter CA, Risley EA, Nuss GW. 1962. Carrageenan-induced edema in hind paw of the rat as an assay for anti-inflammatory drugs. Proc. Soc. Exp. Biol. Med. 111, 544-547.
21. Gupta M, Mazumder UK, Gomathi P, Thamil Selvan V. 2006. Anti-inflammatory evaluation of leaves of Plumeria acuminate. BMC Compl. Altern. Med. 6, 1-6.
22. Olivera de Melo JO, Truiti MCT, Muscara MN, Bolonheis SM, Dantas JA, Caparroz-Assef SM, et al. 2006. Anti-inflammatory activity of crude extract and fractions of Nectandra falcifolia leaves. Biol. Pharm. Bull. 29, 2241-2245.

The antibacterial capacity of marine bacteria isolated from Sponge *Acanthellacavernosa* collected from Lombok Island

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ABSTRACT

An increase in infectious disease, especially in Indonesia, will increase the demand for antibiotics. Marine bacteria are some of the potential resources for pharmaceuticals. More than 33 % of potent bacteria that produce antibiotics were isolated from sponges. This study found a potent antibiotic producer from the sponge-associated bacteria. Sponge collection, bacteria isolation, extraction and characterization of potent active compounds were carried out for this study. Approximately 59 single strains of bacteria were isolated from this sponge, 45 strains showed activity against *Escerichia coli*, *Staphylococcus aureus* and *Vibrio eltor*. The chemical separation of the potent strain *Bacterium* sp Lb 10%.2.1.1.b, using n-phase column chromatography revealed 7 active fractions (7, 8,9, 10, 11,14 and 15). The GC-MS analysis of fraction-F9 indicated some phenolic compounds such as 4-n-nonyl phenol, methyl 3-(3,5-ditertbutyl-4-hydroxyphenyl)propionate; acetosyringone; 2,4-Bis(1-phenylethyl) phenol; 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester; tri(2-Ethylhexyl) trimellitate andoleamide.

Keywords : antibacterial, sponge-associated bacteria, phenolic compounds

INTRODUCTION

Indonesian is known for having rich marine biodiversity, such as sponges; about 850 species are found in eastern Indonesian seas (Mapstone, 1990). Since sponges are becoming known as top bioactive producers, research investigating drugs from these marine biota is flourishing. The biggest problem in developing a drug based on a sponge's resources is the difficulty of improving the mass culture of sponge biomass. Their complicated and unstable chemical structure makes them difficult to synthesize. Several researchers reported a role of the relationship between the host and a symbiotic microorganism in producing a secondary metabolite through mediated mechanisms. Sponge-associated microorganisms play an important role in the biosynthesis of the host's secondary metabolite [5,6].

Investigating microorganisms for antibiotic producers is a sophisticated way to solve the problem in developing drugs from sponges. A study on *A. cavernosa* with its associated fungus *Fusarium* sp. reported that both of them produced succinic acid that is active against pathogenic bacterial and larval settlement [11].This study showed the mutual relationship between the host and its symbiont in secondary metabolite production. Another study about the potency of *Acanthella c*. and the sponge's surface of associated microorganisms was the producing antiinfective and antiparasitic compound kalahinol. Changing the bacterial community on this sponge's surface has optimized the antiparasitic activity and increased the kalahinol production [4].

The capacity of Indonesian *A. cavernosa* and its microbial symbiont to produce antibacterial compounds was investigated to obtain the data and compared to the previous work in a different sampling location. Associated bacteria were isolated, antibacterial activity was screened and selected bacterial extracts were fractionated. A separation technique using open column chromatography and GC –MS analysis were done to profile the secondary metabolite compounds.

MATERIALS AND METHODS

Isolation of sponge-associate bacterial sample

The sponge *A. cavernosa* voucher was collected from Teluk Kode, Lombok, Nusa Tenggara on 11 June 2014. Direct plating was applied for isolating the symbiotic bacteria [7]. Approximately 1 cm^3 was washed with sterile sea-water, macerated and strirred in 5 mL of sterile seawater. An aliquote (100 µL) sponge solution was diluted in 900 µL sterile seawater, transferred to the second tube until the dilution rate was 10^{-4}.Approximately 100 µL sponge solution at every concentration was poured into a 10% and a 100% marine agar plate and spread. After 1-2 weeks incubation at room temperature (28-30°C), the single colony was isolated and transferred to the 100% marine agar medium. The isolated bacterial strains were stored in 20% glycerol stock at -20°C.

Cultivation and antibacterial screening test

Each bacterial strain was cultured in 5 mL marine broth and incubated at 28°C, 150 rpm. After 3 days, the bacterial broth was centrifuged at 6000 rpm to separate biomass and supernatant. The supernatant was extracted using ethyl acetate, while pellets were extracted using acetone. Organic solvent was removed using a rotary evaporator. The dried extract was weighed and stored in a refrigerator before using for an antibacterial test.

The pellet and supernatant extract were diluted in methanol with a concentration of approximately 100 µL. The antibacterial test was carried out using the agar diffusion method [1]. Gram-positive bacteria used for bioindicators were *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* ATCC 6633, purchased from the Microbiology Laboratory the Indonesian University in 10 May 2014. The potent strain was sent to the INACC Laboratory for molecular characterization.

Mass cultivation of the potent bacterial strain and chemical separation.

The strain with highest antibacterial activity continued to be used for further analysis. About 10 L of bacterial culture was set up for antibacterial substance analysis. Approximately 10 L marine broth media was prepared with the pH adjusted to around 7.8. About 10 mL of the selected culture was poured into 10 L marine broth media, incubated in a rotary shaker incubator with a temperature of 28 °C, at 110 rpm for 72 hours. Bacterial broth was harvested and centrifuged at 6000 rpm, 4 °C for 15 minutes. The pellet and supernatant were separated and extracted using an organic solvent. The supernatant was extracted using ethyl acetate and evaporated to get the extract. The pellet was extracted using methanol and partitioned using ethyl acetate.

The ethyl acetate extract of pellet was chromatographed using the n-phase solvent system. Hexane - ethyl acetate and methanol was gradually applied to the silica gel column. Each fraction was collected and evaporated for antibacterial testing. The same method was applied to the supernatant extract.

Gas Chromatography- Mass spectroscopy (GC-MS) analysis.

GC-MS analysis was conducted at the Regional Health Laboratory, Jakarta. The potent open-column fraction was injected into Agilent Technologies 7890 GC-Mass with autosampler and 5975 Mass Selective Detector and Chemstation Data System. This instrument was set to electron impact using ionization mode with electron energy 70eV. The column used for analysis was a capillary column HP Ultra 2L, length (m) 30x0.25 (mm) I.D. X 0.25 (µm) film thicknesses. The oven temperature was set initially at 70 °C, rising at 3 °C/min to 150 °C, held for 1 minute and finally rose for 20°/min to 280 °C where it was held for 26 minutes. The injection port temperature was 250 °C, with an ion source temperature of 230 °C, at the interface temperature of 280 °C and the quadrupole temperature of 140 °C. The carrier gas was helium with a column flow of 1.0 µL.

RESULTS& DISCUSSION

Isolation of potent bacteria

The isolation of *A. cavernosa*-associated bacteria has resulted in 58 bacterial strains. The nutrition that was contained in 10% marine agar broth is suggested as the minimum to get the bacterial strains with a high ability to inhibit pathogenic bacteria. Almost all of the bacterial extracts showed activity against *S. aureus*, *B subtilis* and *E. coli*. Table 1 shows the result of the antibacterial screening test.

Table 1. Result of antibacterial screening of bacterial extracts

No	Extract bacterial code	Diameter inhibition against (mm)		
		S. aureus	*B. subtillis*	*E. coli*
1	S.Lb.10%.1.12.2	8,65	10,45	9,4
2	S.Lb.10%.1.3.10	9,075	11,415	8,35
3	S.Lb.10%.1.12.3	10,175	10,55	8,65
4	S.Lb.10%.1.12.5	10,25	9,775	10,3
5	S.Lb.10%.1.3.1	8,8	12,9	10,475
6	S.Lb.10%.2.1.1b	14,85	11,75	8,45
7	S.Lb.10%.1.12.10	10,2	10,25	10,1
8	S.Lb.10%.1.5.3	10,225	8,75	7,7
9	S.Lb.10%.1.12.8	9,2	11,575	9,8
10	S.Lb.10%.1.3.5	8,3	9,05	8,3
11	S.Lb.10%.2.1.6	10,325	9,85	7,2
12	S.Lb.10%.1.12.9	9	7,425	0
13	P.Lb.10%.1.3.10	9,75	10,05	9,25
14	P.Lb.10%.1.12.3	9,6	4,1	9,55
15	P.Lb.10%.1.3.1	7,3	10	7,6
16	P.Lb.10%.1.12.5	12,6	9,65	6,8
17	P.Lb.10%.1.5.3	8,625	9,4	8,55
18	P.Lb.10%.1.12.8	9,1	9,15	0
19	P.Lb.10%.2.1.6	7,6	9,775	0
20	P.Lb.10%.1.12.9	11,075	10,95	8
21	P.Lb.10%.1.3.5	11,25	9,1	7,8
22	P.Lb.10%.2.1.1b	9,225	12,2	8,5
23	P.Lb.10%.1.12.10	8,75	8,1	0
24	P.Lb.10%.1.12.2	10,45	10,3	9,325
25	S.Lb.100%.1.3.1	11,1	9,25	9,3
26	S.Lb.100%.1.5.4	8,45	10,525	8,9
27	S.Lb.100%.2.1.10	9,6	8,85	8,2
28	S.Lb.100%.2.1.9	9,5	11,225	0
29	S.Lb.100%.1.12.6	11,15	8,925	8,1
30	S.Lb.100%.1.12.3b	10,575	11,6	0
31	S.Lb.100%.1.12.1b	11,625	12,275	7,8
32	S.Lb.100%.1.12.3a	7,5	9,3	7,2
33	P.Lb.100%.1.3.1	7,425	9,875	8,7
34	P.Lb.100%.1.5.4	7,2	0	8,3
35	P.Lb.100%.2.1.10	9,75	0	0
36	P.Lb.100%.1.12.1b	9,375	11,025	8,9
37	P.Lb.100%.2.1.9	7,675	6,9	0
38	P.Lb.100%.1.12.3a	9,1	9	0
39	P.Lb.100%.1.12.6	10,25	8,1	0
40	P.Lb.100%.1.12.3b	8,575	10,35	0
	Ampicillin	14,65	16,6	37,5

S:supernatant, P:pellet

Almost 90% bacterial extract actively inhibits gram positive and negative bacterial growth. 24 extracts belong to bacteria that were isolated from 10% marine agar, while 16 extracts were from 100% marine agar. The most potent extract from strain Lb.10% 2.1.1 was confirmed for further analysis. Molecular characterization of strain Lb.10%2.1.1 using 16 SrDNA partial gene sequencing showed it to be 99 % similar to *Bacterium* FJAT-17799.

Separation of potential extract

The extraction of 10 L bacterial culture resulted in 1.996 g dark brown supernatant extract and 1.25 g pellet extract. The open column chromatography of the supernatant extract using the n-hexane-ethyl acetate gradient system revealed 15 fractions. Open column separation of the pellet extract using n-phase chromatography contained 6 fractions. All of open column fractions were tested for antibacterial activity, as described in Table 2.

Table 2. The antibacterial activity of column chromatography fractions.

Sample	Weight (mg)	Diameter inhibition in (100µg) against	
		Sa (mm)	Bs (mm)
Extract supernatant	1996.0	12	11,45
extract Pellet	1250.0	10,45	11,95
S. Fraction 1	179.6	9,8	12,05
S. Fraction 2	34.2	10,3	11,925
S. Fraction 3	1.1	11,225	10,2
S. Fraction 4	187.6	11,65	7,65
S. Fraction 5	50.5	10,05	7,975
S. Fraction 6	8.1	11,35	8,05
S.Fraction 7	11.9	18,85	18,35
S. Fraction 8	20.8	15,4	7,6
S. Fraction 9	13.5	12,725	8,15
S.Fraction 10	183	14,0	11,65
S.Fraction 11	20.7	13,725	13,875
S.Fraction 12	37.7	11,825	7,7
S.Fraction 13	162.7	10,65	7,5
S.Fraction 14	77.9	13,725	8,1
S.Fraction 15	700.3	15,35	15,15
P. Fraction 1	8.4	14,2	8,65
P. Fraction 2	23.5	10,75	8,75
P. Fraction 3	5.4	14,45	16,7
P. Fraction 4	6.6	11,3	15,775
P.Fraction 5	133.4	10,725	6,95
P. Fraction 6	16.6	10,825	6,95
Kanamycin		35,95	33,95
Ampicillin		44,45	33,6

The most potent anti-gram positive bacteria from the supernatant separation were fractions 7, 8,9, 10, 11,14 and 15, showed by the diameter inhibition range of 12-18 mm. The remaining fraction showed moderate anti-gram positive bacteria against *S. aureus* and *B. subtillis*.

Among 6 pellet fractions, high activity was shown by fractions 1 and 3 and moderate by fractions 2,4,5 and 6. Compared to the positive control kanamycin and ampicillin, all the fractions were weaker. The analysis of a single substance was needed to get a better conclusion.

GC-MS analysis of active fraction

GC-MS analysis of supernatant active fraction SF9 showed some phenolic and benzene compounds such as:

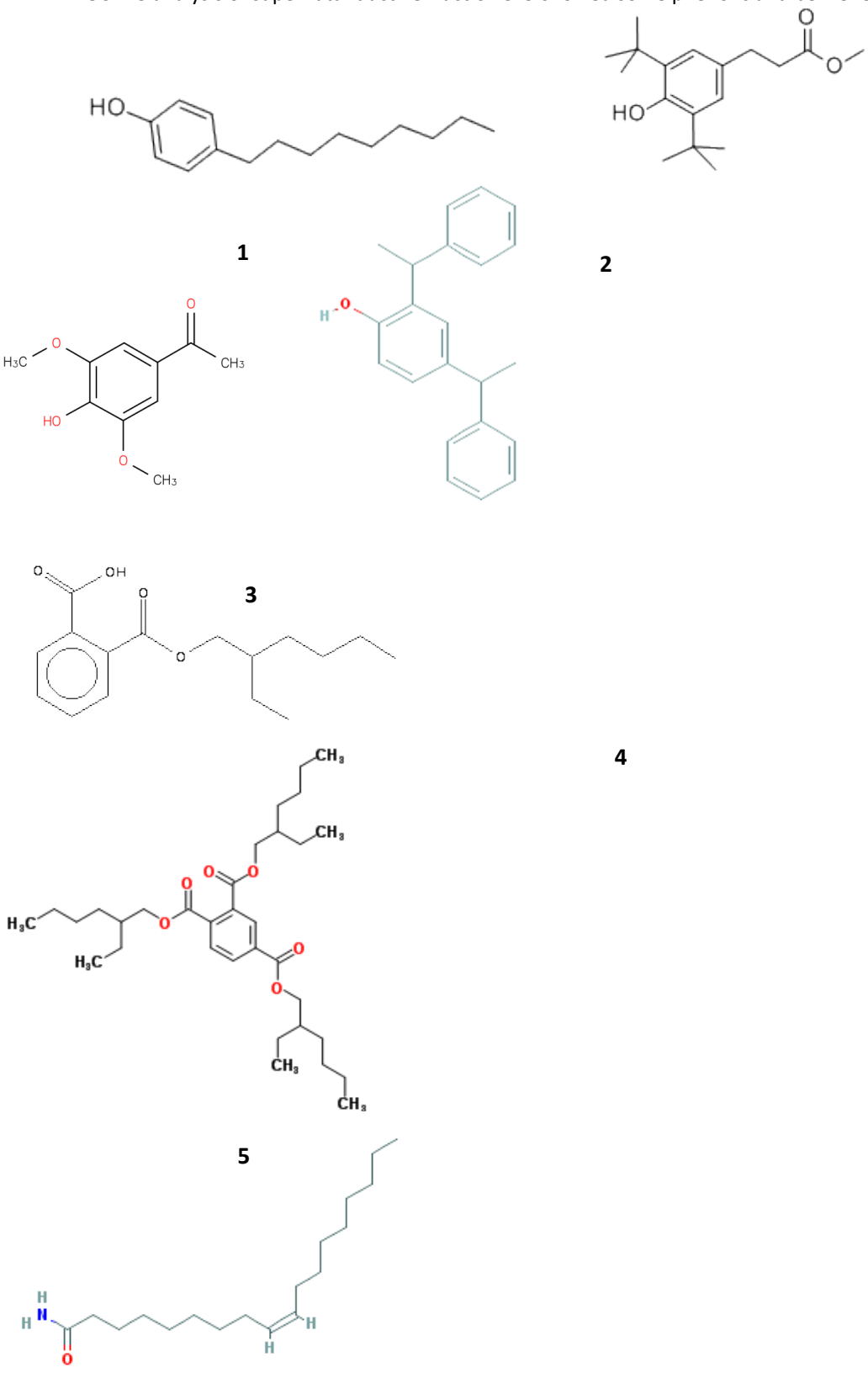

Figure 1. The compounds in active fraction SF9 [2,8,9,12,13,14]

Compound 1, 4-nonylphenol1, appeared at a retention time of 29.468 minutes. It has the molecular weight 202.36 g/mol. The reported activity was anti-estroghenic and weevil pheromone [12]. Compound 2 was methyl 3-(3,5-ditertbutyl-4-hydroxyphenyl)propionate with the retention time of 31.792 minutes and molecular weight of 292.34 g/mol. The previous study reported this compound confirmed for antibacterial and antioxidant/radical-scavenging activity. This compound is also found in the oil of the plant *Jatropacurcas* [8]. Compound 3, with retention time of 34.391 minutes, is acetosyringone. It was widely used for analgesic, antipyretic, anti-inflammatory and anti-asthmatic drugs. This compound was also isolated in many plants including the seagrass *Pasedoniaoceanica* [13]. Compound 4. (retention time of 34.895 minutes) was named as 2,4-Bis(1-phenylethyl) phenol with the molecular weight of 302.409 g/mol. This compound was also isolated from the plant *Zanthoxylumintegrifoliolum* and reported as having anti-inflammatory activity [2]. Compound 5 appeared at retention time of 35.074 minutes was 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester. This compound showed cytotoxic activity. This was previously isolated from marine *Streptomyces sp*. with the molecular weight of 278.34 g/mol [9]. **Compound 6 was tri(2-ethylhexyl) trimellitate with retention time of 45.3 minutes. It has molecular weight of** 546.774 and showed anticancer activity. Compound 7 (retention time of 36.39 minutes) was oleamide**,** the fatty acid amide, that showed activity as a hypnotic agent (modular neuro function)[14]. Some active fractions should be analyzed for characterizing their unknown active substances.

CONCLUSION

Investigation of *A. cavernosa*- associated bacteria, *Bacterium* FJAT-17799 showed potential antibacterial agents. Several active compounds detected by GC-MS spectrum were 4-nonylphenol, methyl 3-(3,5-ditertbutyl-4-hydroxyphenyl)propionate, acetosyringone, 2,4-Bis(1-phenylethyl) phenol, 1,2-benzenedicarboxylic acid, mono(2-ethylhexyl) ester and **tri(2-Ethylhexyl) trimellitate and**oleamide. All of these compounds were reported to have biological activity and show promise for the pharmaceutical industry.

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REFERENCES

1. Bauer AW, Kirby WM, Sherris JC, Turek M. 1966. Antibiotic susceptibility testing by a standardized single disk method. Am. J. Clin. Pathol. 45, 493-498.
2. Chen JJ, Chen PH, Liao CH. Huang SH, Chen IS. 2007. New Phenyl propenoids, Bis (1-phenyl ethyl) phenols, bisquinolinone alkaloid, and anti-inflammatory constituents from zanthoxylum integrifolidum J. Nat. Pro. 70(9), 1444-1448.
3. Mapstone GM. 1990. Reef corals and sponge of Indonesia. A video-based learning module . Result of the Indonesian-Dutch Snellius-11 expedition. Indonesian Institute of Sciences-Netherlands Marine Research Foundation. Unesco, France, 53-59.
4. Muffler K, Ulber B. 2005. Downstream processing in marine biotechnology. Adv. Biochem. 43, 61-87
5. Piel J (2004) Metabolites from symbiotic bacteria. Nat. Prod. Rep. 21,519–538.
6. Thiel V. Imhoff JF. 2003. Phylogenetic identification of bacteria with antimicrobial activities isolated from Mediterranean sponges. Biomol. Eng. 20, 421–423.
7. Hirose Y. 2003. Personal Communication. In training course of Conservation and Sustainable Use of Tropical Marine Biological Diversity at Marine Biotechnology Institute Co. Ltd. Kamaishi Laboratory Japan. October 2002-July 2003
8. Tongpoothorn M. Chauthoi S, Sriuttha M, Saosang K, Viriyachai R. 2012. Bioactive properties and chemical constituents of methanolic extract and its fraction from JatrophaUreas oil. J. Ind. Crop. 36(1), 437-444.
9. Krishnan K, Mani A, Jasmine S. 2014. Cytotoxic activity of bioactive compound 1,2-benzene dicarboxylic acid, mono2-ethyl hexyl ester extracted from 2 marine derived *Streptomyces sp.* VII SJK8. Int. J. Mol. Cell. Med. Autumn. 3(4), 246-254.
10. Iwase H, Oiso S, Kariyazono H, Nakamura K. 2014. Biological effects of the plasticizer Tris (2-Ethylhexyl) trimellitate. Clin. Pharmacol. Biopharmaceut. S2:004. DOI:10.4172/2167-065X.S2-004.

11. Yang LH. 2006 Antifouling compounds from the marine sponge Acanthellacavernosa and its associated microbes Ph. D. thesis on Atmospheric, Marine and Coastal Univ. Hongkong University of Science and Technology
12. http://www.chemicalbook.com/
13. http://www.ebi.ac.uk/chebi/searchId.do?chebiId=CHEBI%3A2404.
14. Mendelson WB. Basile AS. 2001. The Hypnotic actions of the fatty acid amide, oleamide. Neuropsychopharmacology. 25 (55), S36-S39.

Cytotoxic and genotoxic assessment of *Canarium odontophyllum* Miq. (dabai) bark extract against human colorectal HCT116 cell line

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ABSTRACT

The aim of this study was to assess the cytotoxic and genotoxic effect of extracts from stem bark of *C. odontophyllum* against human colorectal cancer cell line HCT116. The IC_{50} values of the aqueous, methanol, and acetone extracts against HCT116 cells whereas acetone extract against normal human colon cell line CCD-18co were determined using the MTT assay. The concentration of the extracts used ranged from 12.5 to 200 µg/ml and treatment times were at 24, 48 and 72 hours. Annexin V-FITC/PI labelling assay was employed to determine mode of HCT116 cell death by the acetone extract at 48 hours. The DNA damage of HCT116 cell was detected using alkaline comet assay at IC_{10} and IC_{25} values of the acetone extract for 30 minutes of treatment exposure. The data findings showed that acetone extract exhibited the highest cytotoxic effect against HCT116 cells compared to methanol and aqueous extracts at 24, 48 and 72 hours. Toxicity of acetone extract was tested against CCD-18co cells at 24 and 48 hours but at 72 hours, CCD-18co cells proliferated. Apoptosis assessment using annexin V-FITC/PI labelling assay revealed that the primary cell death was via apoptosis after 48 hours treatment. Low doses of acetone extract from stem bark of *C. odontophyllum* showed significant DNA damage in HCT116 cells with tail moment of 6.187 ± 0.718 A.U and 7.877 ± 0.142 A.U respectively. Acetone extract from stem bark of *C. odontophyllum* has high potential in the development of anti-cancer agent against HCT116 cells with no cytotoxic effect against human colon fibroblast cells.

Keywords: cytotoxicity, *Canarium odontophyllum*, colorectal cancer, HCT116, apoptosis, DNA damage

INTRODUCTION

Colorectal cancer or colon cancer is defined as an uncontrollable cell growth at the lower part of the digestive system that is the large intestine [1]. Colorectal cancer is one of the main causes to high mortality and morbidity rate of cancer. Moreover, colorectal cancer is a type malignant cancer that always occurs in developing countries. Colorectal cancer falls in second highest mortality and morbidity rate followed by lung cancer, gastric cancer and breast cancer. In Malaysia, colorectal cancer mostly occurs in male and third in frequency among females. Colorectal cancer mostly occurs among the age group of above 50 years old [3].

Plant derived anti-cancer agents play an important role in cancer chemotherapy [4]. Additionally, plant derived anti-cancer agents are known to be safer and give lesser side effects when in comparison with these synthetic anti-cancer agents available [5]. *Canarium odontophyllum* Miq. is a type of plant that is known as 'dabai' or 'Borneo olives'. It can be found in Sarawak, Malaysia especially in Sibu, Sarikei & Kapit [6]. It belongs in the Burseraceae family and *Canarium* L. genus [7]. The fruit of *C. odontophyllum* is oval with a purplish skin and has a single seed along with a hard and thick endocarp [8]. The stem bark of *C. odontophyllum* is greenish grey or yellowish brown in colour with a scaly surface whereas the inner stem bark is reddish brown with a soft texture [9]. Based on the phytochemical screening done, it was found that extracts from stem bark of *C. odontophyllum* contain flavonoid, tannin, saponin, terpenoid and phenolic compound [10].

Damage to DNA always occurs from endogenous and exogenous agents such as reactive oxygen species (ROS) from cellular metabolism and ultraviolet light from the sun [11]. Chemical carcinogens, radiation and genotoxic anti-

cancer agents can cause DNA damage [12]. When there is DNA damage, the damage itself will cause cell cycle arrest where it can lead to DNA repair or cell death via apoptosis [13]. Therefore the objective of the present study is to investigate the mechanism of cell death and to determine the genotoxic effect of extracts from the stem bark of *C. odontophyllum* against human colorectal cell line HCT116.

MATERIALS AND METHODS

Plant Material

Stem bark of *Canarium odontophyllum* Miq. was obtained from Sarawak, Malaysia. All plant parts were identified and authenticated by Mr. Sani Miran and deposited in the Herbarium of the Universiti Kebangsaan Malaysia (UKM), Bangi, Selangor, Malaysia with a voucher specimen number of UKMB 40052.

Preparation of Plant Extracts

The stem bark of *Canarium odontophyllum* was extracted in using acetone, methanol and aqueous. To prepare a stock extract solution of 100 mg/mL, 100 mg of acetone and methanol extract were dissolved in absolute DMSO whereas for aqueous extract, distilled water was used as the diluent. The solution was mixed well until the solution was completely dissolved. All extracts were sterilized by passing through a 0.22 µM membrane filter and were stored in air-tight jars at -20°C refrigerator until further use.

Preparation of Cell Culture

HCT116 and CCD-18co were obtained from American Type Culture Collection (ATCC) (Rockville, MD USA). HCT116 cell line (ATCC Number: CCL-247 ^{TM}) was cultured in McCoy 5A media (1x) (Sigma Aldrich, USA) whereas CCD-18co (ATCC Number: CRL-1790^{TM}) was cultured in EMEM (Eagle's Minimum Essential Medium) (1x) (Sigma-Aldrich, USA). McCoy 5A and CCD-18co media were enriched with 10% fetal bovine serum. In this study all incubations were performed at a high humidity environment of 5% carbon dioxide (CO_2) and at a temperature of 37°C. The cultured cells were cultured up to 70-90% confluence of cells. Trypsin-EDTA (0.25% Trypsin / 0.03% EDTA) was added followed by addition of the media to inactivate the trypsin solution and centrifuged at 1000 rpm for 3 minutes. Subculture of cells was done every 2 to 3 days for HCT116 cell line and 4 to 5 days for CCD-18co cells.

Evaluation of Cytotoxic Activity

MTT assay [14] was performed in the determination of IC50 value of the tested compounds. A total volume of 200 µl of cell suspension at 5.0 x 10^4 cells/mL was seeded and incubated for 24 hours before being treated with the extracts. Menadione was used as a positive control and negative control comprised the untreated cells (HCT116 cells with media only). A total volume of 200 µl of sample treatment was added and incubated for 24, 48 and 72 hours. After treatment incubation period, 20 µL of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) at 5 mg/ml was added and incubated for 4 hours. Next, 200 µL of DMSO solution was added to each well incubated for another 15 minutes. The plate was shaken on an automatic mixer for 5 minutes and the absorbance was read using an ELISA plate reader with a test wavelength of 570 nm. The percentage of cell viability was calculated using the formula given below:

$$\text{Cell viability (\%)} = \frac{\text{Mean of treated sample}}{\text{Mean of negative control}} \times 100\%$$

The percentage of cell viability against the concentration of test compounds graph was plotted to obtain IC_{50} value which is the concentration of sample that inhibits cell growth.

Determination of mode of cell death

Flow cytometry annexin V-FITC/PI assay was used to determine the mode of cell death induced by the treatment compound. A total volume of 3 mL of HCT116 suspension cells at 5 x 10^4 cells/mL were seeded in the 6-well microtitre plate. The cells were incubated for 24 hours and treated with acetone extract at 25 µg/mL, 50 µg/mL, 100 µg/mL and 200 µg/mL. A total volume of 3 mL sample treatment was added and incubated for 48 hours. Next, the cells were washed with 1 mL of PBS solution and trypsinized with 500 µl of trypsin-EDTA before they were centrifuged at 2500 rpm for 5 minutes. The supernatant was discarded and the process was repeated 3 times. A total of 200 µL of annexin binding buffer (1x) was added into the tube. A total volume of 5 µL of annexin V-FITC (eBioscience, Austria) was added into the tube and left for 15 minutes in ice prior to addition of 10 uL of propidium iodide (eBioscience, Austria) at 20 µg/mL. The

tubes were incubated in ice condition for another two minutes and then a total of 200 µl of annexin binding buffer (1x) (eBiosience, Austria) was added and transferred into a falcon tube to be analysed by flow cytometry (BD FASCCanto II) with Cell Quest software (BD Sciences, America).

Alkaline Comet Assay

Alkaline comet assay was used to detect DNA damage [15] at IC_{10} and IC_{25} of acetone extract. Menadione was used as the positive control at IC_{25} value. A total of 3 mL of HCT116 suspension cell at 5 x 10^4 cells/mL was incubated for 24 hours and were then treated with the extract and incubated for 30 minutes. The wells were washed twice with PBS solution and trypsinized to be centrifuged at 2500 rpm for 5 minutes. The supernatant was discarded and this process was repeated. The low melting point agar (LMPA) and normal melting point agar (NMPA) (Sigma Aldrich, USA) were preheated until the agar melted. When the NMPA reached a temperature of 37 °C, a total of 100 µL of NMPA was pipetted onto a frosted slide and a coverslip (50 mm X 22 mm) was placed on top of the slide and was then taken out slowly after the agar hardened. Next, about 80 µL of LMPA was added into each sample tube on top of the prepared NMPA slide and a coverslip was placed slowly onto the two layers of gel. The coverslip was placed in a coplin jar filled with the lysing solution and cooled at 4°C for 24 hours before the slides were placed on the electrophoresis tank. Electrophoresis buffer was added into the tank to immerse the slide for 20 minutes. The electrophoresis process was carried out for 20 minutes at 25 V and 300 mA after which the slides were rinsed with neutralizing buffer three times for every 5 minutes. The slides were then stained with 50 µL of ethidium bromide (Sigma Aldrich, USA) at 20µg/mL. For each slide, 50 single and isolated cells were analysed for any DNA damage under a fluorescent microscope (Biochrom ASYS, UK).

Statistical Analysis

All data were analysed by using SPSS Software Version 22. All data were done in triplicate and were expressed as the mean ± S.E.M. from three different experiments. One-way ANOVA was used to measure statistical differences between the mean in all experiments. The statistical difference was indicated with value $p < 0.05$.

RESULTS AND DISCUSSION

From Table 1, acetone extract showed a higher cytotoxic effect compared with methanol extract in 24 hours of treatment with no cytotoxic effect observed by the aqueous extract. At 48 hours, the acetone extract displayed the lowest in contrast with methanol extract and aqueous extract. The IC_{50} values of all three extracts at 48 hours were lower than the IC_{50} values at 24 hours. The IC_{50} values obtained at 72 hours were found to be slightly higher than the IC_{50} values at 48 hours. However, there was no significant difference indicated between these values. From Table 2, it was found that when acetone extract was treated against CCD-18co, no cytotoxic effect was observed at 24 and 48 hours. However, when treatment of acetone extract was prolonged to 72 hours, CCD-18co cells proliferated at the lower doses. As shown in Figure 1, menadione however, showed prominent cytotoxic activity against HCT116 and CCD-18co cells with IC_{50} value of 8 µM and 20 µM.

Based on Figure 2, there is an apparent decrease in percentage of viable HCT116 cells after 48 hours treatment of dose-dependent acetone extract from stem bark C. *odontophyllum.* One-way ANOVA analysis revealed that there is a significant difference ($p<0.05$) of percentage of viable cells when treated with high doses (100 µg/ml and 200 µg/ml) of acetone extract which are at 30.3 ± 1.474 % and 4.4 ± 1.68 % respectively when in comparison with the percentage of viable cells of the negative control (89.367 ± 3.531 %). A significant ($p<0.05$) increased in the percentage of apoptotic cells after treatment with acetone extract can be observed at 100 µg/ml (58.9 ± 1.158 %) and 200 µg/ml (88.53 ± 1.763 %) when compared to negative control (5.667 ± 2.234 %). A significant ($p<0.05$) increase in necrotic cells can only be observed at 100 µg/ml with 10.8 ± 2.451 % compared to the negative control (4.767 ± 1.378 %) The positive control, goniothalamin, displayed significant percentage of viable, apoptotic and necrotic cell population at 25.767 ± 3.733 %, 58.333 ± 4.694 % and 15.9 ± 1.00 %, respectively. Figure 3 showed the distribution of HCT116 cells with acetone extract treatment in 48 hours and it was found that at high doses, most of the HCT116 cells were in the Q3 quadrant which represented the late apoptosis state.

HCT116 cells were treated with acetone extract from stem bark of *C. odontophyllum* at 8µg/ml (IC_{10} value) and 15µg/ml (IC_{25} value) for 30 minutes. Figure 4 presented tail moment of HCT116 cells after treatment with acetone extract at IC_{10} value and IC_{25} value for 30 minutes. One-way ANOVA analysis displayed a significant ($p<0.05$) increased of HCT116 cell tail moment at both concentrations when compared to the negative control (1.080 ± 0.239 A.U.) which were 6.187 ± 0.718 A.U and 7.877 ± 0.142 A.U, respectively. The value of tail moment increased as the concentration increased, however, there was no significant difference ($p>0.05$) between these values. The positive control, menadione at IC_{25}

exhibited significant ($p<0.05$) tail moment of HCT116 cells at 12.863 ± 0.441 A.U. The percentage of DNA tail of HCT116 cells after treatment with acetone extract at IC_{10} and IC_{25} value for 30 minutes can be observed in Figure 5. According to one-way ANOVA analysis, there was a significant difference ($p<0.05$) between percentage of DNA tail of treated HCT116 cells and the negative control (7.866 ± 0.939 %) at 15.799 ± 0.673 % and 18.432 ± 0.751 % respectively. The percentage of tail intensity increases as the concentration increases, however, there was no significant difference ($p>0.05$) between these values. The positive control, menadione, at IC_{25} revealed significant ($p<0.05$) percentage of tail intensity in HCT116 cells at 47.422 ± 2.02 %.

Table 1. Comparison of IC_{50} values of extracts against HCT116. The IC_{50} value of aqueous, methanol and acetone extract from stem bark of *C. odontophyllum* against HCT116 cell line at 24, 48 and 72 hours of treatment. Data represents the mean of triplicates from 3 different experiments ± S.E.M.

Extract / Time of treatment exposure	IC_{50} value		
	Aqueous extract	**Methanol extract**	**Acetone extract**
24 hours	> 200 µg/mL	65 ± 2.89 µg/mL	50 ± 10.69 µg/mL
48 hours	112 ± 6.11 µg/mL	30 ± 4.73 µg/mL	25 ± 5.20 µg/mL
72 hours	160 ± 5.46 µg/mL	45 ± 7.64 µg/mL	40 ± 2.89 µg/mL

Table 2. Comparison of IC_{50} values of acetone extract against CCD-18co from stem bark of *C. odontophyllum* at 24, 48 and 72 hours of treatment. Data represents the mean of triplicates from 3 different experiments ± S.E.M.

Time of treatment exposure	IC_{50} value
24 hours	> 200 µg/mL
48 hours	> 200 µg/mL
72 hours	> 200 µg/mL

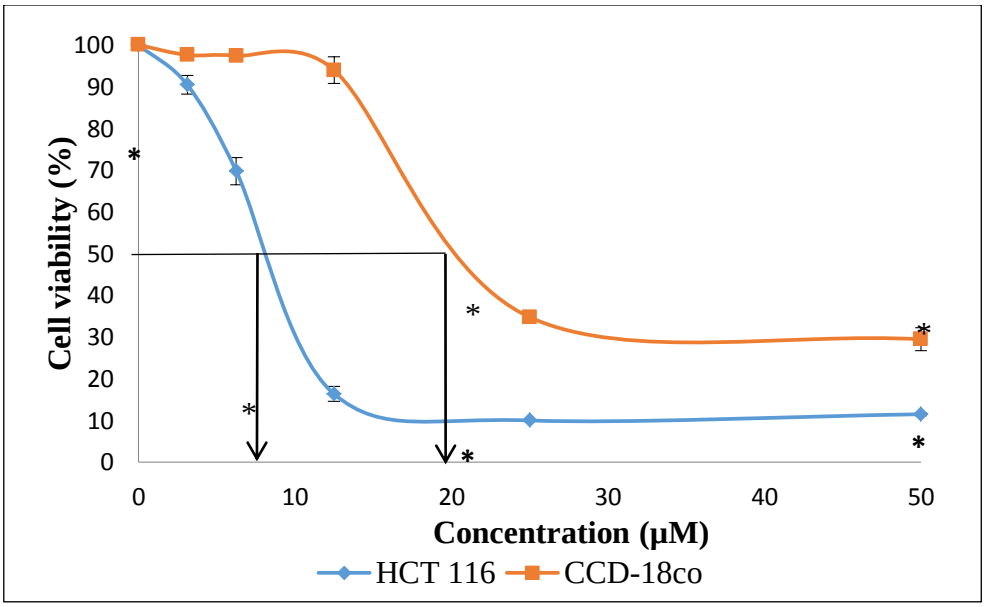


Figure 1. Cell viability of HCT116 and CCD-18co cells after exposure of Menadione. Cell survival is expressed as a percentage relative to the negative control (untreated). Menadione at concentration ranging from 0 – 50 Mm exhibited cytotoxic activity against HCT116 and CCD-18co cells at 24 hours of treatment exposure. Each point represents the mean of triplicates from 3 different experiments ± S.E.M.

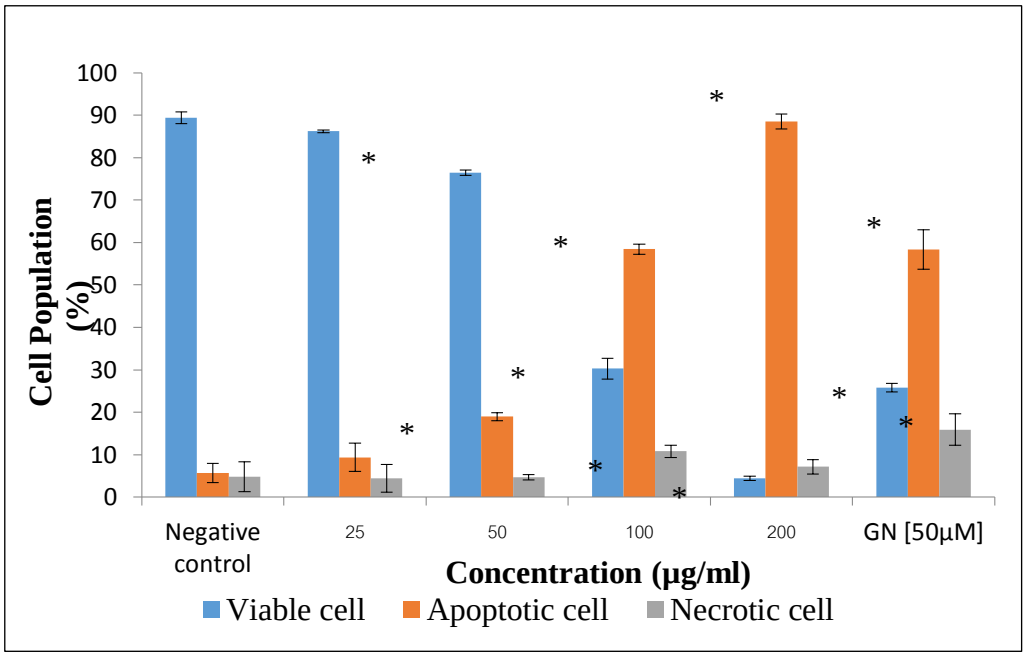
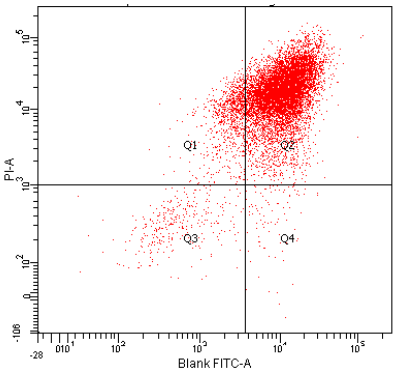
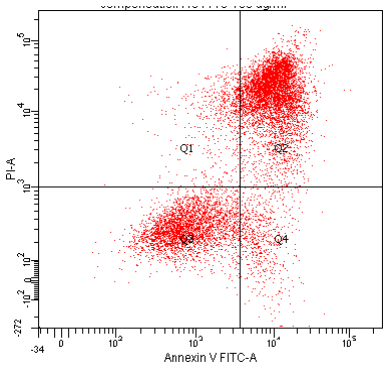


Figure 2. Percentage of cell population of HCT116 after 48 hours treatment with of acetone extract from stem bark of *C. odontophyllum* at concentration ranging from 25 – 200 µg/ml for 48 hours. Each data represents the mean of triplicates from 3 different experiments ± S.E.M.

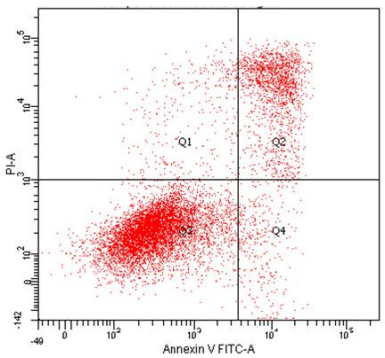
200 µg/mL acetone extract



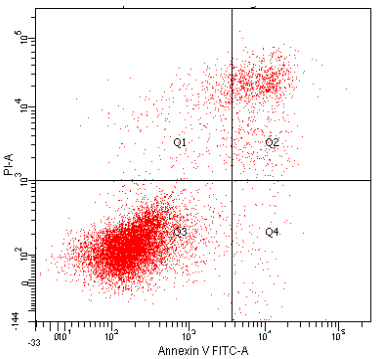
100 µg/mL acetone extract



50 µg/mL acetone extract



25 µg/mL acetone extract



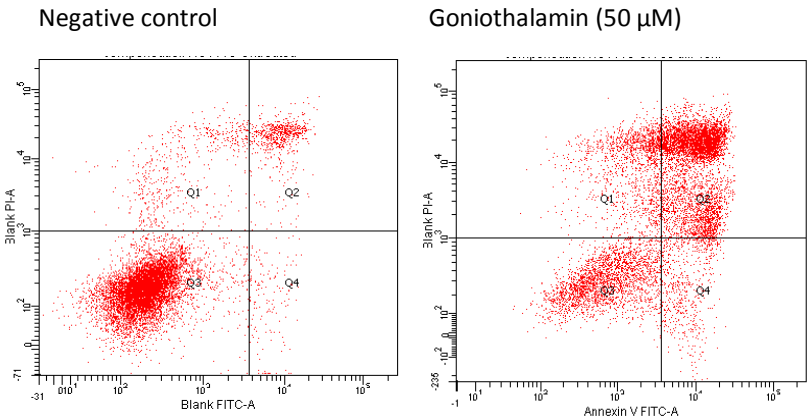


Figure 3. Cytogram of HCT116 cells induced with acetone extract from stem bark of *C. odontophyllum* at concentration ranging from 25 – 200 µg/mL after 48 hours of treatment.

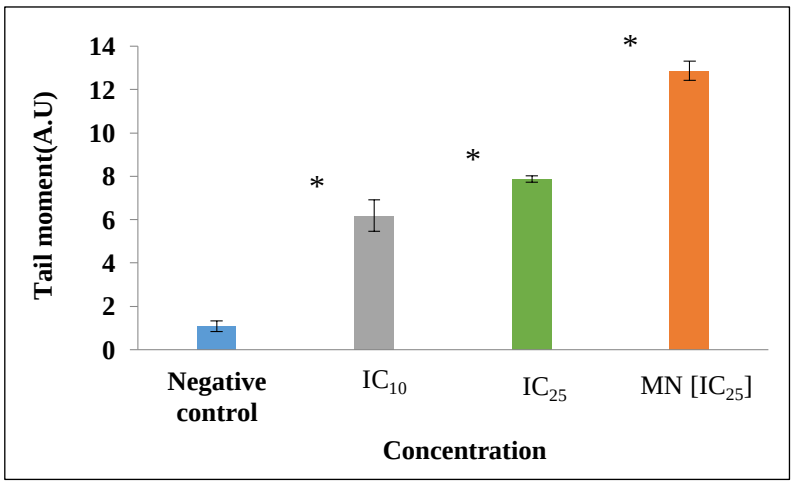


Figure 4. Tail moment of HCT116 cells after 30 minutes of treatment with acetone extract from stem bark of *C. odontophyllum.* Each data represents the mean of triplicates from 3 different experiments ± S.E.M.

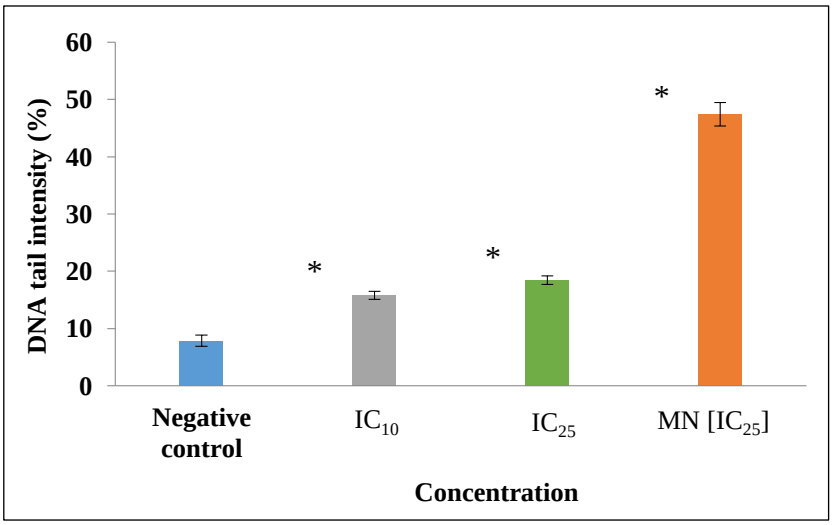


Figure 5. DNA tail intensity of HCT116 cells after 30 minutes of treatment with acetone extract from stem bark of *C. odontophyllum.* Each data represents the mean of triplicates from 3 different experiments ± S.E.M.

CONCLUSIONS

Out of all three extracts, acetone extract exhibited the most potent cytotoxic activity with a significantly higher cytotoxic effect than aqueous extract but the cytotoxicity between acetone and methanol extract showed no significant difference. This data findings correlate with [10,16]. which displayed that acetone extract from the stem bark of *C. odontophyllum* exerted the highest cytotoxic effect against HCT116 cells and as well as acetone extract from the leaves of *C. odontophyllum* The cytotoxic activity of a plant against cancer cells is attributed to their phytochemical properties [17]. The potent cytotoxic effect exerted by acetone extract may be caused by non-polar terpenoid compounds. For example, a few terpenoid derivatives from *Rhizoma curcumae* were found to have antiproliferative properties towards a few cancer cell lines [18]. Polyphenolic compounds such as flavonoids may also contribute to the antitumor activity. Flavonoids are known to have beneficial biological effects that include anti-inflammatory, anti-allergic, antimicrobial, anticarcinogenic and antitumor effects [19]. Based from the results obtained, acetone extract was chosen to proceed with the rest of the experiments as it is considered to be the most potent out of all extracts tested. When acetone extract was treated against normal colon cell line CCD-18co, no cytotoxic effect was observed at 24 and 48 hours. However, when treatment of acetone extract from stem bark of *C. odontophyllum* was prolonged to 72 hours, CCD-18co cells proliferated at lower doses. This may be due to the presence of compounds that contribute to the mitogenic activity of the cells [20]. Hence, acetone extract from stem bark of *C. odontophyllum* exhibited selective toxicity towards colorectal cancer cell line HCT116.

Mode of cell death assessment of acetone extract revealed that the primary cell death of HCT116 cells was via apoptosis after 48 hours treatment. There was an increase of apoptotic cells and a decrease of viable cells with increasing concentrations of acetone extract from stem bark of *C. odontophyllum*. It is said that plant-derived polyphenolic compounds act as antitumor compounds and have apoptosis-inducing properties in cancer cells [21]. Acetone extract from stem bark of *Cephaltaxus griffithii* Hook f. induced apoptosis towards HeLa cells [22]. In addition, the mode of cell death of HL-60 cells after treatment with ethyl acetate extract from stem bark of *Cudrania tricuspidata* was also via apoptosis [23]. Apoptosis was also the primary cell death of HCT116 cells after treatment with ethanol extract from sporophyll of *Undaria pinnatifilda* [24].

The percentage of viable cells obtained are different in these two assays. This may be due to the difference in the end point measurement of these assays [25]. MTT assay is based on the involvement of active mitochondria in living cells to produce succinate dehydrogenase enzyme in order to reduce MTT salt to formazan whereas flow cytometry annexin V-FITC/PI assay is based on the detection of exposed phosphatidylserine on the outer part of the membrane in dead cells [14,15]. Besides that, MTT assay can only measure living cells but not dead cells. Flow cytometry annexin V-FITC/PI assay was able to measure percentage of both living and dead cells in a known amount of cells. The differences between these two assays may be contributing to the deviation of results obtained. Cytotoxic effect induced by acetone extract from stem bark of *C. odontophyllum* towards HCT116 cells may be caused by DNA damage and the detected genotoxicity might be the early mechanism of cell death via apoptosis. Alkaline comet assay was used to detect genotoxic effect of tested compound by measuring its DNA damage at single cell that can be observed under a fluorescent microscope with a comet head (nucleus) and its tail (DNA fragments) [26]. The concentrations used in this study were at IC_{10} and IC_{25} values because a lower and non-cytotoxic concentration was used to dodge any false positive result of dying or dead cells [27]. Among the frequently used comet parameters, percentage of DNA in tail and tail moment could offer the most precise result for the degree of damage [28].

In this study, IC_{10} and IC_{25} of acetone extract from stem bark of *C. odontophyllum* showed significant ($p<0.05$) DNA damage in HCT116 cells after 30 minutes treatment. DNA is the key target by most cytotoxic anticancer drugs whether it acts directly through reactive metabolites or indirectly through the incorporation into DNA nucleotide analogues or by blockade of DNA-metabolizing function such as DNA polymerase or topoisomerase [29]. Nonetheless, the mechanism and type of DNA lesion of HCT116 cells induced by acetone extract from stem bark of *C. odontophyllum* need further investigation to truly understand the mechanism of DNA damage that leads to apoptosis.

In conclusion, acetone extract from stem bark of *C. odontophyllum* exerts the most potent cytotoxic effect compared to aqueous and methanol extracts towards human colorectal cancer cell line HCT116 with no cytotoxic effect against human colon fibroblast cell line CCD-18co. Acetone extract demonstrated cell death through apoptosis and DNA damage was observed in colorectal cancer cell line HCT116. As a consequence, it has potential in the development of anticancer agent against colorectal cancer.

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REFERENCES

1. Underwood JCE, Cross SS. General and Systemic Pathology. Churchill Livingstone: Elsevier; 2009. p. 395.
2. Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. Nature. 2012; 487(7407), 330-337.
3. Malaysian Gastrointestinal Registry Report 2009. http://www.crc.gov.my/wpcontent/uploads/documents/report/20100301_nerReport. Accessed 5 September 2014.
4. Creţu E, Trifan A, Vasincu A, Miron A. 2012. Plant-derived anticancer agents-curcumin in cancer prevention and treatment. Revista medico-chirurgicala a Societăţii de Medici şi Naturalişti din Iasi. 116(4), 1223.
5. Ahmed M, Khan MI, Khan MR, Muhammad N, Khan AU. Role of medicinal plants in oxidative stress and cancer. Nature. 2013; 2,641.
6. Azlan A, Nasir NNM, Amom Z, Ismail A. 2009. Physical properties of skin, flesh, and kernel of *Canarium odontophyllum* fruit. J. Food. Agric. Environ. 7, 55-57.
7. Lau CY. Development of indigenous fruit crops in Sarawak. A case study on dabai fruit. In Proceedings of the National Conference on New Crops and Bioresources. 2009, 15–20.
8. Chew LY, Khoo HE, Amin I, Azrina A. Lau CY. 2012. Analysis of phenolic compounds of dabai (*Canarium odontophyllum* Miq.) fruits by high-performance liquid chromatography. Food Anal. Method. 5(1), 126-137.
9. Mogana R, Wiart C. 2011. Canarium L.: a phytochemical and pharmacological review. J. Pharm. Res. 4(8), 2482-2489.
10. Basri DF, Mohd MAAR, Meng CK, Latif ES, Huyop FZ. 2014. Cytotoxic activity of stem bark extracts from *Canarium odontophyllum* Miq. (dabai) against human colorectal carcinoma HCT 116 cell line. Am. J. Plant Sci. 5(26), 3925.
11. Wouters MD, Van Gent DC, Hoeijmakers JH, Pothof J. 2011. MicroRNAs, the DNA damage response and cancer. Mutat. Res. 717(1), 54-66.
12. Roos WP, Kaina B. 2013. DNA damage-induced cell death: from specific DNA lesions to the DNA damage response and apoptosis. Cancer Lett. 332(2), 237-248.
13. Liao W, McNutt MA, Zhu WG. 2009. The comet assay: a sensitive method for detecting DNA damage in individual cells. Methods. 48(1), 46-53.
14. Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods. 65(1), 55-63.
15. Vermes I, Haanen C, Steffens-Nakken H, Reutellingsperger C. 1995. A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. J. Immunol. Methods. 184(1), 39-51.
16. Basri DF, Shabry ASM, Meng CK. 2015. Leaves extract from *Canarium odontophyllum* Miq. (dabai) exhibits cytotoxic activity against human colorectal cancer cell HCT116. Nat. Prod. Chem. Res. 3(2), 166.
17. Kashani LMT, Majdzadeh M, Khanavi M, Taghizadeh M, Sadati N, Kahkeshani N, Ostad SN. 2014. Cytotoxic activity of selected Iranian traditional medicinal plants on colon, colorectal and breast cancer cell lines. Archives of Breast Cancer. 1(3), 19-22.
18. Lu JJ, Dang YY, Huang M, Xu WS, Chen XP, Wang, YT. 2012. Anti-cancer properties of terpenoids isolated from Rhizoma Curcumae–A review. J Ethnopharmacol. 143(2), 406-411.
19. Kanadaswami C, Lee LT, Lee PPH, Hwang JJ, Ke FC, Huang YT, Lee MT. 2005. The antitumor activities of flavonoids. In vivo. 19(5), 895-909.
20. Ahmad NH, Rahim RA, Mat I. 2010. *Catharanthus roseus* aqueous extract is cytotoxic to Jurkat leukaemic T-cells but induces the proliferation of normal peripheral blood mononuclear cells. Trop. Life Sci. Res. 21(2), 101.
21. Hadi SM, Asad SF, Singh S, Ahmad A. 2000. Putative mechanism for anticancer and apoptosis-inducing properties of plant-derived polyphenolic compounds. IUBMB Life. 50, 167- 171.
22. Moirangthem DS, Talukdar NC, Kasoju N, Bora U. Antioxidant, antibacterial, cytotoxic, and apoptotic activity of stem bark extracts of *Cephalotaxus griffithii* Hook. f. BMC Complement Altern Med. 2012; 12(1): 30.
23. Seo WG, Pae HO, Oh GS, Chai KY, Yun YG, Chung HT, Kwon TO. 2001. Ethyl acetate extract of the stem bark of *Cudrania tricuspidata* induces apoptosis in human leukemia HL-60 cells. Am J Chin Med. 2001; 29(2): 313-320.

24. Nishibori N, Itoh M, Kashiwagi M, Arimochi H, Morita K. In vitro cytotoxic effect of ethanol extract prepared from sporophyll of *Undaria pinnatifida* on human colorectal cancer cells. Phytother Res. 2012; 26(2): 191-196.
25. Chan KM, Rajab NF, Ishak MH, Ali AM, Yusoff K, Din LB, Inayat-Hussain SH. Goniothalamin induces apoptosis in vascular smooth muscle cells. Chem Biol Interact. 2010; 159:129-140.
26. Hartmann A, Agurell E, Beevers C, Brendler-Schwaab S, Burlinson B, Clay P, Tice RR. 2003. Recommendations for conducting the in vivo alkaline Comet assay. Mutagenesis. 2003; 18(1): 45-51.
27. Chauvel-Lebret DJ, Auroy P, Tricot-Doleux S, Bonnaure-Mallet M. Evaluation of the capacity of the SCGE assay to assess the genotoxicity of biomaterials. Biomaterials. 2001; 22(13): 1795-1801.
28. Olive PL, Banáth JP. The comet assay: a method to measure DNA damage in individual cells. Nat Protoc. 2006; 1(1): 23.
29. Roos WP, Kaina B. DNA damage-induced cell death: from specific DNA lesions to the DNA damage response and apoptosis. Cancer Lett. 2013; 332(2): 237-248.

Antioxidant and melanogenesis stimulating activities of different pigment extracts for grey hair treatment

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ABSTRACT

The purpose of this study was to investigate the antioxidant and melanogenesis stimulating activities of different pigments from plant extracts e.g. *Clitoria ternatea* L., *Zea mays var. ceratina*, *Syzygium cumini* (L.), *Hibiscus sabdariffa* Linn., *Caesalpinia sappan* L. and *Antidesma puncticulatum* Miq. All ethanolic extracts were also evaluated for the antioxidant activity by using DPPH radical scavenging assay. The melanogenesis stimulating activity of all pigment extracts were determined by using the stimulation of tyrosinase enzyme activity by tyrosinase activity assay, and the melanocyte proliferation of these extracts at different concentrations of 10 -500 µg/ml was tested using mouse melanoma cells ($B_{16}F_{10}$) by MTT assay. For the antioxidant activity, the ethanolic extract of *S. cumini* exhibited the highest antioxidant activity with the EC_{50} about 151.67 µg/ml. From the results of stimulating activity of tyrosinase enzyme, the ethanolic extract of *C. sappan* had the highest stimulating activity and the value was 96 % while *S. cumini* showed the lowest stimulating activity and the value was 26%. Among all tested extracts, the extracts of *C. ternatea* and *A. puncticulatum* showed strong stimulating on melanocyte proliferation with the proliferation index (P.I.) at 1.5 and 1.4, respectively. To summarize, the results of this study suggest the potential activity of these plant extracts for further study on grey hair treatment application.

Keywords: Antioxidant activity, Melanogenesis stimulating activities, Melanocyte proliferation, Grey hair treatment

INTRODUCTION

Natural hair color, which is based on the complex genetic control, is dependent on amount and classification of melanin. It is produced by the follicular melanocytes which derived from the neural crest cells located in the hair follicles and produces the content of melanins by the melanogenesis pathway. Melanogenesis, a complicated pigment biosynthesis, involves the oxidative reaction of tyrosine to be either brown/black eumelanin or yellow/red pheomelanin, depending on the cysteine or glutathione existing. These produced melanins are packed into granules known as melanosomes and then transferred to the cortical keratinocytes. The onset of white hair in Thailand are at the late 30 years old and in the mid of 40 years old **[1-3]**. Currently, there is no treatment procedure for this condition and the masking of white hairs with hair dye is popularly used. Nevertheless, the hair masking with hair dye has been reported to have the numerous harmful effects, such as dermatitis, hair loss and cancer **[4]**. Many researchers have investigated the low toxic compounds which can induce the melanogenesis pathway. For the development of gray hair prevention agent, a screening program was carried out to find a potential stimulant of melanogenesis from the natural resources by using cultured murine B_{16} melanoma cells with theophylline as a reference drug **[2, 5-7]**. It was shown that, theophylline could enhance the pigmentation in cultured murine B_{16} melanoma cells without any effects on cell proliferation **[2, 5-7]**.

Moreover, Somvong *et al.* investigated antioxidant and melanogenesis stimulating activity of Thai traditional medicinal plant extracts included aqueous, ethyl acetate, methanol and hexane extracts of *Tiliacora triandra*, *Centella asiatica*, *Clitoria ternatea*, *Morus alba* and *Pueraria mirifica*. The ethyl acetate extract of *T. triandra* had the strongest stimulating activity with % stimulation of 94.34. The study on melanocyte proliferation of these extracts showed that methanol extract of *C. ternatea* and aqueous extract of *T. triandra* had strong stimulating activities with proliferation index (P.I.) of 1.7 and 1.6, respectively **[1]**. In this study, the antioxidant and melanogenesis stimulating activities of different pigments were investigated in medicinal plant extracts e.g. *Clitoria ternatea* L., *Zea mays var. ceratina*, *Syzygium cumini* (L.), *Hibiscus sabdariffa* Linn., *Caesalpinia sappan* L. and *Antidesma puncticulatum* Miq.

MATERIALS AND METHODS

Materials

2,2-diphenyl-1-picrylthydrazyl (DPPH) (Sigma Aldrich, USA), Ascorbic acid, α-tocopherol (Nam Siang Co., Ltd., Bangkok, Thailand) were used. All herbs were harvested from Agricultural and Food Technology Department, Thailand Institute of Scientific and Technological Research. All other chemicals were of standard chemical grade.

Preparation of crude ethanol extracts

All medical plants e.g. *Clitoria ternatea* L., *Zea mays var. ceratina*, *Syzygium cumini* (L.), *Hibiscus sabdariffa* Linn., *Caesalpinia sappan* L. and *Antidesma puncticulatum* Miq were prepared by maceration. Initially, 500 g of herb powders was accurately weighed, mixed with 70% ethanol for 4 nights, filtered through Whatman paper No. 41 and rinsed in the same solvent. The solvent was removed under reduced pressure using a rotary evaporator (Heidolph, Hei-VAP Precision) at 45 ^{0}C.

Determination of DPPH radical scavenging activity

The effect of pigment extracts on the DPPH radical scavenging activity was adapted from Somvong & Prasitpuriprecha [1] and Kriengsak *et al* [9]. Frist, 50 µg of extract was accurately weighed and then dissolved in 1 ml of 20% DMSO. After that, the solution of extract (50 µg/ml) was mixed with 100 µM DPPH in absolute methanol and the solution was adjusted to the final volume about 2000 µl. The sample was incubated at 37 ^{0}C for 20 min and then the absorbance of solution was evaluated by using a microplate reader (Sunrise, Tecan Co., Austria) at 517 nm. The absorbance of sample and control was calculated on the effective concentration at 50% (EC_{50}) according to the following equation: EC_{50}= [(Abs. control – Abs. sample)/ Abs. control] X100. The sample was performed in triplicate.

Determination of stimulation of tyrosinase enzyme activity

The stimulation of tyrosinase enzyme activity was tested by tyrosinase activity assay adapted from Somvong & Prasitpuriprecha [1], Choi *et al* [8] and Jeon *et al* [10]. First, the sample was diluted with distilled water at 50 mg/ml. Second, the diluted extract was mixed with tyrosinase enzyme (100 µg/ml) in phosphate buffer pH 6.8 and then incubated at 37 ^{0}C for 10 min. After that, the samples was added with 2 mM tyrosine and then incubated at 37 ^{0}C for 40 min. After incubation, the absorbance of solution was determined by using a microplate reader (Sunrise, Tecan Co., Austria) at 450 nm. The tyrosinase stimulation was calculated according to the following equation : % stimulation = [(A-B)/A] x 100 where A was the absorbance of extract solution, and B was the absorbance of control. The sample was performed in triplicate.

Determination of stimulation of melanocyte proliferation using mouse melanoma cells ($B_{16}F_{10}$)

The stimulation of melanocyte proliferation was tested by MTT assay with mouse melanoma cells ($B_{16}F_{10}$) using the method of Somvong & Prasitpuriprecha [1], Matsuda *et al* [2], Itoh et al. [6] and Jung *et al* [7]. Frist, mouse melanoma cells ($B_{16}F_{10}$) were seeded at the density of 1.5 x 10^4 cells/well in 96-well plates and incubated at 37 ^{0}C for 24 h under 5% CO_2 atmosphere for cell adhesion. Second, the cell was diluted with completed medium DMEM at concentration in the range of 500- 10 µg/ml. After that, 100 µl of different concentrations of all extracts were added in the 96 well plate system and then incubated at 37 ^{0}C for 72 h. After incubation, 50 µl of MTT in PBS at 1 mg/ml was added to the medium in each well and incubated for 4 -6 h. Medium and MTT were then removed from the well and solubilized with 100 µl of DMSO. The absorbances of all samples were assessed using a microplate reader (Sunrise, Tecan Co., Austria) at 570 nm. The proliferation index (P.I.) was calculated according to the following equation: proliferation index (P.I.) = [Mean absorbance of sample well x 100] /Mean absorbance of control. The sample was performed in triplicate.

RESULTS

The DPPH radical has been widely used in the model system to investigate the scavenging activities of several natural compounds such as phenolic compounds, anthocyanins, or crude extracts of plants. DPPH radical was scavenged by antioxidants through the donation of hydrogen, forming the reduced DPPH-H•. The color changed from purple to yellow after reduction, which could be quantified by its decrease of absorbance at wavelength 517 nm. **Table 1** shows the radical-scavenging activity of the different pigment extracts e.g. *Clitoria ternatea* L., *Zea mays var. ceratina*, *Syzygium cumini* (L.), *Hibiscus sabdariffa* Linn., *Caesalpinia sappan* L. and *Antidesma puncticulatum* Miq.using the DPPH coloring method. The result was found that the ethanolic extract of *S. cumini* exhibited the highest antioxidant activity with the EC_{50} about 151.67 µg/ml, but *Z. ceratina* showed the lowest antioxidant activity and the EC_{50} value was 973.33 µg/ml. From the stimulating activity of tyrosinase enzyme of all extracts at 50 mg/ml, the ethanolic extract of *C. sappan* had the strongest stimulating activity with % stimulation of 96 %. Nevertheless, *S. cumini* showed the lowest stimulating activity (26%) as shown in **Table1**.

Table 1 Antioxidant and stimulation of tyrosinase activities of various pigment extracts

Pigment extracts	Part used of the studied plants	Antioxidant activity (EC_{50}, µg/ml))	Tyrosinase stimulation (%)
1.*Clitoria ternatea* L.	Flower	754.00 ± 30.05	84.97 ± 0.64
2.*Zea mays ceratina*	Fruit	973.33 ± 46.19	91.11 ± 0.43
3.*Syzygium cumini* L.	Fruit	151.67 ± 7.57	27.11 ± 3.46
4.*Hibiscus sabdariffa* Linn	Flower	749.67 ± 29.26	87.93 ± 0.49
5.*Caesalpinia sappan* L.	Wood	712.00 ± 14.03	95.75 ± 0.06
6.*Antidesma puncticulatum* Miq	Fruit	226.67 ± 7.64	81.08 ± 0.31
7. Trolox (Antioxidant standard)	-	129.67 ± 2.08	-388.14 ± 17.34

Fig. 1 showed the melanocyte proliferation of various pigment extracts at different concentrations of 10 -500 µg/ml**.** It was indicated that the increased concentrations of all pigment extracts resulted in the increased melanocyte proliferation with the proliferation index (P.I.) in the range of 1.0 – 1.5. Among all tested extracts, the extracts of *C. ternatea* and *A. puncticulatum* showed strong stimulating activity on melanocyte proliferation of all concentrations and the proliferation index (P.I.) was in the range of 1.3 – 1.5 at concentrations about 100 – 500 µg/ml. Nonetheless, the lowest stimulating activity of melanocyte proliferation was obtained from the extract of *Zea mays var. ceratina***,** and the proliferation index (P.I.) was less than 1.1 of all concentrations.

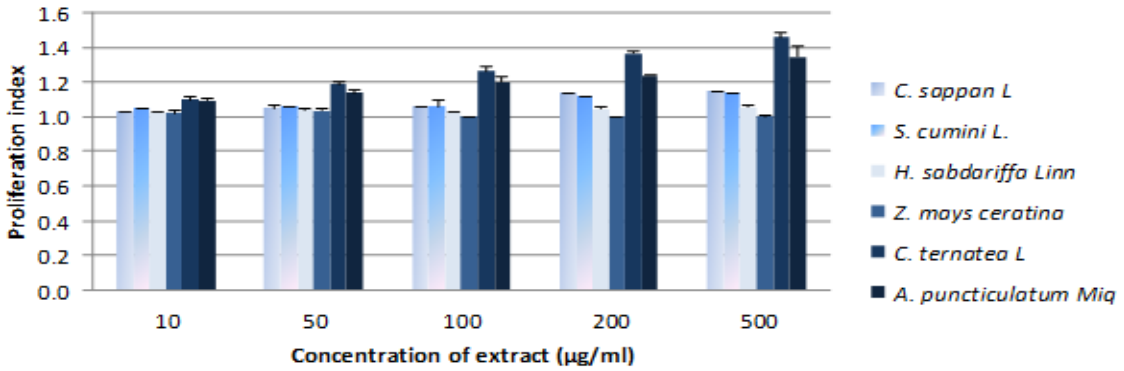

Fig.1 Melanocyte proliferation of various pigment extracts at different concentrations of 10 -500 µg/ml

4. CONCLUSIONS

In conclusion, the ethanolic extract of *S. cumini* exhibited the highest antioxidant activity with the EC_{50} about 151.67 µg/ml and the extracts of *C. ternatea* and *A. puncticulatum* showed strong stimulating activity on melanocyte proliferation of all concentrations. The increased concentrations of all pigment extracts resulted in the increased melanocyte proliferation with the proliferation index (P.I.) in the range of 1.0 – 1.5. Therefore, the effect of pigment extracts from Thai medicinal plant had melanogenesis stimulation and was able to be improved for anti-gray hair application in the future.

ACKNOWLEDGEMENTS

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REFERENCES

1. Somvong K, Prasitpuriprecha C. 2012. Antioxidant and Melanogenesis Stimulating activities of Some Thai Traditional Medicinal Plant Extracts for Grey Hair Treatment. The 4th Annual Northeast Pharmacy Research Conference "Pharmacy Profession in Harmony. Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand. 125 -134.
2. Matsuda H, Hirata N, Kawaguchi Y. 2006. Melanogenesis Stimulation in Murine B_{16} Melanoma Cells by Kava (*Piper methysticum*) Rhizome Extract and Kavalactones. Biol. Pharm. Bull. 29(4), 834—837.
3. Petra CA, Rupert O, Katharina S. 2006. Towards a free radical theory of graying melanocyte apoptosis in the aging human hair follicle is an indicator of oxidative stress induced tissue damage. The FASEB. 20, 908-920.
4. Kinlen LJ, Harris R, Garrod A. 1977. Use for hair dyes by patients with breast cancer: a case-control study. British medicinal Journal. 2, 366-368.
5. Hideaki M, Noriko H, Yoshiko K. 2004. Melanogenesis stimulation in Murine B16 melanoma cells by Piper nigrum leaf extract and its lignin constituents. Biol. Pharm. Bull. 27, 1611-1616.
6. Itoh T and Furuichi Y. 2005. Hot-water extracts from Adzuki Beans (Vigna angularis) stimulate not only melanogenesis in cultured Mouse B16 Melanoma cells but also pigmentation of hair color in C_3H mice. Biosci. Biotechnol. Biochem. 69 (5), 873–882.
7. Jung GD, Yang JY, Song SS. 2001.Stimulation of melanogenesis by glycyrrhizin in B_{16} melanoma cells. Exp. Mol. Med. 33, 131-135.
8. Choi HK, Lim YS, Kim YS. 2008. Free-radical-scavenging and tyrosinase-inhibition activities of Cheonggukjang samples fermented for various times. Food chemistry. 106, 564-568.
9. Kriengsak T, Unaro jB, Kevin C. 2006. Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. Journal of Food Composition and Analysis. 19, 669–675.
10. Jeon SH, Kim KH, Koh JU. 2005. Inhibitory Effects on L-dopa Oxidation of Tyrosinase by Skin-whitening Agents. Bull. Korean Chem.Soc. 26 (7), 1135-1137.

In Vitro stimulant activity study on dermal fibroblast collagen synthesis of peptides isolated from pigeon peas, chick peas and soya beans

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ABSTRACT

Peptides extracted from pigeon pea, chick peas, and soya bean by using alkaline, pepsin and alcalase-flavourzyme. This work aims to study the bioactivity of peptides extracted from native pulses and soya bean to prepare for cosmetic application. Antioxidant activities were measured by DPPH assay, compared to ascorbic acid. The EC_{50} value of peptide extracts presented their results in the range of 32 to 806 µg/ml which were higher than ascorbic acid (EC_{50}= 1.37 µg/ml). The inhibition on dopa oxidase activities of mushroom tyrosinase presented in the range between (IC_{50}) 41 to 810 µg/ml, considered weaker than arbutin (IC_{50} = 1.91 µg/ml). The human dermal fibroblasts (ATCC CRL-1474) were treated with peptide extracts at various concentration and determined cytotoxicity by MTT assay. The IC_{50} values were found more than 1000 µg/ml. The stimulating effect on collagen type I synthesis of dermal fibroblasts was evaluated by ELISA test kit. Alkaline peptide extracts were found higher in stimulating collagen type I synthesis than pepsin and alcalase-flavourzyme. The activities at concentration of 200 µg/ml were exhibited in the range of 53.37±1.03% to 79.68±1.57%. This effect was nearly the same as the activity of ascorbic acid (59.51±3.17%) at concentration of 50 µg/ml. The results showed that peptides were extracted with alkaline, gave highest activity and were suitable as active ingredient for cosmetic development. Further studies will focus on cosmetic products development using peptide extracted from the native pulse, investigate for anti-wrinkle properties and apply to volunteer.

Keywords: collagen synthesis, peptide, pigeon pea, chick pea and soya bean

INTRODUCTION

Skin dryness, an accelerated decreasing of collagen network and collagen synthesis in the dermis, considers hallmarks of skin aging due to ultraviolet light from the sun (photoaging) and occurring as consequence of the passage of time. Collagen type I and III present in the highest level in human skin. Currently, synthesis of short peptides has been used as active ingredient in cosmetic application to stimulate the collagen formation synthesis in people skin. Natural biopeptide from soya bean used in cosmetic application as an anti-aging and stimulating collagen synthesis agent.[1]

Soya bean *(Glycine max* L.), a well known edible bean crop, has been used as a source of human food with its high quality protein and other nutrients for hundreds of years. While chick pea *(Cicer arietinum* L) and pigeon pea (*Cajanus cajan* (L) Millsp), native pulse, found in Asia, used as sources of human food according to their high content of protein, however their utilization still less extensively used than soya bean.

The present study takes up to evaluate the antioxidant, anti-tyrosinase, and collagen stimulating effect of pigeon pea, chick pea compare to peptides from soya bean. The peptides were extracted by using alkaline, pepsin, Alcalase and Flavourzyme in order to gain scientific evidence, supporting for peptides from native pulse. It will use as skin treatment agent, targeting on collagen alteration and whitening effects on humans skin which similarly to soya bean.

MATERIALS AND METHODS

Plant materials

Soya beans were purchased from supermarket in Bangkok, Thailand, chick and pigeon peas were taken from a paddy farm in Nakonratchasima province. These beans and peas were pulverized into coarse powder, and then defatted and removed toxic peptide by exhaustedly soxhlet with hexane and acetone, respectively.

Extraction

Alkaline process

The defatted beans and peas as powder (1.00 kg) were extracted with 10 L of 0.2 % NaOH for 1 hr. with occasional stirring. The supernatant were filtered and centrifuged at 7,000 rpm, temp. 10 ^{o}C for 10 mins. The supernatant solution was adjusted to pH 4.5 with 0.5 N HCl, and then centrifuged at 10,000 rpm, temp. 10 ^{o}C for 20 mins. The precipitate was kept in a freezer temperature at -20 ^{o}C.

Pepsin process

The defatted beans and peas powder 1.00 kg was extracted with 10 L of DI water and adjusted to pH 2.5 with 0.5 N HCl, then added pepsin in amount of 3.35 grams with occasional stirring for 6-7 h at temp. 37 ^{o}C. After that, adjusted pH 9.5 with 1 N NaOH, made volume to 1,500 ml with DI water and heat at 85 ^{o}C for 30 min. The supernatant were filtered through Wathmann No 1 and centrifuged at 7,000 rpm, 4 ^{o}C for 30 min. The solution was centrifuged again at 10,000 rpm, temp.4 ^{o}C for 30 mins. The precipitates were kept in a freezer for their activity testing.

Alcalase and Flavourzyme

The defatted beans and peas powder 1.00 kg was extracted with 10 L 0.1 M tris-base buffer pH 8 at temp.50 ^{o}C for 1 hr with occasional stirring. The reaction was added 0.4 grams of slcalase 2.4 L FG and heat at temp. 50 ^{o}C for 1 hr. Then the reactions were added with 300 mg of flavourzyme and heat at temp.50 ^{o}C for 2 hrs. with occasional stirring. The reaction was heated up to 100 ^{o}C for 15 mins. to stop the activity of alcalase and flavourzyme. The supernatants were filtered through filter paper (Whatman No.1) and centrifuged at 7,000 rpm, temp. 4 ^{o}C for 30 mins. At the final step, the solution was centrifuged again at 10,000 rpm, temp.4 ^{o}C for 30 mins, and the precipitates were kept in a freezer until use.

Scavenging of Diphenyl-picrylhydrazyl (DPPH) Radicals Assay

The free radical scavenging activity of crude peptide extracts were analyzed by the DPPH assay [2]. Peptides were dissolved in phosphate buffer pH 9.5 and filtered through 0.45 µm of PTFE syringe filter. The amount of 100 µl of various concentrations of samples was reacted with 100 µl of $6x10^{-3}$ M DPPH ethanol solution in a 96-well plate, incubated at temp. 37 ^{o}C for 30 mins. The absorbance of the mixture was measured at 517 nm, using a UV–VIS microplate reader. All experiments were carried out in triplicates.

Inhibition of tyrosinase activity

Determination of tyrosinase inhibition activity was performed by the dopachrome method using L-DOPA as the substrate and described by Iida *et al* [3] with slight modifications. Briefly, crude peptide extract were dissolved in phosphate buffer pH 9.5. The reaction was carried out by using 96-well plate. Mixing of 50 µL of various sample concentrations, 50 µl of phosphate buffer (pH 6.8) and 50 µl of 787 units/ml of mushroom tyrosinase solution in a 96-well plate. The pre-incubation at temp. 37 ^{o}C for 10 min, 50 µl of 160 µg/ml L-Dopa was then added and incubated at temp. 37 ^{o}C for 2 mins. The amount of dopachrome was measured at 492 nm. using a UV–VIS microplate reader.

Determination of cytotoxicity and collagen stimulation

Cell culture

Human dermal fibroblast (ATCC CRL-1474) were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum, 2mM L-glutamine and 100 unit/ml penicillin and streptomycin. The cells were incubated in a humidified 5% CO_2 atmosphere at temp.37^{0} C for 72 hrs.

MTT cytotoxicity test [4]

The cells were seeded in a 96-well plate at a density of 10^{5}cells/ml, and incubated for 24 hrs. The range of peptide samples dissolved in DMSO (200 µl, 500- 4000 µg/ml) were added to the cells and incubated for 24 hrs, and were then washed out. MTT (50 µl, 5 mg/ml) and the medium (150 µl) were added in each well. The cell were then incubated at temp.37^{0} C 5% CO_2 for additional 4 hrs. The medium containing MTT was discarded, and MTT formazan that had been

produced, was extracted with 150 µl of DMSO under 15 mins agitation. The absorbance was read at wavelength 540 nm. The toxicity of extract was indicated by 50% inhibitory concentration (IC_{50}).

Determination of collagen synthesis using ELISA [5,6]

The cells were seeded in a 6-well plate at a density of $2x10^5$ cells/ml, and incubated for 24 hrs. After that the media were removed and added with different concentrations of peptide extracts (50, 100 and 200 µg/ml), incubated continually for 24 hrs. and were then removed. Attached cells were transferred to a micro centrifugal tube using a cell scraper and digested of collagen using pepsin solution in acetic acid (0.1mg/ml 50 mM of acetic acid). Finally, the supernatant was detected for human collagen type I using ELISA test kit (Cosmo Bio Co.LTD). Collagen determination was performed by measuring absorbance at wavelength 412 nm. using microplate reader. Then collagen concentration was calculated by interpolating sample OD value to the standard curve. The percentage of collagen stimulation was generated with the following equation given below:

$$\% \text{ Collagen stimulation} = [(A_C - A_T)/ A_C] \times 100$$

A_C = the collagen concentration of control

A_T = the collagen concentration of test samples

RESULTS

The biological activity of peptides from pigeon pea, chick peas, and soya bean was presented in Table 1. The DPPH radical was used to evaluate the free radical scavenging capacity of antioxidants of peptides. The activities were compared to ascorbic acid. The peptides were extracted using alkaline, pepsin and alcalase-flavourzyme resulted EC_{50} value in the range between 32 to 806 µg/ml, while ascorbic acid gave EC_{50}= 1.37 µg/ml. The activity of peptides, extracted from soya bean and native pulse inhibited on dopa oxidase of mushroom tyrosinase. All peptides were displayed their activity lower than arbutin which high anti-tyrosinase agents, applied as a whitening agent. The inhibition on dopa oxidase activities of mushroom tyrosinase was found IC_{50} in the range between 41 to 810 µg/ml. The activities considered weaker than arbutin (IC_{50} = 1.91 µg/ml).

Table 1. Antioxidant and anti-tyrosinase activities (EC_{50}) of peptide extraction from pigeon pea, chick peas, and soya bean, compared to standard.

Samples	DPPH assay (µg/ml)	Anti-tyrosinase (µg/ml)
Pigeon peas		
Alkaline	61	41
Pepsin	335	560
Alcalase and Flavourzyme	342	810
Chick peas		
Alkaline	221	345
Pepsin	705	795
Alcalase and Flavourzyme	806	805
Soya beans		
Alkaline	32	96
Pepsin	290	210
Alcalase and Flavourzyme	210	229
Ascorbic acid	1.37	-
Arbutin	-	1.91

The cytotoxicity resulted in % survival of dermal fibroblast cell line, IC_{50} value over the test concentrations of 1.0-10 mg/ml. The IC_{50} value of peptides from Soya bean and native pulses were more than 10 mg/ml. According to classification of the cytotoxicity for natural ingredients [7], all of peptides isolated can be classified as potentially non-toxic substances.

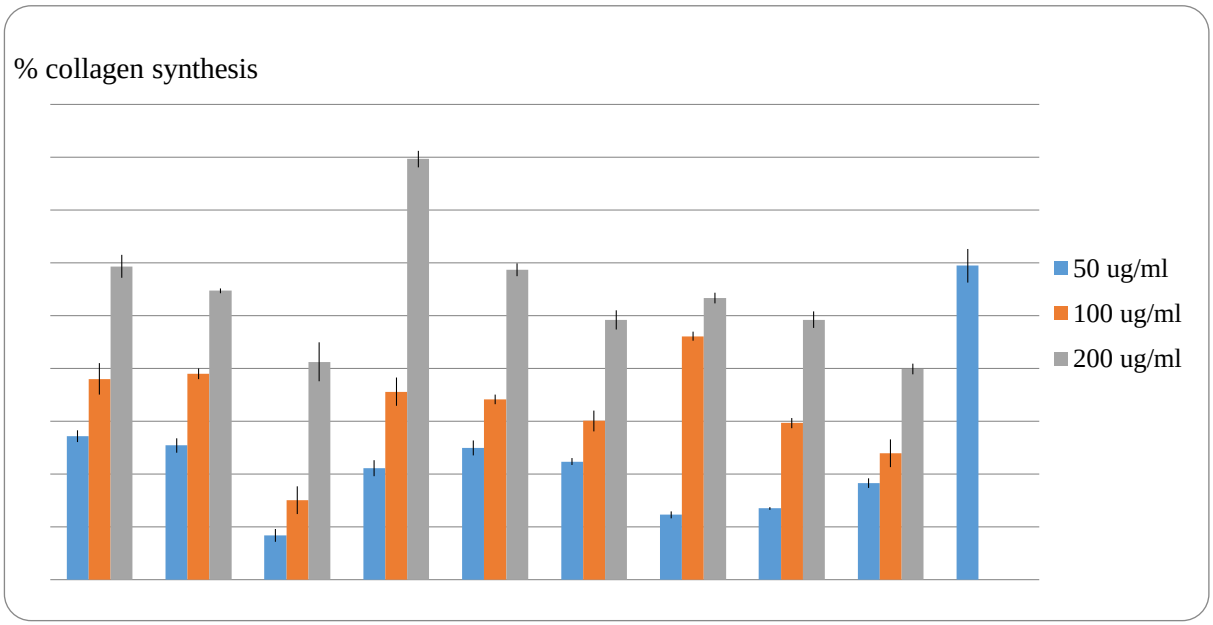

Figure 1. Percentage of stimulant activity on dermal fibroblast collagen synthesis, exposing to peptides : Pigeon peas (C) Chick peas (CI) Soya beans (G) extracted with alkaline , Alcalase and Flavourzyme (A&F) and Pepsin (P) at various concentrations with Normal Skin Dermal Fibroblast

The collagen synthesis stimulating potential of peptides was investigated by using ELISA assay compared to the positive control of ascorbic acid as the results showed in Figure 1. The results revealed that alkaline peptide extracts of soya bean and native pulse exhibited highest activity on stimulating collagen type I synthesis, following by alcalase-flavourzyme and pepsin extracts, respectively. The activities at concentration of 200 µg/ml were found in the range of 53.37±1.03% to 79.68±1.57%, was nearly the same activity of ascorbic acid (59.51±3.17%) at concentration of 50 µg/ml.

CONCLUSION

The results revealed that peptide of pigeon peas extracted by using alkaline exhibited moderate activity of antioxidant and were slightly anti-tyrosinase activity which similar to soya beans. It presented highest activity for stimulating collagen type I synthesis. Although the activity is less than alkaline extracts of soya beans. This activity was nearly the same as ascorbic acid which the active known compound using for stimulation of collagen production in human fibroblasts [8]. The results showed that the peptides of pigeon peas extracted with alkaline gave the highest activity on stimulating collagen synthesis and it is suitable for developing as active agent of cosmetic ingredients. Further studies will focus on cosmetic product development using peptide from this native pulse and investigate for anti-wrinkle properties in volunteer.

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REFERENCES

1. Frei-Andre V, Perrier E. 1999. A comparison of biological activities of new soya biopeptide studied in an *in vitro* skin equivalent model and human volunteers. Int J Cosmet Sci. 21, 299-311.
2. Duan XJ, Zhang WW, Li XM, Wang BG. 2006. Evaluation of antioxidant property of extract and fractions obtained from a red alga, *Polysiphonia urceolata*. Food Chem. 95. 37-43.
3. Iida K, Hase K, Shimomura K, Sudo S, Kadota S, Namba T. 1995. Potent inhibition of tyrosinase activity and melanin biosynthesis from *Rheum officinale*, Planta. Med. 61, 425-428.

4. Mossmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods. 65, 55–63.
5.Wha Kim S, Lee IW, Cho HJ, Cho KH, Han Kim K, Chung JH, et al. 2002. Fibroblasts and ascorbate regulate epidermalization in reconstructed human epidermis. J. Dermatol. Sci. 30, 215-223.
6. Douglas AW, Wanda RO, Dennis AJ, Madeleine D. 1989. Synergistic enhancement of type I and III collagen production in cultured fibroblasts by transforming growth factor-β and ascorbate. FEBS. Lett. 250(2), 541-544
7. Ballantyne B, Cawley TJ. 1999. Toxicology update. J. Appl. Toxicol. 19(4), 291-294.
8. Tiedtke J, Marks O, Morel J. 2007. Stimulation of collagen production in human fibroblasts. Cosmetic Science Technolgy, 15-18

Chemical components of indigenous pigmented-fruits and its potential anti-tyrosinase activity

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ABSTRACT

This research aimed to study the chemical constituents and anti-tyrosinase activity of 5 indigenous pigmented-fruits including *Muntingia calabura* L., *Schleichera oleosa* (Lour.) Oken, *Melodorum fruticosum* Lour., *Lepisanthes rubiginosa* (Roxb.) Leenh. and *Antidesma ghaesembilla* Gaertn. These plants generally found in the northeastern part of Thailand. These indigenous pigmented-fruits were extracted with 50% ethanol and *S. oleosa* showed the highest yield at 13.36%. Tannins and alkaloids were found in all 5 plant extracts whereas flavonoids were not found in *S. oleosa. L. rubiginosa* showed the highest total anthocyanins contents (657.50 ± 0.01 µg/mg). In addition, HPLC analysis showed the peaks of gallic acid in *M. calabura*, the peaks of chlorogenic acid in *S. oleosa* and *L. rubiginosa*, the peaks of vanillin in *A. ghaesembilla*, the peaks of caffeic acid in *S. oleosa* and the peaks of coumaric acid in *L. rubiginosa* and *A. ghaesembilla*. Besides, these plant extracts exhibited high anti-tyrosinase activity, especially *M. calabura* (IC_{50} was 21.30±1.36 µg/ml). The findings from this study provide basic data used in quality control and support these plants to be developed as a health product.

Keywords: chemical components, indigenous pigmented-fruits, anti-tyrosinase activity

INTRODUCTION

Tyrosinase is a kind of oxidoreductase with dinuclear copper ion, belongs to the type-3 copper protein family and is a key enzyme in the biosynthesis of melanin pigments [1]. Melanin plays an essential role in protection against UV injury under normal physiological conditions. However, the excessive level accumulation of melanin can lead to age spots and freckle [2]. As early as last century, skin health and appearance is a major concerned issue for people globally. Pharmaceuticals and traditional remedies have become popular for this purpose. Thus, the development of safe and effective tyrosinase inhibitors is of great concern in the medical and cosmetic industries [3]. Nowadays, only a few compounds such as kojic acid, arbutin, tropolone are used as cosmetic products but these chemical still have the limitation such as rapid decomposition, irritation and carcinogenic effect. Therefore, it is still necessary to search and discover novel tyrosinase inhibitors with higher activities and lower side effects. Herbal plant with great characteristics of high chemical diversity and biochemical specificity is a major resource of natural whitening products [4]. In Thailand, there are a number of plant resources found in the northeast. In this study, 5 indigenous pigmented-fruits which are normally found in northeast area were studied on the chemical components and the inhibitory effect on tyrosinese activity.

MATERIALS AND METHODS

Chemicals Chemicals standard compounds including gallic acid, chlorogenic acid, vannillin, epicatechin gallate, quercetin, caffeic acid, *p*-coumaric acid, ferulic acid were HPLC grade from sigma, USA. Mushroom tyrosinase, kojic acid and L-tyrosine were also obtained from sigma, USA. The other chemicals were analytical grade.

Preparation of the plant extract The fruits of *M. calabura*, *S. oleosa*, *M. fruticosum*, *L. rubiginosa* and *A. ghaesembilla* were collected in May, 2015 in Ubon Ratchathani Province, Thailand. The plant samples were macerated in 50% ethanol for 3 days, filtered through thin cloth and centrifuged at 3000 g, 25 °C for 10 min using a laboratory centrifuge (Kubota, Japan). The clear supernatant was concentrated using a rotary evaporator (ETERA, Japan) at 45-50°C, and then freeze-dried (Christ, Germany).

Chemical component analysis

Phytochemical screening Major phytochemical including alkaloids, tannins, and flavonoids were detected by chemical reaction test. Alkaloids were precipitated from neutral or slightly acidic solutions by reagents containing of heavy metal such as Dragendorff's reagent [5], tannins were tested with ferric chloride solution and flavonoids compound was detected by metallic magnesium and hydrochloric acid in alcohol solutions [6].

HPLC analysis To identify the chemical components of the extract, HPLC analytical system was performed on Agilent Technologies 1200 (AG1100 Autosampler, USA) equipped with isocratic pumping system, four channel in-line vacuum degasser, an autosampler injector and using a C18 column (5 µm, 4.6 x 250 mm). The gradient mobile phase consisted of solution A (acetonitrile: H_2O: phosphoric acid = 79.7:20:0.3) and solution B (0.3% phosphoric acid) at a flow rate of 0.8 ml/min. A concave gradient was applied to the ratio of solution A: solution B from 5:95 to 50:50 in 45 min. A UV-vis detector at wavelength of 210 nm was used. The temperature of analytical room and analysis column was maintained at 25°C.

Determination of anthocyanidins content Total monomeric anthocyanin content was measured following AOAC official method [7]. Briefly, pH 1.0 buffer (potassium chloride, 0.025M) and pH 4.5 buffer (sodium acetate, 0.4M) were prepared. The absorbance of test portion diluted with pH 1.0 buffer and pH 4.5 buffers was determined at both 520 and 700 nm for 20-50 min. The diluted test portions are read versus a blank cell filled with distilled water. Anthocyanin pigment concentration was calculated and expressed as cyanidin-3-glucoside equivalents (mg/g).

Measurement of mushroom tyrosinase inhibition Tyrosinase activity was measured as previously described with slightly modifications [8]. All reactant were dissolved in DMSO, and the determinations were accomplished at 490 nm in a 96-well plate with kojic acid as a positive control. The reaction mixture contained 2 mM L-tyrosine (dissolved in phosphate buffer, pH 6.8), tyrosinase (167 unit/ml) and tested samples, and incubated at 37ºC for 30 min.

RESULTS

The fruit of selected plants with unique color were shown in the Figure 1. These 5 indigenous pigmented-fruits were extracted with 50%ethanol and *S. oleosa* showed the highest yield at 13.36%. *L. rubiginosa* exhibited high anthocyanins contents (657.50 ± 0.01 Cyanidin-3-glucoside equivalents, mg/g). For anti-tyrosinase enzyme activity, *M. calabura showed high inhibition on tyrosinase enzyme with* IC_{50} *at* 21.30±1.36 µg/ml which was better than standard kojic acid (IC_{50} *at* 30.18±2.64 µg/ml) (Table 1). For the phytochemical screening, tannins and alkaloids were found in these 5 plant extracts whereas flavonoids were not found in *S. oleosa* (Table 2). The analysis of chemical constituents showed unique HPLC profiles of these extracts. By comparison of the retention time, many compounds were detected in the extract, i.e., gallic acid in *M. calabura*, chlorogenic acid in *S. oleosa* and *L. rubiginosa*, vanillin in *A. ghaesembilla*, caffeic acid in *S. oleosa* and coumaric acid in *L. rubiginosa* and *A. ghaesembilla* (Table 3 and Figure 2).

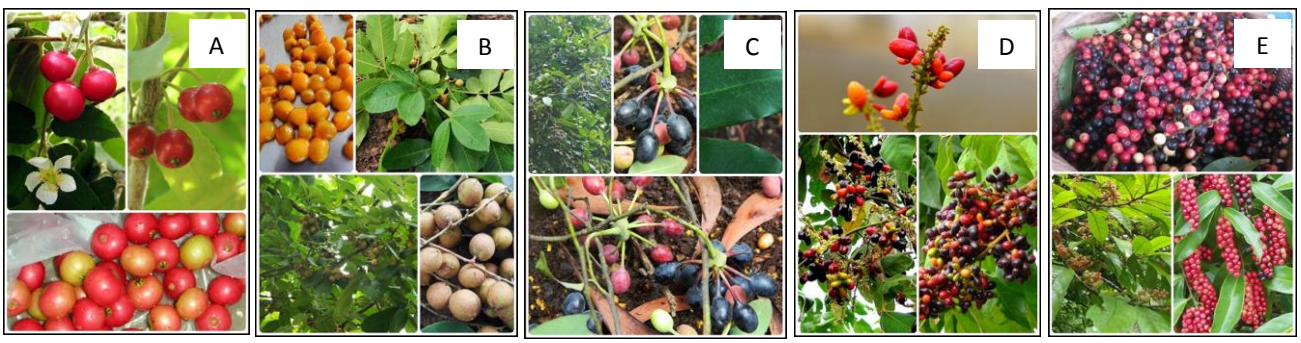

Figure 1. Morphology of *M. calabura* (A), *S. oleosa* (B), *M. fruticosum* (C), *L. rubiginosa* (D), and *A. ghaesembilla* (E).

Table 1. Anthocyanin content, anti-tyrosinase activity and the yield of extraction of 5 indigenous pigmented-fruits

Sample	Anthocyanin content * (Cyanidin-3-glucoside equivalents, mg/g)	Anti-tyrosinase activity * (IC_{50}, ug/ml)	Yield (%)
M. calabura	N.D.	21.30±1.36	4.4
S. oleosa	N.D.	21.47±1.10	13.36
M. fruticosum	164.14 ± 0.01	23.19±2.10	2.55
L. rubiginosa	657.50 ± 0.01	69.14±3.30	4.56
A. ghaesembilla	163.22 ± 0.00	33.29±3.51	6.96
Kojic acid	-	30.18±2.64	-

* Expressed as mean ± SD (n=3)

Table 2. Phytochemical screening of 5 indigenous pigmented-fruits

Chemical	*M. calabura*	*S. oleosa*	*M. fruticosum*	*L. rubiginosa*	*A. ghaesembilla*
Tannins*					
- 0.5% gelatin	-	-	-	-	+
- 1% lead acetate	+	+	+	+	+
- 1% quinine sulfate	+	+	+	+	+
Flavonoids**					
- Shinoda test	-	-	-	+	-
- Pew test	-	-	-	+	-
- acidic condition	-	-	-	+	-
- alkali condition	+	-	+	+	+
Alkaloids***					
- Dragendroff	+	+	+	+	+
- Wagner	+	+	+	+	+
- Hager	+	+	+	+	+
- Kraut	+	+	+	+	+
- Tannic acid	+	-	+	+	+

Positive control: *, ** and *** were tannic acid, quercetin and nicotine, respectively

Table 3. HPLC analysis of 5 indigenous pigmented-fruits

Standard	**Standard Retention time (min)**	**Crude extract (Retention time, min)**
Standard Mix 1	Gallic acid (9.92)	*M. calabura* (9.92), *M. fruticosum* (9.58), *L. rubiginosa* (9.9)
	Chlorogenic acid (19.23)	*S. oleosa* (19.44), *M. fruticosum* (19.19), *L. rubiginosa* (19.17)
	Vanillin (21.58)	*S. oleosa* (21.67) , *L. rubiginosa* (21.59)
	Epicatechin gallate (25.05)	*M. fruticosum* (24.90)
	Quercetin (37.04)	*L. rubiginosa* (37.08)
Standard Mix 2	Caffeic acid (20.04)	*S. oleosa* (20.66)
	Coumaric acid (23.64)	*L. rubiginosa* (23.59), *A. ghaesembilla* (23.93)
	Ferulic acid (25.20)	*L. rubiginosa* (23.93)
Standard Mix 3	Catechin (18.12)	-
	Epigallocatechin (18.64)	-
	Epigallocatechin gallate (21.33)	*S. oleosa* (21.67)

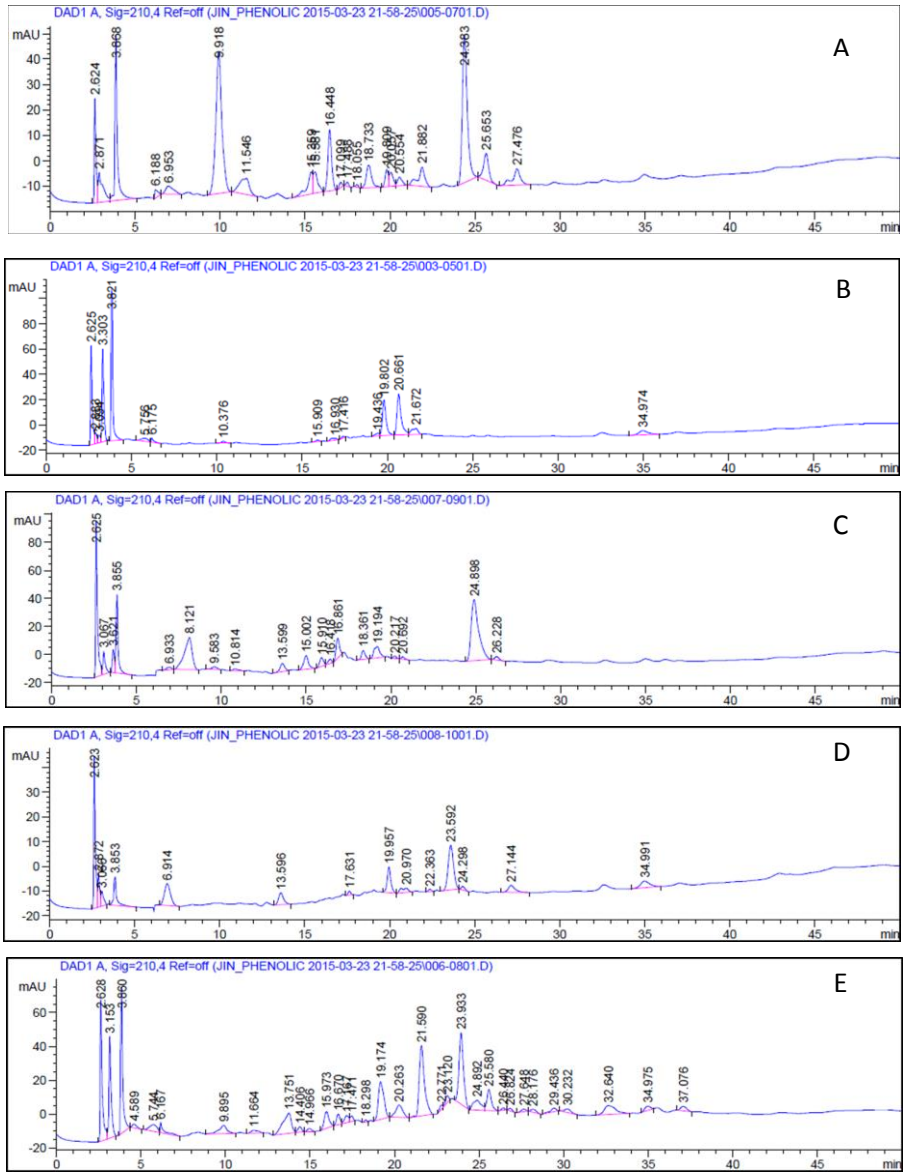

Figure 2. Typical HPLC-chromatograms of *M. calabura* (A), *S. oleosa* (B), *M. fruticosum* (C), *L. rubiginosa* (D), and *A. ghaesembilla* (E)

CONCLUSIONS

The finding provides basic data in quality control and supports these 5 indigenous pigmented-fruits, especially *M. calabura* to be a suitable candidate for the management of hyperpigmentation disorders and as a potent source of whitening agent for the cosmetic industry.

ACKNOWLEDGEMENTS

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REFERENCES

1. Chen QX, Liu XD, Huang H. 2003. Inactivation kinetics of mushroom tyrosinase in the dimethyl sulfoxide solution. Biochemistry (Mosc). 68(6), 644-649.
2. Thanigaimalai P, Hoang TAL, Lee KC, Bang SC, Sharma VK, Yun CY, Roh E, Hwang BY, Kim Y, Jung SH. 2010. Structural requirement (s) of N-phenylthioureas and benzaldehyde thiosemicarbazones as inhibitors of melanogenesis in melanoma B 16 cells. Bioorg. Med. Chem. Lett. 20(9), 2992-2993.
3. Ortiz-Ruiz CV, Berna J, Rodriguez-Lopez JN, Tomas V, Carcia-Canovas F. 2015. Tyrosinase-Catalyzed Hydroxylation of 4-Hexylresorcinol, an antibrowing and depigmenting agent: A kinetic study. J. Agric. Food Chem. 63(31), 7032-7040.
4. Jin W, Wang WY, Zhang YL, Yang YJ, Chu QC, Ye JN. 2013. Determination of phenolic whitening agents in cosmetics by micellar electrokinetic capillary chromatography with amperometric detection. Chinease. Chem. Lett. 24, 636-638.
5. Evans, WC. 2009. Pharmacopoeial and related drugs of biological origin. Trease and Evans'Pharmacognosy 16th. Nottingham, UK: W.B. Saunders.
6. Simonyan, AV, Shinkarenko AL, Oganesyan ET. 1972. Use of the cyanidine reaction in the analysis of flavonoids. Pharm Chem J. 6(10), 689-691.
7. Journal of AOAC International Vol. 88, No.5, 2005, 1273-1278.
8. Aumeeruddy Elalfi Z, Gurib-Fakim A, Mahomoodally MF. 2016. Kinetic studies of tyrosinase inhibitory activity of 19 essential oils extracted from endemic and exotic medicinal plants. S. Afr. J. Bot. 103, 89-94.

Antioxidant and antibacterial activities of *Wedelia trilobata* extract

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ABSTRACT

The aim of this research was to evaluate the antioxidant and antibacterial activities of *Wedelia trilobata* (L.) which is the plant in Asteraceae family and normally found in tropical region of Asia. It has traditionally been used for antipyretic, pain relieving, diabetes and heart disease protection. In this study, *W. trilobata* was separated into 3 parts including flower, leaf and stem and then extracted with 50% ethanol. DPPH assay was used for antioxidant evaluation. The result showed that the flower extract exhibited highest antioxidant activity when compared to the leaf and stem extracts (IC_{50} of 5.50, 11.41 and 21.36 µg/ml, respectively). Moreover, the flower part revealed antioxidative activity closed to vitamin C. In addition, these 3 fractions of *W. trilobata* were tested for antibacterial activity by agar disc diffusion method with 1.30 cm of disc diameter. *Escherichia coli* and *Bacillus cereus* that can cause diarrhea, urinary tract infections, respiratory illness and pneumonia were used in this study. The results showed that *W. trilobata* flower extract showed high antibacterial activity to both *E. coli* and *B. cereus* with clear zone in the range of 1.50-2.57 cm and 2.10-2.13 cm, respectively. Furthermore, these antibacterial results were close to standard drug, Amoxicillin. The findings from this study provide basic data supporting this plant to be developed as a health product.

Keywords: antioxidant, antibacterial, *Wedelia trilobata*

INTRODUCTION

Nowadays, herbal medicine requirement is increasing. Numerous effective antioxidative substances in edible plants have been isolated and identified. In Thailand, many indigenous plants have been utilized as food and medicine [1, 2, 3]. *Wedelia trilobata* (L.), an important herbal medicine, belongs to Apocynaceae family and normally found in tropical region of Asia. It has traditionally been used for antipyretic, pain relieving, diabetes and heart disease protection. There are a few scientific data supported its pharmacological activities. Therefore, in this study, the ethanolic extract of *W. trilobata* was determined in term of DPPH radical scavenging activity and phenolics contents. Antibacterial activity was performed following the disc agar diffusion method. *Escherichia coli* and *Bacillus cereus* which are pathogenic that causes some illnesses such as diarrhea, hemolytic-uremic syndrome (HUS) and kidney failure in human were used in this study [4, 5].

MATERIALS AND METHODS

Plant material and chemical

Wedelia trilobata was collected in October, 2015 from Ubon Ratchathani Province, Thailand. A voucher specimen was kept in the Center for Research and Development of Thai Medicine, Ubon Ratchathani Rajabhat University, Thailand. All chemicals in this study were analytical grade.

Prepatation of ethanolic extract

Wedelia trilobata was separated into 3 parts including flower, leaf and stem and dried in an oven at 45°C for 48 h and then pulverized. The plant powder was separately macerated for 5 days with 50% ethanol at room temperature and filtrated through 0.45 um filter paper. The ethanolic extracts of *W. trilobata* were concentrated at 45°C, using a rotary evaporator under low pressure, then freeze-dried and stored in an air-tight container at -20°C until further used.

Determination of phenolics content

The total phenolics content was determined by the Folin-Ciocalteu method [6]. The extract (5 mg) was dissolved with methanol up to 1 mL, and then the extract solution (0.5 mL) was mixed with 0.25 mL of the 1N Folin-Ciocalteau reagent and 1.25 mL of 20% sodium carbonate. After mixing and standing for 40 minutes at the room temperature, the optical density was measured at 725 nm. The total phenolic contents were expressed as mg tannic acid equivalent (TAE)/g dry basis.

Examination of antioxidant activity

The DPPH free radical-scavenging activity of the extract was determined [7]. 0.1 mM solution of ethanolic DPPH solution was prepared. The various concentrations of the extract dissolved in 50% ethanol were added to 0.2 ml of ethanolic DPPH solution. The absorbance was measured at 515 nm after incubation for 15 min at room temperature. Measurements were performed in triplicate. The percentage of inhibition was calculated using equation:

$$\%\text{inhibition} = [(Abs_{control}-Abs_{sample})/Abs_{control}]*100$$

IC_{50} value was calculated as the concentration of plant extract required to decrease the absorbance at 515 nm by 50% and vitamin C was used as a positive control.

Antibacterial assay

In the present study, *in vitro* antibacterial activity was carried out by the using of disc-diffusion method [8]. Briefly, Petri plates were prepared with 20 ml of sterile Muller Hinton Agar. The standard inoculums using bacterial suspension containing 10^8 CFU/ml were swabbed on the top of the solidified media and allowed to dry for 10 min. Then, prepared extract impregnated discs at the concentrations of 16-512 mg/ml were placed aseptically on the plates. Amoxycilin (50 mg/ml) and 10% DMSO were used as positive and negative controls, respectively. All the plates were then incubated for 24 h at 37ºC. The activity was measured as the clear zone of growth inhibition of agar surface around the discs in centimeter. The assay in this experiment was repeated three times.

RESULTS

Wedelia trilobata was separated into 3 parts including flower, leaf and stem. These fractions were extracted with 50%ethanol and its flowers showed the highest yield at 18.38%. The flower extract also showed high total phenolic contents (622.4±71 mg tannic acid/g). By DPPH assay, the flower extract exhibited the highest antioxidant activity with IC_{50} at 5.50±0.10 µg/ml which was very close to vitamin C (IC_{50} of 4.79±0.12 µg/ml). Besides, the leaf and stem extracts also demonstrated antioxidant activity as shown in Table 1. These three extracts of the plant showed high phenolics content and potent antioxidant activity. The contents of phenolics of *W. trilobata* directly related to its antioxidant activity. This result is in accordance with previous reports that high radical scavenging or antioxidant ability generally had with good correlation higher phenolic content [9, 10]. Moreover, among 3 parts of *W. trilobata*, the flower extract showed high activity against *Bacillus cereus* and *Escherichia coli* by using agar disc diffusion method. Throughout the experiment, the concentrations of the extract at 16-512 mg/ml were used. The maximum zone of inhibition was observed in the flower extract of *W. trilobata* against both of *Bacillus cereus* and *Escherichia coli* (2.17±0.47 and 2.57±0.15 cm, respectively). Amoxycilin at a concentration of 50 mg/ml showed the inhibition zone of 2.50±0.10 and 2.67±0.06 cm to both tested bacteria (Table 2 and Figure 1).

Table 1. Percent yield, total phenolic contents and antioxidant activity of *W. trilobata*

Sample	Phenolics contents (mg tannic acid/g)	Antioxidative activity (IC_{50}, µg/ml)	% yield
W. trilobata (flower)	622.4±71	5.50±0.10	18.38
W. trilobata (leaf)	572.4±35	11.41±0.16	9.33
W. trilobata (stem)	182.5±28	21.36±0.33	8.04
Ascobic acid, Vit.C	-	4.79±0.12	-

Table 2. Antibacterial activity of *W. trilobata*

Sample Concentrations (mg/ml)	Inhibitory effect (Clear Zone, cm)	
	Bacillus cereus	*Escherichia coli*
Flower		
512	2.17±0.47	2.57±0.15
128	2.13±0.59	2.43±0.06
16	2.10±0.53	1.50±0.10
Leaf		
512	2.07±0.06	2.23±0.06
128	1.93±0.06	2.03±0.06
16	1.63±0.06	1.87±0.06
Stem		
512	1.53±0.06	1.70±0.10
128	1.50±0.00	1.53±0.06
16	1.40±0.10	1.40±0.10
Amoxycilin		
50	2.50±0.10	2.67±0.06

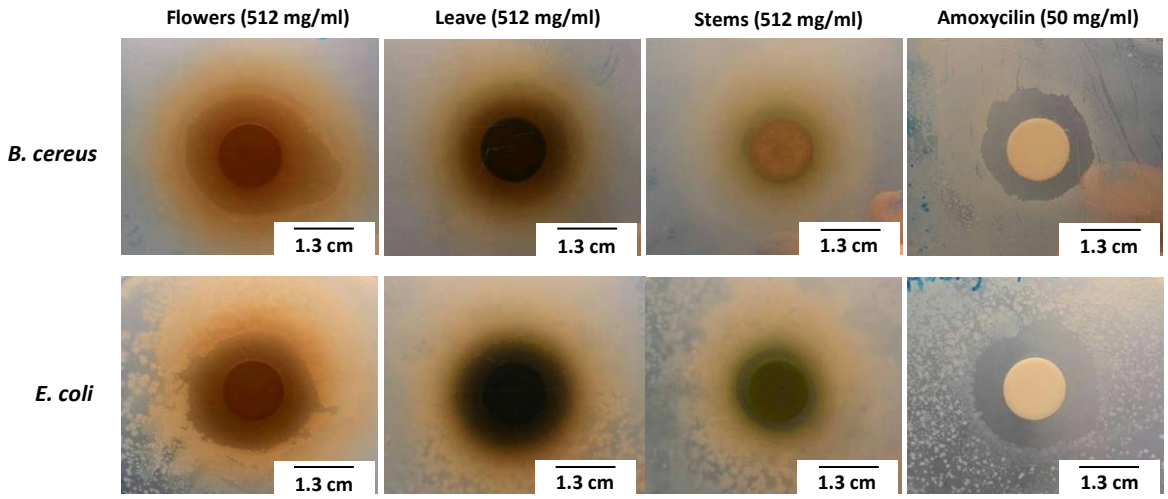

Figure 1. The inhibition zone of the *W. trilobata* extracts against *B. cereus* and *E. coli*

CONCLUSIONS

The findings from this study suggest that the *W. trilobata* has high antioxidant and antibacterial activities, in which the flower extract demonstrated the highest activities and highest yield of extraction. To our knowledge, this is the first report on antioxidant and antibacterial activities of this plant. Therefore, the further investigation on the active compounds and toxicity test of this plant may provide the scientific data to support the potential uses as the health products.

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REFERENCES

1. Sripanidkulchai B, Tattawasart U, Laupatarakasem P, Sripanidkulchai K. 2001. Antimutagencity of eight indigenous medicinal plants. KKU Research Journal. 6(1), 23-33.
2. Nakahara K, Trakultivakorn G, Alzoreky NS, Ono H, Onishi-Kameyama M, Yashida M. 2002. Antimutagenicity of some edible Thai plants, and a bioactive carbazole alkaloid, mahanine, isolated from *Micromelum minutum*. J. Agric. Food. Chem. 50, 4796-4802.
3. Trakoontivakorn G, Nakahara K, Shinmoto H, Takenaka M, Onishi-Kameyama M, Ono H, Yoshida M. 2001. Structural analysis of a novel antimutagenic compound, 4-hydroxypanduratin A, and the antimutagenic activity of flavonoids in a Thai spice, fingerroot (*Boesenbergia pandurata* Schult.), against mutagenic heterocyclic amines. J. Agric. Food. Chem. 49, 3046-3050.
4. Ravan H, Amandadi M, Sanadgol N. 2016. A highly specific and sensitive loop-mediated isothermal amplification method for detection of *Escherichia coli* O157:H7. Microb. Pathog. 91, 161-165.
5. Hariram U, Labbe RG. 2016. Growth and inhibition by spices of growth from spores of enterotoxigenic *Bacillus cereus* in cooked rice. Food Control. 64, 60-64.
6. Lin JY, Tang CY. 2007. Determination of total phenolic and flavonoid contents in selected fruits and vegetables, as well as their stimulatory effects on mouse splenocyte dproliferation. Food Chem. 101, 140-147.
7. Oh HT, Kim SH, Choi HJ, Chung MJ, Ham SS. 2008. Antioxidative and antimutagenic activities of 70% ethanol extract from masou salmon (*Oncorhychus masou*). Toxicol. 22, 1484-1488.
8. Gaydos JM, Harrington BJ. 1982. Agar disk diffusion for the quality control testing of Autobac elution disks. Antimicrob. Agents Chemother. 21(3), 516-518.
9. Liu X, Zhao M, Wang J, Yang B, Jiang Y. 2008. Antioxidant activity of methanolic extract of emblica fruit (*Phyllanthus emblica* L.) from six regions in China. J. Food Compost. Anal. 21, 219-228.
10. Robak J, Dryglewski RJ. 1988. Flavonoids are scavengers of superoxide anion. Biochem. Pharmacol. 37, 83-88.

Aphrodisiac potential of *Citrullus lanatus* cv. sai num peung rind juice in male rats

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ABSTRACT

Citrullus lanatus has been reported to have citrulline and arginine; the contents that could be useful for the improvement of male sexual performances. The aim of this research was to study the aphrodisiac potential of watermelon cv. Sai Num Peung rind juice (WRJ) in normal male rats. WRJ was prepared. Healthy adult male rats were divided into 5 groups; each group consisting of 5 rats. Group 1 received distilled water at the dose of 1 mL/day and served as a control group. Groups 2 to 4 received WRJ at the doses of 0.5, 0.75 and 1 mL/day, respectively, daily for 35 days. Group 5 received Sildenafil citrate at the dose of 60 mg/kg body weight, one hour prior to the experiment and served as a positive control group. We found that WRJ significantly increased mounting frequency and intromission frequency and significantly decreased mounting latency and intromission latency compared to the control ($P<0.05$). The relative organ weight of testes of treated rats was significantly higher than the control group ($P<0.05$). WRJ consumption produced a significant increase in seminiferous tubule diameter, germinal thick cell, spermatogonia, spermatocytes and spermatids and caused a significant decrease in seminiferous tubule lumen compared to the control ($P<0.05$). Thus, our findings support the use of WRJ as a natural aphrodisiac agent to enhance sexual functions in man.

Keywords: Aphrodisiac, Watermelon, *Citrullus lanatus*, Reproductive system, Male rat

INTRODUCTION

Erectile dysfunction (ED) is the common disorder found in elderly man. Sildenafil citrate or Viagra has long been used to improve penile erection and enhance sexual satisfaction [1]. However, there is still questioned about its effectiveness and long-term safety [1]. Thus, the search for novel aphrodisiac agents obtained from natural products is required [1]. Watermelon, *Citrullus lanatus*, is the plant belonging to the family of Cucurbitaceae. The plant is generally consumed as fruit or vegetable in many countries [2]. Watermelon has been reported to have antioxidant [3], antidiabetic [4], anticancer [5] and uterine smooth muscle relaxant activities [2,6]. Previous reports indicated that watermelon contains a variety of beneficial compounds including β-carotene, lycopene, phenols, flavonoids and vitamins [7]. Additionally, citrulline and arginine have isolated from watermelon and these two amino acids could be useful for the improvement of male sexual performances [8,9]. Therefore, the aim of this research was to investigate the aphrodisiac potential of watermelon cv. Sai Num Peung rind juice (WRJ) in normal male rats.

MATERIALS AND METHODS

WRJ preparation

Fresh fruits of watermelon were obtained from the local market in Nakhon Ratchasima, Thailand. The fruits were cleaned by tab water and peel was removed by the peeler. Rind was separated from flesh and blended by household electrical blender. WRJ was filtered through a filter paper. The filtered juice was placed in the glass bottles and pasteurized in a covered water bath with the temperature of 72^{o}C for 15s. The juice was then kept in a refrigerator (4^{o}C) until use.

Animal preparation

Healthy male Wistar rats (350-400 g) were used in this present study. They were maintained in accordance with the guidelines of the Committee on Care and Use of Laboratory Animal Resources, National Research Council of Thailand. Animals were divided into 5 groups of 5 animals each. Group 1 received distilled water at the dose of 1 mL/day and served as a control group. Groups 2 to 4 received WRJ at the doses of 0.5, 0.75 and 1 mL/day, respectively, daily for 35 days. Group 5 received Sildenafil citrate at the dose of 60 mg/kg body weight, one hour prior to the experiment and served as a positive control group.

Study on sexual behavior

Sexual behavioral examination was carried out on 35 days of WRJ treatment. Single male rat was gently dropped in 60×50×40 cm glass cages and acclimatized for 5 min. Then, a receptive female was presented to male by placing it gently into the same cage. The sexual parameters were recorded by a digital video camera and calculated as follows [10]:

1. time from the introduction of female into the cage of the male up to the first mount or Mounting Latency (ML)
2. time from the introduction of the female up to the first intromission by the male or Intromission Latency (IL)
3. time from the first intromission of a series up to the ejaculation or Ejaculatory Latency (EL)
4. number of mounts before ejaculation or Mounting Frequency (MF)
5. number of intromission before ejaculation or Intromission Frequency (IF)

The female rats used for mating test were made receptive by hormonal treatment [10].

Histological preparation

The rats were humanely killed by cervical dislocation under CO_2 anesthesia [6]. Testes were removed, cleared from adipose tissue, weighted and fixed in 10% neutral buffer formalin for histological preparation [11,12]. The specimens were observed under the microscope as previously reported by [11,12].

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). The significance of difference was analyzed using one-way analysis of variance (ANOVA). P value <0.05 was considered statistically significant.

RESULTS

Effects of WRJ on sexual behavior

The results obtained with the test for general mating behavior show that oral administration of WRJ was able to significantly increase in the MF, IF, EL_1 and EL_2 and caused a significant decrease in the ML and IL compared to control ($P<0.05$). Standard reference drug, sildenafil citrate, significantly increased the MF, IF, EL_1 and EL_2 and decreased in the ML and IL when compared with the control animals ($P<0.05$) (Table 1). In this present study, sildenafil citrate was used to evaluate the quantitative value and not to compare the mechanisms of action.

Table 1. Effect of WRJ on sexual behavior in male rats

Parameters	Treatments				
	Control	WRJ (0.5 ml/day)	WRJ (0.75 ml/day)	WRJ (1 ml/day)	Sildenafil citrate (60 mg/kg)
MF	8.00±1.58	14.00±5.77	28.33±4.48*	35.02±2.89*	49.67±1.88*
IF	9.40±0.08	19.00±7.09	38.33±3.28*	45.42±3.28*	62.01±7.02*
ML (in sec)	214.33±10.80	195.33±12.09	170.33±12.91*	152.20±14.33*	122.36±15.24*
IL (in sec)	200.24±6.98	177.67±9.86	125.67±6.17*	133.31±7.20*	116.62±5.63*
EL_1 (in sec)	200.67±8.07	221.29±2.68	240.20±3.61*	259.01±2.08*	275.00±2.10*
EL_2 (in sec)	267.44±4.15	270.15±2.99	286.21±5.17*	298.00±6.08*	315.00±6.91*

MF = mounting frequency, IF = intromission frequency, ML = mounting latency, IL = intromission latency, EL_1 = ejaculatory frequency in first series, EL_2 = ejaculatory frequency in second series. Values are expressed as mean ± SEM, $n = 5$. *P value <0.05 was considered statistically significant when compared with the control group.

Effects of WRJ on testicular weight and testicular histology

The relative organ weight of the testes of rats received WRJ at the dose of 1 mL was significantly higher than the control (P<0.05). Additionally, WRJ produced a significant increase in seminiferous tubule diameter, germinal thick cell, spermatogonia, spermatocytes and spermatids and caused a significant decrease in seminiferous tubule lumen compared to the control (P<0.05). However, WRJ consumption did not affect the number of Leydig cells (P>0.05) as shown in Table 2 and Figure 1.

Table 2. Effects of WRJ on testicular weight and testicular histology of male rats.

Parameters	Treatments			
	Control	WRJ (0.5 ml/day)	WRJ (0.75 ml/day)	WRJ (1 ml/day)
Testicular weight (%)	0.96±0.03	0.95±0.00	0.99±0.04	1.47±0.18*
Spermatogonia	76.33±16.42	79.66±2.18	108.66±11.31*	150.00±14.29*
Primary spermatocytes	72.33±5.49	93.75±4.34*	99.14±6.50*	125.01±2.98*
Secondary spermatocytes	68.16±7.16	91.08±8.26*	118.33±6.60*	126.83±9.16*
Spermatids	97.66±4.04	110.33±4.80	148.41±5.69*	164.00±18.08*
Leydig cells	83.66±9.27	80.66±6.88	84.00±7.37	85.33±22.06
STD (µm)	703.81±36.24	1078.91±68.85*	1287.56±53.86*	1425.55±53.46*
STL (µm)	264.59±22.12	170.70±28.66*	183.36±12.94*	145.57±6.48*
GTC (µm)	253.87±12.64	379.57±47.05*	462.32±19.47*	563.04±47.09*

Data are expressed as Mean ± SEM, *n* = 5, *P value <0.05 was considered statistically significant when compared with the control group. STD = seminiferous tubule diameter, STL =seminiferous tubule lumen, GTC =germ thick cell.

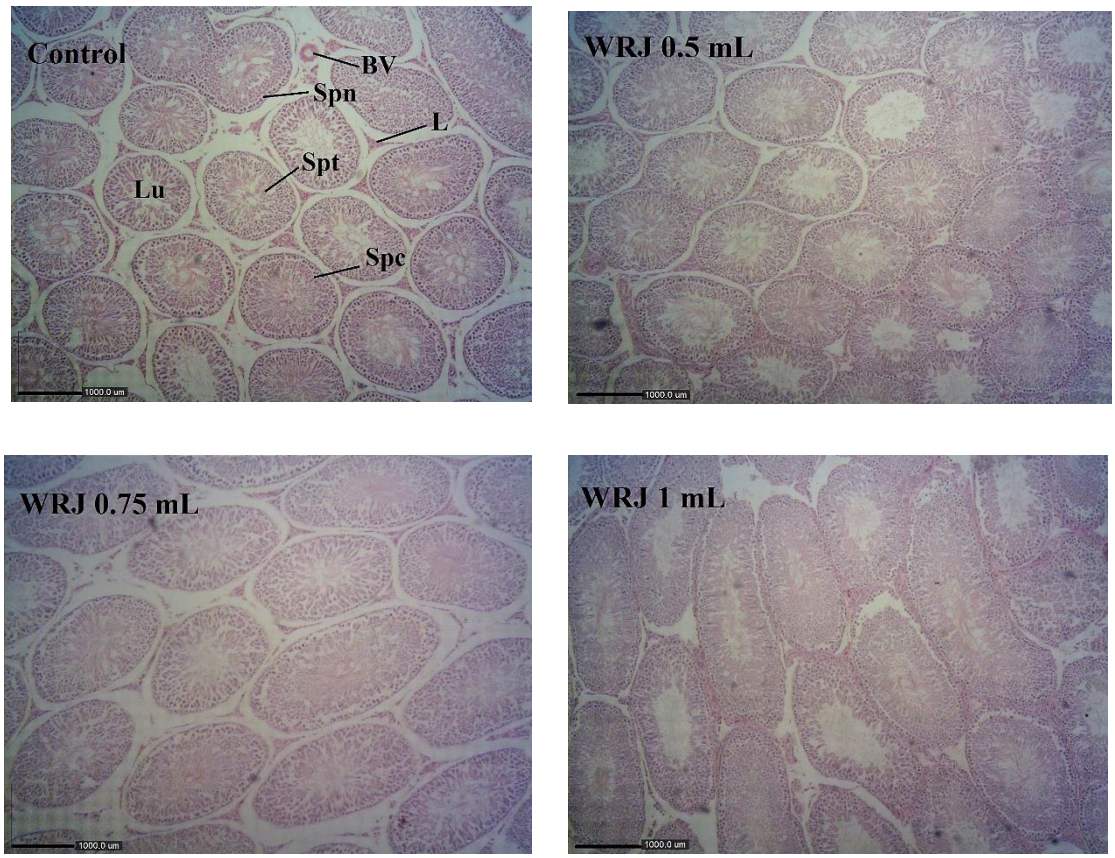

Figure 1. Effects of WRJ on testicular histology of male rats (Scale bar = 1000 µm). BV = blood vessel, Spn = spermatogonia, Spt = spermatids, Spc = spermatocytes, L = Leydig cells, Lu = seminiferous tubule lumen.

CONCLUSIONS

Our data indicate that WRJ consumption increased sexual activity of male rats. In addition, testicular weight, spermatogonia, spermatocytes, spermatids, seminiferous tubule diameter, seminiferous tubule lumen and germ thick cell observed in the juice treated rats were significantly higher than the control group. Thus, our results support the use of WRJ as a natural aphrodisiac agent to enhance sexual functions in man.

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REFERENCES

1. Sandroni P. 2001. Aphrodisiacs past and present: a historical review. Clin. Auton. Res. 11, 303-307.
2. Jayaprakasha GK, Chidambara Murthy KN, Patil BS. 2011. Rapid HPLC-UV method for quantification of L-citrulline in watermelon and its potential role on smooth muscle relaxation markers. Food Chem. 127, 240-248.
3. Tlili I, Hdider C, Lenucci MS, Ilahy R, Jebari H, Dalessandro G. 2011. Bioactive compounds and antioxidant activities during fruit ripening of watermelon cultivars. J. Food Comp. Anal. 24, 923-928.
4. Ahn J, Choi W, Kim S, Ha T. 2011. Anti-diabetic effect of watermelon (*Citrullus vulgaris* Schrad) on streptozotocin-induced diabetic mice. Food Sci. Biotechnol. 20, 251-254.
5. Rahmat A, Rosli R, Zain WNIWM, Endrini S, Sani HA. 2002. Antiproliferative activity of pure lycopene compared to both extracted lycopene and juices from watermelon (*Citrullus vulgaris*) and papaya (*Carica papaya*) on human breast and liver cancer cell lines. J. Med. Sci. 2, 55-58.
6. Munglue P, Eumkep G, Wray S, Kupittayanant S. 2012. The effects of watermelon (*Citrullus lanatus*) extracts and L-citrulline on rat uterine contractility. Reprod. Sci. 20, 437-448.
7. Kim S-J, Matsushita Y, Fukushima K, Aoki D, Yagami S, Yuk HG, Lee SC. 2014. Antioxidant activity of a hydrothermal extract from watermelons. LWT-Food Sci. Technol. 59, 361-368.
8. Cormio L, Siati MD, Lorusso F, Selvaggio O, Mirabella L, Sanguedolce F, Carrieri G. 2011. Oral L-citrulline supplementation improves erection hardness in men with mild erectile dysfunction. Urology. 77, 119-122.
9. Moody JA, Vernet D, Laidlaw S, Rajfer J, Gonzalez-Cadavid NF. 1997. Effects of long-term oral administration of L-arginine on the rat erectile response. J. Urol. 158, 942-947.
10. Ågmo A. 1997. Male rat sexual behavior. Brain Res Protoc. 1, 203-209.
11. Türk G, Sönmez M, Aydin M, Yüce A, Gür S, Yüksel M, Aksu EH, Aksoy H. 2008. Effects of pomegranate juice consumption on sperm quality, spermatogenic cell density, antioxidant activity and testosterone level in male rats. Clin. Nutr. 27, 289-296.
12. Mishra RK, Singh SK. 2008. Safety assessment of *Syzygium aromaticum* flower bud (clove) extract with respect to testicular function in mice. Food Chem. Toxicol. 46, 3333-3338.

Antioxidant properties of 5 herbal plants based of pharmacophore modeling

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ABSTRACT

Malaysian medicinal plants are known to exert therapeutic effects. We have evaluated some species namely *Moringa oleifera, Clinacanthus nutans, Rhodomyrtus tomentosa, Arctium lappa* and *Sonneratia alba* for their antioxidant properties assisted by using pharmacophore modeling approach. We displayed five major compounds from each plant namely: 3-caffeoylquinic, benzylglucosinolate, kaempferol, leucodelphinidin and quercetin (*Moringa oleifera*), adenosine, arctigenin, arctiin, kaempferol, and solasonine (*Arctium lappa*), 2-cis-entadamine A, phaeophytin, clinamides B, isovetaxin, and vitexin (*Clinancathus nutans*), 5-hydroxymethylfurfural, alphitolic acid, betulin, mtheyl gallate, oleanolic acid (*Sonneratia alba*) and lupeol, rhodomyrtosone A, rhodomyrtosone B, rhodomyrtosone C and rhodomyrtosone D (*Rhodomyrtus tomentosa*).

Keywords: Pharmacophore Modeling, antioxidant properties, *Moringa oleifera, Clinacanthus nutans, Rhodomyrtus tomentosa, Arctium lappa, Sonneratia alba,* ligand-based pharmacophore.

INTRODUCTION

Antioxidant refers to compound which is able to scavenge, cease formation or counteracting the damaging action of oxidants on cells [1]. They act as reducing agent, hydrogen donor, singlet oxygen quencher and metal chelator. Naturally, our body produces free radicals in small amount for particular functions such as modulation of inflammation, killing pathogens, detoxifying toxins and producing signaling molecules [2,3]. Even though the generation of these free radicals is kept in check by defense and repairs system, the uncontrolled generation of free radical species particularly of oxygen species and decreased in antioxidant protection within cells cause oxidative stress to healthy body cells [4]. Excess free radicals participate in various chemical reactions subsequently produce more reactive species of oxygen, nitrogen and sulphur which is linked to many chronic diseases like cancers, cardiovascular diseases, neurological disorder, auto-immune deficiency diseases and degenerative disorders associated with aging [5].

Considering the extensive damages arise from oxidative stress on human health, it is uncommon that antioxidant is one of the interest compound in the study of plant medicinal value as it provides protection against various oxidative stress-related diseases. In plants, polyphenols such as flavonoids, phenolic acids, stilbenes, coumarins, lignin and lignins are the interest compounds that possess free radical scavenging activity [6]. In this study antioxidant properties are identified based on pharmacophore modeling; the focus is on five plants from South Asia namely *Moringa oleifera, Clinacanthus nutans, Rhodomyrtus tomentosa, Arctium lappa* and *Sonneratia alba*.

These plants have been well known for centuries as alternative medicine to cure various diseases. Among the prominent uses of *C. nutans* are as cure for various types of cancers and skin inflamation, *M. olifera* and *R. tomentosa* as anti-diabetic [7,8]. Meanwhile, *A. lappa* has been studied for is anti-inflammatory which can prevent or treat gout attack and *S. alba* as anti-diabetic [9]. The five major compounds found in each plant are as follow respectively: 3-caffeoylquinic, benzylglucosinolate, kaempferol, leucodelphinidin and quercetin (*M .oleifera*), adenosine, arctigenin, arctiin, kaempferol, and solasonine (*A. lappa*), 2-cis-entadamine, phaeophytin, clinamides B, isovetaxin, and vitexin (*C. nutans*), 5-hydroxymethylfurfural, alphitolic acid, betulin, methyl gallate, oleanolic acid (*S. alba*) and lupeol, rhodomyrtosone A, rhodomyrtosone B, rhodomyrtosone C and rhodomyrtosone D (*R. tomentosa*).

Pharmacophore modeling method simulates the search of potential and promising drugs candidates by virtual screening. Pharmacophore modelling provides useful structural and chemical information for future development of more potent molecules [10]. Ligand-based pharmacophore model plays a major role in searching drugs and treatment of certain diseases. Pharmacophore modelling has been applied in combination with other molecular modelling technique [11].

A virtual screening approach was divided into two parts; ligand-based screening and structure-based screening. Ligand-based screening is 2D or 3D chemical structures of known actives molecules of model which use established selected compounds of interest from a database. The virtual screen was based on concept of common features and hypothetical geometry of the experimented compounds.

MATERIALS AND METHOD

Training Set

A chemical feature-based 3D pharmacophore model was built within the LigandScout 3.12 OMEGA software. The training set of antioxidant compounds was selected from published data. The selection is essential for generating pharmacophore model. The training set was manipulated for optimization and drawn using ACD/ChemSketch. The optimized training set was listed in Figure 1.

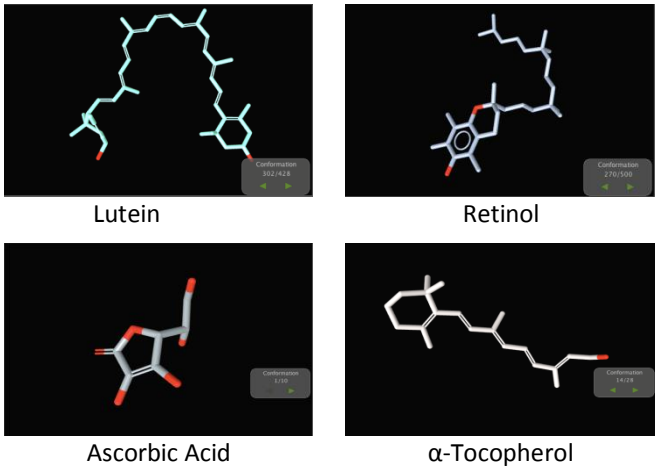


Figure 1. List of Training Set

Hypothesis generation

The ligand-based pharmacophore modelling approach is carried out to evaluate the fit value of compounds isolated from 5 different herbal plants which are *Moringa oleifera, Rhodomyrtus tomentosa. Clinacanthus nutans, Artium lappa* and *Sonneratia casiolaris*. A pharmacophore model is generated from selected antioxidant compounds of a set of training sets. The selected test set which consist of components from the 5 herbal plant which are phaeophytin, 2-cis-entadamine A, clinamide B, vetaxin, isovetaxin, benzylglucosinolate, kaemferol,lLeucodelphinidin, quecertin, betulin, oleanolic acid, aphitolic acid, 5- hydroxymethylfurfural, methyl galate, arctigenin , solasonine, arctiin , adenosine, lupeol, and rodomentosa A,B,C and D.

RESULTS AND DISCUSSION

Pharmacophore model generated based on the selected training set (lutein, α-tocopherol, retinol and ascorbic acid) and the features obtained in the pharmacophore model are shown in Figure 2 and Table 1. Along obtaining the proximity of the training set with the test set (3-caffeoylquinic, benzylglucosinolate, kaempferol, leucodelphinidin, quercetin, adenosine, arctigenin, arctiin, kaempferol, solasonine, 2-cis-entadamine, phaeophytin, clinamides B, isovetaxin, vitexin, 5-hydroxymethylfurfural, alphitolic acid, betulin, methyl gallate, oleanolic acid, lupeol, rhodomyrtosone A, rhodomyrtosone B, rhodomyrtosone C and rhodomyrtosone D, 10 models of ligand based score fit values are confirmed as shown in Table 2 based on the configuration of each chemical structure respectively. 0-1 are the ligand based score fit value; whereby the closest to 1 is the best and top ligand based score fit value and best to be evaluated. The pharmacophore model feature is evaluated based on model 1. Table 3 shows the fit value and pharmacophore features of training set and test set based on the generated pharmacophore model and Figure 3 (i-xxviii) illustrates the pharmacophore interaction of the test sets and training sets.

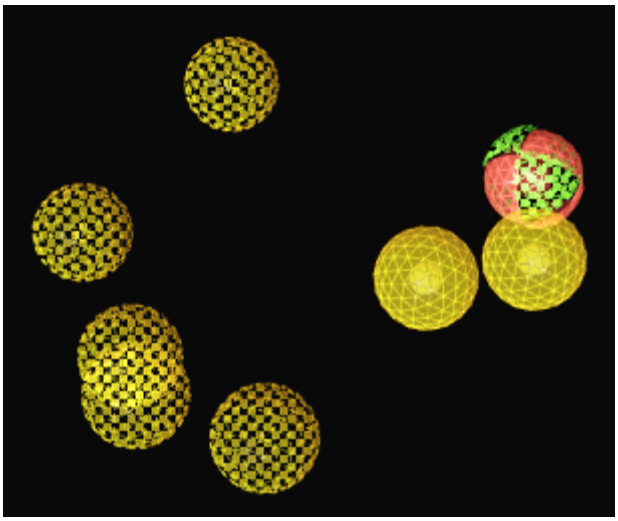


Figure 2. Pharmacophore model

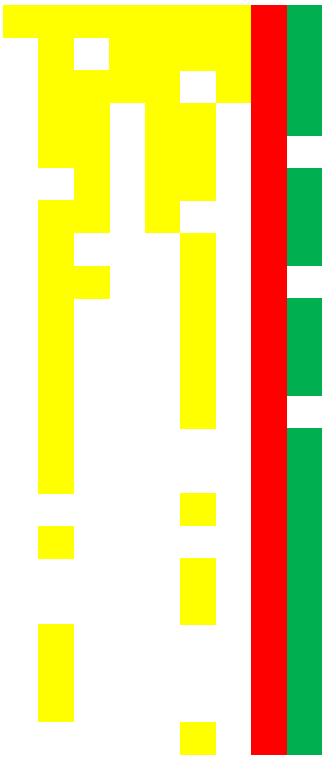
Table 1. Features of the Pharmacophore Model

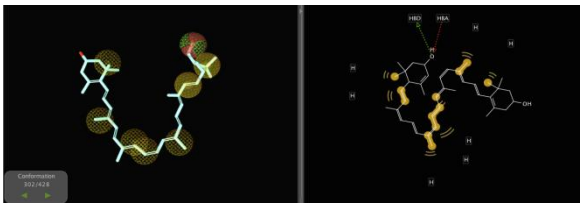
Features	Interactions
	Hydrophobic
	Hydrogen Bond Acceptor
	Hydrogen Bond Donor

Table 2. Number of Models Generated and Score Fit Values

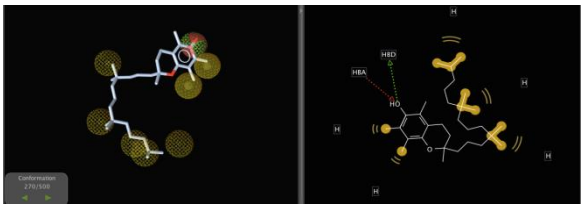
Models	Score Fit Value
1	0.6559
2	0.2961
3	0.2938
4	0.2902
5	0.2900
6	0.2841
7	0.2754
8	0.2506
9	0.2497
10	0.2451

Table 3. Fit Value and Pharmacophore Features of Training Set and Test Set

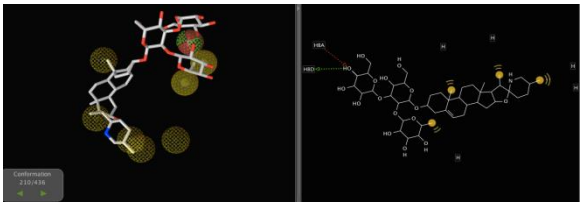
Name	Type	Pharmacophore Features	Pharmacophore Fit-Value
Lutein	Training		73.6600
α-Tocopherol	Training		62.5400
Solasonine	Test		58.7900
Betulin	Test		56.6100
Phaeophytin	Test		48.4900
Arctiin	Test		46.8200
Lupeol	Test		45.4900
Arctigenin	Test		44.4400
Retinol	Training		42.8400
Rhodomyrtosone B	Test		42.7200
Oleanolic Acid	Test		42.3600
Aphitolic Acid	Test		42.3500
Rhodomyrtosone A	Test		36.7200
Quercetin	Test		35.4700
3- caffeoylquinic	Test		35.4700
Rhodomyrtosone C	Test		35.4600
2-cis- Entadamide	Test		35.4600
Benzylglucosinolate	Test		35.4500
Vitexin	Test		35.4400
Kaempferol	Test		35.3300
Isovetaxin	Test		35.3300
5-hydroxyxethylfuran	Test		34.9900
Clinamide B	Test		33.0200
Ascorbic Acid	Training		0.0000
Methyl Gallate	Test		0.0000
Rhodomyrtosone D	Test		0.0000
Leucodelphinidin	Test		0.0000
Adenosine	Test		0.0000



(i)



(ii)



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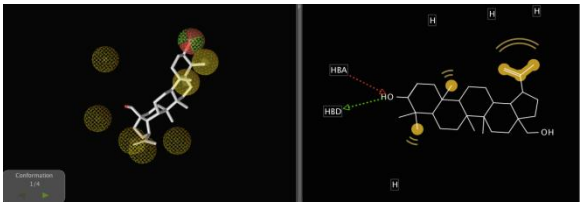

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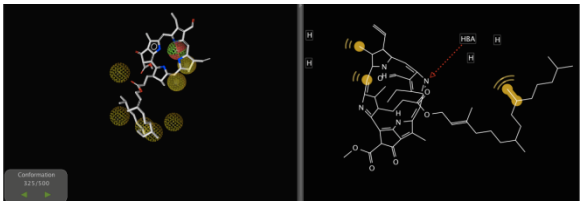

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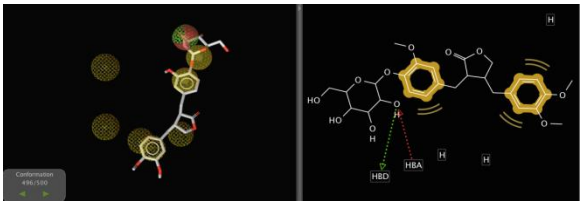

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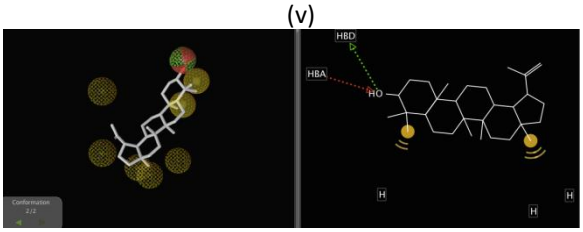

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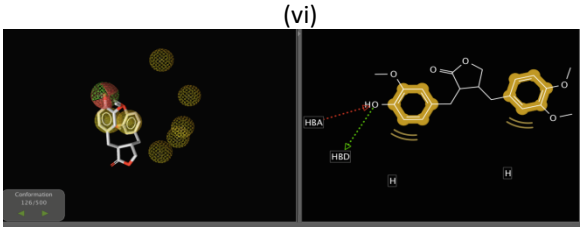

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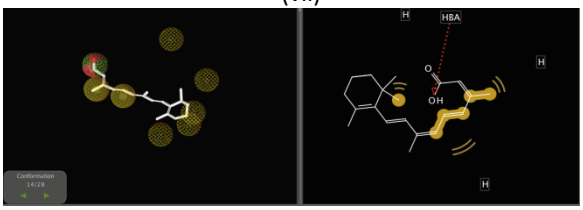

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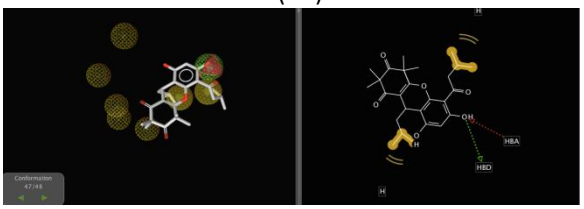

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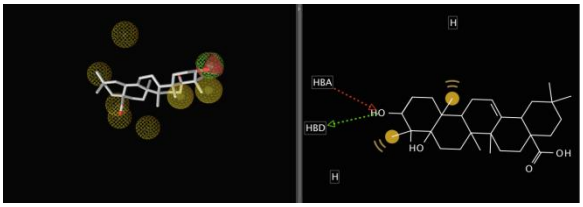

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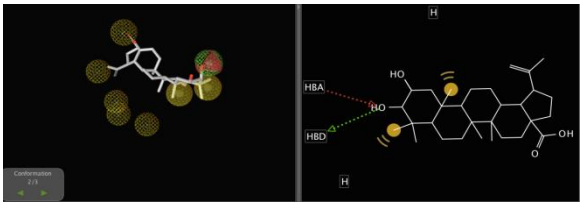

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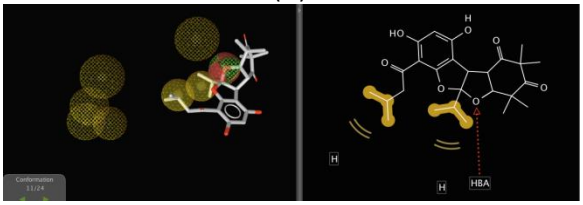

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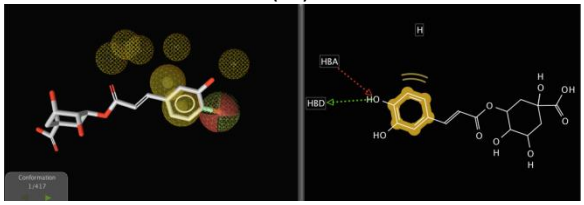

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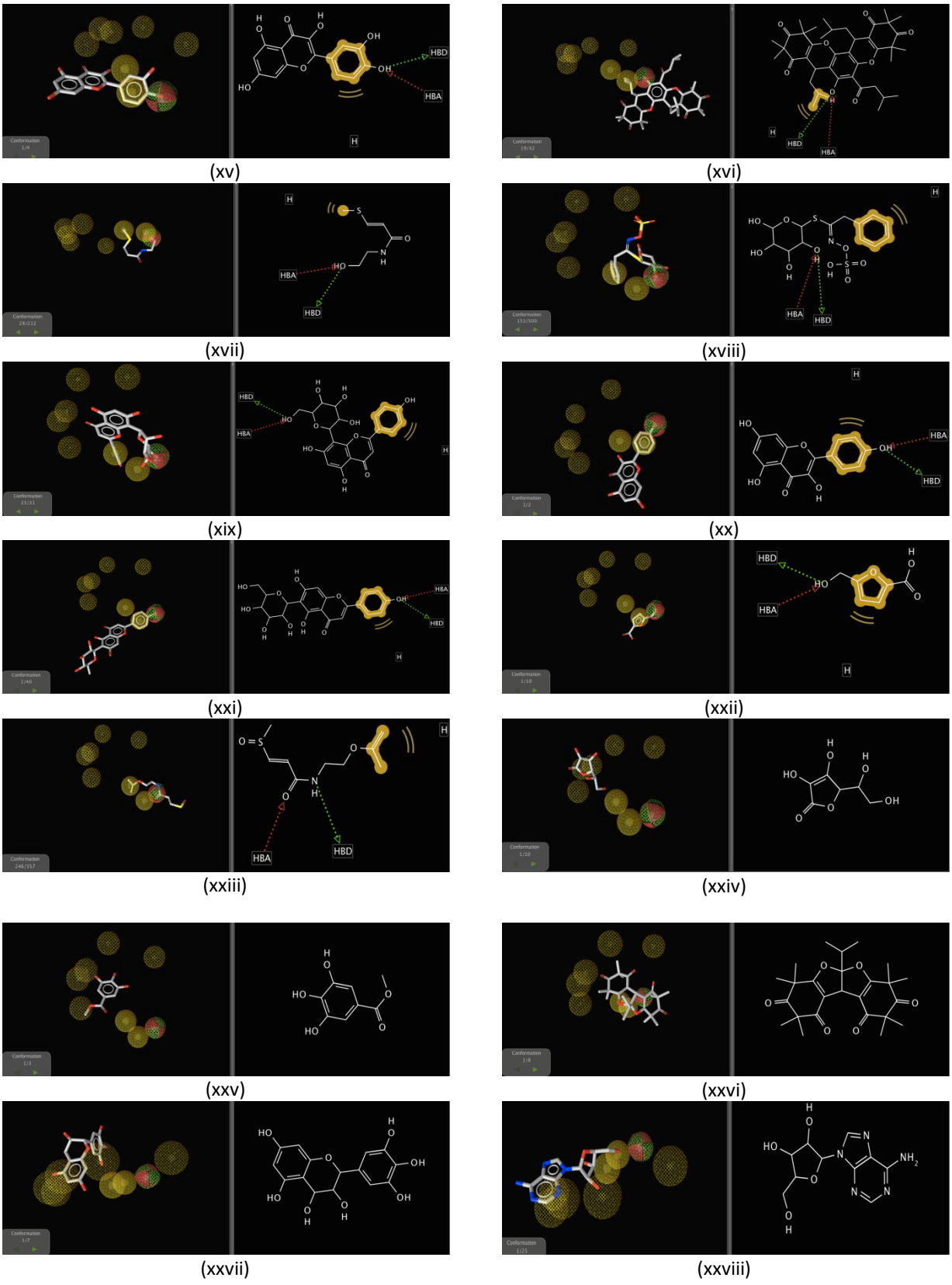

Figure 3. Pharmacophore Interaction of Training Set and Test Set

Ligand based drug design is where the chemical structure and pharmacophore features proximity are obtained by aligning the pharmacophore model generated based on selected training set with the test set using Ligand Scout 3.12 Omega Software. There are several pharmacophore features; whereby through generating the pharmacophore model features such as hydrogen bond acceptor (HBA), hydrogen bond donor (HBD) and hydrophobic (Hy) are obtained. From the results obtained for an antioxidant remedy the presence of these chemical features are important.

Based on Table 1 and Figure 2, Hy is the main chemical feature represents a compound containing the properties as an antioxidant. The Hy interaction is evaluated by the presence of methyl functional group in an aromatic structured compound. HBD interaction is obtained due to the presence of hydroxyl group, sulphate group and imine group whereas for HBA interaction obtained due to the presence of carbonyl group, hydroxyl group, thioether group and ether group.

Model 1 from the generated pharmacophore based on Table 2 was chosen to be evaluated because the fit value of model 1 is the closest to 1. Through this model, it is found that 6 test sets have better antioxidant property than Retinol and Ascorbic Acid. Solosonine, betulin, phaeophytin, arctiin, lupeol and arctigenin are in between three training sets which can be observed as a potential possessing antioxidant properties. There are also found that 14 test set possesses a mild antioxidant properties as shown in Table 3 and Figure 3 in between α-tacopherol and ascorbic acid due to lack a hydrophobic and hydrogen bond acceptor features which represents the properties of an antioxidant. Further training set and 4 other test sets are not compatible for antioxidant properties due to the structure scaffold and absents of the chemical features.

CONCLUSION

In conclusion, the presence of hydrophobic, hydrogen bond donor and hydrogen bond acceptor are the chemical features for an antioxidant remedy. Based on the results obtained solasonine, arctiin and arctigenin from *Arctium lappa*, belutin from *Sonneratia alba* and lupeol from *Rhodomyrtus tomentosa* possess the properties of antioxidant. As for the other 14 chemical constituents are to possess mild antioxidant properties which can be enhanced through combining compounds that possess the features of hydrophobic.

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REFERENCES

1. Irshad M, Chauduri PS. 2012. Oxidant-antioxidant system: Role and significance in human body. Indian J. Exp. Biol. 40, 1233-1239.
2. Davies M. 2012. Free radicals, oxidants and protein damage. Australian Biochemist. 43, 8-12.
3. Kunwar A, Priyadarsini KI. 2011. Free radicals, oxidativestress and important antioxidants in human health. J. Med. Allied Sci. 1, 53-60.
4. Lobo V, Patil A, Phatak A, Chandran N. 2010. Free radicals, antioxidants and functional foods: Impact on human health. Pharmacogn. Rev. 4, 118-126.
5. Carocho M, Ferreira IC. 2013. A review on antioxidants, prooxidants and related controversy: natural and synthetic compounds, screening and analysis methodologies and future perspectives. Food Chem. Toxicol. 51, 15-25.
6. Sharma SK, Singh L, Singh S. 2013. A review on medicinal plants having antioxidant potential. Indian J. Res. Pharmacy Biotechnol. 1, 404-409
7. Yoke, K. Y., Jun, J. T., Soek, S. H., Siau, H. M., Cheng, G. L. E. Hoe, S. C. & Ahmad, Z. 2013, "Clinacanthus nutans extracts are antioxidant with antiproliferative effect on cultured human cancer cell lines", Evidence-Based Complementary and Alternative Medicine.
8. Limsuwan, S., Kayser, O., & Voravuthikunchai, S. P, 2012, Antibacterial Activity of Rhodomyrtus tomentosa (Aiton) Hassk. Leaf Extract against Clinical Isolates of Streptococcus pyogenes. Evidence-Based Complementary and Alternative Medicine, Volume 2012, Article ID 697183, 6 pages, doi:10.1155/2012/697183, pp. 1-6.
9. Morada, N. J., Metillo, E. B., Uy, M. M., & Oclarit, J. M, (2011), Anti-diabetic Polysaccharide from Mangrove Plant, Sonneratia alba, 2011 International Conference on Asia Agriculture and Animal: IPCBEE vol.13 (2011) © (2011)IACSIT Press, Singapore,1-4.
10. Kaur, P., Sharma, V., & Kumar, V. (2012). Pharmacophore Modelling and 3D-QSAR Studies on N3-Phenylpyrazinones as Corticotropin-Releasing Factor 1 Receptor Antagonists. International Journal of Medicinal Chemistry Volume 2012 (2012), Article ID 452325, 13 pages http://dx.doi.org/10.1155/2012/452325, 13.
11. Caporuscio, F., & Tafi, A. (2011). Pharmacophore Modelling: A Forty Year Approach and Its Modern Synergies. Curr Med Chem 2011;18(17), pp.2453-53.

Chemical analysis and antioxidant properties of polysaccharides extracted from the mycelium of *Boletus colossus* Heim.

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ABSTRACT

Boletus colossus Heim. is one of the higher fungi which has been reported as an important source of bioactive compounds. This study aimed to investigate the chemical composition and inhibitory effect on antioxidant activity of crude polysaccharides from the mycelium of *Boletus colossus* Heim. The mycelium of *B. colossus* Heim. was extracted with cold and hot water. Polysaccharides were precipitated from the aqueous solution by 95% ethanol. These extractions produced four fractions of crude extracts: cold water polysaccharide extract (CWPE), cold water extract (CWE), hot water polysaccharide extract (HWPE) and hot water extract (HWE). The chemical compositions of crude extracts such as total carbohydrate, reducing sugar and total polysaccharide content were determined. The highest total carbohydrate (213.81 mg glucose/ g dry mycelia weight) and polysaccharide contents (185.15 mg glucose/ g dry weight) were found in HWPE. Conversely, CWE showed the highest reducing sugar content (about 47.97 mg glucose/ g dry weight). Analysis of these extracts by FT-IR showed the spectra of polysaccharide compounds in α and β-configurations. DPPH radical scavenging capacity, reducing power and total phenolic content have been used to evaluate the value of antioxidant activities. HWE showed the highest percentage of DPPH inhibition (IC_{50} 85.59 µg/mL) and reducing power. Also, the highest total phenolic content was observed from the HWE (69.66 mg gallic acid/ g crude extract). Therefore, these results indicated that all crude extracts from *B. colossum* Heim. mycelium could potentially be used as natural antioxidants.

Keywords: Antioxidant activity, *Boletus colossus* Heim., mycelium

INTRODUCTION

Boletus colossus Heim. (Bolete mushroom) is an edible ectomicorrhizal mushroom group. It recently has been popular as a source of bioactive compounds used in agriculture for controlling insect or weed. However, other bioactive metabolites in mushroom have been reported. Mushrooms have also been reported as organisms with antioxidant activity. This is correlation with their phenolic and polysaccharide compounds [1]. Among various naturally occurring substances, polysaccharide extracts from mushrooms may prove to be one of the useful candidates in the search for effective, non-toxic substances with antioxidant property [2,3]. Since the growth rate of *B. colossus* is very slow, and it takes a long time to cultivate in the green house. Thus it is expensive to obtain fruiting bodies. Therefore, using a submerged culture to produce effective substances from cultured mycelia might be the alternative way to overcome this problem. However, the chemical composition and the antioxidant properties of this mushroom have rarely been studied. Therefore, the objectives of this study were to investigate the chemical composition of crude polysaccharides extracted from *B. colossus* mycelium and their inhibitory effect on antioxidant activity.

MATERIALS AND METHODS

Materials

The mycelia of *Boletus colossus* Heim. in submerged culture was obtained from Department of Horticulture, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University.

Extraction of polysaccharides from *B. colossuss* mycelia

The extraction methods which used were the modified methods of Tsai *et al*. [2]. The freeze-dried mycelia (10 g for each method) from submerged culture was ground to powder at room temperature, and then extracted with deionized water by maceration at 30°C for 24 h and hot water by reflux at 95°C for 6 h. The extracted solutions were filtered and concentrated 6-fold under a vacuum. Polysaccharides were precipitated by adding 95% ethanol in ratio 1:4 (v/v) at 4°C overnight. The mixture was centrifuged at 5,000 rpm for 15 min. The supernatant and precipitate were separated. The precipitated polysaccharides were dried using freeze-dryer, while the supernatant was evaporated to dryness and freeze-dried to a powder form. These extractions yielded four fractions of crude extracts: cold water polysaccharide extract (CWPE), cold water extract (CWE), hot water polysaccharide extract (HWPE) and hot water extract (HWE).

Determination of total carbohydrate content

The total carbohydrate content was determined by the modified phenol-sulfuric acid method according to Ammar *et al.* [4]. Briefly, each extract 0.20 mL was mixed with 5% (w/v) phenol 0.20 mL and concentrated H_2SO_4 1.0 mL. The reaction mixture was kept at room temperature for 10 min and then cooled in water for 20 min. The solution was taken to measure the absorbance at 490 nm. The total carbohydrate content was calculated by comparing with the standard curve of D-glucose

Determination of reducing sugar

The reducing sugar content was determined using the modified method of Ammar *et al.*[4]. Three milliliter of 3,5-Dinitrosalicyclic acid (DNS) 1% (w/v) was added into 3.0 mL of each extract. The mixture solution was warmed at 90°C on water bath for 5 min. 40% (w/v) of sodium potassium tartrate 1.0 mL was added. The solution was kept at room temperature for 5 min and the absorbance was measured at 575 nm. The reducing sugar was calculated with D-glucose as a standard. Total polysaccharide content was the subtraction of reducing sugar content from total carbohydrate content.

Characterization of extracts by Infrared spectroscopy (FTIR)

All extracts were subjected to FTIR spectroscopy at 400-4000 cm^{-1} by ATR technique.

DPPH radical scavenging capacity assay

DPPH assay was measured by the modified method of Anita *et al.* [5]. One milliliter of 0.2 mM DPPH in methanol was added into 2.0 mL of each extract solution at various concentrations. The mixture was shaken vigorously and left to stand for 30 min in the dark. The absorbance was measured at 514 nm. Butylated hydroxytoluene (BHT) was used as positive control. The percentage of DPPH inhibition was calculated.

Reducing power assay

Reducing power assay was measured by the modified method of Isabel *et al*. [6]. Each extract at various concentrations 2.5 mL was mixed with 0.2 M phosphate buffer (pH 6.6) 2.5 mL and 1% (w/v) $K_2Fe(CN)_6$ 2.5 mL. The reaction was kept at 50°C for 20 min and then 10% (w/v) of trichloroacetic acid 2.5 mL was added. The mixture solution was centrifuge at 650 rpm for 10 min. Five milliliter of upper solution was mixed into 5.0 mL of DI water and 0.1% (w/v) ferric chloride 1.0 mL. The absorbance was measured at 700 nm against blank. BHT was used as positive control.

Determination of total phenolic content

The total phenolic content was determined by the modified Folin-Ciocalteu method of Thetsrimuang *et al.* [7]. Briefly, 0.5 mL of each extract in methanol was added into 10% (v/v) Folin-Ciocalteu reagent 0.5 mL. The reaction was left at room temperature for 3 min and then 35% (w/v) Na_2CO_3 0.5 mL was added. The mixture solution was adjusted to 5.0 mL with DI water. The absorbance of the mixture was read at 725 nm after left to stand in the dark for 90 min. The quantification was determined based on a standard curve of gallic acid. The amount of total phenolic content was expressed as mgGAE/g crude extract.

RESULTS

The extraction of *B. colossuss* mycelia by hot and cold water produced four fractions of extracts which called as HWPE, HWE, CWPE and CWE. Each extract was freeze-dried to powder form, resulting in brownish solid. The percentage of crude extracts of HWPE, HWE, CWPE and CWE was 24.54, 15.80, 20.91 and 19.22, respectively.

The total polysaccharide content could not be directly measured because there are mixed complex and combination of various monosaccharide, oligosaccharide. Therefore, total polysaccharide content was the subtraction of reducing sugar content from total carbohydrate content. The chemical analysis of extracts was carried out using phenol-sulfuric acid method, data as shown in Table 1. HWPE showed higher polysaccharide content than CWPE about two times.

Table 1. Total carbohydrate, total reducing sugar and total polysaccharide contents of extracts from *B. colossus* Heim. mycelia

Extracts	(mg glucose/g dry weight)			Total phenolic content (mgGAE/g crude extract)
	Total carbohydrate	Total reducing sugar	Total polysaccharide	
HWPE	213.81±5.58	28.66±2.66	185.15	21.03±0.96
HWE	76.52±2.59	45.93±0.69	30.59	69.66±1.47
CWPE	103.08±2.21	13.79±3.94	89.29	47.28±2.80
CWE	72.26±6.06	47.94±1.59	24.32	62.30±1.91

The analysis of polysaccharides by FTIR spectra of four crude extracts are shown in figure 1 (a-d). The strong functional groups found were at wave number 3200-3400 cm^{-1} (O-H stretching), 2927-2929 cm^{-1} (C-H stretching), 1654 and 1560 cm^{-1} (protein), 1000-1200 cm^{-1} (pyranose ring), 844 cm^{-1} (α-configuration) and 891 cm^{-1} (β-configuration). However, these polysaccharides may contain α and β-configuration.

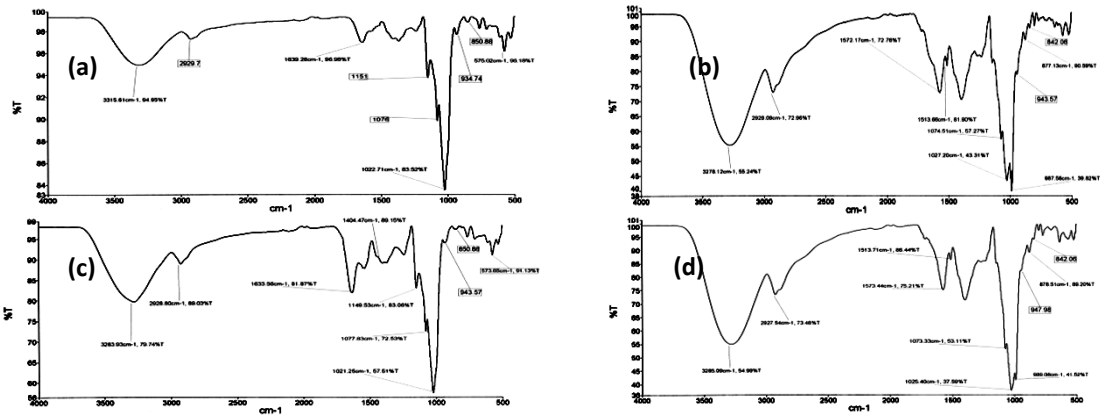

Figure 1. FTIR spectra of extracts from *B. colossus* Heim. (a) HWPE (b) HWE (c) CWPE and (d) CWE

Antioxidant activity of extracts

The antioxidant activities of mycelia extracts from *B. colossus* Heim. were evaluated by DPPH radical scavenging capacity, reducing power and total phenolic content. By DPPH assay, the antioxidant activity was found in the order of HWE > CWE > CWPE > HWPE. HWE had the highest antioxidant activity with IC_{50} 85.59 μg/mL as shown in figure 2. Reducing powers of four extracts from *B. colossuss* mycelia increased with the increasing of concentrations as shown in figure 2. The hot water extraction seemed to provide higher activity than cold water extraction. HWE showed the highest reducing power activity than other extracts at all concentrations. In addition the results showed high correlation between DPPH and reducing power activities of all extracts. The total phenolic content of extracts that determined by Folin-Ciocalteu method were in range 21.0-69.7 mgGAE/g crude extract as shown in Table 1. HWE and CWE showed higher phenolic content than HWPE and CWPE.

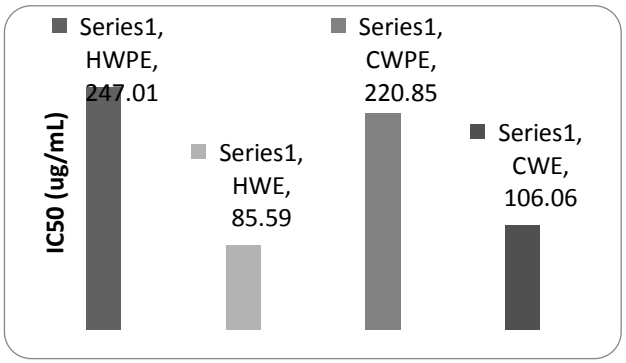

Figure 2. IC_{50} of extracts from *B. colossuss* mycelia by DPPH radical scavenging capacity assay

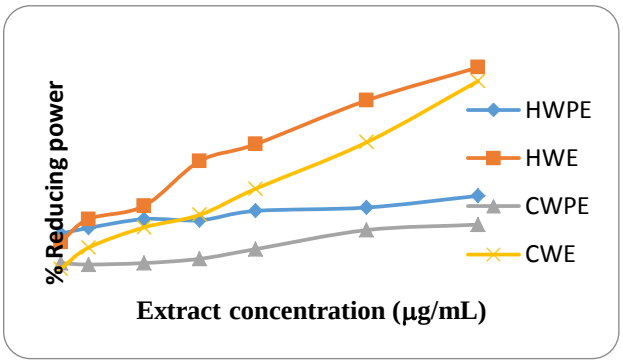

Figure 3. Comparison of reducing power activities of extracts from *B. colossuss* mycelia

CONCLUSIONS

All crude extracts from *B. colossuss* mycelia showed antioxidant activities. The hot water extraction exhibited higher value of total polysaccharide content and antioxidant activity including total phenolic content. Therefore, these results indicated that all extracts from *B. colossum* Heim. mycelia could potentially be used as natural antioxidants.

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REFERENCES

1. Mau JL, Tsai SL, Tseng YH, Huang SJ. 2005. Antioxidant properties of hot water extracts from *Ganoderma tsugae* Murrill. LWT. 38, 589-597.
2. Tsai MC, Song TY, Shih PH, Yen GC. 2007. Antioxidant properties of water-soluble polysaccharides from *Antrodia cinnamomea* in submerged culture. Food Chem. 104, 1115-1122.
3. Song TY and Yen GC. 2002. Antioxidant Properties of *Antrodia camphorate* in Submerged Culture. J. Agric. Food Chem. 50, 3322-3327.
4. Ammar AA, Berhe AA and Ghezzehei TA. 2013. A new method for rapid Determination of carbohydrate and total carbon concentration using UV Spectrophotometry. Carbohydr. Polym. 97, 253-261.
5. Anita K, Maja K, Miomir N, Dragica J, Nina T and Leo VJLD. 2011. Antioxidative activities and chemical characterization of polysaccharides extracted from the basidiomycete *Schizophyllum commune*. LWT-Food Sci. Technol. 44, 2005-2011.
6. Isabel CFRF. Paula B, Miguel V and Lillian B. 2007. Free-radical scavenging capacity and reducing power of wild edible mushrooms from northeast Portugal: Individual cap and stipe activity. Food Chem. 100, 1511-1516.
7. Thetsrimuang C, Khammuang S and Sarnthima.R. 2011. Antioxidant Activity of Crude Polysaccharides from Edible Fresh and Dry Mushroom Fruiting of *Lentinus sp*. Strain RJ-2. Int. J. Pharmacol. 7(1), 58-65.

Comparative study of antioxidant activity and total phenolic content of ethanolic extracts from local medicinal plants in Narathiwat province

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ABSTRACT

The study was to compare the antioxidant activity and total phenolic content of ethanolic extracts from local medicinal plants (Mon-Ngo, *Rhodomyrtus tomentosa*, *Gynochthodes sublanceolata*, *Litsea glutinosa* , and *Molineria latifolia*) in Narathiwat, Thailand. The total phenolic content of the ethanolic extracts were measured by Folin-Ciocalteu method and the antioxidant activity was measured by DPPH radical scavenging assay. The total phenolic contents were found in the range of 29.800 ± 0.117-652.500 ± 5.000 mgGAE/g extract. Mon-Ngo showed the highest of total phenolic content (652.500 ± 5.000 mgGAE/g extract). The IC_{50} values were found in the range of 4.202 ± 0.014-387.140 ± 3.402 µg/mL while compared of the reference standards ascorbic acid and BHT (6.693 ± 0.042 µg/mL and 15.836 ± 0.070 µg/mL). Mon-Ngo showed the lowest of IC_{50} value (4.202 ± 0.014). Mon-Ngo was rich in total phenolic content and good property of antioxidation.

Keywords: Antioxidant Activity, Total Phenolic Content, Ethanolic Extracts, Local Medicinal Plants, Narathiwat

INTRODUCTION

Free radical or reactive oxygen species are causative agents in the etiology of variety of aging and many of diseases [1] including cancer, artherosclerosis, cardiovascular disease, inflammatory diseases [2-4] and neurodegenerative disorders [5]. Antioxidants are molecules which are capacity in habiting oxidative mechanisms that lead chronic diseases [6-7]. Antioxidant are secondary metabolites of plants [8]. The most of antioxidants which are found in plants, vegetables and fruits contain phenolic compounds [9]. Beta-carotene, vitamin C and vitamin E are widely used as antioxidants [10]. Medicinal plants that contain phenolic compounds have been reported to showed good efficiency of antioxidant activity [11-12]. The use of plants and plants products are increasing in population [13] for decreasing the risk various of diseases and health production [14]. The objective of this study was to evaluate the total phenolic content and antioxidant activity of the ethanolic extracts of Mon-Ngo, *Rhodomyrtus tomentosa*, *Gynochthodes sublanceolata*, *Litsea glutinosa*, and *Molineria latifolia* in Narathiwat, Thailand. Because the littles are study or research on local medicinal plants in Narathiwat. The future in this study, it is helpful to promote the uses of local medicinal plants in the food, pharmaceutical and cosmetic. The total phenolic content was determined by Folin-Ciocalteu method [15]. The antioxidant activity was determined by 2,2-Diphenyl-1-picryhydrazyl (DPPH) radical scavenging assay [16-19] which were used ascorbic acid [20] and 2,6-Di-tert-butyl-4-methy-phenol (BHT) [22-23] as a standards.

MATERIALS AND METHODS

Chemicals and Reagents

All the chemicals and reagents were of analytical grade. Folin-Ciocalteu's phenol reagents (Merck, Germany), Gallic acid (Merck, Germany), Sodium carbonate (Merck, Germany), 2,2-Diphenyl-1-picryhydrazyl (DPPH) (Sigma-Aldrich, USA), L-Ascorbic acid (Sigma-Aldrich, USA), 2-6-Di-tert-butyl-4-methyl-phenol (BHT) (Sigma-Aldrich, USA), Absolute ethanol (Avantor performance materials, Malaysia)

Plant materials

All of selected local medicinal plants were listed in Table 1.

Table 1. Scientific names, plants part and location of selected local medicinal plants

Scientific name	Plant parts	Location
Mon-Ngo*	Tubers	Sukhirin, Narathiwat
R. tomentosa	Leaves	Ra-ngae, Narathiwat
G. sublanceolata	Leaves	Yi-ngo, Narathiwat
L. glutinosa	Leaves	Mueang, Narathiwat
M. latifolia	Leaves	Yi-ngo, Narathiwat

* not find Scientific name

Plants extraction

Plant samples (Mon-Ngo, *R. tomentosa*, *G. sublanceolata*, *L. glutinosa*, and *M. latifolia*) were collected during late April to September 2015 from Narathiwat, Thailand. The plant materials were washed, air-dried, dried in a hot air oven at 60 ˚c for 3 days and then coarse powdered by using grinding machine. Some 50 g of coarse powdered plant materials was extracted at room temperature with 250 mL of 95% ethanol under constant shaking for 3 days. The extracts were filtered through Whatman No.1 filter paper and evaporated in rotator evaporator (Heidolph, Schwabach, Germany) to obtain the crude extracts. The ethanolic extracts were kept in freeze at -20 ˚c and dried by freeze dryer (Labcanco, USA). All ethanolic extracts were calculated percentage of yield.

Determination of the total phenolic content

The total phenolic content of all ethanolic extracts were determined using Folin-Ciocalteu colorometric method as described in Hou et al. (2003) [15] with some modifications. Brief, 0.2 mL of sample (2,000 ppm) was mixed with 2.5 mL of distilled water and 0.2 mL of folin-Ciocalteu's phenol reagent. After 3 min, 2.0 mL of 7% (W/V) sodium carbonate was added. The mixture solutions were incubated in the darkness at room temperature for 90 min, after which the absorbance were measured at 750 nm using an UV-Vis spectrophotometer (Madison, WI 53711, USA). The total phenolic content was determined using a calibration curve prepared with gallic acid. The estimation of the total phenolic content was carried out in triplicate. The results were mean ± standard deviations and expressed as milligram of gallic acid equivalent per gram of extract (mg GAE/g extract) were calculate by the following equation:

$$\text{mg GAE/g extract} = \frac{C \cdot V \cdot DF \cdot 10^{-3}}{M}$$

mg GAE/g extract = total phenolic content, C = the concentration of gallic acid solution established from the calibration curve (ppm), V = the volume of extract (mL), M = the weight of ethanolic extract (g extract)

Determination of the DPPH radical scavenging activity

The free radical scavenging activity of the ethanolic extract was determined using DPPH method as describe in Schlesier et al. (2002) [16]. Brief, 0.1 mM solution of DPPH in absolute ethanol was prepared. Different dilutions (0-600 µg/mL) of the extract 500 µL was mixed with 500 µL of DPPH solution. The mixture solution was incubated in darkness at room temperature for 30 min. The change in colour from deep violet to light yellow was then measured at 515 nm using a microplate reader (Biochrome, Cambridge, UK). Ascorbic acid and BHT were used as a standards. All tests were carried out in triplicate. The results were mean ± standard deviation and expressed the percentage scavenging activity of the DPPH free radical was calculated by using the following equation:

$$\%\text{Scavenging activity} = \frac{((\text{Abs}_{\text{control}} - \text{Abs}_{\text{blank}}) - (\text{Abs}_{\text{sample}} - \text{Abs}_{\text{blank}})) \times 100}{\text{Abs}_{\text{control}} - \text{Abs}_{\text{blank}}}$$

Blank = Absolute ethanol (500 µL), Control = Absolute ethanol (500 µL) plus DPPH solution (500 µL, 0.1 mM), Median Inhibition Concentration (IC_{50}) values of samples required to scavenge DPPH radical by 50% was calculated from linear regression.

Statistical analysis

All the measurements were performed in triplicate and data reported as mean ± Standard Deviation (SD). Duncan's New Multiple Range Test (MDRT) was carried out in order to test any significant difference ($p<0.01$) between the ethanolic extracts.

RESULTS AND DISCUSSION

Percentage of yield of ethanolic extracts

The percentage of yield (%yield) of the ethanolic extracts and its color were shown in Table 2. Mon-Ngo (tubers) had the highest %yield (18.46), followed by *L. glutinosa*, *R. tomentosa*, *G. sublanceolata* and *M. latifolia* were found in the range 1.37-2.74.

Table 2. Colour and %yield of ethanolic extracts

Plant samples	Colour of extract	%yield
Mon-Ngo (tubers)	Dark red	18.46
R. tomentosa (leaves)	Light green	1.92
G. sublanceolata (leaves)	Dark brown	2.2
L. glutinosa (leaves)	Dark green	2.74
M. latifolia (leaves)	Dark green	1.37

Total Phenolic Content

A linear calibration curve of gallic acid with R^2 value of 0.9996 was obtained . Mean of total phenolic content of ethanolic extracts (Table 3) measured using the linear regression (Y = 0.005X + 0.0015). The total phenolic content were found in the range of 29.800 ± 0.117-652.500 ± 5.000 mgGAE/g extract . The ethanolic extracts of Mon-Ngo (tubers) contain significantly highest total phenolic content (652.500 ± 5.000 mgGAE/g extract, P-value < 0.01), followed by *R. tomentosa* (leaves, 174.833 ± 1.155 mgGAE/g extract), *L. glutinosa* (leaves, 88.833 ± 1.155 mgGAE/g extract), *M. latifolia* (leaves, 32.983 ± 0.115 mgGAE/g extract) and *G. sublanceolata* (leaves, 29.800 ± 0.117 mgGAE/g extract). Mon-Ngo was rich in total phenolic content.

Table 3. Total Phenolic Content of ethanolic extracts

Plant samples	Total Phenolic Content (mgGAE/g extract)
Mon-Ngo (tubers)	652.500 ± 5.000d
R. tomentosa (leaves)	174.833 ± 1.155c
G. sublanceolata (leaves)	29.800 ± 0.117a
L. glutinosa (leaves)	88.833 ± 1.155b
M. latifolia (leaves)	32.983 ± 0.115a

Values represent Mean ± SD (n = 3), the same letter are not significantly different (P-value < 0.01)

DPPH radical scavenging activity

The median inhibition concentration (IC_{50}) (Table 4) were found in the range of 4.202 ± 0.014-553.817 ± 6.263 µg/mL while the IC_{50} value of the reference standards (ascorbic acid and BHT) were 6.693 ± 0.042 µg/mL and 15.836 ± 0.070 µg/mL, respectively. The ethanolic extracts of Mon-Ngo (tubers) had the lowest IC_{50} (4.202 ± 0.014 µg/ml), followed by *R. tomentosa* (leaves) (18.089 ± 0.039 µg/ml), *L. glutinosa* (leaves, 27.820 ± 0.071 µg/ml), *M. latifolia* (leaves, 140.508 ± 0.316 µg/ml) and *G. sublanceolata* (leaves, 387.140 ± 3.402 µg/ml), respectively. Statistical analysis was found

mean IC_{50} of Mon-Ngo and ascorbic acid not significantly different ($p<0.01$). Mean IC_{50} of *R. tomentosa* and BHT not significantly different ($p<0.01$). Mon-Ngo was demonstrated good property antioxidation.

Table 4. Median Inhibition Concentration (IC_{50}) of ethanolic extracts

Plant samples	IC50 (µg/ml)
Mon-Ngo (tubers)	4.202 ± 0.014a*
R. tomentosa (leaves)	18.089 ± 0.039b*
G. sublanceolata (leaves)	387.140 ± 3.402
L. glutinosa (leaves)	27.820 ± 0.071
M. latifolia (leaves)	140.508 ± 0.316

Values represent Mean ± SD (n = 3), *a : significant difference with ascorbic acid, b: significant difference with BHT (P-value < 0.01).

CONCLUSIONS

The total phenolic contents were found in the range of 29.800 ± 0.117-652.500 ± 5.000 mgGAE/g extract. In DPPH radical scavenging assay, the IC_{50} value were found in the range of 4.202 ± 0.014-387.140 ± 3.402 µg/mL while the IC_{50} value of the reference standards ascorbic acid and BHT were 6.693 ± 0.042 µg/mL and 15.836 ± 0.070 µg/mL. The ethanolic extract, Mon-Ngo (tuber) had the highest total phenolic content (652.500 ± 5.000 mgGAE/g) and lowest IC_{50} value (0.042 µg/mL). Therefore, Mon-Ngo was rich in total phenolic content and demonstrated good property antioxidation.

REFERENCES

1. Alho H, Leinonen J. 1999. Total antioxidant activity measured by chemiluminescence method. Met. Enzymol. 299, 3-15.
2. Chew YL, Lim YY, Omar M, Khoo KS. 2008. Antioxidant activity of three edible seaweeds from two areas in South East Asia. J. Food Sci. Tech. 41(6), 1067-1072.
3. Liao KL, Yin MC. 2000. Individual and combined antioxidant effects of seven phenolic agents in human erythrocyte membrane ghosts and phosphotidylcholine liposome system: Impertance of the partition coefficient. J. Agri. Food Chem. 48, 2266-2270.
4. Franco R, Schoneveld O, Georgakilas AG, Panayiotidis MI. 2008. Oxidative stress, DNA methylation and carcinogenesis cancer letters, 266, 6-11.
5. Auroma OI. 1998. Free radicals, oxidative stress, and antioxidants in human health and disease. J. Am. Oil. Chem. Soc. 75, 199-212.
6. Wong C, Li H, Cheng K, Chen F. 2006. A systematic survey of antioxidant activity of 30 Chinese medicinal plants using the ferric reducing antioxidant power assay. Food. Chem. 97, 705-711.
7. Cardador-Martinez A, Loacra-Pina G, Oomah BD. 2002. Antioxidant activity in common beans (*Phaseolus vulgaris* Li.). J. Agri. Food Chem. 50(24), 6975-6980.
8. Walton NJ, Brown DE. 1999. Chemicals from plants: Perspectives on plant secondary products. Imperial College press: London, UK.
9. Annonymous. 2002. Indian herbal pharmacopoeia, revised new edition, Indian drug manufractures association. Muibai, 79-87.
10. Mccall MR, Frei B. 1999. Can antioxidation vitamins materially reduce oxidative damage in humans? Free Radic. Biol. Med. 26, 1034-1035.
11. Zheng W, Wang SY. 2001. Antioxidant activity and phenolic compounds in selected herb. J. Agri. Food Chem. 49(11), 5165-5170.
12. Brown JE, Rice-Evans CA. 1998. Luteolin-rich artichoke extract protects low density lipoprotein from oxidation *in vitro*. SFRR. 29, 247-255.
13. Eisenberg DM, Ressler RC, Foster C, Norlock FE. 1993. Unconventional medicine in the United State: prevalence, costs and patterns of use. NEJM. 328, 242-252.
14. Conforti F, Sosa S, Marrelli M, Menichini F, Statti GA, Uzunov D, et al. 2009. The protective ability of Mediterranean dietary plants against the oxidative damage: The role of radical oxygen species in inflammation and the polyphenol, flavonoids and sterol contents. J. Agri. Food Chem. 112, 587-594.
15. Hou WC, Lin RD, Cheng KT, Hung YT, Cho CH, Chen CH, Hwang SY, Lee MH. 2003. Free radical – scavenging activity of Taiwanese native plant. Phytomedicine. 10(2/3), 170.

16. Schlesier K, Harwat M, Bohm V, Bitsch R. 2002. Assesment of antioxidant activity by using different in vitro methods. SFRR. 36, 177-187.
17. Kano M, Takayanagi T, Harada K, Makino K, Ishikawa F. 2005. Antioxidative activity of anthocyanins from purple sweet potato *Ipomoera batatas* cultivar Ayamurasaki. Biosci. Biotechnol. Biochem. 69, 979–988.
18. Karioti A, Hadjipavlou-Litina D, Mensah ML, Fleischer TC, Skaltsa H. 2004. Composition and antioxidant activity of the essential oils of *Xylopia aethiopica* (Dun) A. Rich. (Annonaceae) leaves, stem bark, root bark, and fresh and dried fruits, growing in Ghana. J. Agri. Food. Chem. 52, 8094–8098.
19. Mimura T, Yazaki K, Sawaki K, Ozawa T, Kawaguchi M. 2005. Hydroxyl radical scavenging effects of guaiacol used in traditional dental pulp sedation: Reaction kinetic study. JBR. 26, 139–145.
20. Ricci D, Fraternale D, Giamperi L, Bucchini A, Epifano F, Burini G, et al. 2005. Chemical composition, antimicrobial and antioxidant activity of the essential oil of *Teucrium marum* (Lamiaceae). J Ethnopharmacol. 98, 195–200.
21. Mimica-Dukic N, Bozin B, Sokovic M, Simin N. 2004. Antimicrobial and antioxidant activities of *Melissa officinalis* L. (Lamiaceae) essential oil. J. Agri. Food Chem. 52, 2485–2489.
22. Sokmen, M., Serkedjieva, J., Daferera, D., Gulluce, M., Polissiou, M., Tepe, B., et al. (2004). In vitro antioxidant, antimicrobial, and antiviral activities of the essential oil and various extracts from herbal parts and callus cultures of Origanum acutidens. J AGRI FOOD CHEM, 52, 3309–3312.

Bioactivity assessments of *Vitis vinifera* cv. Ribier (Pok Dum) seeds prepared by supercritical CO_2 and ethanol extraction method

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ABSTRACT

Grape seed extracts (GSE) prepared from *Vitis vinifera* cv. Ribier (Pok Dum) is a by-product of the winery and grape juice industry. Therefore, this research aimed to study the method of extraction and bioactivity assessments from GSE. Conventional extraction with 95% ethanol and supercritical fluid carbon dioxide extraction (SCF-CO_2) with 95% ethanol co-solvent were compared. GSE were assessed for their biological activities on anti-oxidant enzyme-like activity (superoxide dismutase, SOD), anti-tyrosinase activity (DOPA-oxidase inhibitory effect) and cytotoxic property (MTT assay). Our results showed that percentage yield of GSE samples extracted by conventional extraction with 95% ethanol (GSECE) at 11.09% and GSE samples extracted by supercritical fluid carbon dioxide extraction (GSESCF) at 12.86%. SOD-like activity result of both GSECE and GSESCF samples were exhibited SOD-like activity at 98.0425% (SD ± 0.0154) and 95.5562% (SD± 0.0285), respectively. The inhibition rate of tyrosinase activity of both GSECE and GSESCF samples were 83.6250% (SD ± 0.7312) and 91.2870% (SD± 0.8541), respectively. Interestingly, the result on MTT assay of both GSECE and GSESCF samples were non-cytotoxic to L929 (mouse fibroblasts), HepG2 (human hepatocellular carcinoma cells) and NHDF (normal human dermal fibroblasts) when tested for 24 hours. Their 50% inhibitory concentration (IC_{50}) values were greater than 2,000 µg/ml.

Keywords: Grape seed extract (GSE), *Vitis vinifera* cv.Ribier (Pok Dum), SCF-CO_2, SOD assay, MTT assay, L929, HepG2, NHDF

INTRODUCTION

Natural food such as vegetables and fruits contain phytochemicals which are antioxidants including vitamins such as vitamim A, vitamim C, vitamin E and selenium, flavonoids and carotenoids. The flavonoids are well known for health-promoting properties. They have been used for prevention of various pathophysiological conditions, including hypertension, atherosclerosis and cancer [1]. One interesting source of antioxidants is grapes. The seeds of grapes are known to be rich in polyphenols that have potent antioxidant activity. The phytochemicals in grape seeds help to protect the heart, inhibit the oxidation of LDL cholesterol, reduce the level of homocysteine. The major active ingredients found in the grape seeds are oligomeric proanthocyanidins which *in vitro* antioxidant activity is greater than vitamin C and vitamin E by 20 and 50 times, respectively. Extracts of grape seeds contain a rich mixture of monomeric flavan-3-ols, phenolic acids and oligomeric proanthocyanidins [2] such as catechin, epicatechin, and procyanidin B2 [3]. The most abundant of these phytocompounds are the oligomeric proanthocyanidins (OPCs). Extensive research suggests that grape seed extract (proanthocyanidins) or OPCs may be beneficial in many areas of health due to their antioxidant effect. Grape seed extract has the ability to bind to collagen and it also promotes youthful skin, cell health, elasticity and flexibility [4]. Supercritical carbon dioxide fluid extraction (SCF-CO_2) method has received much interest in its use and further development for industrial applications [5]. SCF-CO_2 extraction is not only an environmental friendly technology, but also offers some advantages over conventional methods that uses carbon dioxide (CO_2) as a solvent which is non-toxic, nonflammable and non-polluting solvent for the extraction of natural products. Our study was conducted with the aim to investigate anti-oxidant, anti-tyrosinase and cytotoxic properties of grape seed extracts from *Vitis vinifera* cv.Ribier (Pok Dum) prepared by conventional extraction with 95% ethanol and supercritical fluid carbon dioxide extraction (SCF-CO_2) with 95% ethanol co-solvent.

MATERIALS AND METHODS

Sample preparation and extraction

Grape seeds were collected from unutilized or by-products of food processing industry in Nakhon Ratchasima province, Thailand. After drying seeds from *Vitis vinifera cv.* Ribier (Pok Dum) in a hot air oven at 55°C , grape seeds were thoroughly crushed to powder. One hundred grams of grape seed powders were extracted with 95% ethanol conventional extraction. While other one hundred grams of grape seed powders were fed into supercritical carbon dioxide fluid extraction (SCF-CO_2) apparatus.

Figure 1. Dried seeds of *Vitis vinifera cv.* Ribier (Pok Dum)

Determination of antioxidant enzyme-like activity: **superoxide dismutase (SOD)**

SOD activity was measured using SOD assay kit-WST (Sigma Aldrich, Switzerland). The assay was based on the reduction rate of superoxide anion radicals ($O_2^{\bullet-}$) by SOD. The $O_2^{\bullet-}$ were generated in the system by xanthine oxidase (XO) activity. The water-soluble tetrazolium (WST) salt produced a water-soluble formazan dye up on reduction with a superoxide anion. The rate of the reduction with $O_2^{\cdot-}$ was linearly related to the XO activity, and was inhibited by SOD. Therefore, inhibition activity of SOD or SOD-like materials can be determined by a colorimetric method. The WST working solution was prepared by dilution of 1 ml of WST solution with 19 ml of buffer solution. Then, the enzyme solution was prepared by centrifugation of the enzyme tube for 5 sec and mixed well using pipetting. Fifteen (microliter (µl) of enzyme solution was added to 2.5 ml of dilution buffer. The SOD standard was prepared at various concentrations of 100 µg/ml. Sample solution concentration 1mg/ml was added (20 µl) to each well sample and blank of 96-well microplate and mixed with 200 µl of WST working solution and followed by adding 20 µl of enzyme working solution to each sample and blank. The wells were mixed thoroughly and incubated at 37°C for 20 min. After incubation, the absorbance was determined at 440 nm [7]. The SOD activity (% inhibition rate) was calculated by using the following equation.

$$\text{SOD activity (inhibition rate \%)} = \frac{\{[(A_{blank}\ 1 - A_{blank}\ 3) - (A_{sample} - A_{blank}\ 2)]}{(A_{blank}\ 1 - A_{blank}\ 3)\} \times 100}$$

Anti-tyrosinase activity assay

Inhibition of tyrosinase activity was analyzed according to the method of Morita (1994) [6] with some modifications. L-DOPA solution was prepared at 0.32 mg/5ml in 20 mM sodium phosphate buffer (pH 6.8), while mushroom tyrosinase was prepared at 314.8 unit/ml. Tyrosinase enzyme was added to each sample dilution in 96-well microplate, and incubated at room temperature for 10 minutes. Finally, L-DOPA was then added and the plate was left for 10 minutes at room temperature. The absorbance was measured at 492 nm using microplate reader. Kojic acid (0.0142/10 ml dissolved in 20% ethanol) was used for positive control. Tyrosinase inhibition (%) was calculated as following equation:

$$\%\text{Tyrosinase inhibition} = \frac{(A\text{-}B) - (C\text{-}D)}{(A\text{-}B)} \times 100$$

A = absorbance of blank solution with enzyme
B = absorbance of blank solution without enzyme
C = absorbance of sample solution with enzyme
D = absorbance of sample solution without enzyme

Cytotoxic activity assay

The cytotoxic activity of GSE samples was assessed using MTT method against L929, HepG2 and NHDF cell lines. Cells were grown in DMEM medium supplied with 10% fetal bovine serum (FBS) at 37°C in humidified atmosphere containing 5% CO_2. On experimental , cell line at density of $1x10^5$ cells/ml was seeded onto 96 well plate and incubated at 37˚C of 5% CO_2 for 24 hours prior to being treated with various concentrations of GSE samples. Five different concentrations of each GSE sample (4000, 2000, 1000, 500, 250 µg/ml concentrations) dissolved in ethanol (EtOH) were added to cells whereas concurrent vehicle control was included. The treatments were performed for 24 hours and in triplicates. After treatment, the cells were washed with 1x HBSS before adding 200 µl of MTT (1.25 mg/ml^{-1}) to each well. Cells were incubated in dark at 37ºC in humidified incubator containing 5% CO_2 for 3 h. At the end of 3 h incubation time, MTT-containing media was discarded by pipetting. The formazan crystals formed in cells were washed once with 1x HBSS and then dissolved by adding 200 µl of DMSO. The absorbance value was measured at 570 nm by micro-plate reader system [8]. The Cytotoxicity criteria from MTT assay were used to calculate the percentage viability of the cells using following equation**:**

$$\% \text{ Viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of untreated cells}} \times 100$$

A graph of absorbance (y axis) plotted against sample concentration (X-axis) was constructed. The cytotoxicity of the GSFs were presented as 50% inhibitory concentration (IC_{50}), the concentration of test sample required to reduce the absorbance to half (50%) that of the control.

RESULTS AND DISCUSSION

Percentage of yield of GSE

By two methods of extraction, the GSE by SCF-CO_2 extraction with co-solvent gave higher yield (12.86%) than the GSE samples by 95% methanol extraction (11.09%). As shown in **Figure 2.**

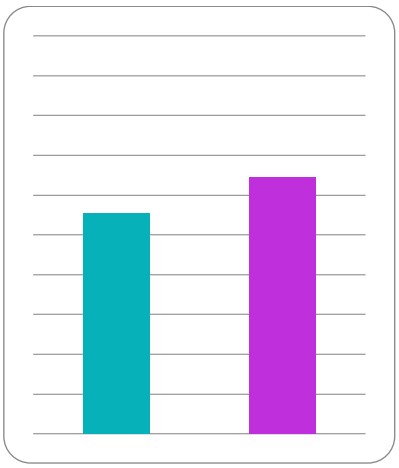

Figure 2. Yield percentage of GSE samples from different extractions.

GSECE = GSE samples extracted by conventional extraction with 95% ethanol
GSESCF = GSE samples extracted by supercritical fluid carbon dioxide extraction

Anti-tyrosinase activity of GSE

GSE samples were assay with spectrophotometer (Tecan Group Ltd, Swizerland) the reading with and without extract were taken at 475 nm. With the help of above given formula percentage
of inhibition was calculated. All the percentage inhibition values were given in the **Table1** below. Inhibition of tyrosinase by kojic acid (positive control). GSE samples presented strong inhibition of tyrosinase with comparable with those of kojic acid (positive control). The greatest inhibition rate of tyrosinase activity was 91.29% found in GSE by SCF-CO_2 extraction with co-solvent. According to results presented in **Table1**.

Table1. Tyrosinase inhibition percentage of GSE samples from different extractions (mean ±SD ,n=3).

Sample	% Inhibition
GSECE	83.625 ± 0.06212
GSESCF	91.287 ± 0.05414
Kojic acid	68.4117 ± 0.0242

GSECE = GSE samples extracted by conventional extraction with 95% ethanol
GSESCF = GSE samples extracted by supercritical fluid carbon dioxide extraction

SOD activity of GSE

The antioxidant activity of GSE samples by using SOD assay, it can be seen that GSE samples prepared by different extractions exhibited varying degrees of antioxidant activity. GSE samples by 95% methanol extraction were found to give the maximum antioxidant activity (97.84%) inhibition. According to results presented in **Table2.**

Table2. SOD inhibition percentage of GSE samples from different extractions (mean ±SD ,n=3).

Sample	% Inhibition
GSECE	97.8406 ± 0.0549
GSESCF	95.5562 ± 0.0285

Cytotoxicity of GSE

GSE samples were non-cytotoxic to L929 (mouse fibroblasts), NHDF (normal human dermal fibroblasts) and HepG2 (human hepatocellular carcinoma cells) in wide range of concentrations from 250 to 4000 µg/ml when tested for 24 hours using the MTT assay comparable with positive control (Mitomycin C, MMC). The most effective cell growth inhibition activity and extremely low IC50 were observed in L929, NHDF and HepG2 by GSE samples, with IC50 values ranging from 2,100 to 2,500 µg/ml, 3,300 to 3,500 µg/ml and 2,600 to 3,700 µg/ml respectively. According to results presented in **Table3.**

Table 3. Cytotoxicity of GSE samples from different extractions (mean ±SD ,n=3).

Sample	Cell lines (IC_{50}µg/ml)		
	L929	NHDF	HepG2
GSECE	2175.42 ± 0.10	3359.73 ± 0.12	3783.83 ± 0.06
GSESCF	2568.21 ± 0.11	3588.20 ± 0.03	2653.87 ± 0.06
MMC	1.59 ± 0.13	1.08 ± 0.10	1.47 ± 0.08

CONCLUSIONS

In view of the extensive use of GSE in human dietary supplements, it is of great importance to investigate their bioactivity including anti-oxidant, anti-tyrosinase and cytotoxic activities. Our current results demonstrated that GSE samples extracted by conventional extraction with 95% ethanol and GSE samples extracted by supercritical fluid carbon dioxide extraction with co-solvent of Thai grape *Vitis vinifera* cv.Ribier (Pok Dum) seeds possessed these pharmacological activities and non-cytotoxic to L929, HepG2 and NHDF cell lines when tested by MTT assay. Therefore, these GSE may be a good natural ingredient for development of food supplement and cosmetic products.

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REFERENCES

1. Rice-Evans CA, Miller N, Paganga G. 1997. Antioxidant properties of phenolic compounds. Trends Plant Sci. 2(4), 152 -159.
2. Gonzalez-Manzano S, Rivas-Gonzalo JC, Santos-Buelga C. 2004. Extraction of flavan-3-ols from grape seed and skin into wine using simulated maceration. Anal. Chim. Acta. 513(1), 283–289.
3. Guo I, Xu L. 1996. Procyanidin: a plant medicine with many uses. World Phytomed. 11(5), 196–204.
4. Fine AM. 2000. Oligomeric proanthocyanidins complexs: history, structure and phytopharmaceutical applications. Altern. Med. Rev. 5(2), 144- 151.
5. Masamoto Y, Iida S, Kubo M. 1980. Inhibitory effect of Chinese crude drugs on tyrosinase. Plant Med. 40, 361-365.
6. Morita H, Kayashita T, Kobata H, Gonda A, Yakeya K, Itokawa H. 1994. Pseudostellalin A-C, new tyrosinase inhibitory cyclic peptides from Pseudostellaria heterophylla. Tetrahedron. 50, 6797-6804.
7. Comhair A, Erzurum C. 2010. Redox control of asthma: molecular mechanisms and therapeutic opportunities. Antioxid. Redox Signal. 12, 93-124.
8. Sieuwerts AM, Klijn JG, Peters HA, Foekens JA. 1995. The MTT tetrazolium salt assay scrutinized: how to use this assay reliably to measure metabolic activity of cell cultures *in vitro* for the assessment of growth characteristics, IC50-values and cell survival. Eur. J. Clin. Chem. Clin. Biochem. 33(11), 813-23.

Cytotoxic, cyto-protective and apoptotic activities of ethanolic extract of Thai indigenous mushroom *Russula alboareolata*

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ABSTRACT

Russula alboareolata Hongo (Russulaceae family) is Thai indigenous mushroom which is mostly found in the northeastern region. It has been consumed as foods and used in the treatments of various diseases. Although many benefits have been claimed, few scientific reports are available in the literature. In this study, we evaluated the bioactivities of the *Russula alboareolata* (*R alboareolata*) ethanolic extract. The results indicated a slight harmful property of *R alboareolata* extract when tested using water-soluble tetrazolium salt (WST-1) assay against L929 mouse fibroblast (IC_{50} = 634.97 ± 105.97 µg/ml), HepG2 human hepatocellular carcinoma (IC_{50} = 1099.65 ± 76.74 µg/ml), MCF-7 human breast adenocarcinoma (IC_{50} = 652.86 ± 58.59 µg/ml) and HeLa human cervical carcinoma (IC_{50} = 506.33 ± 20.45 µg/ml) cells. Interestingly, the *R alboareolata* extract enhanced the cell survival in mitomycin C (MMC, 10 µg/ml) treated-mouse macrophage RAW 264.7 cells (ATCC TIB-71) by 10% when tested at100 µg/ml. The apoptotic activity was evaluated at various *R alboareolata* extract concentrations on L929, HeLa and HepG2 cells using the mitochondrial membrane potential (MMP) assay by a double-field fluorescence JC-1 and Hoechst 3342 staining and analysed by Cell Imaging Analyser (INCell 2200, GE Healthcare, UK). Valinomycin (1µg/ml) was used as a positive control. A marked decrease in the red/green fluorescent ratio indicated apoptotic property. Following 24-hr treatment, results showed that valinomycin clearly induced apoptosis in L929, HeLa and HepG2 cells at 43.86, 36.85 and 40.00%, respectively. Similar apoptotic activity was observed at 77.20, 73.69 and 30.00% in L929, HeLa and HepG2 when respectively exposed to *R alboareolata* extract at 600, 500 and 1,000 µg/ml. The data of our study suggest that *R alboareolata* extract possessing a slight cytotoxic, cyto-protective and apoptotic properties when evaluated under specified experimental conditions in L929, HeLa , HepG2 and RAW 264.7cell lines.

Keywords: *Russula alboareolata*, cytotoxicity, cyto-protectivity, apoptosis, L929, HeLa, HepG2, MCF-7, RAW 264.7 cells

INTRODUCTION

Russula mushrooms are belonging to the family of Russulaceae that are ectomycorrhizal families in the order Agaricalse [1]. Their shapes resemble of an umbrella. There are clear cap and stem with the gills underneath the cap. The mushroom is fresh, soft, fragile and perishable. It was reported that there are around 750 worldwide species of *Russula* [2]. The distributions of *Russula* species are in the several countries including the United States of America, Sweden, France, Norway, Madagascar, Italy, Belgium, Taiwan, China, Japan and Thailand [3]. Some *Russula* mushrooms have an established histories of the uses in traditional medicines for the treatments of various diseases such as *Russula cyanoantha* and *Russula nobilis* for treatment of fever, *Russula luteotacta* for wound healing, *Russula delica* and *Russula parazurea* for the treatments of gastritis and high blood pressure, *Russula acrifolia* has been used for treatments of skin cancer and *Russula luteotacta* has been used as sleep promoting agent [4]. In Thailand, *Russula* mushrooms were found in 17 provinces of the northeastern part [1,5] and some of them have been used for cooking and treatments of various diseases for a long time [6]. However, there are still some *Russula* mushrooms of Thailand those have never been studied on biological properties. Therefore, we carried out this study focusing on *Russula alboareolata* (in Thai "เห็ดขาวดิน-ขาวแป้ง , น้ำแป้ง") with the aim to evaluate its biological properties including cytotoxic, cyto-protective and apoptotic activities on normal and cancer cell lines. The information obtained from this study can be new scientific data to support the

ethnomedical usages and/or for development of beneficial health supplement products from *R alboareolata* in the future.

MATERIALS AND METHODS

Mushroom samples

Fresh samples of *R alboareolata* (Figure 1.) were collected from natural habitat in rainy season during August-October 2013-2014 from three provinces including Kalasin, Mukdahan and Yasothon in the Northeastern part of Thailand**.** The physical characteristic of the mushroom was identified by the mushroom specialist, Mr.Winai Klinhom of Medicinal Mushroom Museum, Faculty of Science, Mahasarakham University, Mahasarakham, Thailand.

Figure 1. Morphology of *Russula alboareolata* Hongo

Extraction of mushroom

Collected *R alboareolata* mushrooms were dried in hot air oven at 50˚C for 18-20 hours and ground using an electric grinder. Dried samples were macerated in 95% ethanol (plant: solvent ratio 1:10 w/v) with the sonication for 1 hour. The ethanolic extract solutions were filtered and evaporated using the rotary evaporator to yield ten dried *R alboareolata* mushroom crude extract. The crude extract was stored in darkness at -20˚C until utilization.

Cell culture and maintenance

All cell lines used in this study were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). They were grown as adherent in Dulbecco's Modified Eagle Medium (DMEM, GIBCO®) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS, GIBCO®) and 1% (v/v) penicillin-streptomycin (GIBCO®). The cells were propagated in tissue culture flask (Corning®) at 37ºC in humidified atmosphere incubator with 5% CO_2, sub-culturing every 2-3 days by trypsinization with 1 to 2 ml of 0.25 % trypsin-EDTA to allow detachment of cells prior to adding of fresh culture medium. Then, cell suspension was aspirated and dispensed into new culture flasks. On experimental day, cells were harvested by trypsinization as explained before in Hank's balanced salt solution (HBSS, GIBCO®) or phosphate buffered saline (PBS, GIBCO®). They were plated in 96 well-plates at a density of 1 x 10^5cells/well and incubated for 24 hours before treatments.

Determination of cytotoxic activity of extract

The cytotoxic property of *R alboareolata* extract was evaluated by using 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1, 3-benzene disulfonate (WST) assay (BioVision, Milpitas, CA, USA). Its principle is based upon a conversion of tetrazolium salt to soluble formazan crystals by succinate dehydrogenase in the mitochondria of metabolically active cells. Hence in the dead cells, this reaction will not be occurred. In our study, the cytotoxic activity of *R alboareolata* extract was performed on normal cell line i.e. mouse fibroblasts (L929, ATCC® CCL-1) and three transformed or cancer cell lines including human hepatocellular carcinoma (HepG2, ATCC®HB-8065), human breast adenocarcinoma (MCF-7, ATCC®HTB-22™) and human cervical carcinoma (HeLa, HeLa,ATCC®CCL-2) cells.

Each cell line at density of $1x10^5$ cells/ml was seeded onto 96 well-plate and incubated at 37˚C of 5% CO_2 for 24 hours prior to being treated with various concentrations of *R alboareolata* extract for 24 hours. By the end of treatment, 100 µl of WST solution was added to cells in each well. The plates were kept in darkness for 30 mins before measuring absorbance at 450 nm by the microplate reader system. Values of the three independent experiments obtained from WST assay were used to calculate the percentage viability of the cells using the equation demonstrated below. A graph of absorbance (Y-axis) plotted against sample concentration (X- axis) was constructed. The cytotoxicity of *R alboareolata* extract was presented as 50% inhibitory concentration (IC_{50}), the concentration of test sample required to reduce the absorbance to half (50%) that of the negative control.

$$\% \text{ Viability} = \frac{\text{Absorbance of treated cells (with extract)}}{\text{Absorbance of untreated cells (without extract)}} \times 100$$

Determination of cyto-protective activity of extract against MMC toxicity

The cyto-protective activity of *R alboareolata* extract was carried out on the mouse monocyte macrophage RAW 264.7 (ATCC TIB-71™). Prior to experimentation, the toxicity of *R alboareolata* extract on this cell line was evaluated at 125, 250, 500, 1,000 and 2,000 µg/ml concentrations using the WST assay described above. The extract concentration that yielded % cell viability greater than 80% was selected for cyto-protective activity study. For experimentation, 100 µl of RAW 264.7 cells at density of $1x10^5$ cells/ml were seeded onto 96 well-plate and incubated at 37˚C of 5% CO_2 for 24 hours. The standard mutagen mitomycin C (MMC) at 10 µg/ml concentration was used as cytotoxic-inducer. Overnight culture medium was removed from the plate and then replaced with 200 µl of *R alboareolata* at 125 µg/ml in the presence of MMC for 24 hours incubation. By the end of treatment time, cell viability was assessed as previously described for WST assay. Data were expressed as percentage of cell viability of untreated control (untreated) cells *versus* treated cells (in presence of extract, extract + MMC and MMC alone).

Determination of cell apoptotic activity of extract by mitochondrial membrane potential (MMP) assay

Apoptotic activity of *R alboareolata* extract was determined by MMP assay using JC-1 fluorescent method. The L929, HeLa and HepG2 cells at density of $1x10^5$ cells/ml were seeded onto 24 well plate and incubated at 37˚C of 5% CO_2 for 24 hours. Media were removed and replaced with 1,000 µl of fresh media containing extract at 600, 500 and 1,000 µg/ml for L929, HeLa and HepG2, respectively. Cells were further incubated at 37˚C for 24 hours. Valinomycin (1µg/ml) was used as apoptotic inducer and kept as a positive control. Unlike treatments of extract, the treatment of valinomycin was performed for 2 hours due to its high toxicity. Following treatment period, cells were harvested by trypsinization and collected by centrifugation at 1,500 rpm for 4 min at 4˚C. Cells were transferred to 1.5 ml microcentrifuge tubes and then stained with JC-1 reagent as procedure described by supplier (Sigma Chemicals, St.Louis, MO, USA). Apoptotic cells of L929, HeLa and HepG2 were analyzed by fluorescent imaging system using the InCell 2200 Analyser (GE Healthcare, UK) with Cy3 (excitation 542/ emission 697) and FITC (excitation 475/ emission 525) filters. For a precision of apoptotic cell analysis, the dye Hoechst 3342 (Sigma Chemicals, St. Louis, MO, USA) was utilized as a co-nuclear (DNA) counterstain.

RESULTS AND DISCUSSION

Cytotoxicity of *R alboareolata* extract

Results obtained from three independent experiments demonstrated the IC_{50} values of *R alboareolata* extract at 634.97 ± 105.97, 506.33 ± 20.45, 1099.65 ± 76.74 and 652.86 ± 58.59 µg/ml for L929, HeLa, HepG2 and MCF-7 cells, respectively. Regarding the classification of the cytotoxicity for natural ingredients described by Farshad H. Shirazi (2004) [7], *R alboareolata* extract was potentially harmful (100 µg/ml < IC_{50} < 1,000 µg/ml) to L929, HeLa and MCF-7 cells and was potentially non-toxic (IC_{50}>1,000 µg/ml) to HepG2 cells.

Cyto-protectivity of *R alboareolata* extract

Before cell protection against MMC was undertake on RAW 264.7 mouse monocyte macrophage cells, the initial cytoxicity screening of *R alboareolata* extract was examined by using WST-1 assay. The cytotoxicity results were illustrated in Table 1.

Table 1. Cytoxicity screening of *R alboareolata* extract on RAW 264.7 cells evaluated by WST-1 assay.

R. alboareolata extract Concentration (µg/ml)	Cell viability (%)
125	85.18
250	65.39
500	59.68
1,000	43.53
2,000	36.33

As reported in Table 1, treatment of *R alboareolata* extract at various concentrations ranging from 125 to 2,000 µg/ml produced cell toxicity in a dose-dependent manner. Therefore, to avoid cytotoxicity effect caused by the extract, the lowest dose at 125 µg/ml was chosen for cyto-protective activity study.

Summary of results on cyto-protective activity of *R alboareolata* extract against MMC treatment in RAW 264.7 cells for 24 hours was shown in Table 2. It was found that the sole treatment of *R alboareolata* extract at 125 µg/ml did not cause the toxicity to cells (% cell viability = 90.84 ± 3.00). In contrary, treatment of MMC at 10 µg/ml resulted in a reduction of % cell viability of RAW 264.7 cells to 22.49 ± 0.18 (Table 2.). It was clearly shown that cytotoxic effect of MMC was attenuated in the presence of *R alboareolata* extract. This was evident by an increase in cell survival rate from 22.49 ± 0.18 to 32.53 ± 3.00 % (Table 2.). The results suggest a cyto-protective ability of *R alboareolata* extract against MMC induced cell death.

Table 2. Percent viability of RAW 264.7 cells following treatments with *R. alboareolata* extract (125 µg/ml), mitomycin C (MMC, 10 µg/ml) and a combination of *R. alboareolata* extract and MMC was determined by WST-1 assay.

Treatment	* % Cell viability
Untreated	100
R. alboareolata extract	90.84 ± 3.00
Mitomycin C (MMC)	22.49 ± 0.18
R. alboareolata + MMC	32.53 ± 3.00

* Data were expressed as mean ± SD of tripicate experiments.

Apoptotic activity of *R alboareolata* extract

Apoptosis is the pharmacodynamics endpoint of anticancer drug therapy as this phenomenon ensures that no cancer resistance to chemotherapy will occur [8]. Moreover, apoptosis is an autonomous dismantled process to remove individual components of cells and avoids inflammatory effect normally associated with necrosis; thus no toxicity to the normal surrounding cells will occur when cells undergo apoptosis [8,9]. To investigate whether the cytotoxic effect of the *R. alboareolata* extract was due to apoptosis, we treated the L929 (non-transformed cell line) and HeLa and HepG2 (transformed or cancerous cell lines) at their IC_{50} concentrations for 24 hours.

In this study, we used the mitochondrial membrane potential (MMP) assay to evaluate the apoptotic activity of the *R. alboareolata* extract. This mitochondrial disruption includes changes in the membrane potential and alterations to the oxidation–reduction potential of the mitochondria. The alterations in the membrane potential are presumed to be due to the opening of the mitochondrial permeability transition pore (MPTP), allowing passage of ions and small molecules. Probes that detect MMP are positively charged, causing them to accumulate in the electronegative interior of the mitochondrion. Changes in the MMP can be measured by a variety of fluorescent techniques whereas in our study the membrane-permeant JC-1 dye was employed. Following JC-1 staining, cells were analyzed by Cell Imaging Analyser (InCell® 2200, GE Healthcare, UK) using the green (FITC, excitation 475/emission 525) and red (Cy3, excitation 542/ emission 697) filters.

It was reported that JC-1 dye is more specific for mitochondrial versus plasma membrane potential and more consistent in its response to depolarization than some other cationic dyes such as DiOC6(3) and rhodamine 123 [10]. Therefore, JC-1 dye is widely used in apoptosis studies to monitor mitochondrial health [11]. The dye exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~529 nm) to red (~590 nm). Consequently, mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. The potential-sensitive color shift is due to concentration-dependent formation of red fluorescent J-aggregates. Regarding this principle, we demonstrated images of apoptotic activity induction by *R. alboareolata* extract on L929 (mouse fibroblasts), HeLa (human cervical carcinoma) and HepG2 (human hepatocellular carcinoma) cells in Figure 2.

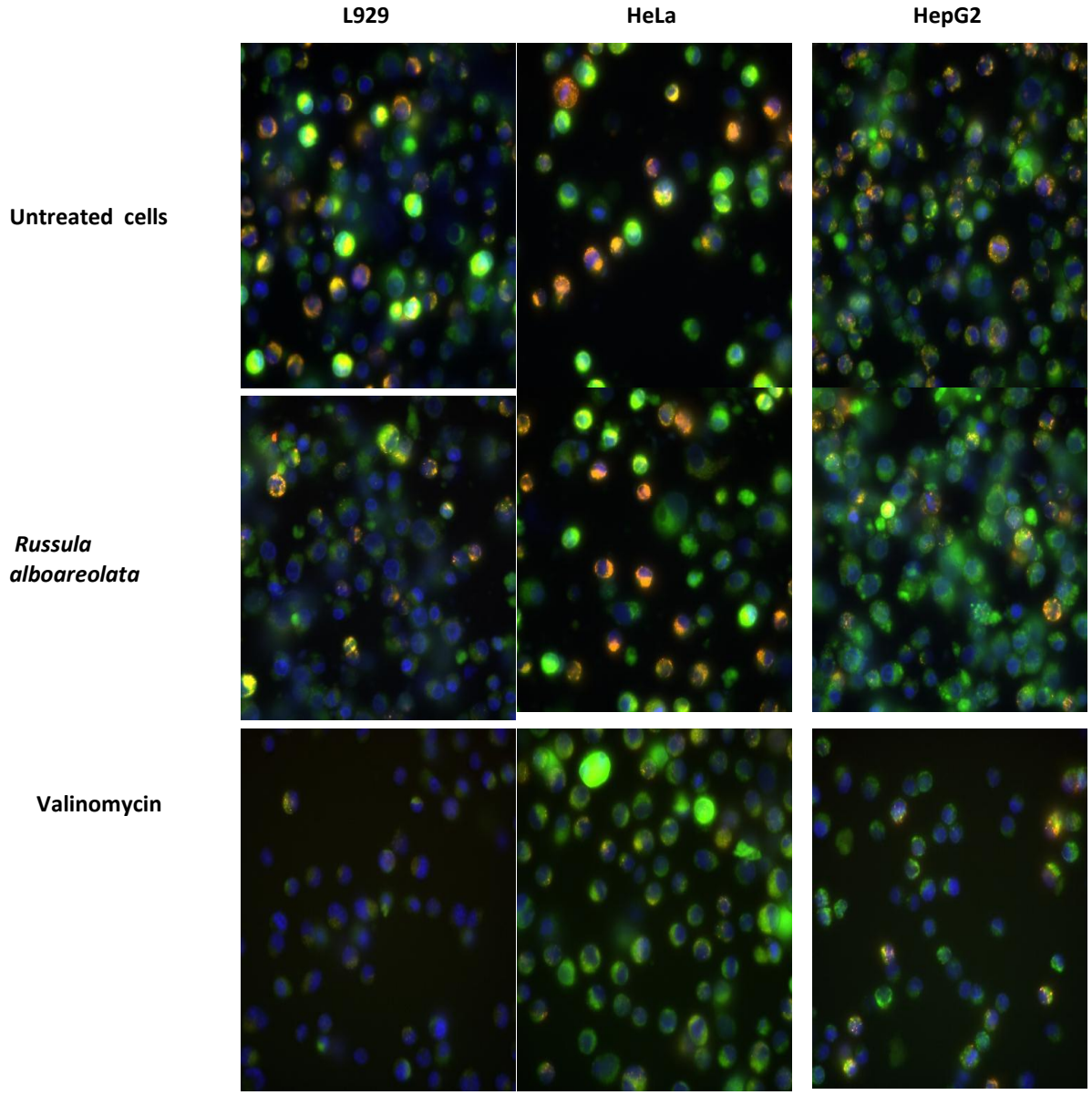

Figure 2. Effect of *Russula. alboareolata* extract on mitochondrial membrane potential (MMP) alterations occurred in L929, HeLa and HepG2 following treatment for 24 hours at concentrations of 600, 500 and 1,000 µg/ml extract, respectively. Valinomycin (positive control) was tested at 1 µg/ml for 2 hours. Images of cells were analyzed by JC-1 and Hoechst 3342 dye staining.

Regarding results demonstrated in Figure 2, L929, HeLa and HepG2 cells were also stained with Hoechst 3342 dye. Hoechst 3342 is a part of a family of blue fluorescent dyes used commonly to stain DNA in cells [12-13]. Our aim of staining cells with JC-1 and Hoechst 3342 for apoptotic cells analysis since JC-1 dye will stain on cell membrane whereas Hoechst 3342 stain for nuclear DNA. Therefore, the location and number of individual cells were clearly identified during cell analysis/scoring. In this study, at least 1,000 cells were scored for each cell line and treatment.

Table 3. exhibited a summary of ratio of red to green fluorescence, % mitochondria health and % apoptosis obtained from each cell line and treatment. The ratio depends only on the membrane potential and not on other factors such as mitochondrial size, shape, and density, which may influence single-component fluorescence signals. Use of fluorescence ratio detection, therefore allows us to make comparative measurements of membrane potential and

determine the percentage of mitochondria (% mitochondria health) within L929, HeLa and HepG2 cells that respond to *R. alboareolata* extract (Table 3).

Table 3. Summary of apoptotic effects on L929, HeLa and HepG2 cells after treatments with valinomycin and *R. alboareolata* extract mitochondrial membrane potential (MMP) alteration and JC-1 stain.

Sample	Fluorescent Intensity (Red/Green)			% Mitochondria health			% Apoptosis		
	L929	HeLa	HepG2	L929	HeLa	HepG2	L929	HeLa	HepG2
Untreated	0.57	0.19	0.10	100.00	100.00	100.00	-	-	-
Valinomycin	0.32	0.12	0.06	56.14	63.15	60.00	43.86	36.85	40.00
Russula alboareolata	0.13	0.05	0.07	22.80	36.31	70.00	77.20	73.69	30.00

Note: Valinomycin treatment performed at concentration of 1 µg/ml for 2 hours.
Russula alboareolata extract treatments performed at 500, 600 and 1,000 µg/ml for HeLa, L929 and HepG2 cells, respectively and for 24 hours.

Following treatments, HeLa, L929 and HepG2 cells were co-stained with Hoechst 33342 nucleic acid dye. Hoechst 33342 is a popular cell-permeant nuclear counterstain that emits blue fluorescence when bound to dsDNA. Therefore in this study, this dye was employed to distinguish condensed pycnotic nuclei in apoptotic cells of L929, HeLa and HepG2. Regarding data of % apoptosis (Table 3), we found that valinomycin (1µg/ml) clearly induced apoptosis in L929, HeLa and HepG2 cells as indicated by a marked decrease in the red/green fluorescence ratio to 0.32, 0.12 and 0.06, respectively. The percentages of mitochondria (% mitochondria health) of the three tested cell lines were also illustrated and they were used to calculate % apoptosis of each cell lines and treatment as expressed in Table 4. Based on these results, *R. alboareolata* extract possesses apoptotic activity against both normal (L929) and cancerous cells (HeLa and HepG2). However, we were unable to compare the degree of apoptotic induction by *R. alboareolata* extract among these three cell lines due to the different concentrations used.

CONCLUSIONS

The ethanolic extract of *Russula alboareolata* mushroom was slightly harmful to L929 (IC_{50} = 634.97 ± 105.97), HeLa (IC_{50} = 506.33 ± 20.45) and MCF-7 (IC_{50} = 652.86 ± 58.59 µg/ml) cells and was potentially non-toxic to HepG2 (IC_{50} = 1099.65 ± 76.74). When tested at125 µg/ml, it exhibited cyto-protective effect against MMC-induced cell death in RAW 264.7 cells. The apoptotic effect of *R alboareolata* extract was seen in both the normal (L929) and cancer cells (HeLa and HepG2) when evaluated by mitochondrial membrane potential (MMP) assay. Further studies including active constituents and *in vivo* experiments are required if this mushroom will be used as a novel dietary supplement and/or botanical-drug for chemoprevention.

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REFERENCES

1. Neha J, Veena P. 2013.Diversity analysis of ectomycorrhizal genus *Russula* using RAPD markers. Octa J. Env. Res.1, 332-335.
2. Joshi S BR, Stephenson SL. 2012. The current status of the family *Russulaceae* in the Uttarakhand Himalaya, India. Mycosphere. 3, 486-501.
3. Po-Tsang Lee M, Ming-Ling Wu, Wei-Jen Tsai, Jiin Ger, Jou-Fang Deng, Hsiao-Min Chung. 2001. Rhabdomyolysis: an unusual feature with mushroom poisoning. Am. J. Kidney Dis. 38, 1-5.
4. กลิ่นหอม วินัย, กลิ่นหอม อุษา. 2554. เห็ดเป็นอาหารเพื่อสุขภาพตามภูมิปัญญาของหมอพื้นบ้าน.พิมพ์ครั้งที่ 1.กรุงเทพมหานคร; โรงพิมพ์องค์การสงเคราะห์ทหารผ่านศึกษา.
5. Manassila M, Boonkerd N, Rodtongb S, Teaumroonga N. 2005. Phylogenetic diversity of wild edible *Russula* from Northeastern Thailand on the basis of internal transcribed spacer sequence. Sci. Asia.3, 323-328.
6. กลิ่นหอม วินัย, กลิ่นหอม อุษา. 2551. 57 เห็ดเป็นยาแห่งป่าอีสาน.พิมพ์ครั้งที่ 1.กรุงเทพมหานคร; มูลนิธิสุขภาพไทย.
7. Shirazi FH. Si, Ahmadi N, Kamalinejad M. 2004. Evaluation of northern Iran *Mentha pulegium l.* cytotoxicity. DARU.12, 106-110.
8. Gallardo-Escárate C, Álvarez-Borrego J, Brand EV, Dupré E, Río-Portilla MA. 2007. Relationship between DAPI-fluorescence fading and nuclear DNA content: An alternative method to DNA quantification. Biol. Res. 40, 29-40.
9. Elmore S. 2007. Apoptosis: a review of programmed cell death. Toxicol. Pathol. 35, 495-516.
10. De Proost I, Pintelon I, Brouns I, Kroese ABA, Riccardi D, Kemp PJ, et al. 2008. Functional live cell imaging of the pulmonary neuroepithelial body microenvironment. Am. J. Respir. Cell Mol. Biol. 39, 180-189.
11. Perry SW, Perry J, Norman P, Barbieri J, Brown EB, Gelbard HA. 2011. Mitochondrial membrane potential probes and the proton gradient: a practical usage guide. Biotechniques. 50, 98–115.
12. Latt SA, Stetten G, Juergens LA, Willard HF, Scher CD. 1975. Recent developments in the detection of deoxyribonucleic acid synthesis by 33258 Hoechst fluorescence. J. Histochem. Cytochem. 23, 493–505.
13. Latt, SA, Stetten, G. 1976. Spectral studies on 33258 Hoechst and related bisbenzimidazole dyes useful for fluorescent detection of deoxyribonucleic acid synthesis. J. Histochem. Cytochem. 24, 24–33.

The development of *Phyllanthus emblica* and *Zanthoxylum limonella* feminine hygiene wash

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ABSTRACT

The purpose of this study was to develop an external feminine hygiene wash comprising the patented mix-extract from fruits of *Phyllanthus emblica* and *Zanthoxylum limonella* to benefit from its anti-oxidant, anti-inflammatory, anti-tyrosinase and anti-microbial effects. The feminine hygiene wash was formulated in 4 formulas and the mix-extract was added to the selected formula. The product was clear yellow solution. It had suitable viscosity and when rubbed with water gave smooth and fine foam. The product stability was tested using heating and cooling method at 45 ^{o}C 24 hours and 4 ^{o}C for 24 hours for 6 cycles. The feminine hygiene wash was active against *Staphylococcus aureus* (DMST 8013 and DMST 8840), *S. epidermidis* (DMST 12228), *Streptococcus pyogenes* (DMST 17020) and *Candida albicans* (DMST 90028 and DMST 10231) using tissue disc agar diffusion method.

Keywords: Feminine hygiene wash, *Phyllanthus emblica*, *Zanthoxylum limonella*

INTRODUCTION

The external feminine hygiene wash is a mild and non-irritant personal care product for intimate area, which should have pH 3.8 -4.5 as normal vaginal pH [1]. An external feminine hygiene wash comprising the mix-extract from fruits of *Phyllanthus emblica* and *Zanthoxylum limonella* was developed to benefit from the mix-extract's anti-oxidant, anti-inflammatory anti-tyrosinase and anti-microbial effectiveness.

Phyllanthus emblica L. (EUPHORBIACEAE) is known as 'Ma-Kham Pom' in Thailand. Its edible fruits are rich in Vitamin C and contain tannins, phyllembic acid, gallic acid, ellagic acid, trigalloylglucose, terchebin, corilagan and emblicol . The ethanolic extract form fruits of *P. emblica* have antimicrobial activity against gram positive and gram negative bacteria together with fungal strains including *Candida albicans*. The extract was analgesic, anti-inflammatory and anti-diarrheal [2].

Zanthoxylum limonella Alston (RUTACEAE) is known as 'Ma-Khwaen' in Thailand. Its edible fruits are used as spice in northern Thai food. The fruit contains alkaloids, tannins, flavonoids, phenolic compounds, steroids, fixed oil, volatile oil and glycosides. It has antibacterial and antioxidant activities [3-5].

The patented mix fruit extract of *P. emblica* and *Z. limonella* was proved to be anti-oxidant, anti-inflammatory anti-tyrosinase and anti-microbial effective [6, 7]. The developed feminine hygiene wash comprising the mix-extract was assessed for stability under heating and cooling method at 45 ^{o}C 24 hours and 4 ^{o}C for 24 hours for 6 cycles; and for antimicrobial effectiveness using a tissue disc agar diffusion method.

MATERIALS AND METHODS

Plant materials

Fruits of *P. emblica* and *Z. limonella* were collected, dried and powdered by the Agricultural Technology Department of Thailand Institute of Scientific and Technological Research (TISTR).

Preparation of the extracts

P. emblica extract was prepared by macerated 500 g of the fruit powder with ethanol-water for 4 nights, filtered through Whatman paper No.41 and rinsed with the same solvent. The solvent was removed under reduced pressure using a rotary evaporator at 45 °C. *Z. limonella* was extracted with another proportion of ethanol-water for 3 nights, filtered, rinsed and evaporated under reduced pressure. The crude extracts were freshly mixed at the patented appropriate ratio to be effective against tested microbial.

Formulation of the external feminine hygiene wash

Four different formulas of the feminine hygiene wash for external use were developed from a master formula (as shown in Table 1.). The mix-extract was added to the selected formula to make formula 5 as the following method. Solutions A and B and C were separately prepared before mixing together. Solution A was allantoin heated at 60-70 ˚C until clear, and then added sodium laureth sulfate to mix. Solution B was the mixture of cocamidopropyl- betaine, propylene glycol, PEG-7, phenoxy ethanol and lactic acid. Solution C was the solution in water of the patented mix-extract of *P. emblica* and *Z. limonella*. Thoroughly mixed solution B and C, and then added the mixture into solution A. Observed the pH and viscosity of the product. The viscosity was measured using Brookfield viscometer (DV-II+Pro).

Table 1. Master formula of the patented feminine hygiene wash

Ingredients	Function	Master formula
		% w/w
Allantoin	Natural soothing,antiirritant, skin protectant	0.1-1
Phenoxy ethanol	Preservative	0.5-2
Lactic acid	Natural antiseptic	0.5-2
Propylene glycol	Cosolvent, Humectant	1-3
PEG-7	Cosolvent	1-3
Cocamidopropyl- Betaine	Amphoteric surfactant	5-15
Sodium laureth sulfate	Anionic Surfactant	20-30
Sodium chloride	Thickening adjustment	q.s.
Fragrance	Flavoring agent	q.s.
RO Water q.s. to	Vehicle	100

Stability test

The heating and cooling test was used for stability assessment of the feminine hygiene wash. The product was kept in a hot air oven at 45°C for 24 hrs and turned to 4°C for 24 hrs in a refrigerator for 6 cycles, and then observed the appearance, viscosity and pH of the product.

***In vitro* anti-microbial assessment of the product:**

A tissue disc agar diffusion assay was performed against *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus pyogenes and Candida albicans.* Stock of microorganism was prepared by cultivation on agar, and then separated to sterile water and adjusted to the concentration of 0.5 McFarland. The microorganism was distributed evenly over the nutrient agar surface in a Petri dish and let dry for a while in aseptic condition before placing tissue paper discs of 1-cm diam. on the agar culture. Sample solution was prepared by stirring 1-2 g of the feminine hygiene wash with water (1:1) for 1 min. 20 µl of sample was pipette onto the tissue disc for 1-min contact before removing the tissue off. Incubated at 37 ˚C for 18-24 hrs, and then observed the clear zones of inhibition.

RESULTS

The developed feminine hygiene washes were clear discolored solution of pH 4. They had the smell of lactic acid. Formula 2 provided rougher foam than the others when rubbed with water. Formula 3 was selected according to its smooth and fine foam with suitable texture. The mix-extract from fruits of *P. emblica* and *Z. limonella* was added to the selected formula to make formula 5, which was clear yellow solution of pH 4 (as shown in Figure 1).

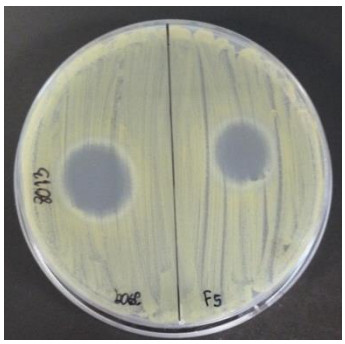
Figure 1. Feminine hygiene wash products formula 3 (F3) and formula 5 (F5)

The appearances (color, thickness, odor and pH) of the developed feminine hygiene washes formula 3 and formula 5 were stable under heating and cooling test at 45 °C 24 hrs and 4 °C for 24 hrs for 6 cycles. They were active against *Staphylococcus aureus* (DMST 8013 and DMST 8840), *S. epidermidis* (DMST 12228), *Streptococcus pyogenes* (DMST 17020) and *Candida albicans* (DMST 90028 and DMST 10231) using tissue disc agar diffusion method (as shown in Table 2).

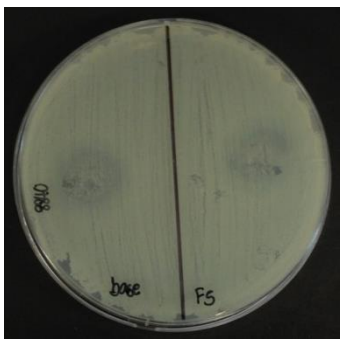
Table 2. Microbial inhibition of the feminine hygiene wash products (formula 3 and 5) *via* tissue diffusion method

Microorganism	Clear zones of inhibition (mm)	
	Formula 3	Formula 5
Staphylococcus aureus (DMST 8013)	+1.9	+1.5
Staphylococcus aureus (DMST 8840)	-/+	-/+
Streptococcus pyogenes (DMST 17020)	+1.8	+1.8
Staphylococcus epidermidis (DMST 12228)	+1.9	+2
Candida albicans (DMST 90028)	+1.5	+1.5
Candida albicans (DMST 10231)	+2	+2

Note: - no zone of inhibition -/+ no clearly zone of inhibition + clearly zone of inhibition

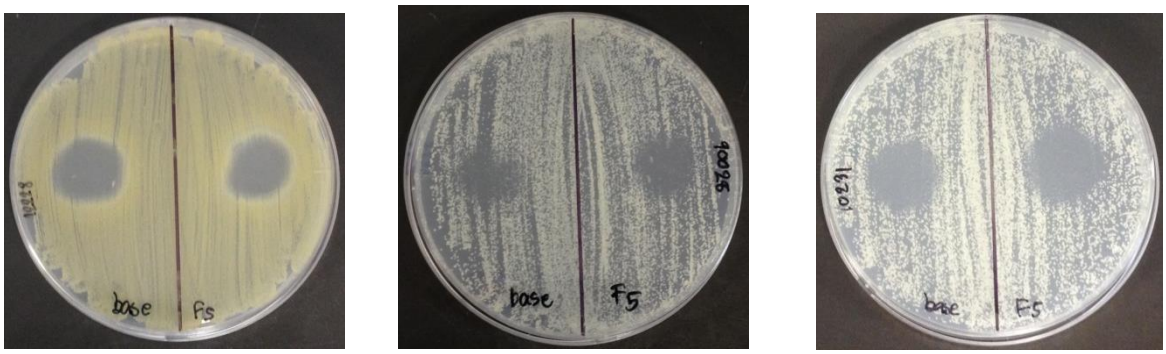


Staphylococcus aureus DMST 8013



Staphylococcus aureus DMST 8840

Streptococcus pyogenes DMST 17020



Staphylococcus epidermidis DMST 12228 *Candida albicans* DMST 90028 *Candida albicans* DMST 10231

Figure 2. Microbial inhibition of the feminine hygiene wash product formula 3 (left) and formula 5 (right)

CONCLUSIONS

The developed feminine hygiene wash formula 5 comprising the patented mix-extract from fruits of *P. emblica* and *Z. limonella* was clear yellow solution of pH 4 which gave smooth and fine foam. The product was stable under 6 cycles of the freeze – thaw method and was active against tested microbial.

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REFERENCES

14. Feminine hygiene – Caring for your intimate Health. [cited 2014 Oct 7]. Available from : http://www.intimina.com/blog/feminine-hygiene/
15. Moazzem Hossen SM, Sarkar R, Mahmud S, Abdul Aziz NM. 2014. Medicinal potential of *Phyllanthus emblica* Linn. Fruits Extracts: Biological and Pharmacological activities. BJPR. 4(12), 1486-99.
16. Tangjitjaroenkun J, Supabphol R, Chavasiri W. 2012. Antioxidant effect of *Zanthoxylum limonella* Alston. J. Med. Plants Res. 6(8), 1407-14.
17. Arun Kumar KV, Paridhavi M. 2011. Evaluation of physicochemical parameters on the fruit of *Zanthoxylum limonella* Alston (Family - Rutaceae). IJPC. 2, 1-3.
18. Charoenying P, Laosinwattana C, Phuwiwat W, Lomratsiri J. 2008. Biological activities of *Zanthoxylum limonella* Alston fruit extracts. KMITL Sci. J. 8, 12-5.
19. Potduang B, Fungsin B, Keeta I, Ketmanee N, Intarangsie A, Soradech S, et al. 2012. Biological activities of a patented mix extracts from fruits of *Phyllanthus emblica* and *Zanthoxylum limonella.* Thai J. Pharm. Sci. 36, 44-47.
20. Khayungarnnawee A, Soradech S, Potduang B, Sematong T, Laovitthayanggoon S, Keeta I, et al. 2012. Anti-inflammatory activity of fruit extracts from *Phyllanthus emblica* and *Zanthoxylum limonella.* Thai J. Pharm. Sci. 36, 38-40.

The development of *Phyllanthus emblica* and *Zanthoxylum limonella* facial mask powder

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ABSTRACT

A facial mask powder comprising the patented mix-extract of *Phyllanthus emblica* and *Zanthoxylum limonella* fruits was developed to benefit from its properties as the anti-oxidant, anti-inflammatory, anti-tyrosinase and anti-microbial. The facial mask powder was formulated in 4 formulas which were different in the amount of diluents and humectants. The selected formula 4 gave softer and smoother skin after the proper masking. The product was stable after 6 cycles of heating and cooling (45 °C 24 hours and 4 °C 24 hours). The 20% EtOH extract from the facial mask powder was antityrosinase effective (IC_{50} = 3.53 mg/ml). The anti-microbial assessment using tissue disc agar diffusion method revealed that the 40% diluted solution of 60% EtOH extract from the mask was active against tested microbial.

Keywords: Facial mask powder, *Phyllanthus emblica*, *Zanthoxylum limonella*

INTRODUCTION

Facial masks are products that provide deep nourishment to the skin. They hydrate the skin, drawn out the impurities and reduce excess oil. Facial masks generally need to remain on the skin about 10-15 minutes before washed out with water. A facial mask contains many ingredients such as essential oils, vitamins, clay and herbs [1, 2].

Phyllanthus emblica L. (EUPHORBIACEAE) and *Zanthoxylum limonella* Alston (RUTACEAE) are known in Thailand as 'Ma-Kham Pom' and 'Ma-Khwaen' respectively. Their edible fruits were extracted with water-ethanol and the patented mix fruit extract was proved to have anti-oxidant, anti-inflammatory, anti-tyrosinase and anti-microbial activities [3, 4].

In this study, we aimed to develop the facial mask powder comprising the patented mix-extract from fruits of *P. emblica* and *Z. limonella* to benefit from the mix-extract's effectiveness as anti-oxidant, anti-inflammatory, anti-tyrosinase and anti-microbial. The product assessments were performed on stability, antityrosinase and antimicrobial.

MATERIALS AND METHODS

Plant materials

The fruits of *P. emblica* and *Z. limonella* were collected and powdered by the Agricultural Technology Department of Thailand Institute of Scientific and Technological Research (TISTR).

Preparation of the extracts

P. emblica fruit powder 500 g was extracted by macerated with ethanol-water for 4 nights, filtered, rinsed and evaporated at 45 °C under reduced pressure using a rotary evaporator (Heidolph, Hei-VAP Precision). *Z. limonella* was extracted with another proportion of ethanol-water for 3 nights, filtered, rinsed and evaporated under reduced pressure. The crude extracts were kept in a refrigerator.

Preparation of facial mask powder

Four different formulas of the facial mask powder were developed from a master formula (as shown in Table 1.) with varying diluents (kaolin or talcum) and glycerin, and then evaluated the best formula. The selected formula 4 comprising the patented mix-extract from fruits of *P. emblica* and *Z. limonella* was prepared by weighing the extracts in a patented proportion to be effective against tested microbial, and then mixed with water, talcum and glycerin. The product was dried at 45°C in a hot air oven, and then ground into powder.

Table 1. Patented formula of the facial mask powder

Ingredients	Function	Master formula %w/w	Formula 1	Formula 2	Formula 3	Formula 4
P. emblica extract	Active ingredient	0.1-1	A	A	A	A
Z. limonella extract	Active ingredient	0.1-1	B	B	B	B
Talcum	Diluent	40-60	-	50	-	C
Kaolin	Diluent	40-60	50	-	45	-
Glycerin	Humectant	1-10	-	-	5	5
Water	Vehicle	q.s. to 100	39	39	39	39

Evaluation of facial mask powder

Stability of the facial mask powder formula 4 was assessed under accelerated condition of 4°C 24 hrs alternate with 45°C 24 hrs, for 6 cycles. The physical properties was monitored on appearances (color, odor, powder agglomeration)

Antityrosinase assessment of the facial mask powder formula 4 was performed using by the Dopachrome micro-plate method as described by Potduang *et al.* [5]. Sample solution was prepared by shaking 10 g of the facial mask in 10 ml of 20% ethanol (1:1) for 2 min, filtered through a Butchner funnel and syringe filter membrane. Concentration of the sample solution was determined by pipette 0.5 ml of the filtrate into a test tube, dried in a vacuum centrifuge evaporator , weighed the dried extract and then x2 to give the concentration in g/ml. Mixed 50 µl of the sample solution with 50 µl of 314.8U/ml mushroom tyrosinase enzyme in buffer and 100 µl of 0.02 M sodium phosphate buffer (pH 6.8). After 10 min, added 50 µl of 0.34 mM L-Dopa (Sigma) in buffer, mixed well, measured the absorbance at 492 nm by a micro-plate reader, and re-measured after 2 min. All samples were run in triplicate compared to Kojic acid. Calculated the enzyme inhibition from the equation:

% tyrosinase inhibition = [(A - B) - (C - D)]×100/ (A - B)

The absorbance differences are represented as A = control (L-Dopa + enzyme), B = blank 1 (L-Dopa), C = reaction mixture (enzyme + sample + L-Dopa) and D = blank 2 (sample + L-Dopa). Calculated the IC_{50} using the resulted linear equation of %tyrosinase inhibition *vs.* log concentration.

In vitro anti-microbial assessment of the facial mask powder formula 4 was performed using a tissue disc agar diffusion assay against *Staphylococcus aureus* (DMST 8013); *S. aureus* (DMST 8840); *S. epidermidis* (DMST 12228); *Streptococcus pyogenes* (DMST 17020); *Propionibacterium acnes* (DMST 14916) and *Candida albicans* (DMST 90028 and DMST 10231). The stock of microorganism was cultured on agar and then separated to sterile water and adjusted to the concentration of 0.5 McFarland. The microorganism was spread over the agar culture in a Petri dish, and left for a while in aseptic condition before placing tissue paper discs of 1 cm diam. on the agar surface. Sample solution was prepared by shaking the mask 5 g in 5 ml of 60% ethanol (1:1) for 2 min, filtered through a Butchner funnel and syringe filter membrane. Pipette 0.4 ml of the filtrate into a test tube, dried in a vacuum centrifuge evaporator; weighed the dried extract and then shook with 1 ml sterile water to make a 40% diluted sample solution. 20 µl of the sample solution was pipette onto the tissue disc for 15-mins contact with the agar culture before removing the tissue off. The clear zone of inhibition was observed after incubated at 37°C for 18-24 hrs.

RESULTS

The facial mask powder formula 1 and 3, which used kaolin as diluents, gave gray and bad odor. Talcum was used as diluents in formula 2 and 4, resulted in yellow powder with better odor. Glycerin was added in formula 3 and 4 to give softer and smoother feeling. The facial mask powder formula 4 was selected as the best formula.

The selected facial mask powder formula 4 was a fine yellow powder which provided moist, smooth and softening skin after 15-min masking method. It was stable under heating and cooling test (4°C 24 hrs and 45°C 24 hrs for 6 cycles with no change in the odor and color, and no agglomeration appeared. The 20% EtOH extract from the facial mask powder was antityrosinase effective (IC_{50} = 3.53 mg/ml) as shown in Table 2. The facial mask powder was active against tested microbial as shown in Table 3 and Figure 1.

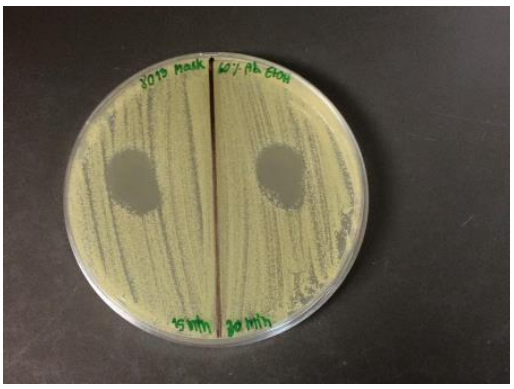
Table 2. Antityrosinase effectiveness of the facial mask powder formula 4

Treatment	Concentration of sample (µg /ml)	Log concentration	%Tyrosinase inhibition	IC_{50} (µg /ml)
Facial mask powder	31.25	1.4949	0.00	3.53×10^3
	62.50	1.7959	6.82	
	125.00	2.0969	12.50	
	250.00	2.3979	18.18	
	500.00	2.6990	31.82	
Kojic acid	3.90	0.5911	19.57	0.02×10^3
	7.80	0.8921	30.43	
	15.60	101931	45.65	
	31.25	1.4949	58.70	
	62.50	1.7959	73.91	

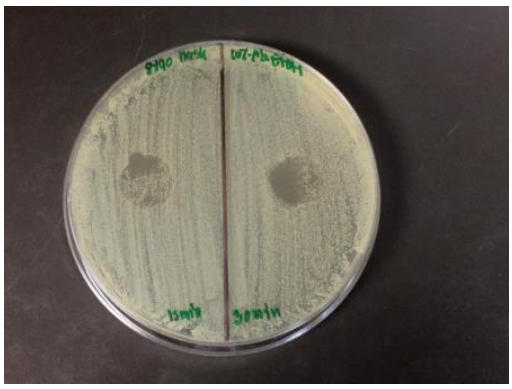
Table 3. Microbial inhibition of the selected facial mask powder formula 4

Microorganisms	Clear zones of inhibition
Staphylococcus aureus (DMST 8013)	+
Staphylococcus aureus (DMST 8840)	+
Streptococcus pyogenes (DMST 17020)	+
Staphylococcus epidermidis (DMST 12228)	+
Propionibacterium acnes (DMST 14916)	-/+
Candida albicans (DMST 90028)	-/+
Candida albicans (DMST 10231)	-/+

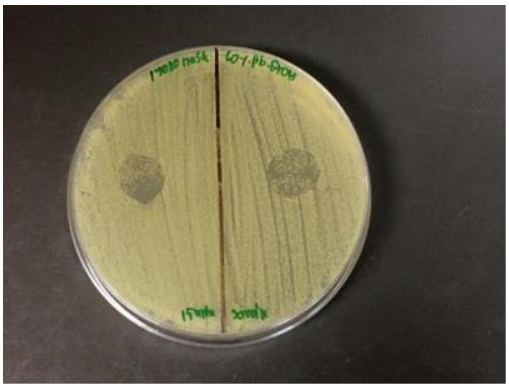
Note: - no zone of inhibition -/+ no clearly zone of inhibition + clearly zone of inhibition



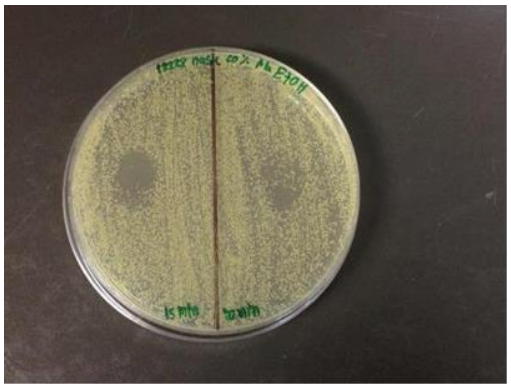
Staphylococcus aureus DMST 8013



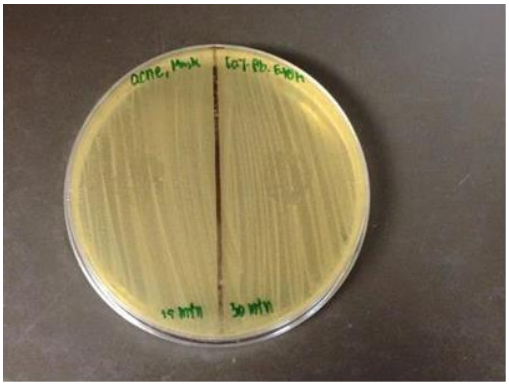
Staphylococcus aureus DMST 8840



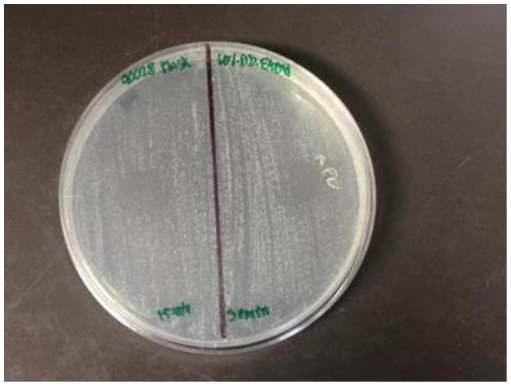
Streptococcus pyogenes DMST 17020



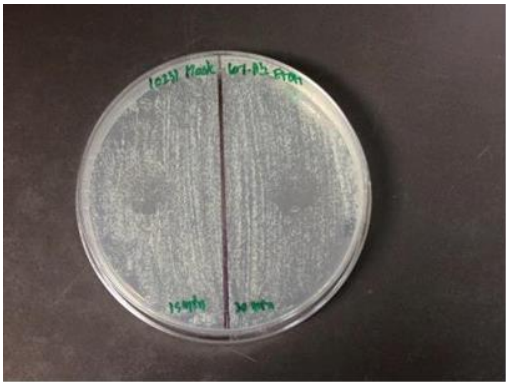
Staphylococcus epidermidis DMST 12228



Propionibacterium acnes DMST 14916



Candida albicans DMST 90028



Candida albicans DMST 10231

Figure 1. Clear zones of inhibitions of the *P. emblica* and *Z. limonella* facial mask powder formula 4

CONCLUSIONS

The stable facial mask powder formula 4 contained the patented mix-extract from fruits of *P. emblica* and *Z. limonella* which was anti-oxidant, anti-inflammatory anti-tyrosinase and anti-microbial effective. The product had appropriated diluents and humectants which provided moist smooth and tender skin after 15-mins masking process. The 20% EtOH extract from the facial mask powder was antityrosinase effective (IC_{50} = 3.53 mg/ml). The anti-microbial assessment using tissue disc agar diffusion method revealed that the 40% diluted solution of 60% EtOH extract from the mask was active against tested microbial.

ACKNOWLEDGEMENTS

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REFERENCES

1. The benefits behind facial masks revealed. [internet]. [cited 2014 Oct 1]. Available from: http://newhope360.com/science/benefits-behind-facial-masks-revealed
2. Facial mask. [internet]. [cited 2014 Oct 1]. Available from : http://spas.about.com/od/facialtreatments/a/Facial-Masks.htm
3. Potduang B, Fungsin B, Keeta I, Ketmanee N, Intarangsie A, Soradech S, et al. 2012. Biological activities of a patented mix extracts from fruits of *Phyllanthus emblica* and *Zanthoxylum limonella.* Thai J. Pharm. Sci. 36, 44-47.
4. Khayungarnnawee A, Soradech S, Potduang B, Sematong T, Laovitthayanggoon S, Keeta I, et al. 2012. Anti-inflammatory activity of fruit extracts from *Phyllanthus emblica* and *Zanthoxylum limonella.* Thai J. Pharm. Sci. 36, 38-40.
5. Potduang B, Meeploy M, Giwanon R, Benmart Y, Kaewduang M, Supatanakul W. 2008. Biological activities of *Asparagus racemosus.* Afr. J. Trad. Complement. Altern. Med. 5(3), 230-237.

Effect of nobiletin on the transport of salazosulfapyridine through Caco-2 cell monolayers

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ABSTRACT

Salazosulfapyridine (SASP) is effective for inflammatory bowel diseases (IBDs), including ulcerative colitis and Crohn's disease. Patients with IBD require high doses of SASP to achieve therapeutically effective intestinal concentrations because SASP is immediately secreted into the mucosal lumen. Nobiletin is a polymethoxyflavone component found in citrus peel. In experiments using Caco-2 cell monolayers grown on Transwell inserts, nobiletin inhibited the apical efflux of SASP and decreased the ratio of basolateral-to-apical and apical-to-basolateral apparent permeability (P_{appBA}/P_{appAB}). Ko143, breast cancer resistance protein (BCRP) inhibitor, and MK571, multidrug resistance-associated protein 2 (MRP2) inhibitor, inhibited the apical efflux of SASP, whereas quinidine, p-glycoprotein (multidrug resistance 1: MDR1) inhibitor, had no significant effect. Nobiletin appeared to inhibit the apical efflux of SASP, thus inhibiting BCRP and MRP 2. Our results suggest that nobiletin administered in conjunction with SASP enhances the therapeutic effects of these drugs in patients with IBD.

Keywords: nobiletin, salazosulfapyridine, inflammatory bowel diseases, BCRP, MRP 2

INTRODUCTION

Salazosulfapyridine (SASP) is used in the treatment of inflammatory bowel diseases (IBDs) [1]. The choice and use of SASP as the therapeutic drug has tended to decrease because of the side effects, such as agranulocytosis or myeloablation, caused by sulfapyridine, a by-product of SASP [2]. Recently, the selection and use of 5-aminosalicylic acid (5-ASA), which is an active moiety obtained from SASP by the action of colonic bacteria, and its derivatives has increased as the therapeutic drug of choice for patients with IBD[3]. However, SASP was frequently selected depending on the patient's condition because there is a difference between the therapeutic effects of SASP and 5-ASA. SASP still is an important drug for patients with IBD [4].

Patients with IBD require high doses of SASP to achieve therapeutically effective intestinal concentrations because SASP is immediately secreted into the mucosal lumen [5]. High doses of SASP increase sulfapyridine levels, which can lead to dose-dependent intolerable adverse reactions. If the SASP dose could be reduced by inhibiting the excretion of SASP from intestinal cells, it would reduce the adverse reactions associated with the high doses of SASP required for IBD treatment.

Flavonoids are plant-derived polyphenolic compounds widely distributed in the diet and have been reported to have many beneficial effects on health [6]. Some flavonoids are well-known modulators of the cellular transport of various substances mediated by breast cancer resistance protein (BCRP), multidrug resistance-associated protein 2 (MRP2), and p-glycoprotein (multidrug resistance 1: MDR1) inhibitor [7-10]. Our previous study indicated that it was possible to decrease doses of 5-ASA by co-administration with quercetin, one of the flavonoids, owing to the decrease in excretion of AC-5-ASA; this compound is the main metabolite of 5-ASA in intestinal cells[11]. Nobiletin, which is one of the flavonoid found in citrus peel, is a polymethoxyflavone component and has a structure similar to quercetin. Fruits or beverages containing nobiletin are very popular as health foods because of its beneficial capabilities, such as the prevention of hyperglycemia, high blood pressure, cancer, and dementia [12]. The intake of nobiletin through dietary sources appears to be relatively easy.

The purpose of this study was to investigate whether nobiletin administered in conjunction with SASP could enhance the therapeutic effects of these drugs in patients with IBD.

MATERIALS AND METHODS

Materials

Transwell inserts were purchased from Corning Costar (Cambridge, MA, USA), tissue culture dishes from Becton Dickinson Com. (Falcon, USA), and nobiletin was provided by Dr. Choshi and Dr. Hibino of Fukuyama University. SASP was purchased from LKT Lab, Inc. (MN, USA) (Figure 1); Ko143 from SIGMA; MK571, quinidine and other chemicals were purchased from Wako Pure Chemical Co. (Osaka, Japan). The XG-C18M-5 column was purchased from Nomura Chemical Co. (Aichi, Japan).

Cell cultures

Caco-2 cells were purchased from Riken (no. RCB0988) and maintained in Dulbecco's modified eagle medium (DMEM) containing 16% fetal calf serum (FCS) and penicillin–streptomycin–amphotericin B. Cells were cultured at 37°C in an atmosphere of 5% CO_2 and 95% relative humidity. The cells were sub-cultured once a week.

Efflux of SASP from Caco-2 cells

The suspended Caco-2 cells were seeded on 6-well polycarbonate Transwell inserts (0.4 µm mean pore size, 4.7 cm^2 growth area) at a density of 5×10^4 cells/well and then cultured for three weeks in DMEM containing 16% FCS. The monolayers with transepithelial electric resistance > 250 Ωcm^2 were used for transport studies. Nobiletin or transporters inhibitors were added to the apical and basolateral chambers. After 10 min, SASP at a final concentration of 200 µM was then added to either the apical or basolateral chamber. After incubation for 30, 60, 90, and 120 min at 37°C, a 50 µL sample from the chamber opposite to the drug addition underwent HPLC analysis as described below. The apparent permeability coefficient (P_{app}) was determined for both the apical (A) to basolateral (B) (P_{appAB}) and basolateral (B) to apical (A) (P_{appBA}) directions. The P_{app} value was calculated as follows:

$$P_{app} = (\Delta Q/\Delta t) \times [1/ (A \times C_0)]$$

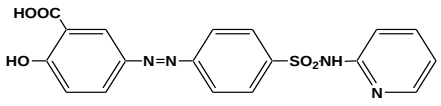
Where ΔQ is the amount of SASP in the receiver chamber, Δt is a function of time per second, A is the Caco-2 cell growth area (4.7 cm^2), and C_0 is the initial concentration of SASP applied in the donor chamber.

HPLC analysis

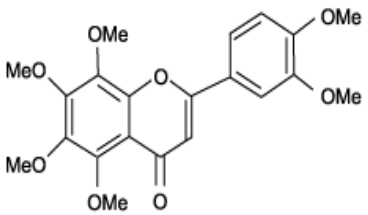
Concentration of SASP was determined by the HPLC system equipped with an UV-8020 detector (373 nm, Tosoh Co., Japan) and a CCPD pump. A 50 µL aliquot of sample was injected into XG-C18M-5 column maintained at 40°C. A mobile phase of methanol (40%) and 20 mM phosphate buffer solution (pH 7.0) with 1.0 M tetramethylammonium hydroxide solution (1 %) was used to separate SASP. The flow rate of the mobile phase was 1.0 mL/min, and SASP appeared at 7.5 min. The quantitative determination of SASP was based upon the integration of fluorescence peak areas.

Statistical analysis

The data in figures are given as the mean ± S.E. of four to five experiments. Differences among the mean values were assessed by Dunnett's test using Stat-100 (BIOSOFT, UK) or Student's t-test. A P value of <0.05 was considered significant.



Salazosulfapyridine (SASP)



nobiletin

Figure 1. Structures of salazosulfapyridine(SASP) and nobiletin

RESULTS

Effect of nobiletin on SASP efflux

The transcellular transport of SASP was determined using Caco-2 cell monolayers grown on Transwell inserts (Figure 2). The P_{appBA} value was 6.34 ± 0.642 × 10^{-6} cm/s and the P_{appAB} value of SASP was 0.279 ± 0.062 × 10^{-6} cm/s. The SASP efflux ratio (P_{appBA}/P_{appAB}) was 22.72, indicating that SASP permeability from apical-to-basolateral side was lower than that from the opposite direction (Table 1).

The effect of nobiletin on SASP efflux was examined using Caco-2 cell monolayers (Figure 2). Nobiletin increased the apical-to-basolateral transport of SASP and decreased transport from the opposite direction. The P_{appBA} value was 4.816 ± 0.197 × 10^{-6} cm/s and 3.823 ± 0.131 × 10^{-6} cm/s, respectively, and the P_{appAB} value was 0.505 ± 0.117 × 10^{-6} cm/s and 0.789 ± 0.158 × 10^{-6} cm/s at concentrations of 10 μM and 50 μM of nobiletin, respectively. Nobiletin decreased the SASP efflux ratio (P_{appBA}/P_{appAB}) to 9.54 and 4.85 at concentrations of 10 μM and 50 μM, respectively (Table 1).

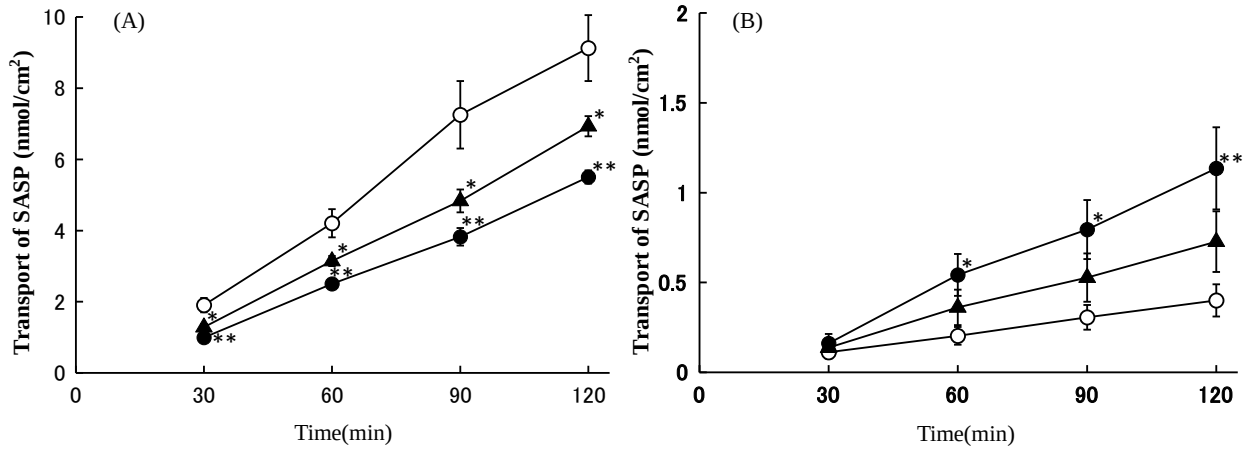


Figure 2. Effect of nobiletin on the efflux of SASP through Caco-2 cell monolayers. Basolateral-to-apical (A) and apical-to-basolateral (B) permeation of 200□M SASP were investigated in the presence or absence of 10 or 50 □M nobiletin. Open circles represent the data without nobiletin. Triangles and closed circles represent the data with nobiletin 10 and 50 □M, respectively. Each value represents the mean ± S.E. of four or five experiments. **P*<0.05.,***P*<0.01.

Table 1. The apparent permeability coefficient (P_{app}) of SASP through Caco-2 cell monolayers treated with or without nobiletin.

SASP	$P_{app}\times10^{-6}$(cm/sec)		$\frac{P_{app(B\rightarrow A)}}{P_{app(A\rightarrow B)}}$
	B→A	A→B	
Cont	6.340±0.642	0.279±0.062	22.72
Nobiletin 10 μM	4.816±0.197*	0.505±0.117	9.54
Nobiletin 50 μM	3.823±0.131**	0.789±0.158**	4.85

Basolateral-to-apical (A) and apical-to-basolateral (B) permeation of 200□M SASP were investigated in the presence or absence of 10 or 50 □M nobiletin. P_{app} entries are mean ± S.E. of four or five experiments. **P*<0.05.,***P*<0.01.

Effect of transporters inhibitors on SASP efflux

To investigate the active transport of SASP, the effect of transporter inhibitors on SASP transport was examined in the presence of Ko143 (BCRP inhibitor), MK571 (MRP2 inhibitor), quinidine (MDR1 inhibitor) (Figure 3 and Table 2). Ko143 and MK571 decreased the apical-to-basolateral transport of SASP and increased transport from the opposite direction, but quinidine had no significant effect on transport in either direction. The SASP efflux ratios (P_{appBA}/P_{appAB}) in the presence of Ko143 and MK571 were 6.61 and 3.95, respectively. The ratio in the presence of quinidine was 19.37,

which was similar to 16.97 in the absence of inhibitor. BCRP and MRP2 appeared to be responsible for efflux inhibition of SASP from Caco-2 cells. The inhibitory effect of MRP2 was greater than that of BCRP.

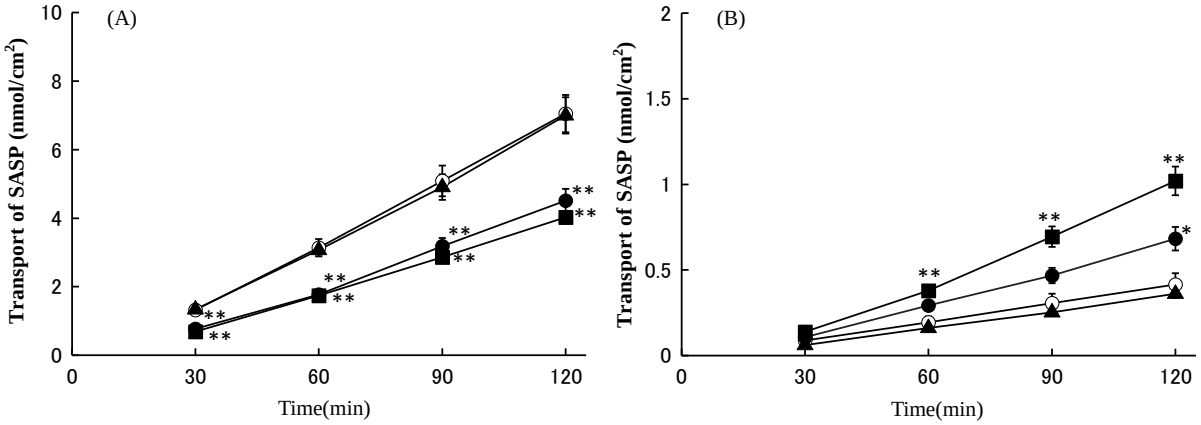


Figure 3. Effect of transporter inhibitors on the efflux of SASP through Caco-2 cell monolayers. Basolateral-to-apical (A) and apical-to-basolateral (B) permeation of 200□M SASP were investigated in the presence or absence of transporter inhibitors. Open circles represent the data without transporter inhibitors, and closed circles, squares and triangles represent the data with 2□M Ko143, 50□M MK571, and 50 □M quinidine, respectively. Each value represents the mean ± S.E. of three or four experiments. **P*<0.05.,***P*<0.01.

Table 2. The apparent permeability coefficient (P_{app}) of SASP through Caco-2 cell monolayers treated with or without transporter inhibitors.

SASP	$P_{app}\times10^{-6}$(cm/sec)		$\frac{P_{app(B\rightarrow A)}}{P_{app(A\rightarrow B)}}$
	B→A	A→B	
Cont	4.900±0.378	0.289±0.046	16.97
Ko143	3.133±0.237**	0.474±0.048*	6.61
MK 571	2.797±0.118**	0.701±0.058**	3.95
Qunidine	4.861±0.370	0.251±0.018	19.37

Basolateral-to-apical (A) and apical-to-basolateral (B) permeation of 200□M SASP were investigated in the presence or absence of transporter inhibitors. P_{app} entries are mean ± S.E. of three or four experiments. **P*<0.05, ***P*<0.01.

CONCLUSIONS

Nobiletin, a popular flavonoid, inhibited the SASP apical efflux. SASP appeared to be pumped out by BCRP and MRP2 transporters, which were expressed at the apical side of the intestinal cell. Nobiletin appeared to inhibit the SASP apical efflux by inhibiting BCRP or MRP2 transporters. Our results suggest that nobiletin administered together with SASP enhances the therapeutic effects of these drugs in patients with IBD.

ACKNOWLEDGEMENTS

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REFERENCES

1. Van Hees PA, Van Lier HJ, Van Elteren PH, Driessen M, Van Hogezand RA, Ten Velde GP, et al. 1981. Effect of sulphasalazine in patients with active Crohn's disease: a controlled double-blind study. Gut. 22, 404-409.
2. Stein RB, Hanauer SB. 2000. Comparative tolerability of treatments for inflammatory bowel disease. Drug Saf. 23, 429-448.
3. Masuda H, Takahashi Y, Nishida Y, Asai S. 2012. Comparison of the effect of mesalazine and sulfasalazine on laboratory parameters: a retrospective observational study. Eur. J. Clin. Pharmacol. 68, 1549-1555
4. Martínez González J, Busto Bea V, Mesonero Gismero F, Parejo Carbonell S, Garrido E, López San Román A. 2013. Rescue therapy with sulfasalazine prior to immunosuppressive or biological agents in ulcerative colitis poorly controlled with mesalazine. Gastroenterol Hepatol. 36, 7-10.
5. Zamek-Gliszczynski MJ, Bedwell DW, Bao JQ, Higgins JW.2012. Characterization of SAGE Mdr1a (P-gp), Bcrp, and Mrp2 knockout rats using loperamide, paclitaxel, sulfasalazine, and carboxydichlorofluorescein pharmacokinetics. Drug Metab Dispos. 40, 1825-33.
6. KUHNAU J. 1976. The flavonoids. A class of semi-essential food components: their role in human nutrition. World Rev Nutr Diet. 24, 117-191
7. Yoshimura S, Kawano K, Matsumura R, Sugihara N, Furuno K. 2009. Inhibitory effect of flavonoids on the efflux of N-acetyl 5-aminosalicylic acid intracellularly formed in Caco-2 cells. J Biomed Biotechnol. 2009, 467489.
8. Van Zanden JJ, De Mul A, Wortelboer HM, Usta M, Van Bladeren PJ, Rietjens IM, et al. 2005. Reversal of in vitro cellular MRP1 and MRP2 mediated vincristine resistance by the flavonoid myricetin. Biochem Pharmacol. 69, 1657-1665.
9. Shang S, Morris ME. 2003. Effects of the flavonoids biochanin A, morin, phloretin, and silymarin on P-glycoprotein-mediated transport. J. Pharmacol. Exp. Ther. 304, 1258-1267
10. Zhang S, Yang X, Morris ME. 2004. Flavonoids are inhibitors of breast cancer resistance protein (ABCG2)-mediated transport. Mol. Pharmacol. 65, 1208-1216.
11. Kamishikiryo J, Matsumura R, Takamori T, Sugihara N. 2013. Effect of quercetin on the transport of N-acetyl 5-aminosalicylic acid. J. Pharm. Pharmacol. 65, 1037-1043.
12. Assini JM, Mulvihill EE, Huff MW. 2013. Citrus flavonoids and lipid metabolism. Curr. Opin. Lipidol. 24, 34-40.

Antimutagenic and anti-oxidized LDL properties of *Pluchea indica* tea

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ABSTRACT

Pluchea indica is a widespread medicinal plant that grows naturally in many countries of Southern and South-Eastern Asia, including Thailand. The leaves of this plant are capable of treating dysentery, rheumatism, leucorrhea, bad breath and body odor. This study aimed to determine the antimutagenicity and oxidized-LDL inhibitory effects of *P. indica* tea. Mutagenic and antimutagenic activities of this plant were assessed by Ames test using *Salmonella typhimurium* TA98 and TA100 and somatic mutation and recombination test (SMART) against urethane in *Drosophilla melanogaster*. In addition, the inhibitory effect on oxidized-LDL was also investigated via thiobarbituric acid reactive substance (TBARS) assay. It was found that no mutagenic activity for both tests was observed. Moreover, *P. indica* tea at 0.625 mg/plate also showed the greatest antimutagenicity (62.9% of inhibition in TA98 and 55.2% of inhibition in TA100). In the same way, *P. indica* tea at the highest concentration used in this experiment (25 mg/ml) remarkably inhibited the mutagenicity of 20mM urethane in SMART test. Interestingly, the inhibitory effect of *P. indica* tea extract on the oxidized-LDL was apparent. IC_{50} against TBARS of oxidized-LDL was calculated with a significant value by 0.654±0.004 mg/ml. Conclusively, the findings suggest the mutagenesis and oxidized-LDL inhibitory capacity and support the potential utilization of *P. indica* tea in functional food development.

Keywords: *Pluchea indica*, Antimutagenicity, Ames test, SMART, Oxidized-LDL

INTRODUCTION

Pluchea indica Less. is a shrub of the family Asteraceae and is widespread in Southeast Asia. In Thailand, various parts of *P. indica* have been used in traditional medicine. The leaves are capable for treating dysentery, rheumatism, leucorrhea, bad breath and body odor. The roots are used to treat fever, lumbago, indigestion and headache [1-3]. A collection of preliminary data from community enterprise in Khlung district, Chantaburi province, Thailand has found that the locals unite made liquid soap, spa body scrub powder and facial scrub. *P. indica* has also been used in form of herbal tea product (dried leaves), but there are problems about the lack of scientific evidence to support its consumption. Therefore, we are interested in the study of antimutagenicity and inhibitory effect on the oxidized-LDL of *P. indica* tea.

MATERIALS AND METHODS

Preparation of extract

P. indica Less. herbal tea (dried leaves) was purchased from community enterprise at Khlung district, Chantaburi province, Thailand. Then, it was ground to a powder using a grinder. Powdered sample (100g) was added to boiling distilled water (1L), boiled for 30min. Water extract was filtered by Whatman No.1 with vacuum pump and then centrifuged at 3,000 rpm for 10min at 25°C. The remaining residues were re-extracted with boiling distilled water as describe above. The collected filtrate was evaporated by rotary evaporator and dried in a freeze-dryer. All extracts were weighed, stored at -20°C and protected from light until used.

Mutagenic and antimutagenic assays by Ames test

Aminopyrene (AP) treated with nitrite in acid solution was used as a positive mutagen since it gave direct-acting mutagenicity in the condition similar to that occurring during stomach digestion [4]. AP treated with nitrite in acid solution (0.06 and 0.12 µg/plate) was used as a positive mutagen on strain TA 98 and TA 100, respectively. *Salmonella typhimurium* tester strains were histidine-dependent strains (His^-) TA98 and TA100 and were manipulated as suggested by [5]. The untreated sample (100 µL) was mixed with 500 µL of 0.5 M phosphate buffer (pH 7.4) and 100 µL of each tester strain to make the final volume as 700 µL. The mixture was incubated at 37^0C in a shaking water bath for 20 min as suggested by [6]. After incubation, 2 ml of top agar containing 0.5 mM L-histidine and 0.5 mM D-biotin (45^0C) was added, mixed well and poured onto a minimal glucose agar plate. The plate was rotated to achieve uniform colony distribution and incubated at 37^0C in darkness for 48h. Numbers of His^+ revertant colonies were counted. Positive mutagenic effect was considered when the number of induced revertants increase in a dose response relationship manner when at least two doses were higher than spontaneous revertants and at least one dose gave rise to twice over the spontaneous revertants [7]. The tube containing distilled water was used to determine spontaneous reversion. The results were expressed as mean±SD of histidine (His^+) revertants per plate.

A volume of 100 µL of each tester strain was added to the test tube containing 500 µL sodium phosphate–potassium buffer, 25, 50 or 75 µL of *P. indica* tea and 25 µL nitrite-treated AP. Distilled water was adjusted to the final volume of 700 µL. The mixture was treated as described in the mutagenic assay. The percentage of inhibition was calculated as following: %Inhibition = *(A-B)/(A-C)* x 100 where *A* is the number of histidine revertants per plate induced by nitrite treated AP, *B* is the number of histidine revertants per plate induced by nitrite treated AP in the presence of each sample and *C* is the number of spontaneous histidine revertants per plate. The percentage of inhibition is classified as strong, moderate, weak and negligible effect if it is higher than 60%; 60– 41%; 40–21% or 20–0%, respectively [8].

Mutagenic and antimutagenic assays by SMART test

Virgin females of Oregon wing flare strain (*ORR/ORR*; *flr³/TM3*, *Ser*) were mated with males of multiple wing hair strain (*mwh/mwh*) on regular medium to produce *trans*-heterozygous larvae of improved high bioactivation cross. Both strains were maintained on the regular medium modified from the formula of Roberts [9] which had propionic acid (0.01 mL) as a preservative.

Appropriate amount of *P. indica* tea was added to regular medium at different concentrations (12.5, 25, 50 and 100 mg/mL) and it was homogenized. Each medium was used as an *experimental medium* for mutagenicity testing of each sample. 20 mM Urethane (URE) was substituted for DI water in the regular medium and was used as a *positive control medium*. An *experimental medium containing URE* was prepared by adding each concentration of sample (12.5, 25 and 50 mg/mL) into the positive control medium and homogenized. The sample provided more than 50% survival rate of adult flies was considered. This medium was used for co-administration study.

To obtain a statistical assessment of antimutagenicity, the frequencies of total spots per wing were compared in pairs (that is, URE alone versus URE plus sample; URE plus sample versus URE plus sample) using the non-parametric Mann–Whitney U-test [10]. Level of significance was set for $P < 0.05$. To allow a quantitative comparison, the percentage of inhibition was determined for each category of spot. The percentage of inhibition was also calculated as following $100(a - b)/a$ where *a* is the frequency of spots induced by URE alone and *b* is the frequency of spots induced by URE in the presence of sample [11].

Plasma and lipoprotein preparation

The rest of the plasma was measured by pooling plasma samples collected from blood specimens of diabetic patients that provided from the medical laboratories, Health Science Center, Burapha University. The rest blood of patients was collected in evacuated tubes. Sodium fluoride (NaF) (5 mmol/L) was used as an anticoagulant and antioxidant when samples were intended for LDL isolation. Blood was centrifuged at 800xg, at 4^0C for 15 min and separated plasma was employed to prepare LDL. Plasma was removed and kept at -80^0C for further analysis.

Isolation of LDL

Low density lipoprotein (LDL) fractions were separated by the sequential density gradient ultracentrifugation method, which is modified from Niki [12] method. 80 ml plasma fraction obtained by low-speed centrifugation was mixed with KBr (3.81 g). Then, it was ultracentrifuged in Beckman Polyallomer centrifuge tubes at 150,000xg for 8h at 4^0C. Sliced tube and discarded the top fraction. Bottom fraction (80 ml) was mixed with KBr (5.27 g). It was ultracentrifuged again and kept top fraction (10 ml; density 1.040 g mL^{-1}). It was repeated 3 times. After that, it was dialyzed against 12L PBS for 48h at 4^0C (two changes of 6L each) then sterilized though a 0.45 µm filter. Protein content was determined by Bradford's assay.

Determination of total protein content

Dissolved 10 mg of Coomassie Brilliant Blue G-250 in 5 mL 95% ethanol was added into 10 mL of 85% (w/v) phosphoric acid. The mixture was diluted to 100 mL when the dye was completely dissolved, then filtered through Whatman No.1 paper before use. Unknown was diluted if necessary to obtain between 5 and 10 µg protein in at least one assay tube containing 10µL of samples. An equal volume of 0.2% albumin was added to each sample and vortexed. 0.2% albumin was administered to standards as well if this option is used. Standards contained a range of 1 to 10 µg protein (0.2% albumin) in 10 µL volume. 1 mL dye reagent was added and incubated for 5 min. The absorbance was measured at 595 nm. A standard curve of absorbance versus micrograms protein was prepared and calculated.

Preparation of oxidized-LDL

To oxidize LDL, LDL (5 mg) was dissolved in PBS (1 mL) containing 10 µM $CuSO_4$. Consequently, it was transferred to 3.5 cm sterile plastic culture dish and incubated for up to 48h at 37 °C, dialyzed against 12L PBS for 48h at 4^0C (two changes of 6 L each). Protein content was also determined by Bradford's method.

SDS-PAGE analysis of LDL

Assembly kit for the preparation of gels and the separated proteins was employed properly. Then the samples were mixed with water and dye to heat at 95^0C for 5 min, marker and protein samples were loaded into the wells. Proteins were separated in an electric field by electric current 80 watt for 90 min or until the color of sample buffer moved down at the bottom of the gel. The gel was stained with Commassie Brilliant blue for 30min. It was washed with destain solution for 1-2h or until the bands of protein were clear.

Measurement of thiobarbituric acid-reactive substances (TBARS) formation from oxidized-LDL

TBARS were monitored following the procedure previously described. Briefly, 50 µg of LDL was added into 150 mM NaCl 1.5 mL. A volume of 200 µL of sample and 400 µL of thiobarbituric acid (TBA) reagent containing 1.5g trichloroacetic acid (TCA), 0.0375g TBA, 2.2 ml of 2 mM butylated hydroxytoluene (BHT) in 70% ethanol and 2.5 mL of 1M HCl were mixed into 1.5 mL microtube and 6 µL of 50 mM BHT in 70% ethanol was then added. Solution mixture was incubated for 15 min at 95^0C in a water bath and then cooled in ice-cold water for 30 min. After cooling, the test tube was centrifuged at 3,000xg for 15 min at 4^0C. The upper layer was transferred into a 96-well microplate and measured the absorbance at 535 nm with a microplate reader. Assays were performed in triplicate. The calculation was based on standard curve prepared with different concentration of MDA (0–50 µM) diluted with distilled water. The results were expressed in malondialdehyde standard curves prepared with malondialdehyde (MDA) and TBARS were expressed as mg of malondialdehyde/g dry matter.

RESULTS

No mutagenic activity of *P. indica* tea was observed for *Salmonella typhimurium* both TA98 and TA100 strains without metabolic activation (Table 1). The inhibitory effect of *P. indica* tea on the mutagenicity of positive mutagen is shown in Table 2. The sample presented no killing effect. In contrast, it inhibited the mutagenicity of the reaction product of AP-nitrite model in the absence of metabolic activation with moderate (41–60%) to strong (>60%) inhibition on both strains. Dose 0.625 mg/plate, *P. indica* tea exhibited strong and moderate inhibitory activity against AP-nitrite model on *S. typhimurium* TA98 (62.9%) and TA100 (55.2%), respectively.

Table 1. Mutagenic activity of *P. indica* tea by Ames/Salmonella test

Sample	Histidine revertants (colonies/plate)	
	TA 98	TA 100
H_2O	25.5±3.5	109.0±4.6
AP-nitrite	1202.3±58.1	806.3±14.7
0.156 mg/plate	22.7±2.1	110.0±6.2
0.312 mg/plate	33.3±0.6	129.3±6.1
0.625 mg/plate	36.5±0.7	135.3±20.2

Table 2. Antimutagenic property of *P. indica* tea using Ames/Salmonella test

Sample	Histidine revertants (colonies/plate)			
	TA 98	% Antimutagenicity	TA 100	%Antimutagenicity
AP-nitrite + tea 0.156 mg/plate	704.5±29	41.4	418.0±50.9	48.20
AP-nitrite + tea 0.312 mg/plate	517.5±16.3	57.0	392.5±16.3	51.30
AP-nitrite + tea 0.625 mg/plate	445.5±34.6	62.9	361.5±24.7	55.2

P. indica tea was not toxic in 48h larval feeding except at 100 mg/mL. However, at 50 mg/ml of sample seemed to moderately decrease of % survival rate. We therefore decided to select two less concentrations for antimutagenic study. The frequency of total spots per wing recorded in the sample controls was 0.00–0.15 (data not shown). In the negative control series the frequency of spots was 0.325 (Tables 3–4). The data given in Table 4 demonstrate that the different concentrations of *P. indica* tea were not equally effective in significantly reducing the mutagenicity of urethane. In general the different processing, when effective, significantly reduced not only the total spot frequency but also the twin spot frequency. Single spots are produced either by mitotic recombination or by somatic mutation, deletion or other changes. Twin spots are produced exclusively by mitotic recombination occurring between the proximal marker *flr* and the centromere. One of the possible antimutagenic components found in *P. indica* tea are quercetin [1] that could protect dealkylated nucleotides from further ethyl methanesulfonate alkylation by forming the strong hydrogen bonds with O^6-guanine and O^4-thymine via B ring hydroxyl group (bond lengths lower than 2.5 A˚) [13].

Table 3. Number of total spots/wing from mutagenic assay by SMART test of *P. indica* tea

Sample concentration	% Survival	Total spots/wing (n=40)
H_2O	100	0.325
20 mM URE	86	19.950[a]
12.5 mg/mL	98	0.575
25 mg/mL	90	0.300
50 mg/mL	60	0.375
100 mg/mL	35	0.525

[a] significantly different from spontaneous mutation at $P < 0.05$

Table 4. Number of total spots/wing when *P. indica* tea was incubated with URE using SMART test

Sample concentration	% Survival	Total spots/wing (n=40)
H_2O	100	0.325
20 mM URE	86	19.950[a]
12.5 mg/mL	95	12.750[b]
25 mg/mL	25	11.175[b]

[a, b] significantly different from spontaneous mutation at $P < 0.05$

It is now widely accepted that oxidative modification of LDL affects the metabolism of lipoproteins, leading to the accumulation of atherosclerotic lesion in the arterial intima. Figure 1 expresses an SDS-PAGE profile of LDL, the intensity of the main LDL greatly increases in favor of high molecular weight aggregation that cannot penetrate the separating gel and size of the protein are 157 KDa and 62 KDa. Plasma lipid peroxidation is always represented in term of LDL oxidation that is followed by the TBARs assay. This method was measured collectively end aldehyde products formed from peroxidized fatty acid. Figure 2 depicts TBARs production upon incubation of LDL (100 µg/ml) in the presence of increasing concentrations (0-0.5 mg/mL) of *P. indica* tea. TBARs represent malondiadehyde (MDA) in the peroxidative chain reaction. A dose-dependent inhibitory effect of *P. indica* tea was apparently observed. *P. indica* tea attenuated TBARs values from 4.53±0.001 to 1.21±0.001 µg MDA/mg protein of LDL in a concentration-dependent manner with the IC_{50} against TBARS of LDL by 0.654±0.005 mg/mL.

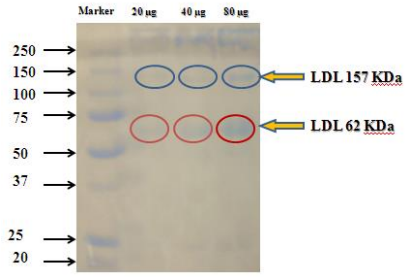

Figure 1 SDS-PAGE (denaturing conditions) profile of LDL. Lane 1: marker; Lane 2: LDL 20 µg/mL; Lane 3: LDL 40 µg/mL; Lane 4: LDL 80 µg/mL.

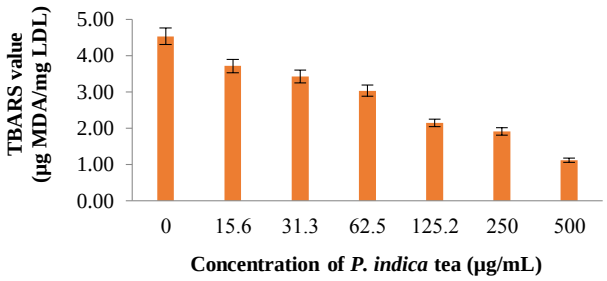

Figure 2 TBARS produced upon incubation of LDL (100 µg/mL) with 10 µmol/ml $CuSO_4$ in the presence of increasing concentrations (0–0.5 mg/mL) of *P. indica* tea. Data are expressed as mean±SD of 3 independent experiments in which each condition was analyzed in triplicates.

CONCLUSIONS

In conclusion, our study has shown that *P. indica* tea inhibits the genotoxic effects of AP-nitrite and urethane. This suggests *P. indica* tea contains a mixture of many antimutagens. Co-treatment experiments by SMART test showed that direct protective mechanism is involved in an 'activation' process to give antimutagenic effect. Additionally, it also displays the inhibitory effect against oxidized-LDL that possesses atherosclerotic and coronary vascular protective agents.

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REFERENCES

1. Buapool D, Mongkol N, Chantimal J, Roytrakul S, Srisook E, Srisook K. 2013. Molecular mechanism of anti-inflammatory activity of *Pluchea indica* leaves in macrophages RAW 264.7 and its action in animal models of inflammation. J. Ethnopharmacol. 146, 495–504.
2. Sen T, Dhara AK, Bhattacharjee S, Pal S, Nag Chaudhuri AK. 2002. Antioxidant activity of the methanol fraction of *Pluchea indica* root extract. Phytother. Res. 16(4), 331-335.
3. Thoobbucha N, Petchlert C. 2015. Free radical scavenging capacity and modulative effect on antioxidant enzyme activity of *Pluchea indica* Less. extract. Naresuan-Payao J. 8(2), 74-79.
4. Kangsadalampai K, Butryee C, Manoonphol K. 1996. Direct mutagenicity of the polycyclic aromatic hydrocarbon-containing fraction of smoked and charcoal-broiled foods treated with nitrite in acid solution. Food Chem. Toxicol. 35, 213–218.
5. Maron DM, Ames BN. 1983. Revised methods for *Salmonella* mutagenicity test. Mutat. Res. 444, 451–461.
6. Yahagi T, Degawa M, Seino Y, Matsushima T, Nagao M. 1975. Mutagenicity of carcinogenic azo dyes and their derivatives. Cancer Lett. 1, 91–96.
7. De Serres FD, Shelby MD. 1979. Recommendations on data production and analysis using the Salmonella/microsome mutagenicity assay. Mutat. Res. 64, 159–165.
8. Calomme M, Pieters L, Vlirtnck A, Berghe VD. 1996. Inhibition of bacterial mutagenesis by citrus flavonoids. Planta. Med. 62, 222–226.
9. Roberts DB. 1986. Basic Drosophila care and techniques. In: Roberts DB, editor. Drosophila: a practical approach. Oxford: IRL Press. 1-38.
10. Frei H, Wurgler FE. 1988. Statistical methods to decide whether mutagenicity test data from *Drosophila* assays indicate a positive, negative, or inconclusive result. Mutat. Res. 203, 297-308.

11. Abraham SK, Singh SP, Kesavan PC. 1998. In vivo antigenotoxic effects of dietary agents and beverages co-administered with urethane: assessment of the role of glutathione *S*-transferase activity. Mutat. Res. 413, 103–110.
12. Niki E. 2000. Lipid peroxidase. In: Taniguchi N, Gutteridge JMC, editors. Experimental Protocols for Reactive Oxygen and Nitrogen Species, Oxford University Press. 156-157.
13. Mladenovic´ M, Matic´ S, Stanic´ S, Solujic´ S, Mihailovic´ V, Stankovic´ N, Katanic J. 2013. Combining molecular docking and 3-D pharmacophore generation to enclose the in vivo antigenotoxic activity of naturally occurring aromatic compounds: Myricetin, quercetin, rutin, and rosmarinic acid. Biochem. Pharmacol. 86, 1376–1396.

Role of herbal mixture on *in vitro* antioxidant activity and eNOS activation in human endothelial cells

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ABSTRACT

Nitric oxide (NO) produced by endothelial nitric oxide synthase (eNOS), is crucial for maintaining vascular endothelial health and function. Oxidative stress and aging, important risk factors of atherosclerosis and cardiovascular diseases result in eNOS uncoupling and endothelial dysfunction. In this study, herbal mixture of *Etlingera pa*vieana (Zingiberaceae family) and *Butea superba* (Leguminoceae family) was designed and investigated *in vitro* antioxidant activity using DPPH and superoxide radical scavenging assay. The effects of this herbal mixture on the activation of eNOS and cell viability were also determined in human umbilical vein endothelial cell line (EA.hy 926 cells). The mixture of *E. pavieana* water extract and *B. superba* ethanol extract (ratio of 1:1) scavenged DPPH and superoxide radical in a dose-dependent manner with EC_{50} values of 110.9±7.5 and 271.3±28.7 □g/mL, respectively. We also found that the herbal mixture caused increase in eNOS phosphorylation without cytotoxicity against endothelial cells. It could be concluded that mixture of *E. pavieana* water extract and *B. superba* ethanol extract might improve endothelial dysfunction by decreasing oxidative stress and promoting NO production in endothelial cells. The data suggest that the mixture might be useful in development of food supplement for preventing atherosclerosis.

Keywords: *Etlingera pavieana*, *Butea superba*, Antioxidant activity, eNOS, endothelial cell

INTRODUCTION

Endothelial nitric oxide synthase (eNOS)-catalyzed nitric oxide (NO) is involved in regulating of vascular tone by causing vasodilation through the generation of cGMP in the vascular smooth muscle cells. It also possesses anti-atherosclerotic properties by inhibiting platelet aggregation and preventing leukocyte adhesion to vascular endothelium [1].

Cardiovascular diseases most often results from atherosclerosis and is a leading cause of death of noncommunicable diseases (NCDs) [2]. Endothelial dysfunction is an early biomarker of atherosclerosis. Reduction in NO bioavailability is cardinal feature of endothelial dysfunction. Oxidative stress plays an important role in atherosclerosis by promoting eNOS uncoupling and endothelial dysfunction. Thus, decreasing oxidative stress and enhancing eNOS activation are promising strategies for prevention of atherosclerosis [3].

Etlingera pavieana (Pierre ex Gagnep.) R.M.Sm. is a plant belonging to the family Zingiberaceae and its rhizome has been showed significant antioxidant activity [4] and inhibitory effect on NO production in LPS-induced macrophages [5]. *Butea superba* Roxb. (Leguminoceae family) is Thai medicinal plant used for treating impotence and increasing rejuvenation [6, 7]. Tuber of the plant possesses various biological activities such as antioxidant, antimicrobial and acetylcholinesterase inhibitory activity [6-8]. In our ongoing study, various plants were screened for their antioxidant activity and eNOS activation property. The herbal mixture of *E. pavieana* and *B. superba* was designed and investigated for desired properties. The *in vitro* antioxidant activity of this herbal mixture was evaluated using DPPH and superoxide radical scavenging assay. The eNOS activation was evaluated by monitoring the level of phosphorylated eNOS in human endothelial cells.

MATERIALS AND METHODS

Preparation of plant extracts and herbal mixture

Ground rhizome of *E. paviena* was added to boiling distilled water (1:10), further heated for 30 min, and then filtered through Whatman No.1 paper and the procedure repeated. The combined filtrate was evaporated and freeze-dried. Ground tuber root of *B. superba* was macerated in 95% ethanol (1:10) at room temperature for 5 days and filtered through Whatman filter paper No.1. The plant residue was then re-extracted with ethanol twice as described above. Filtrates were pooled and evaporated in vacuo. The extracts were kept at -20^{o}C, finally protected from light until used. The herbal mixture was prepared by mixing the water extract of *E. paviena* rhizome and ethanol extract of *B. superba* in a ratio of 1:1.

DPPH radical scavenging assay

DPPH radical scavenging assay was performed according to the method of Srisook et al. [9].

Superoxide radical scavenging assay

Superoxide radical scavenging assay was performed according to the method of Jain and Agrawal [10].

Cell viability test by MTT assay

Human umbilical vein endothelial cell line (EA.hy 926 cells) was purchased from ATCC. Cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and incubated at 37 ^{o}C in 5% CO_2 atmosphere. Endothelial cells were plated into a 24-well plate (5X10^4 cells/well). Media containing the herbal mixture were added to wells and further incubated for 24 h. The cell viability was assessed as method described by Srisook et al. [11].

Determination of eNOS phosphorylation by Western blot analysis

Cells were plated into a 60-mm plate (3X10^5 cells) and treated with herbal mixture for 1 h. Cells were scraped in ice-cold eNOS lysis buffer (320 mM sucrose, 200 mM HEPES, 1mM EDTA). Collected cells were sonicated on ice and their protein concentrations were determined using Quick Start Bradford assay kit (Bio-Rad, USA). Equal amount of protein was separated on 10% SDS-PAGE and transferred onto PVDF membrane. After blocking for 1 h, the membrane was incubated with antibodies specific for p-eNOS-Ser1177 and eNOS (Cell Signalling Technology, USA) at 4^{o}C for overnight and then incubated with HRP-conjugated secondary goat anti-rabbit antibodies for 1 h. at room temperature. Specific protein bands on the PVDF membranes were visualized on X-ray film activated by ECL.

RESULTS AND DISCUSSION

DPPH radical scavenging assay has been widely used to screen free radical scavenging activity of natural plant antioxidants [9]. The herbal mixture of *E. pavieana* and *B. superba* dose-dependently scavenged DPPH radical (Figure 1). However, DPPH radical scavenging activity of herbal mixture (EC_{50} value=110.9±7.5 g/mL) was less than a positive control gallic acid (EC_{50} value=6.1±0.4 g/mL). Superoxide is a major reactive oxygen species formed under oxidative stress. In vascular endothelium, superoxide was generated from various enzyme systems including NADPH oxidase and uncoupled eNOS [12]. Superoxide avidly reacts with NO to form potent oxidizing agent peroxynitrite which further oxidized tetrahydrobiopterin BH_4, a NOS cofactor, leading to eNOS uncoupling, consequently reduction in NO bioavailability and endothelial dysfunction [3]. The study showed that amount of superoxide radical was also scavenged by the herbal mixture in a dose-dependent manner with EC_{50} value of 271.3±28.7g/mL, while an EC_{50} value of gallic acid was 36.5±2.0 g/mL (Figure 2).

In addition to regulation of eNOS activity by intracellular Ca^{2+} level, activation of eNOS is mediated by phosphorylation of the enzyme. Phosphorylation of Ser1177 residue stimulates the flux of electrons through the reductase domain and thus enzyme activity [13]. As shown in Figure 3, the treatment with herbal mixture increased the phosphorylation of eNOS compared to control cells which exposed to vehicle 0.2% DMSO. The cell viability of human umbilical vein endothelial cell line (EA.hy 926 cells) was not affected by the herbal mixture at concentration up to 100 g/mL (Figure 4). A similar observation on eNOS activation by plant extract has been reported in treatment of black current extract [14].

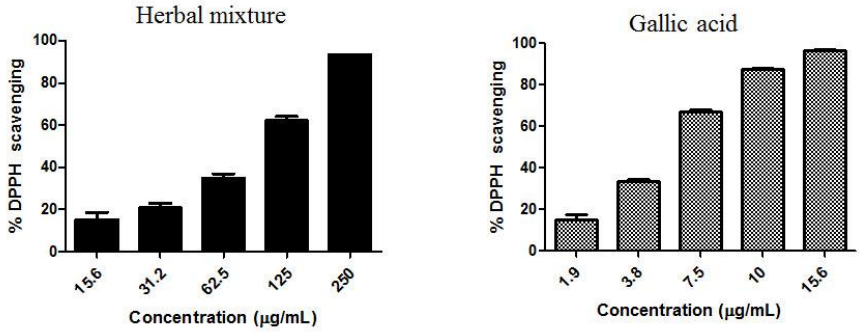

Figure 1. DPPH radical scavenging activity of herbal mixture and gallic acid at various concentrations. Each column shows the mean ± SEM. of at least 3 experiments with triplicate samples.

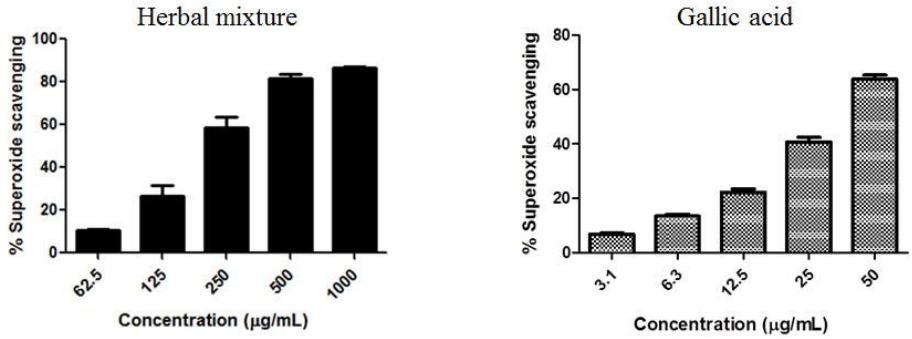

Figure 2. Superoxide radical scavenging activity of herbal mixture and gallic acid at various concentrations. Each column shows the mean ± SEM. of at least 3 experiments with triplicate samples.

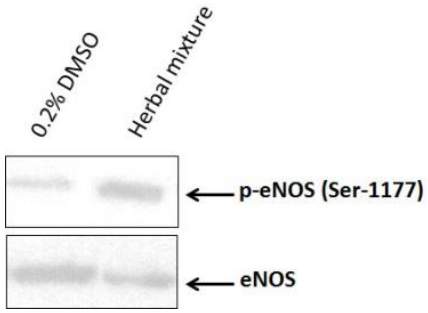

Figure 3. Effect of herbal mixture on phosphorylation of eNOS. The EA.hy 926 cells were treated with 100 µg/mL of herbal mixture for 1 h. and quantified the level of p-eNOS-Ser1177 and eNOS by Western blot analysis. 0.2 % (v/v) DMSO was used as vehicle. The immuno-blot picture shown is a representative of three separate experiments.

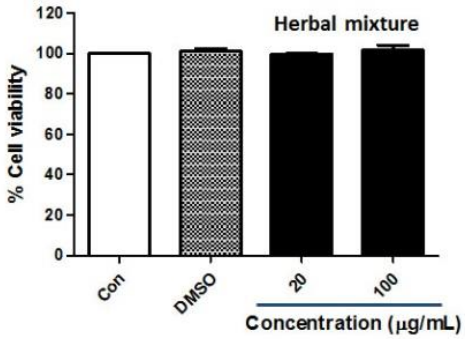

Figure 4. Effect of herbal mixture on EA.hy 926 cell viability. Cells were treated with 20 and 100 µg/mL of herbal mixture for 24 h. and determined cell viability by MTT assay as described in the Methods. CON = control cells and DMSO = cells treated with 0.2 % (v/v) DMSO. Each column shows the mean ± SEM. of 3 experiments with triplicate samples.

CONCLUSIONS

The obtained results showed that the herbal mixture of *E. pavieana* water extract and *B. superba* ethanol extract scavenged DPPH and superoxide radicals dose-dependently and increase in eNOS phosphorylation without cytotoxicity against endothelial cells. It implies the vascular protective effect of this herbal mixture by decreasing oxidative stress and promoting NO production in endothelial cells. The data suggest that the mixture might be useful in development of dietary supplement for reducing risk of atherosclerosis and cardiovascular diseases. However, further experiments on identified the active compounds and *in vivo* study are required.

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REFERENCES

1. Li H, Forstermann U. 2013. Uncoupling of endothelial NO synthase in atherosclerosis and vascular disease. Current Opinion in Pharmacology. 13, 161–167.
2. World Health Organization. 2015. Global status report on noncommunicable diseases 2014.
3. Li H, Forstermann U. 2011. Therapeutic effect of enhancing endothelial nitric oxide synthase (eNOS) expression and preventing eNOS uncoupling. Br. J. Pharmacol. 164, 213–223.
4. Srisook K, Nounnang N, Thabthim Y, Srisook E. 2012. A comparative study of antioxidant and anti-tyrosinase activities of rhizomes and leaves of *Etlingera pavieana*. Proceeding of the 4th International Natural Products for Health and Beauty. pp. 551.332-551.335.
5. Srisook E, Palachot, M, Srisook K. 2012. In vitro anti-inflammatory effect of *Etlingera pavieana* rhizomes and its compounds in lipopolysaccharide-induced macrophages. Proceeding of the 4th International Natural Products for Health and Beauty. pp. 551.329-551.331.
6. Prasad M, Padmalatha K, Jayaram K, Raju N, Silva J. 2007. Medicinal plants from deccan ecoregion, India: Traditional knowledge, ethnopharmacology, cultivation, utilization, conservation and biotechnology-opportunities and impediments. Medicinal and Aromatic Plant Science and Biotechnology. 1, 155-208.
7. Chukeatirote E, Saisavoey T. 2009. Antimicrobial property and antioxidant composition of crude extracts of *Pueraria mirifica*, *Butea superba* and *Mucuna macrocarpa*. Maejo Int. J. Sci. Technol. 3, 212-221.
8. Nuengchamnong N, Ruengprapun N, Kanitvittaya S, Sornprasit S, Promhirangul P, Noiarsa P, Sucharay B. 2007. Screening of Thai fruit and herbal beverages for their antioxidant activities. J. Health Sci. 16, 778-785. (In Thai.).
9. Srisook K, Buapool D, Boonbai R, Simmasut P, Charoensuk Y, Srisook E. 2012. Antioxidant and anti-inflammatory activities of hot water extract from *Pluchea indica* Less herbal tea. J. Med. Plants Res. 6, 4077-4081.
10. Jain P, Agrawal R. 2008. Antioxidant and free radical scavenging properties of developed mono- and polyherbal formulations. Asian J. Exp. Sci. 3, 213-220.
11. Srisook K, Srisook E, Nachaiyo W, Chan-In M, Thongbai J, Wongyoo K, Chawsuanthong S, Wannasri K, Intasuwan S, and Watcharanawee K. 2015. Bioassay-guided fractionation of anti- inflammatory agents from *Clerodendrum inerme* (L.) Gaertn. leaves and its mechanistic action through the suppression of iNOS and COX-2 pathways in LPS-induced RAW 264.7 macrophages. Journal of Ethnopharmacology. 165, 94-102.
12. Ugusman A, Zakaria Z, Hui C, Nordin N. 2011. *Piper sarmentosum* inhibits ICAM-1 and Nox4 gene expression in oxidative stress-induced human umbilical vein endothelial cells. BMC Compl. Altern. Med. 11:31 (8 pages).
13. Forstermann U, Sessa W. 2012. Nitric oxide synthases: regulation and function. Eur. Heart J. 33, 829–837.
14. Edirisinghe I, Banaszewski K, Cappozzo J, McCarthy D, Burton-Freeman B. 2011. Effect of black currant anthocyanins on the activation of endothelial nitric oxide synthase (eNOS) *in vitro* in human endothelial cells. J. Agri. Food Chem. 59, 8616–8624.

Effect of *Cissus quadrangularis*, *Pluchea indica* and *Clerodendrum serratum* extracts on expression of cyclooxygenase proteins

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ABSTRACT

Several Thai herbal medicines have been safely and effectively used for treatment of hemorrhoid, a disease of enlarged anal veins associated with inflammation. *Cissus quadrangularis* L. (CQ), *Pluchea indica* (L.) Less (PI) and *Clerodendrum serratum* Moon var. wallichii Clarke (CS) are commonly used for hemorrhoid treatment. To investigate the anti-inflammatory effect of CL, PI and CS crude extract, we examined cyclooxygenase (COX)-1 and COX-2 protein expression using western immunoblotting technique. MTT cell viability test indicated that the ethanoic extract of CQ, PI and CS showed minimum toxicity in HeLa cells at a concentration of 3.13, 1.56 and 3.13 µg/ml, respectively. In LPS-activated HeLa cells, COX-2 protein expression did not change with the treatment of CL, PI and CS extract. Similar findings were obtained with COX-1 protein. Our results suggest that these medicinal plant extracts may mediate anti-inflammatory effect through different pathways. Alternatively, the inhibition does not change protein expression but could involve enzyme activity, which should be further investigated.

Keywords: *Cissus quadrangularis* L., *Pluchea indica* (L.) Less, *Clerodendrum serratum* Moon, anti-inflammation, cyclooxygenases

INTRODUCTION

Hemorrhoid is a common disease characterized by enlarging of veins in the anal canal, in which can cause bleeding and pain if ruptures. The lack of exercise, low-fiber diets and modern-day lifestyle, are ones of the major factors contributing to the increasing prevalence of hemorrhoid. Hemorrhoid can be manifested by chronic inflammation of tissues in the rectal area causing pain, discomfort and embarrassment to patients. Treatments for hemorrhoid depend on the disease stage. Many Thai herbal medications have been commonly used for treatment of hemorrhoid due to their safety and low cost. *Cissus quadrangularis* L. (CQ), in Vitaceae family, is the only herbal remedy for hemorrhoid that is listed in the National List of Essential Medicine (NLEM). While CQ has been intenstively studied for its mechanism of action, other herbs such as *Pluchea indica* (L.) Less (PI) and *Clerodendrum serratum* Moon var. wallichii Clarke (CS) are also widely and effectively used. It was reported that CQ and PI elicited its anti-inflammatory effect through inhibition of nitric oxide (NO), lipoxygenase (LO) and cyclooxygenase (COX) pathways [1-4]. They inhibited prostaglandin E_2 production, EPP-induced ear edema and carrageenan-induced paw edema in animal models. In contrast, CS has only few studies on its anti-inflammatory action. COX is an important enzyme that produces many inflammatory mediators from arachidonic acids, particularly PGE_2. It is also a direct target of non-steroidal anti-inflammatory drugs (NSAIDs), which are effectively used for combating inflammation and pain. Thus, in the present study, we investigate the effect of PI and CS, compared with CQ, on the expression of COX-1 and COX-2 proteins in order to explore the mechanism of action of three commonly used herbal medicines for hemorrhoid treatment.

MATERIALS AND METHODS

Ethanol extraction of crude extract from plant samples

Pluchea indica (L.) Less (PI), *Clerodendrum serratum* Moon var. wallichii Clarke (CS) and *Cissus quadrangularis* L. (CQ) were cut into small pieces followed by drying in a hot air oven at 50°C for 3-5 days. 5 g of dried plants was extracted with 95% ethanol above boiling water for 15 min and the filtrate was consequently collected. Then, the residue was

replenished with ethanol and the extraction was repeated. All filtrates were combined and the ethanol was evaporated. The dried crude extract was then stored at 4°C and protected from light.

Cell culture and MTT cell viability assay

HeLa cells were cultured in D ulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 4.5 g/l D-glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 1% nonessential amino acids. The cells were maintained in a humidified chamber at 37°C, 5% CO_2. To perform MTT assay, HeLa cells ($1.5x10^4$ cells) were seeded in a 96-well plate at 100 µl. Next day, cells were treated with crude extract of PI, CS and CQ dissolved in DMSO for 24 h. Subsequently, 10 µl of MTT solution (2 mg/ml, Sigma) was added to each well and incubated in the humidified incubator for 2 h. After removing the media, DMSO was added to dissolve the formazan product, which is generated by mitochondria of viable cells, and the absorbance was measured at 550 nm after 2 h of incubation at room temperature. % Cell viability was calculated as percentage of the absorbance of the treatments subtracted with that of cell-free control compared with DMSO.

Western immunoblotting

To measure the expression of COX proteins, HeLa cells ($3x10^5$ cells) were cultured in a 6-well plate until 70% confluency. Cells were washed with phosphate buffer saline (PBS) and replaced with serum-free media. After 24 h, crude extracts of PI, CS and CQ at the minimum toxicity concentrations were used to treat cells for another 24 h. When appropriate, lipopolysaccharide (LPS, Sigma) activation of inflammation was induced at 30 min upon treatments. Subsequently, the cells were lysed in lysis buffer containing 60 mM Tris-HCl buffer, pH 6.8, 2% SDS and protease inhibitors, with sonication at 130 watts, 40% amplitude for 5 sec with 5-sec pause (5 times). The samples were then centrifuged at 12,000 xg for 10 min at 4°C (Haraeus Biofuge 15R). Thereafter, the supernatant was collected and used for BCA protein concentration assay (Thermo Scientific). 30 µg of total protein lysates were then subjected to SDS-PAGE using 10% acrylamide gel, and, afterwards, transferred to PVDF membrane. The protein blot was subsequently blocked for non-specific binding with 5% skimmed milk in Tris buffer saline (TBS) containing 0.1% tween 20 (TBS-T), at room temperature for 1 h. Next, the blot was probed with rabbit anti-COX-1 and COX-2 antibodies (1:1000, Cell Signaling Technology) in 5% BSA/TBS-T, overnight with gentle shaking at 4°C. The secondary antibody was goat anti-rabbit IgG conjugated with horseradish peroxidase (HRP) (1:10,000, KPL), which was probed for 1 h at room temperature. The protein bands were detected with enhanced chemiluminescence (ECL, Thermo Scientific) and X-ray film (Thermo Scientific).

RESULTS

To study the effect of our interested plant extracts on the expression of inflammatory COX proteins, we first investigated the toxicity of the ethanoic extracts on HeLa cells. Upon treatments of different concentrations of the extracts for 24 h, the cells were tested with MTT cell viability assay. The results showed that these ethanol extracts affected HeLa cell survival in a concentration-dependent manner compared to the vehicle control DMSO (Figure 1). However PI, CS and CQ at the concentration of 3.13, 1.56 and 3.13 µg/ml, respectively, elicited the least toxicity effect on the cells. Furthermore, at the high dose 400 µg/ml, PI, CS and CQ caused 85, 60, 59% cell survival, respectively, suggesting that PI is possibly less toxic to the cells than the others. When PI concentration was doubly increased to 800 µg/ml, the comparable survival effect (approximately 60 %) was then obtained. The minimum toxicity concentration was subsequently used for examining the expression of COX-1 and COX-2 proteins using western immunoblotting technique. However, we found that the 65 kDa protein bands of COX-1 in HeLa cells did not change with the treatments at the concentration tested (Figure 2). Likewise, in HeLa cells activated with LPS, these extracts had no effect on the expression of COX-2 protein, which is well known for its principal role in inflammation (Figure 3). Moreover, these finding is consistent with our quantitative reverse transcriptase (RT) PCR (data not shown).

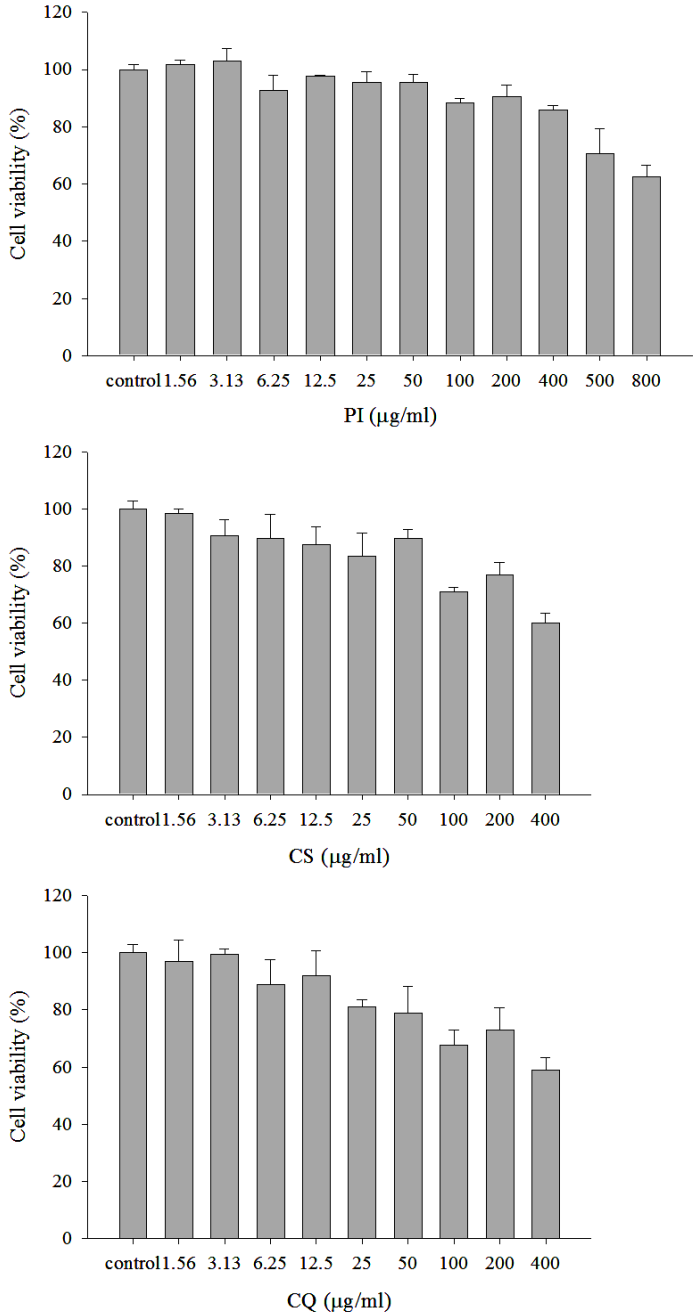

Figure 1. Effect of PI, CS and CQ extracts on viability of HeLa cells. HeLa cells were treated with different concentrations of PI, CS and CQ extracts for 24 h and assessed with MTT cell viability assay. % Cell viability was compared with cells treated with DMSO as a control.

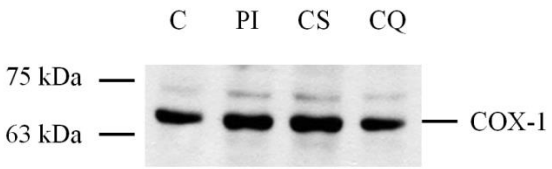

Figure 2. Effect of PI, CS and CQ extracts of COX-1 protein expression. HeLa cells were treated with DMSO (C), PI (3.13 µg/ml), CS (1.56 µg/ml) or CQ (3.13 µg/ml) extracts for 24 h. Total lysate were analyzed with western immunoblotting using specific monoclonal antibody against COX-1 protein (1:1,000, Cell Signaling Technology).

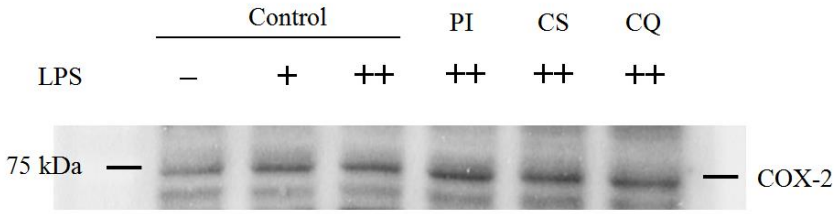

Figure 3. Effect of LPS and PI, CS and CQ extracts on COX-2 protein expression. HeLa cells were treated with DMSO as a control or extracts of PI (3.13 µg/ml), CS (1.56 µg/ml), CQ (3.13 µg/ml). Upon the treatments, cells were activated with LPS to promote inflammation. The symbols "+" and "++" indicate the concentration of LPS; 1 and 5 µg/ml, respectively. The equally loaded cell lysates were subjected to western immunoblotting and detected for COX-2 protein expression using anti-COX-2 antibody (1:1,000, Cell Signaling Technology).

CONCLUSIONS

Our study showed that ethanoic extracts of PI, CS and CQ did not change the protein expression of COX-1 and COX-2 in HeLa cells. Interestingly, previous studies showed that PI and CQ inhibited PGE_2 production [1, 3], an important inflammatory mediator produced by COXs. Therefore, our findings suggest that the mechanism of anti-inflammation of these plants might not take place at the protein expression level but could involve the enzyme activity. Moreover, these ethanol plant extracts might mediate anti-inflammatory effect through different pathways. Further studies are required to investigate the exact mechanism and of these herbal medicines commonly used for treatment of hemorrhoids.

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REFERENCES

1. Bhujade AM, Talmale S, Kumar N, Gupta G, Reddanna P, Das SK, Patil MB. 2012. Evaluation of *Cissus quadrangularis* extracts as an inhibitor of COX, 5-LOX, and proinflammatory mediators. J. Ethnopharmacol. 141, 989-996.
2. Panthong A, Supraditaporn W, Kanjanapothi D, Taesotikul T, Reutrakul V. 2007. Analgesic, anti-inflammatory and venotonic effects of *Cissus quadrangularis* Linn. J. Ethnopharmacol. 110, 264-270.
3. Buapool D, Mongkol N, Chantimal J, Roytrakul S, Srisook E, Srisook K. 2013. Molecular mechanism of anti-inflammatory activity of *Pluchea indica* leaves in macrophage RAW 264.7 and its action in animal models of inflammation. J. Ethnopharmacol. 146, 495-504.
4. Sen T, Ghosh TK, Chaudhuri AK. 1993. Studies on the mechanism of anti-inflammatory and anti-ulcer activity of *Pluchea indica*-probable involvement of 5-lipoxygenase pathway. Life Sci. 52, 737-743.

Effect of sterilized conditions on rice callus induction and anti-aging properties

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ABSTRACT

This study investigated concentration and incubation time effects of Clorox on growth and biological activities of callus during surface sterilization processes. In order to induct callus from rice seeds, the seeds were sterilized under different concentrations of Clorox (10 – 40%) and incubation time (10 – 40 minutes). Then, the sterilized rice seeds were inoculated on agar solidified Murashige and Skoog medium supplemented with 2,4-D, NAA and Kn for callus induction. After 21 days, rice callus was collected and extracted with ethanol. The extract was evaluated for total phenolic content and anti-aging properties by using DPPH radical scavenging, anti-inflammatory, anti-tyrosinase, anti-collagenase, and keratinocytes proliferation-promoting assays. The results showed that increasing Clorox concentration and incubation time reduced total phenolic content and most anti-aging activities, except anti-collagenase activity. The best condition of rice-seed surface sterilization was 30% Clorox for 30 minutes which showed complete surface sterilization (0% contamination) and high total phenolic content (27.40mg GAE/ml), DPPH radical scavenging (36.56%), superoxide dismutase activity (50.11%), nitric oxide inhibition (65.14%), anti-tyrosinase (69.06%), anti-collagenase (38.61%), and keratinocyte proliferation (34.44%). In summary, higher Clorox concentration and incubation time reduce the risks of contamination in rice-callus induction and also affect bioactivities of rice callus. Furthermore, rice-callus extract has anti-aging potential for use as an active ingredient in cosmetic and pharmaceutical applications.

Keywords: Anti-aging activities, Clorox concentration, Clorox incubation time, Rice callus, Sodium hypochlorite, Surface sterilization, Total phenolic content

INTRODUCTION

Rice (*Oryza sativa* L.) is the world's major food and has many health benefits because it contains high amounts of phytochemical constituents including γ-oryzanols, phenolics, flavonoids, etc. [1, 2]. These active compounds are also synthesized and accumulated in cultured rice callus. Ludwig and coworker reported that the extract of rice callus can improve the human-skin barrier function with anti-aging activities [3]. Recently, plant-cell cultures are an alternative technique for the production of active ingredients for pharmaceutical and cosmetic uses as they provide contaminant-free biosustainable systems. As well, the production of desired compounds can be easily expanded to an industrial scale. During rice-callus induction process, it is easily contaminated with fungi, yeast and bacteria due to rice seeds high-opportunity infect with microorganisms from the environment. Sodium hypochlorite (NaOCl) is the most common agent used for surface sterilization in plant-tissue culture techniques. It is a strong oxidizer with broad spectrum antimicrobial activity and reduces losses during germination caused by pathogens [4, 5]. NaOCl ionizes to sodium cation (Na^+) when dissolved in water and hypochlorite anion (ClO^-) is in equilibrium as hypochlorous acid (HOCl) [6]. The HOCl damages microbe-cell membranes, proteins and nucleic acids by oxidative degradation upon contact [7]. Moreover, NaOCl affects metabolism and germination of plant seeds due to its strong oxidizing property, which makes it highly reactive with amino acids, nucleic acids, amines, and amides [8]. The general reaction between NaOCl and amino acids produces the respective aldehyde, NH_4Cl and CO_2 [9]. Thus, during NaOCl sterilization, NaOCl may also react with some active constituent in callus resulting in changes in their pharmaceutical properties. Therefore, the optimal condition for callus induction against the sterilized condition should be investigated.

The purpose of this study was to examine the effect of Clorox concentration and incubation time during surface sterilization on the growing and biological activities of rice callus. Various Clorox concentration and incubation time were used as treatment conditions for rice-seed surface sterilization. Then, rice callus was inducted with hormone-

supplemented Murashige and Skoog medium. The callus was determined for their total phenolic content and anti-aging potential including antioxidant (DPPH radical-scavenging and superoxide- dismutase activities), anti-inflammatory, anti-tyrosinase, anti-collagenase, and keratinocytes proliferation-promoting activities.

MATERIALS AND METHODS

Plant material and surface sterilization of rice seed

Plant material

In this investigation, Mun-pu rice, which is a pigmented rice variety, was chosen to induce the callus. The sample was cultured in Chiang Rai, Thailand and collected on December, 2013.

Optimal Clorox concentration

In this study, sodium hypochlorite was derived from Clorox household bleach solution (5.25 % NaOCl), which is a common brand used in plant-tissue culture laboratories. Dehusked rice seed was sterilized with Clorox at various concentrations including 10, 20, 30, and 40% for 30 minutes. Then, rice seed was rinsed three times with autoclaved distilled water and dried with autoclaved tissue paper. The seed was cultured on medium for callus induction.

Optimal Clorox incubation time

The optimal incubation duration was determined by immersing the rice seed in 30% Clorox at various incubation times including 10, 20, 30, and 40 minutes. Rice seeds were rinsed three times with autoclaved distilled water and dried with autoclaved tissue paper. The seeds were cultured on medium for callus induction.

Induction of rice callus

For the establishment of callus cultures, the sterilized rice seed was inoculated on agar solidified basal Murashige and Skoog (MS) medium (pH 5.8) and supplemented with 2,4-D (2 mg/L), NAA (1 mg/L), and Kn (0.2 mg/L). The contamination and explants response toward callus formation was observed after 14 days of incubation. The non-contaminated callus was collected after 21 days.

Extraction of rice callus

Callus (1g) was extracted with 10 ml ethanol and sonicated for 2 hours. Then, the callus extract was filed and stored at 4°C for future use.

Total phenolic content (TPC)

Total phenolic content was analyzed using the Folin-Ciocalteu assay [10]. The extract was mixed with deionized water, Folin-Ciocalteu reagent, and 7% sodium carbonate. Then, mixtures were incubated for 60 minutes before measured absorbance at 750 nm and expressed as gallic-acid equivalents (µg GAE/mL extract).

Antioxidant by using DPPH radical scavenging assay (DPPH)

The scavenging activity of 1,1-diphenyl-2-picrylhydrazyl free radicals was determined following Vichit and Saewan [10]. The extract was mixed with 0.1 mM DPPH solution and incubated for 30 minutes at room temperature. Absorbance was measured at 515 nm. The scavenging activity was derived as follows:

$$\text{DPPH scavenging activity (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

Antioxidant by using superoxide dismutase activity (SOD)

The supernatant of treated cells with 50% extract was collected. WST working solution and enzyme working solution were added and then mixed thoroughly. The mixture was incubated at 37°C for 20 minutes and measured absorbance at 450 nm. The SOD activity was calculated using the following equation:

$$\text{SOD activity (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

Anti-inflammation by using nitric-oxide inhibition (NO)

The culture medium of treated cells with 50% extract and 1 µg/ml lipopolysachharide was combined with sulfanilamide solution and incubated for 5 minutes at room temperature. Then, N-1-napthylethylenediamine

dihydrochloride solution was added and incubated for 5 minutes. The absorbance of the solution was measured at 540 nm. The percentage of nitric oxide inhibition was calculated using the following equation:

$$\text{Inhibition of nitric oxide (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

Anti-tyrosinase activity (ATR)

Anti-tyrosinase assays were described by Saewan et al. [11] using a modified dopachrome method with L-DOPA as the substrate. Extract was added to 0.1 M phosphate buffer (pH 6.8) and 1 mM L-DOPA. The mixture was incubated for 10 minutes at 37°C. Then, 200 unit/ml mushroom tyrosinase was added into the sample solution and incubated for 15 minutes at 37°C. Absorbance was measured at 475 nm and calculated as:

$$\text{Inhibition of tyrosinase (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

Anti-collagenase activity (ACL)

Anti-collagenase activity was designed to screen MMP-1 inhibitors using a thiopeptide as a chromogenic substrate. Extract was added with 153 mU/µl MMP-1 and 1.3 µM NNGH inhibitor. They were incubated for 60 minutes at 37°C. Then, 100 µM substrate was added and absorbance was measured at 412 nm. The percentage of collagenase inhibition was calculated as:

$$\text{Collagenase inhibition (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

Keratinocytes proliferation promoting (KPP)

The extract was added to human-epidermal keratinocyte culture and incubated for 72 hours at 37°C in a 5% CO_2 humidified incubator. Then, the culture medium was removed and 0.1 mg/ml MTT solution was added. After 4 hours of incubation, dimethylsulfoxide was added and incubated for a further 30 minutes. The absorbance was measured at 570 nm and the percentage of keratinocytes' proliferation was calculated as:

$$\text{Keratinocytes' proliferation (\%)} = [(A_{sample} - A_{control})/A_{control})] \times 100$$

Statistical analysis

The obtained data were performed in triplicate and statistically analyzed using the SPSS program version 21 for Windows (SPSS Inc., Chicago, IL, USA), and the differences were considered significant when $p<0.05$. The comparison of data between each sample was analyzed by using One Way Analysis of Variance (ANOVA).

RESULTS

Rice seed was sterilized by Clorox at different concentrations (10, 20, 30, and 40%) and durations (10, 20, 30, and 40 minutes). Sterilized rice seed was induced callus on solidified MS medium supplemented with 2,4-D, NAA, and Kn for 21 days. Then, rice callus was collected and extracted for determined total phenolic content and anti-aging activities. In this study, anti-aging activities of rice-callus extract was determined by using antioxidant, anti-inflammatory, anti-tyrosinase, anti-collagenase, and keratinocytes proliferation activities. Antioxidant activities were determined by two different assays including DPPH radical scavenging (DPPH) and superoxide dismutase (SOD). DPPH assay provides on a hydrogen atom donating ability [12]; whereas, SOD assay detects activity of the most important antioxidant enzyme that acts as catalyze the dismutation of superoxide radical into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) [13]. Anti-inflammation was determined the by inhibition of nitric oxide (NO), which is considered a pro-inflammatory mediator that induces inflammation under abnormal conditions [14]. Anti-tyrosinase activity investigated the depigmentation agents that act as inhibitors of tyrosinase, which is the key enzyme in melanogenesis or melanin-pigment production in the skin [11]. Anti-collagenase activity was determined for matrix metalloproteinase-1 (MMP-1) inhibitors using a thiopeptide as a chromogenic substrate. MMP-1 is fibroblast collagenase, which is primarily responsible for the degradation of collagen in the photo-aging process of human skin [15]. Keratinocytes proliferation-promoting activity is used to prove anti-aging mechanisms because it is a source of material for wound healing [16]. These activities are involved in aging skin in many pathways and can be used to prove the anti-aging potential of natural extract.

Optimal Clorox concentration

The optimal Clorox concentration for rice-seed surface sterilization was performed at various concentrations including 10, 20, 30, and 40% for 30 minutes. The result showed that increasing the Clorox concentration reduced the contamination of rice seed (Table 1). When Clorox concentration was increased from 10 to 20%, the contamination was decreased from 25.71 to 2.14%. Up to 30% of Clorox concentration provided complete surface sterilization. The highest callus induction was found in surface sterilization with 30% Clorox. Whereas, the lowest callus induction was found in 10% Clorox with almost seed contaminated by fungi. Total phenolic content and most anti-aging activities were decreased when Clorox concentration was increased, except for anti-collagenase activity. At 10%, Clorox showed significantly higher TPC, SOD, and NO than other Clorox concentrations. While, the highest ACL was 40% Clorox that may be caused by NaOCl induced some substance against collagenase enzyme. The high correlation between TPC and anti-aging activities was found with SOD (0.748), NO (0.767), TR (0.712), and MPP (-0.721). The most effective Clorox concentration was 30% with complete sterilization and high anti-aging activities. Therefore, this concentration was used for further experiment activities.

Table 1. Effect of Clorox concentration

		Clorox concentration (%)			
		10	20	30	40
Contamination (%)		25.71 ± 4.04^{c}	2.14 ± 1.01^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Callus induction (%)		73.57 ± 3.03^{b}	95.71 ±2.02^{a}	97.86 ± 1.01^{a}	95.71 ± 4.04^{a}
TPC	(µg GAE/ml extract)	36.27 ± 3.25^{a}	29.42 ± 2.81^{b}	26.26 ± 0.59^{b}	19.92 ± 5.41^{c}
DPPH	(% DPPH scavenging)	38.68 ± 2.26^{a}	38.86 ± 1.44^{a}	39.47 ± 2.22^{a}	29.28 ± 3.00^{b}
SOD	(% SOD activity)	58.26 ± 3.85^{a}	52.16 ± 1.99^{b}	50.51 ± 0.65^{b}	40.33 ± 0.45^{c}
NO	(% NO inhibition)	79.70 ± 0.58^{a}	68.47 ± 1.75^{b}	69.93 ± 2.89^{b}	40.90 ± 3.13^{c}
ATR	(% TR inhibition)	71.68 ± 0.18^{a}	72.38 ± 0.69^{a}	69.96 ± 1.57^{b}	66.86 ± 0.82^{c}
ACL	(% MMP inhibition)	32.92 ± 1.83^{c}	33.89 ± 1.13^{c}	38.68 ± 2.95^{b}	54.61 ± 2.17^{a}
KPP	(% Proliferation)	32.61 ± 1.15^{a}	30.45 ± 1.49^{a}	30.07 ± 1.08^{a}	26.58 ± 1.15^{b}

Values represent means ± sd with different letters within the same row being significantly different ($p < 0.05$).

Optimal Clorox incubation time

The optimal Clorox incubation time of rice-seed surface sterilization was performed at 10, 20, 30, and 40 minutes with 30% Clorox. The complete surface sterilization was obtained when rice seed was incubated for 30 and 40 minutes (Table 2). When incubation duration was more than 30 minutes, it was completely sterilized. Clorox incubation at 30 minutes was the highest callus induction and at 10 minutes was the lowest callus induction due to most rice seed being effected by fungal contamination. The shorter incubation periods showed higher SOD, NO, ATR, and KPP. While ACL was increased when the Clorox incubation time was increased. Moreover, the high correlation between total phenolic content and anti-aging activities was found with NO (0.716) and TR (0.817).

Table 2. Effect of Clorox incubation time

		Clorox incubation time (min)			
		10	20	30	40
Contamination (%)		33.57 ± 5.05^{c}	4.29 ± 2.02^{b}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
Callus induction (%)		65.00 ± 5.05^{c}	95.00 ± 1.01^{a}	97.86 ± 1.01^{a}	89.29 ± 3.03^{b}
TPC	(µg GAE/ml extract)	26.29 ± 0.94^{a}	26.81 ± 0.20^{a}	27.40 ± 0.15^{a}	15.67 ± 2.36^{b}
DPPH	(% DPPH scavenging)	36.33 ± 1.23^{a}	34.59 ± 2.46^{a}	36.56 ± 1.97^{a}	29.37 ± 1.97^{b}
SOD	(% SOD activity)	55.95 ± 0.35^{a}	51.60 ± 0.63^{b}	50.11 ± 1.47^{b}	46.32 ± 0.49^{c}
NO	(% NO inhibition)	78.67 ± 2.65^{a}	70.41 ± 5.52^{b}	65.14 ± 4.03^{b}	48.61 ± 1.64^{c}
ATR	(% TR inhibition)	74.57 ± 1.23^{a}	70.74 ± 0.85^{b}	69.06 ± 0.81^{b}	55.94 ± 0.95^{c}
ACL	(% MMP inhibition)	21.35 ± 1.98^{c}	27.27 ± 2.36^{c}	38.61 ± 4.80^{b}	55.72 ± 4.22^{a}
KPP	(% Proliferation)	38.54 ± 0.74^{a}	36.26 ± 1.31^{b}	34.44 ± 0.77^{c}	34.02 ± 0.95^{c}

Values represent means ± sd with different letters within the same row being significantly different ($p < 0.05$).

Overall results showed that total phenolic content and most anti-aging activities were decreased when it was incubated in high Clorox concentration (40 %) and incubation time (40 minutes). These result may be occured due to NaOCl not only destroyes microorganism or microbe cell membranes but it can damages some plant cells. When plant cells were damaged with excess Clorox concentration or incubation time, plant cells were low substance production which affect to antioxidant and anti-aging activities. While, anti-collagenase activity was increased when increased Clorox concentration and incubation time that may be caused by NaOCl induced some substance against collagenase enzyme.

CONCLUSION

The rice-seed surface sterilization studied two factors: concentration and incubation time of Clorox on callus-induction and anti-aging activities of rice callus. The complete surface sterilization of rice seed occurred with more than 30% Clorox and 30 minutes of incubation time. Clorox concentration and incubation duration showed effects on total phenolic content and anti-aging activities. Increasing the Clorox concentration and incubation time decreased the total phenolic content and anti-aging activities, except anti-collagenase activity. Overall, results showed that rice callus has anti-aging potential for use as an active ingredient in cosmetic and pharmaceutical applications.

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REFERENCES

1. Finocchiaro F, Ferrari B, Gianinetti A, Dall'asta C, Galaverna G, Scazzina F, et al. 2007. Characterization of antioxidant compounds of red and white rice and changes in total antioxidant capacity during processing. Mol. Nutr. Food Res. 51, 1006 - 1019.
2. Chung HS, Shin JC. 2007. Characterization of antioxidant alkaloids and phenolic acids from anthocyanin-pigmented rice (*Oryza sativa* cv. *Heugjinjubyeo*). Food Chem. 104, 1670 - 1677.
3. Ludwig P, Bennett S, Gruber J. 2011. Rice meristem stimulates epigenetic rejuvenation: new technologies. South African Pharmaceutical and Cosmetic Review 38, 34 - 37.
4. Bloomfield SF. 1996. Chlorine and iodine formulations. Ascenzi JM, ed. Handbook of Disinfectants and Antiseptics. New York: Marcel Dekker, Inc., 133 - 158.
5. Hong JH, Gross KC. 1989. Surface sterilization of whole tomato fruit with sodium hypochlorite influences subsequent postharvest behavior of fresh-cut slices. Postharvest Biol.Technol. 13, 51 - 58.
6. Macnish AJ, Morris KL, Theije A, Mensink MGJ, Boerrigter HAM, Reid MS, et al. 2010. Sodium hypochlorite: A promising agent for reducing Botrytis cinerea infection on rose flowers. Postharvest Biol. Technol. 58, 262 - 267.
7. McDonnell G, Russell AD. 1999. Antiseptics and disinfectants: activity, action, and resistance. Clin. Microbiol. Rev. 12, 147 - 179.
8. Abdul-Baki AA. 1974. Pitfalls in using sodium hypochlorite as a seed disinfectant in C incorporation studies. Plant Physiol. 53, 768 - 771.
9. Kantouch A, Ardel-Fattah SH. 1971. Action of sodium hypochlorite on α-amino acids. Chemicke Zvesti 25, 222 - 230.
10. Vichit W, Saewan N. 2015. Antioxidant activities and cytotoxicity of Thai pigmented rice. Int. J. Pharm. Pharm. Sci. 7, 329 - 334.
11. Saewan N, Koysomboon S, Chantrapromma K. 2011. Anti-tyrosinase and anti-cancer activities of flavonoids from *Blumea balsamifera* DC. J. Med. Plants Res. 5, 1018 - 1025.
12. Alam MN, Bristi NJ, Rafiquzzaman M, 2013. Review on in vivo and in vitro methods evaluation of antioxidant activity. Saudi Pharm. J. 21, 143 - 152.
13. Assady M, Farahnak A, Golestani A, Esharghian M, 2011. Superoxide dismutase (SOD) enzyme activity assay in *Fasciola* spp. parasites and liver tissue extract. Iran J. Parasitol. 6, 17 - 22.
14. Debnath T, Park S, Kim D, Jo J, Lim B. 2013. Anti-oxidant and anti-inflammatory activities of *Inonotus obliquus* and germinated brown rice extracts. Molecules. 18, 9293 - 9304.
15. Pandel R, Poljšak B, Godic A, Dahmane R. 2013. Skin photoaging and the role of antioxidants in its prevention. International Scholarly Research Notices Dermatology: 1 - 11
16. Diegelmann RF, Evans MC. 2004. Wound healing: an overview of acute, fibrotic and delayed healing. Front. Biosci. 9, 283 - 289.

Anti-oxidation and anti-tyrosinase activity of *Gnetum gnemon* Linn. leaf extract

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ABSTRACT

The *Gnetum gnemon* leave powder was extracted by acetone, ethanol, ethylacetate, hexane, methanol, water, and tris-HCl. The tris-HCl extract provided the highest crude extract, 9.5 ±0.1838 g/100 g. The anti-oxidant activity (DPPH° scavenging) was found in the acetone extract, 38.50±4.47%, and followed by the ethlyacetate extract, 18.65±0.51%. The hexane extract and the acetone extract showed the highest inhibition of lipid oxidation (TBARS), 28.48 ± 4.36%, and 28.25 ±12.74%, respectively. The acetone extract presented the highest peroxide inhibition. It could extend the shelf life of the sunflower oil. The total polyphenol, alpha-tocopherol, and beta-carotene in 100 g of the acetone extract were 1215.06±0.01 mgGAE, 18.96 mg, and 14.09 mg, respectively. The methanol extract showed the highest antityrosinase activity. The total phenolic compound, vitamin E and beta carotene in 100 g of the methanol extract were 773.4 ± 0.031 mg GAE/100 g, 14.54 mg and17.33 mg, respectively. The IC_{50} of the methanol extract and the semi-purified extracts, CT13 and CT15 on the anti-tyrosinase activity were 122.67, 66.67 and 93.33 ppm, respectively. The *Gnetum gnemon* leave extracted with methanol was mixed into cream base and applied to 30 volunteers for 30 days. The results showed the most volunteers (96.6%) preferred to the cream mixed 0.2% (w/w) of the extract. The stability of the *Gnetum gnemon* leave extract cream was performed by freeze- thaw method, 6 cycles. The anti-oxidant activity, anti-tyrosinase activity, viscosity, pH, moisture, and color of the *Gnetum gnemon* leave extract cream were less than 50% after sixth freeze-thaw cycle.

Keywords: *Gnetum gnemon* Linn leave, extract, anti-oxidant, anti-tyrosinase

INTRODUCTION

Gnetum gnemon Linn. or Liang (local name) is the local vegetable of the southern Thailand. It is used as food in many recipes, such as Liang pad kai (Liang leaf fried with egg), Liang loaw ka ti (Liang leaf boiled in coconut milk), etc. In the past, the leaf juice was applied on face to reduce the black spots on the skin [1]. This vegetable consists of high phosphorus, vitamin A, and calcium [1]. C-Glycosylflavones [2], and stilbene derivatives [3] were found in *Gnetum gnemon* leaf. C-Glycosidic moieties are derivatives of apigenin and luteolin [2] and stilbene derivatives present anticancer activity and antidiabetic [3]. However, other bioactivities such as antioxidant activity, alpha-tocopherol, beta-carotene and antityrosinase activity of *Gnetum gnemon* Linn. are interesting. Thus, the studying on antioxidation activity and antityrosinase activity of *Gnetum gnemon* Linn leaf is the objective in this research. In this study, *Gnetum gnemon* Linn leaf was dried, milled, and extracted by percolation method. The methanol extracts were selected to purify by column chromatography. Two active semi-purified fractions (CT13 and C15) were obtained. The activity of anti DPPH radicals, anti-peroxide reaction, and antityrosinase of the methanol extracts and the semi-purified extracts were measured. And the many concentration of the extracts were mixed into the cream base to study the potential used as the whitening and anti-aging agent.

MATERIALS AND METHODS

Gnetum gnemon Linn leaf preparation

Mature *Gnetum gnemon* Linn leaves were dried under shade, 35-40 °C for 7 days. Dried leaf were milled and passed through 100 mesh screening. The powder was packed in aluminium foil bag and stored in chilling temperature.

Extraction

The *Gnetum gnemon* Linn leaf powder was extracted by acetone, ethanol, ethylacetate, hexane, methanol, and hot water with percolation method and by tris-HCl with maceration method for 18 h. The solvents were removed by Vacuum rotary evaporator (RE111, Buchi, Switzerland). The amount of crude extracts and yields were measured and calculated. The antioxidant activity (DPPH^{o}) [3], total polyphenol [5], vitamin E [5], beta carotene [5] and antityrosinase activity and its IC_{50} [4] of the obtained extracts were conducted. The extract provided higher activity was selected to purification step.

Purification of _Gnetum gnemon_ Linn extract

The selected crude extract was purified by column chromatography [6]. The purity was detected by thin layer chromatrogarphy (F254 TLC-plastic plate, Merck) [6]. The obtained single fraction was recovery and its antioxidant activity and antityrosinase activity were conducted.

Optimization of _Gnetum gnemon_ Linn in cream

Mixed 0.1, 0.2, and 0.5% w/w of the selected extract into the cream base which consisted of 1% w/w of carbopol polymer No.940, 95% ethanol, tween 20 and distilled water. The antioxidant activity and antityrosinase activity in the mixed cream were studied. The optimized contents of the extract in the cream were evaluated by 30 volunteers (15 females and 15 males). The *Gnetum gnemon* Linn cream (2 g) was applied on the dorsum of the hand skin 2 times/day for 2 week. The dorsum of hand skin before and after applied the *Gnetum gnemon* Linn cream were evaluated by the digital camera which resolution 12 million pixel. The satisfaction of volunteers was evaluated, also. Koji acid (0.01% w/w) was used to be positive control as antityrosinase agent.

Shelf life test

The shelf life of the *Gnetum gnemon* Linn cream was operated by freeze-thaw cycle method (6).For the one cycle, the cream was incubated at 4^{o} C for 48 hr, immediately then, moved to heat at 45 °C for 48 hr. In this study, the cream was tested for 6 cycles. After each cycle of freeze –thaw test, the antioxidant activity (DPPH^{o}), the antityrosinase activity viscosity (Brookfield visco-meter), pH (pH meter), and colour (colorimeter) of the *Gnetum gnemon* Linn cream were evaluated.

RESULTS

Gnetum gnemon Linn leaf powder (100 g) extracted with acetone, ethanol, ethylacetate, hexane, methanol, hot water, and tris-HCl were 0.43±0.01 g, 1.48±0.01 g, 1.04±0.02 g, 0.70±0.01 g, 1.74±0.03 g, 4.34±0.03 g, and 9.50±0.18 g, respectively. The antioxidant activity and Anti-peroxide reaction activity in sunflower oil were shown on Table 1. The total polyphenol, alpha-tocopherol, and beta-carotene in 100 g of the acetone extract were 1215.06±0.01 mgGAE, 18.96 mg, and 14.09 mg, respectively.

Table 1. Antioxidant activity and Antityrosinase activity of 1 mg crude extract and 100 mg crude extract of *Gnetum gnemon* Linn leaf, respectively

Extracts	Antioxidant activity (%)		Antityrosinase activity (%)
	DPPH	TBAR	
Acetone	38.50 ± 4.47^{a}	28.25 ±12.74^{a}	16.50 ± 3.74^{c}
Ethanol	11.00 ±1.15^{bc}	0.00 ± 0.00^{b}	17.05 ±0.05^{c}
Ethylacetate	18.65 ± 0.51^{b}	20.24 ± 0.00^{ab}	18.65 ± 0.51^{c}
Hexane	14.76 ± 0.90^{b}	28.48 ± 4.36^{a}	14.76 ± 0.90^{d}
Methanol	11.58 ±1.73^{bc}	0.33 ± 0.46^{b}	41.85 ±1.03^{a}
Hot water	3.60 ± 0.38^{cd}	1.83 ±2.58^{b}	23.60 ± 0.18^{b}
Tris-HCl	1.67 ± 0.01^{d}	0.00 ± 0.00^{b}	2.67 ± 0.01^{e}

The values are presented as mean ± S.D (n =3) and statistically significant at $P < 0.05$.

Regarding to the antityrosinase and antioxidant activity, the methanol extract and acetone extract were selected to purify by column chromatography. CT 13 (fraction from the methanol extract) and CT15 (from the acetone extract) provided the antityrosinase activity and antioxidant activity, respectively. The crude extracts (100 mg) provided 0.03g of CT13 and 0.06 g of CT15.

The IC_{50} of the methanol extract and the semi-purified extracts, CT13 and CT15 on the antityrosinase activity were 122.67, 66.67 and 93.33 ppm, respectively and on the antioxidant activity were 98.03, 54.62 and 32.14 ppm, respectively. The obtained results indicated that the methanol extract is suitable to select for further experiment.

The methanol extract was selected to use as a moisturizer and whitening into the cream base. After the 30 volunteers applied the cream mixed the extract 0% wt, 0.2% wt, 0.4% wt on hand skin every day for 2 week. The sensory analysis of cream was evaluated by the five- point hedonic scale scoring test. The results showed on the Figure 1.

On Figure 1, the volunteers significantly preferred to the cream mixed the extract 0.2% wt, which the evaluated scores (1-5) of the texture, odor, color and satisfaction were 3.40±0.68, 2.45±0.99, 2.85±0.58, and 3.1±0.72, respectively.

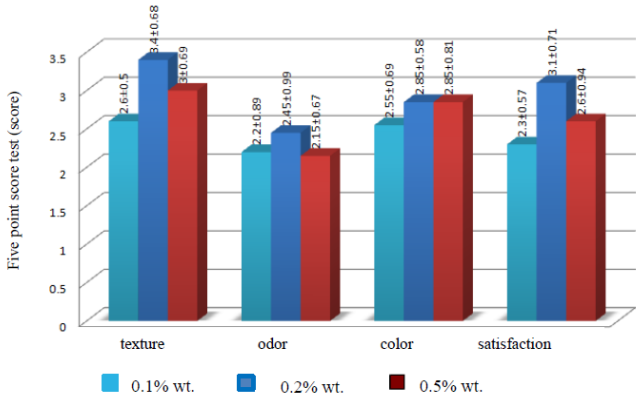

Figure 1. The sensory analysis evaluation of the cream mixed the *Gnetum gnemon* Linn leaf extract 0.1% wt, 0.2% wt, and 0.5% wt by the volunteers.

The results of the comparative study on the sensory analysis of cream mixed the extract 0.2 % wt and koji acid 0.2wt.% by the 30 volunteers (15 females and 15 males) were presented on Figure2. The volunteers (96.6%) significantly preferred to the cream mixed the extract 0.2% wt, with the texture, odor, color and satisfaction evaluated scores (1-5) of 3.33±0.71, 2.63±0.72, 3.00±0.74, and 3.37±0.61, respectively. From the study on the effect of the cream gel mixed the extract on the dorsum of hand skin (before and after) was discovered that the 96.6% of the volunteers applied the cream mixed the extract were satisfied (Fig. 2).

After the 6 cycles of freeze-thaw test were performed, the antioxidant activity ($DPPH^{o}$), antityrosinase activity, viscosity, acidity, moisture, color and odor in the cream mixed the extract were evaluated. The results were showed on the Table 2.

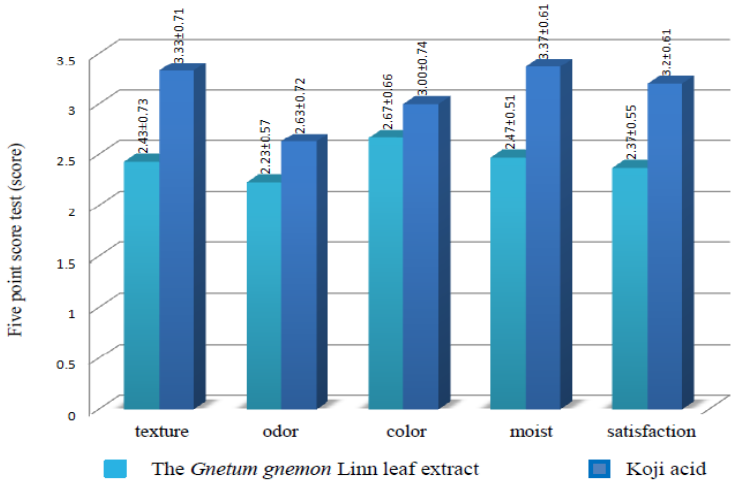

Figure 2. The sensory analysis evaluation of the cream mixed the *Gnetum gnemon* Linn leaf extract and koji acid 0.2% wt by the 30 volunteers.

Table 2. Some Properties of the cream gel mixed the extract of *Gnetum gnemon* Linn leaf before and after the shelf life test.

Properties	Freeze-thaw Test	
	Before	After
Antioxidant activity (%)	96.73±0.13	62.63±0.75
Antityrosinase activity (%)	51.23±0.13	36.44±0.24
Viscosity (cP)	604000	53400
Acidity (%)	5.50±0.40	4.86±0.91
Moisture content (%)	89.74±0.15	80.09±0.11
Color	L = 95.59	L = 95.24
	a = -1.24	a = -1.09
	b = +2.88	b = +3.32
Odor	odor	odor

The values are presented as mean ± S.D (n =3)

CONCLUSIONS

In this study has shown the benefits of the Thai local vegetable "*Gnetum gnemon* Linn leaf". The *Gnetum gnemon* Linn leaf extract the consisted of the total polyphenol, β-carotene and α-tocopherol which these compositions are widely used as the antioxidants and the moisturizers and used as whitening in the anti-aging products such as cream and lotion. When the *Gnetum gnemon* Linn leaf extract was incorporated into the cream as moisturizer and whitening to replace the jojoba oil and koji acid, the 96.6% volunteers were satisfied. All the results indicated that the extract from *Gnetum gnemon* Linn leaf extracted by methanol provide the potential as a natural moisturizer and whitening agent.

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REFERENCES

1. Jullakoa K. 1996. Miang: Queen of southern folk vegetable. Book of the 2nd Alternative Agriculture Assembly "Exhibition of Agriculture and organic food. 81p.
2. Iliya I, Ali Z, Tanaka T, Iinuma M , Furusawa M, Nakaya K, et al. 2003. Stilbene derivatives from *Gnetum gnemon* Linn. Phytochem. 62, 601-606.
3. Chang LW, Juang LJ, Wang BS, Wang MY, Tai HM, Hung WJ, et al. 2011. Antioxidant and antityrosinase activity of mulberry (*Morus alba* L.) twigs and root bark. Food Chem. Toxicol. 49,785-790.
4. Masuda T, Yamashita D, Takeda T, Yonemori S. 2005. Screening for tyrosinase inhibitors among extracts of seashore plants and identification of potent inhibitors from *Garcinia subelliptica*. Biosci. Biotechnol. Biochem. 69,197-201.
5. Chanphrom P. 2007. Antioxidant and antioxidant activities of pigmented rice varieties and rice bran. Thesis of nutrition. Mahidol University.
6. Jinda N, Treenuphab V, Pathummad S. 2000. Crude Extract and Bioactive Activity of *Gnetum gnemon* Linn. Kasetasrt University Research and Development Institute, Bangkok. 90 p.

Anti-oxidation capacity and nutrition value of rice and vegetable salad (Khoa Yum Pak Tai)

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ABSTRACT

Antioxidant activity and phenolic compound of 20 samples of rice and vegetable salad with Boo Doo dressing (Khoa Yum Pak Tai) collected from Chumphon, Surat Thani and Nakon Srithammarat were determined. The results showed that the antioxidant activity determined by DPPH^{O} and FRAP assay of lemongrass and kaffir lime leaf were higher than that of yard long bean and mungbean sprouts, respectively. The values determined by ABTS assay of lemongrass, yard long bean and kaffir lime leaf were not significantly ($P > 0.05$), but significantly less than that of mungbean sprouts ($P \leq 0.05$). The total phenol content of kaffir lime leaf was higher than that of lemongrass, yard long bean, and mungbean sprouts. Rice cooked with noni leave juice presented higher antioxidant activity and total phenol content. The composition of Khoa Yum samples was studied. They composed of cooked rice 40-56%, 5.0-8.2% mungbean sprouts, 2.0-2.5% sliced lemongrass, 1.0-1.56% sliced kaffir lime leaf, 6-10% sliced yard long bean, 4-7% sliced cucumber, 0-3% noni leaf, 0-5% sliced others (Wildbetal Leafbush, *Paederia scandens* (Lour.) Merr. (Pa Hom)leaf, Torch ginger flower and leaf), 0.5-1 fruit of lime, and 1% pomelo. The dried powder ingredients consisted of 4-5% grind dried shrimp, 4% grind dried coconut, and 1-1.5% grind dried chili and 8% Boo Doo sauce. Their nutrition values were 19.4-21.2% protein, 4.51-5.80% fat, 3.43-4.60% Fiber, 55.0-65.40% moisture, and 6.91-15.16% carbohydrate.

Keywords: Antioxidant capacity, nutrition value, rice and vegetable salad, Koa Yum Pak Tai

INTRODUCTION

Khoa Yum Pak Tai or rice and vegetable salad with Boo Doo sauce is the well-known as a traditional folk food of southern provinces in Thailand. This food consist of many local fresh vegetables, herbs and dried powder of ingredients and served with the dressing as a sauce made from the effluent of the salt-fermented fish Doo which boiled together with some herbs [1]. Some fresh local vegetables are a source of anti-oxidant agent, antibacterial agents and high nutrition value [2]. In the way, types of fresh vegetable, dried ingredients in Khoa Yum Pak Tai and their nutrition values were investigated.

MATERIALS AND METHODS

Sample preparation: Rice and vegetable salad (Kho Yum)

Twenty samples of rice and vegetable salad were collected from fresh markets and restaurants in Chumphon province Fig. 1a), Surat Thani province (Fig. 1b), and Nakon Sri thammarat province (Fig. 1c). Their mass of vegetables and dried powder ingredients were identified. After that, 10 gram of vegetable were mixed into 100 ml of the mixture of distilled water and 95% ethanol (1:1 by volume), blended for 1 min, and centrifuged at 8000 rpm for 5 min under 4 °C, respectively. The antioxidant activity as DPPH, FRAP, and ABTS and the nutrition values were determined.

Antioxidant activity assay

The antioxidant activity of each vegetable extract was determined as the ferric reducing / antioxidative power (FRAP) [3], ABTS radical cation decolorization assay [4], and DPPH free radical scavenging activity [5].

Total phenolic compound assay

The total phenolic compound assay was performed by Folin-ciocalteau micro method [6]. The total phenol compound was reported as mg of gallic acid (Sigma, USA) equivalent per gram of sample.

a b c

Figure 1 Rice and vegetable salad (Khoa Yum Pak Tai) in Chumphon (a), Surat Thani (b), and Nakon Sri Thammarat (c)

Determination of nutritional values

The content of protein, ash, dietary fiber, fat and oil, and moisture were determined by the proximate analysis method [7]. The carbohydrate content was calculated by the followed equation.

% Carbohydrate = 100-(%protein + %ash + %fat + %fiber + %moisture)

RESULTS

The compositions of Khoa Yum samples were studied. They composed of 40-56% of cooked rice, 5.0-8.2% of mungbean sprouts, 2.0-2.5% of sliced lemongrass, 1.0-1.56% of sliced kaffir lime leaf, 6-10% of sliced yard long bean, 4-7% of sliced cucumber, 0-3% of noni leaf, 0.5-1 fruit of lime, 1% of pomelo and 0-5% of sliced others such as Wildbetal Leafbush, *Paederia scandens* (Lour.) Merr. (Pa Hom) leaf (Fig.2a), Torch ginger flower and leaf (Fig.2b). The dried powder ingredients consisted of 4-5% of grind dried shrimp, 4% of grind dried coconut, and 1-1.5% of grind dried chili and 8% (w/v) of Boo Doo sauce.

a b

Figure 2. *Paederia scandens* (Lour.) Merr. (Pa Hom) leaf (a) and Torch ginger flower and leaf (b) used as ingredients in Khoa Yum Pak Tai.

The antioxidant activity of the vegetable extracts (on wet basis) determined by DPPH° and FRAP assay of lemongrass and kaffir lime leaf were higher than that of yard long bean and mungbean sprouts, respectively. The values determined by ABTS assay of lemongrass, yard long bean and kaffir lime leaf were not significantly ($P > 0.05$), but significantly less than that of mungbean sprouts ($P \leq 0.05$) The total phenol content of kaffir lime leaf was higher than that of yard long bean, lemongrass, and mungbean sprouts, respectively (Table 1).

On dried basis, the DPPH° value of kaffir lemon leaf and lemongrass were higher than that of yard long bean and mungbean sprouts,respectively (Table 2). The ABTS of mungbean sprouts was significantly less than that of lemongrass, kaffir lime leaf and yard long bean. The total phenol content of kaffir lime leaf was highest while that of lemongrass, yard long bean, and mungbean sprouts were 1.04±0.13, 1.53±0.28, and 0.83±0.14 respectively (Table 2). These obtained results were nearby the antioxidant activity of some local herbs [8].

The nutritional values of collected samples were 19.4-21.2% of protein, 4.51-5.80% of fat, 3.43-4.60% of Fiber, 55.0-65.40% of moisture, and 6.91-15.16% of carbohydrate (Table 3). The dried powder ingredients consisted of 4-5%

of finely dried shrimp, 4% of roasted coconut meal, and 1-1.5% of dried chili powder and 8-15% (w/v) Boo Doo sauce. Their protein contents were higher than the standard recipe[9] and developed recipe by [8]. Regarding on the calories, samples collected from Surat Thani provided the lowest calories because they contained less amount of cooked rice.

Table 1. Antioxidant capacity and total phenolic compound content on wet basis.

Assay	Standard agent	Herbs and vegetables			
		Lemongrass	Kaffir lime leaf	Yard long bean	Mungbean sprouts
DPPH	Ascorbic acid	6.40±2.58^{a}	7.12±1.25^{a}	4.28±2.08^{b}	0.56±0.13^{c}
FRAP	Fe^{2+}	4.93±0.58^{a}	5.41±0.82^{a}	4.53±0.12^{a}	1.38±0.28^{b}
	Ascorbic acid	5.09±0.48^{a}	4.94±0.27^{a}	4.91±0.58^{a}	1.25±0.17^{b}
ABTS	Ascorbic acid	6.26±1.43^{b}	6.76±4.30^{b}	6.53±0.35^{b}	15.01±2.13^{a}
Total polyphenol	Gallic acid	1.04±0.13^{b}	11.20±1.08^{a}	1.53±0.28^{b}	0.83±0.14^{c}

The values are presented as mean ± S.D (n =5) and statistically significant at $P < 0.05$

Table 2. Antioxidant capacity and total phenolic compound content on dried basis.

Assay	Standard agent	Herbs and vegetables			
		Lemongrass	Kaffir lime leaf	Yard long bean	Mungbean sprouts
DPPH	Ascorbic acid	20.65±2.78^{b}	13.08±1.25^{c}	54.73±2.08^{a}	5.56±3.25^{d}
FRAP	Fe^{2+}	21.04±1.18^{c}	13.21±1.02^{d}	49.53±3.65^{a}	34.08±0.35^{b}
	Ascorbic acid	17.09±1.55^{c}	14.01±1.57^{d}	40.27±2.58^{a}	23.50±1.23^{b}
ABTS	Ascorbic acid	22.86±0.43^{b}	23.06±0.03^{b}	75.32±2.65^{b}	115.61±26.03^{a}
Total polyphenol	Gallic acid	3.56±0.02^{b}	31.12±5.18^{a}	12.18±2.04^{b}	17.98±1.28^{c}

The values are presented as mean ± S.D (n =5) and statistically significant at $P < 0.05$

Table 3. Nutrition values of rice and vegetable salad with Boo Doo dressing (Kho Yum Pak Tai).

Nutrition	Content (per 100 grams)		
	Chumphon	Surat Thani	Nakon Sri Thammarat
Protein (g)	21.16±0.54^{a}	19.41±0.43^{a}	19.65±2.45^{a}
Fat (g)	5.80±0.32^{a}	4.51±0.43^{b}	5.78±2.43^{a}
Fiber (g)	4.60±0.31^{a}	3.43±0.48^{b}	3.75±0.43^{b}
Ash (mg)	274.22±4.34^{b}	340.56±7.20^{a}	260.64±10.56^{b}
Moisture (g)	55.03±4.30^{b}	65.40±4.89^{a}	55.40±3.54^{b}
Carbohydrate (g)	13.14±2.10^{b}	6.91±0.48^{c}	15.16±4.21^{b}
Calories (cal)	118.81±1.32^{b}	64.62±1.56^{c}	132.76±0.48^{a}

The values are presented as mean ± S.D for 4, 8, and 10 rates in Chumphon, Surat Thani, and Nakon Sri Thummarat, respectively and statistically significant at $P < 0.05$

CONCLUSIONS

Rice and vegetable salad or Khoa Yum Pak Tai contains high content of total phenolic compound. They significantly presented the antioxidant capacity and the nutrition values. Thus, this food showed potential to be a healthy food because it provides the antioxidant capability, high nutrition value, and low calories.

ACKNOWLEDGEMENTS

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REFERENCES

1. Suttapong S. 2013. The Study of South Entity Culture Wisdom: Khao Yam. Master thesis of Science (Home Economics), Department of Home Economics, Kasetsart University, Bangkok. 136 p.
2. Intarachaimas W, Phetcharat P, Rungphithakchai C, Semae S, Suwannapak T. 2008. Potential of Local Vegetables for "Kao Yam Budu" Mixture. Journal of Yala Rajabhat University. 3, 21-29.
3. Benzie IFF, Strain JJ. 1999. Ferric reducing antioxidant power assay, direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Methods Enzymol 299, 15-27.
4. Rea R, Pellegrinia N, Proteggentea A, Pannalaa A, Yanga M, Rice-Evansa C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Rad. Biol. Med. 26, 1231-1237.
5. Brand-Williams, W, Cuvelier ME, Berset C. 1995. Use of a free radical method to evaluate antioxidant activity, Lebensmittel-Wissenschaft und –Technologie. Food Sci. Technol. 28, 25-30.
6. Biju J, Sulaiman CT, George S, Reddy VRK. 2014. Total phenolics and flavonoids in selected medicinal plants from Kerala. International Journal of Pharmacy and Pharmaceutical Sciences. 6, 406-408.
7. A.O.A.C. 2000. Official Method of Analysis, 17th ed. Association of Official Analytical Chemists. Washington, D.C.
8. Komonmanee B. 2009. Antioxidation capacity, nutrition and suitable storage condition of ready-to-eat budoo kao yum. Master thesis of Science in Food Technology, Maejo University, Chaingmai. 121 p.
9. Kongpan S. 1999. ข้าวยำ จานเดียวครบ. [online] Source http://www.elib-online.com/doctors/food_rice.html. (July 10, 2015).

The effect of *Moringa oleifera* ethanolic leaf extract and its 2 major active components on colon cancer cell viability

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ABSTRACT

Moringa oleifera Lam is an indigenous deciduous plant and has been used for the treatment of various diseases. It possesses many biological activities, including anti-inflammatory, antioxidant, anticancer, hepatoprotective activities. Crypto-chlorogenic acid (CCA) and astragalin (AST) have been found to be major active components in the ethanolic leaf extract. The *M. oleifera* leaf extract (MOL) and its 2 major active components were evaluated for antioxidant effect against hydrogen peroxide and cell viability effect in colon cancer (HT29) cells by MTT assay. CCA and AST at doses of 0.05-0.5 mM significantly suppressed hydrogen peroxide mediated cytotoxicity whereas MOL increased. The MOL showed higher cytotoxicity than its 2 major active components. Both MOL and its 2 major active components decreased the viability of HT29 cells in a concentration-dependent manner. However, low concentration of CCA increased the cell viability more than that of AST and MOL, respectively. These results suggest that the HT29 cell toxicity by the MOL could be attributed to other bioactive components. Moreover, MOL and its bioactive compounds may be used as a cytoprotective agent at low concentrations.

Keywords: Colon cancer, Cell viability, *Moringa oleifera*, HT29 cells

INTRODUCTION

Moringa oleifera Lam, or drumstick tree, known in Thai as Ma-room, is a common plant in South Asia, South East Asia, Mexico and Africa. It belongs to Moringaceae family. It has an impressive range of medicinal uses with high nutritional value. All parts of the plant: root, bark, gum, leaf, pods, flowers, seed and seed oil have been used in the indigenous medicine of South Asia. This plant possesses many biological activities, including anti-inflammatory, antioxidant, anticancer, hepatoprotective activities [1-3]. Recently, isoquercetin, astragalin (AST), and crypto-chlorogenic acid (CCA) were discovered to be major active components in *M. oleifera* ethanolic leaf extract [4].The average amounts of CCA, and AST were found more than that of isoquercetin. Moreover, both CCA and AST have been reported as an antioxidant with anti-inflammatory activity [5-6]. However, the *Moringa oleifera* leaf extract (MOL) and its 2 major active components (CCA and AST) are not yet studied in cultured human colon cancer cells. Therefore, in the present study, we examine the effect of MOL and its 2 components, CCA and AST, on the viability and antioxidant activity against hydrogen peroxide of colon cancer (HT29) cells.

MATERIALS AND METHODS

Preparation of *Moringa oleifera* ethanolic leaf extract

M. oleifera Lam leaves were collected during April to May 2015 from Nakhon Pathom province, the leaves were dried in a hot air oven at 60°C for 24 hours. The dried samples were ground and passed through a 20 mesh sieve. The extractions were performed using the previously reported method [7]. Briefly, *M. oleifera* powder leaves were accurately weighed (5.0 g) and extracted by maceration with 70% ethanol (1 : 10, w/v) for 72 hours at room temperature with occasional shaking. The extraction process was repeated for 5 times. All filtrates were combined, filtered, and dried under vacuum in a rotary evaporator. The dried crude extract was then stored at 4°C and protected from light.

Determination of crypto-chlorogenic acid and astragalin contents in *M. oleifera*leaf extracts

In order to determine the contents of CCA and AST (Signa-Aldrich, Steinheim, Germany) in MOL, Thin layer chromatography was performed using ethyl acetate:formic acid:acetic acid:water, 34:3.5:1.5:7, v/v/v/v as a mobile phase. Densitometric scanning was done using a CAMAG TLC 4 scanner in the reflectance-absorbance mode at 340 nm controlled by visionCAT (CAMAG, Muttenz, Switzerland). Five-point calibration was done for each analysis. The amounts of CCA and AST were calculated from peak area using linear regression from the calibration curve.

Cell culture and MTT cell viability assay

HT29 cells were cultured in D ulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 4.5 g/l D-glucose, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and 1% non-essential amino acids. The cells were maintained in a humidified chamber at 37°C, 5% CO_2. To perform MTT assay, HT29 cells ($2.0x10^4$ cells) were seeded in a 96-well plate at 100 µl. Next day, cells were treated with MOL, CCA or AST dissolved in DMSO for 24 and 48 h. Subsequently, 10 µl of MTT solution (2 mg/ml, Sigma) was added to each well and incubated in the humidified incubator for 2 h. After removing the media, DMSO was added to dissolve the formazan product, which is generated by mitochondria of viable cells, and the absorbance was measured at 550 nm after 2 h of incubation at room temperature. % Cell viability was calculated as percentage of the absorbance of the treatments subtracted with that of cell-free control compared with DMSO.

Oxidative stress assay

HT29 cells were cultured with MOL, CCA or AST in supplemented medium for 4 h. Treated cells then were exposed to 150 µM H_2O_2 for 2 h. The number of viable cells in treated groups after H_2O_2 exposure was measured using MTT assay. Untreated cells served as the negative control. (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, Trolox, used as a positive control.

RESULTS

TLC densitogram of MOL has been recorded (Figure 1). The content of CCA and AST in dried powder of *M. oleifera* leaves (% dry weight) were 0.1310 and 0.056, respectively. To study the antioxidant effect of MOL, CCA and AST in HT29 cells, we examined the cell viability of pre-incubated HT29 cells with MOL, CCA or AST, and then, exposed with H_2O_2. The results showed that CCA and AST, but not MOL, pre-treated cells were able to recover in their viability from oxidative stress (Figure 2). The cell viability in CCA or AST pre-treated cells remained higher than that in MOL pre-treated cells. From calculated contents of CCA and AST in MOL, CCA or AST (0.5 mM) was equivalent to CCA or AST found in 50 or 100 µg/ml MOL, respectively. In those concentrations of MOL, the cell viability was still low. With MTT viability assay, it was possible to assess whether CCA, AST or MOL was able to influence different cell viability profile. When cells were treated with different concentrations of MOL (0.05-0.5 µg/ml) and CCA or AST (0.05-0.5 mM) for 24 h and 48 h, it was showed surprisingly that all treatments increased viable cells (Figure 3). However, MOL and its 2 major active components decreased the viability of HT29 cells in a concentration-dependent manner. The MOL showed higher cytotoxicity than its 2 major active components. Low concentration of CCA increased the cell viability more than that of AST and MOL, respectively.

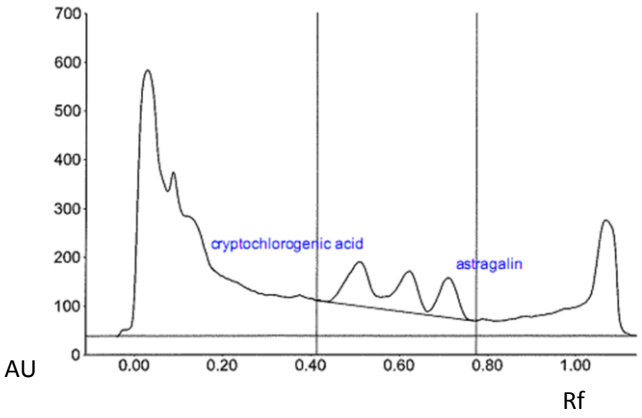


Figure 1. TLC densitogram of MOL for cryptochlorogenic acid and astragalin determination.

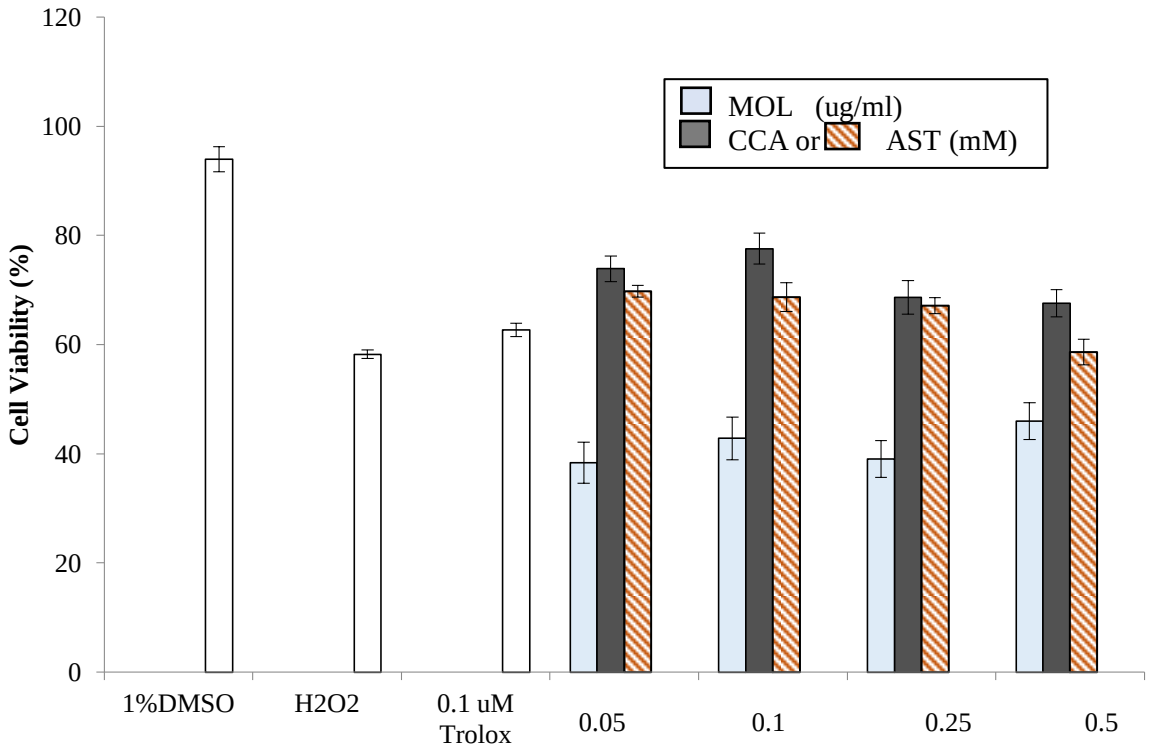
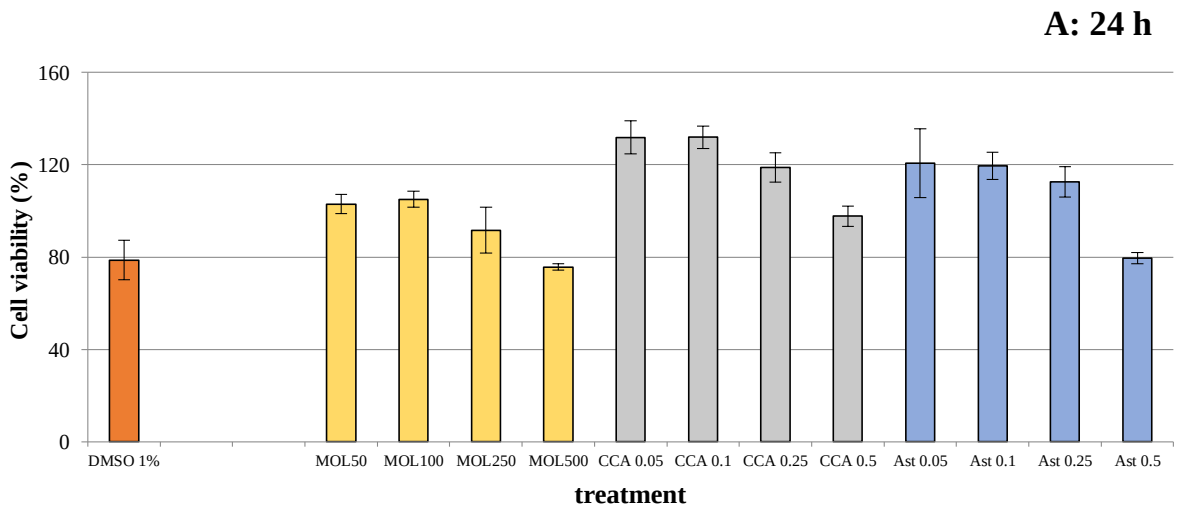


Figure 2. The viability of HT29 cells determined by MTT assay after incubation with 150 µM H_2O_2. Control (DMSO 0.1%, and 0.1 µM Trolox) and MOL (0.05-0.5 µg/ml), CCA or AST (0.05-0.5 mM) pre-treated cells were stressed with H_2O_2. Data are expressed as mean ± SD (n=3).



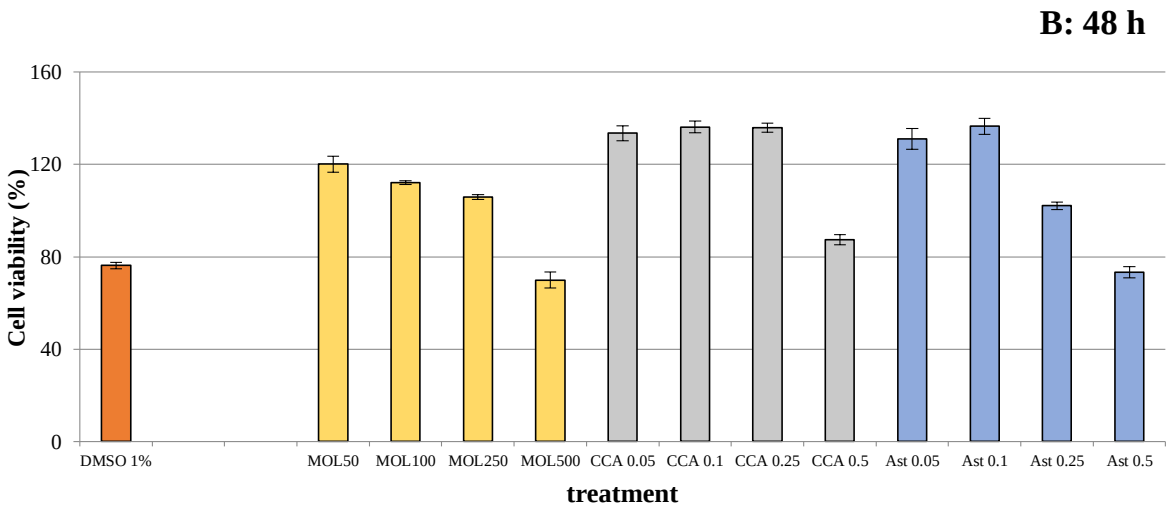


Figure 3. Effect of MOL (0.05-0.5 µg/ml), CCA or AST (0.05-0.5 mM) on viability of HT29 cells. Cells were treated for 24 h (A), and 48 h (B), then assessed with MTT assay. Data are expressed as mean ± SD (n=3).

CONCLUSIONS

The study demonstrated that ethanolic extracts from *M. oleifera* leaves affected on HT29 cell toxicity. The 2 major active components, CCA and AST, may not be directly contributed as a key molecule for cytotoxicity. Moreover, MOL and its bioactive compounds may be used as a cytoprotective agent at low concentrations. Further studies are needed to assess the exact mechanism explaining the reported bioactivities.

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REFERENCES

1. Anwar F, Latif S, Ashraf M, Gilani AH. 2007. *Moringa oleifera*: a food plant with multiple medicinal uses. Phytother. Res. 21(1), 17-25.
2. Jafarain A, Asghari G, Ghassami E. 2014. Evaluation of cytotoxicity of *Moringa oleifera* Lam. callus and leaf extracts on Hela cells. Adv. Biomed. Res. Sep 23;3:194.doi: 10.4103/2277-9175.140668. eCollection 2014.
3. AbdullRazis AF, Ibrahim MD, Kntayya SB. 2014. Health benefits of *Moringa oleifera*. Asian Pac. J. Cancer Prev. 15(20), 8571-8576.
4. Vongsak B, Sithisarn P, Gritsanapan W. 2013. Simultaneous determination of crypto-chlorogenic acid, isoquercetin, and astragalin contents in *Moringa oleifera* leaf extracts by TLC-densitometric method. Evid Based Complement Alternat Med. 2013, 917609. doi: 10.1155/2013/917609. Epub 2013 Feb 25.
5. Cho IH, Gong JH, Kang MK, Lee EJ, Park JH, Park SJ, Kang YH. 2014. Astragalin inhibits airway eotaxin-1 induction and epithelial apoptosis through modulating oxidative stress-responsive MAPK signaling. BMC Pulm. Med. 29, 14, 122. doi: 10.1186/1471-2466-14-122.
6. Vongsak B, Mangmool S, Gritsanapan W. 2015. Antioxidant activity and induction of mRNA expressions of antioxidant enzymes in HEK-293 cells of *Moringa oleifera* leaf extract. Planta. Med. 81(12-13), 1084-1089.
7. Vongsak B, Sithisarn P, Mangmool S, Thongpraditchote S, Wongkrajang Y, Gritsanapan W. 2013. Maximizing total phenolics, total flavonoids contents and antioxidant activity of *Moringa oleifera* leaf extract by the appropriate extraction method. Ind. Crops. Prod. 44, 566-571.

Antioxidant activity of *Nelumbo nucifera* and herbal tea development

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ABSTRACT

Nelumbo nucifera (Lotus) was long been used by Thai traditional doctors for treatment diabetic and chronic disease patients. Many researches referring that the antioxidant activity herb is always good benefit because of inhibiting oxidation reaction. The objectives of this study were to investigate the antioxidant activity and sensory evaluation of developed herbal tea from lotus part used. The antioxidant activity was investigated by DPPH radical scavenging in ethanolic extract from ten parts used of lotus. (petal, stamen, seed, embryo, ovary, leaf, young leaf, petal stalk, bud and root). The results of antioxidant activity were expressed as percentage inhibition by 50% (EC_{50}). The petal extract was exhibited the highest antioxidant activity with EC_{50} 12.78±0.44 µg/ml following by petal stalk and ovary (EC_{50} = 16.89±0.61 and 37.70±1.28 µg/ml. respectively).The sensory acceptable was tested by adding proper herb in original lotus tea to improved color and odor to meet consumer acceptance (rhizome with safflower, petal stalk with jasmine, leaf with pandan, petal with roselle and stamen with chrysanthemum) in one hundred consumers using 9-point Hedonic scale. The average score of modified lotus tea from five parts used are significant difference ($P<0.05$) in all sensory parameter (appearance, color, odor, flavor and overall liking). The highest acceptable sensory test was rhizome part with appearance, color and overall liking by 7.07±1.43, 7.31±1.17 and 7.26±1.43 respectively. The study indicated worthy part of this plant was petal. The future study should be focus on food product development to promote daily consumption.

Keywords: *Nelumbo nucifera*, lotus, antioxidant activity, herbal tea, sensory evaluation

INTRODUCTION

Lotus (*Nelumbo Nucifera*) is a well-known medicinal plant in Asian countries, including Korea, Japan, and China. The plant has its roots firmly in the mud and sends out long stems to which their leaves are attached. The leaves are sometimes, and flowers always rise above the water surface. The beautiful and fragrant flower opens in the morning and petals fall in the afternoon. The Lotus grows best in calm fresh water and blooms year round.

Traditional medicinal uses: Almost all parts of the lotus plant are eaten as vegetable and also used in the indigenous system of medicine. Rhizomes and leaves are used with other herbs to treat sunstroke, fever, diarrhoea, dysentery, dizziness, vomiting of blood, hemorrhoids. Moreover, the whole plant is used as an antidote to mushroom poisoning. The lotus embryonic seeds were used to treat high fever, cholera (in China), nervous disorders and insomnia; the seeds were used to stop vomiting, relieve indigestion and diarrhea or just as a tonic. Petals of lotus flowers were used for syphilis, cosmetic unguents (in Java), while its flower stalk was used with other herbs to treat bleeding from the uterus. The pods contain alkaloids that stop bleeding. [1, 2]

Nelumbo nucifera extracts from leaf, root, seed, and flower are reported to have significant therapeutic benefits and it posseses antidiarrhoeal activity, psychopharmacological, diuretic activity, antipyretic, antimicrobial activities, hypocholesterolemic effect, hypoglycemic, alleviation of hepatic steatosis. Antioxidant activity of various parts of *Nelumbo nucifera* is well established, e.g. leaf, stamen and rhizome. [3, 4] *Nelumbo nucifera* Gaertn. (Thai name: Bua Luang) was long used by Thai traditional doctors for the treatment of diabetic patients. There are many researches

referring that the antioxidant activity herb is always a good benefit for chronic disease patients because of inhibiting the oxidation reaction by reducing advanced glycation endproducts (AGEs).

The Objectives of this study were to investigate the antioxidant activity of ethanolic extract from 10 parts use of *Nelumbo nucifera* Gaertn (petal, stamen, seed, embryo, ovary, leaf, young leaf, petal stalk, bud and root) and to evaluate sensory acceptance of selected developed 5 parts used of lotus tea by proper herbal fortification.

MATERIALS AND METHODS

Sample preparation: The plant materials from 10 parts use of *Nelumbo nucifera* Gaertn (petal, stamen, seed, embryo, ovary, leaf, young leaf, petal stalk, bud and root) were collected from natural source. The plants were dried in well ventilation open air and ground to give the powder. By maceration in methanol, each 100 gm. of the powder leaf in 95 % ethanol was collected every 3 days for 3 times. The extract was filtered through vacuum pump and evaporated by rotary evaporator then keep in -20 °C before reaction testing.

Anti-oxidative reaction: The reduction of 2.2-diphenyl-1-picrylhydrazyl (DPPH) radical by spectrophotometric assay was carried on. Scavenging effect of extract or pure compound on DPPH radical was examined based on the method of Yamasaki et al., 1994. This antioxidant activity testing method is based on chemical test. Butylated hydroxytoluene (BHT) was used as reference standard and positive control. Samples for testing were dissolved in absolute ethanol to obtain a high concentration of 1000 μg/ml. Each sample was further diluted for at least 4 concentrations (two-fold dilutions of final concentration 1, 10, 50,100 μg/ml). Each concentration was tested in triplicate. A portion of sample solution (500 μl) was mixed with an equal volume of 6 x 10^{-5} M DPPH (in methanol) and allowed to stand at room temperature for 30 min. The absorbance (A) was measured at 520 nm. BHT as a positive standard was tested in the same system. The scavenging activity of the samples corresponded to the intensity of quenching DPPH. The results were expressed as percentage inhibition. Effective concentration of sample required to scavenge DPPH radical by 50% (EC_{50}) were obtained by linear regression analysis of dose-response curve plots of % inhibition versus concentration and calculated EC_{50} by prism program. [5]

Sensory evaluation: Acceptance testing was used to determine how much each sample was liked based on a 9-point hedonic scale for a set of attributes: overall liking, flavor, and texture where 9=like extremely and 1=dislike extremely. In addition, 100 consumers were asked what they like and dislike about each sample. Sensory acceptance of selected 5 original and fortified lotus tea were tested.

RESULTS

Ethanolic extraction: The percentage of yield from extractions showed in Figure 1. The highest percentage yield of ethanolic extract is leaf by 13.82 % follow by petal and embryo of by 12.54 and 10.53 % respectively.

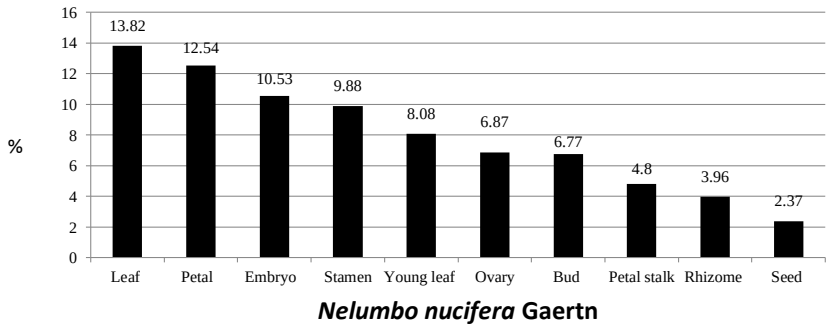

Figure 1. The percentage yield of the Lotus part used by 95% ethanolic extract

Anti-oxidative reaction: The ethanolic extract of petal showed the highest antioxidant activity by DPPH radical scavenging with the EC_{50} 12.78±0.44 μg/ml. following by extracts of petal stalk and ovary (EC_{50} = 16.89±0.61 and 37.70±1.28 μg/ml. respectively).

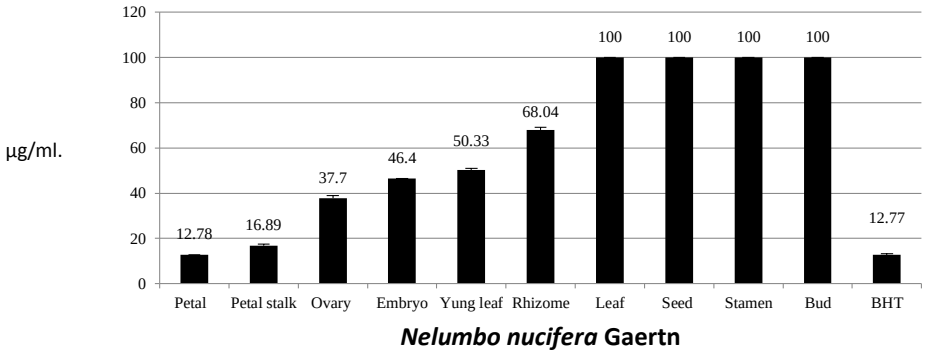

Figure 2. Antioxidant activity of the Lotus part used by DPPH radical scavenging

Sensory evaluation:

The average score of original lotus tea from 5 parts used are significant difference (P<0.05) in odor flavor and overall liking and total score in table 1. The highest average mark was Rhizome part with appearance, color, odor, flavor, overall liking and total score are 6.16±1.63, 6.30±1.47, 6.52±1.76, 5.95±1.98, 6.43±1.54 and 6.27±1.35. The reason of consumer acceptance is good odor and no bitter test.

Table 1. Sensory acceptance of original Lotus tea from selected 5 parts used

Sensory parameter	Part used of Lotus				
	Rhizome	Petal stalk	Leaf	Petal	Stamen
Appearance ^{ns}	6.16 ± 1.63^{ab}	5.98 ± 2.05^{ab}	5.77 ± 2.06^{b}	6.19 ± 2.12^{ab}	6.48 ± 1.81^{a}
Color ^{ns}	6.30 ± 1.47^{ab}	6.00 ± 2.02^{ab}	5.80 ± 2.01^{b}	6.17 ± 2.03^{ab}	6.45 ± 1.68^{a}
Odor *	6.52 ± 1.76^{a}	5.77 ± 1.63^{b}	5.91 ± 2.10^{b}	5.58 ± 1.87^{b}	5.54 ± 1.79^{b}
Flavor*	5.95 ± 1.98^{a}	5.18 ± 2.15^{bc}	4.38 ± 2.25^{d}	4.60 ± 2.30^{bcd}	5.37 ± 2.08^{ab}
Overall liking*	6.43 ± 1.54^{a}	5.76 ± 1.75^{bc}	5.28 ± 2.04^{cd}	5.21 ± 1.84^{d}	5.97 ± 1.71^{ab}
Total Score*	6.27 ± 1.35^{a}	5.74 ± 1.45^{bc}	5.43 ± 1.74^{c}	5.55 ± 1.60^{bc}	5.96 ± 1.39^{ab}

Sample with difference letter in the same row are difference (p< 0.05)
* : Significant difference(p<0.05) ns : Non-significant difference(p>0.05)

Sensory acceptance of selected 5 herbal fortified lotus tea:

The second sensory test follow by adding suitable herb in original lotus tea for color and odor improve to meet consumer acceptance was shown in table 2.

Herbal fortified in lotus tea

- Rhizome add Safflower (Carthamus tinctorius)
- Petal stalk add jasmine (Jasminum Sambac)
- Leaf add Pandan *(Pandanus amaryllifolius*)
- Petal add Roselle *(Hibiscus subdariffa*)
- Stamen add Chrysanthemum *(Chrysanthemum indicum*)

The average score of modified lotus tea from 5 parts used are significant difference (P<0.05) in all sensory parameter such as appearance, color, odor, flavor, overall liking and total score are shown in table 2. The highest average score was rhizome part with appearance, color, overall liking and total score are 7.07± 1.43, 7.31± 1.17, 7.26± 1.62 and 7.15± 1.17 respectively. The reason of acceptance is good color and odor.

Table 2. Sensory acceptance of Herbal fortified Lotus tea from selected 5 parts used

Sensory parameter	Part used of Lotus				
	Rhizome	Petal stalk	Leaf	Petal	Stamen
Appearance *	7.07 ± 1.43[a]	7.01 ± 1.28[a]	6.10 ± 1.77[b]	6.04 ± 1.61[b]	7.00 ± 1.39[a]
Color *	7.31 ± 1.17[a]	6.67 ± 1.46[b]	5.91 ± 1.66[c]	6.06 ± 1.47[c]	6.91 ± 1.42[ab]
Odor *	7.05 ± 1.62[a]	7.23 ± 1.33[a]	6.32 ± 1.73[b]	5.85 ± 1.66[c]	7.06 ± 1.46[a]
Flavor *	7.07 ± 1.65[a]	6.71 ± 1.41[a]	6.07 ± 1.90[b]	5.66 ± 1.92[b]	7.94 ± 1.78[a]
Overall liking *	7.26 ± 1.42[a]	6.83 ± 1.26[a]	6.13 ± 1.56[b]	5.87 ± 1.63[b]	7.11 ± 1.62[a]
Total Score *	7.15 ± 1.17[a]	6.89 ± 1.03[a]	6.11 ± 1.47[b]	5.90 ± 1.37[b]	7.00 ± 1.35[a]

Sample with difference letter in the same row are difference ($p < 0.05$)
* : Significant difference($p<0.05$) ns : Non-significant difference($p>0.05$)

CONCLUSIONS

The ethanolic extract of showed the highest antioxidant activity by DPPH radical scavenging. The highest average score of sensory evaluation was rhizome part with appearance, color, overall liking. The further study should be continue to isolation the active antioxidant compounds from best part of lotus and developed to another health products or promote consumption in daily food and wildly use in food ingredient.

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REFERENCES

1. Kitagaki H, Ono N, Hayakawa K, Kitazawa T, Watanabe K, Shiohara T. 1997. Repeated elicitation of contact hypersensitivity induces a shift in cutaneous cytokine milieu from a T helper cell type 1 to a T helper cell type 2 profile. J. Immunol. 159(5), 2484–2491.
2. Oh JH, Choi BJ, Chang MS, Park SK,.2009. *Nelumbo nucifera* semen extract improves memory in rats with scopolamine-induced amnesia through the induction of choline acetyltransferase expression. Neurosci. Lett. 461(1), 41–44.
3. Ono Y, Hattori E, Fukaya Y, Imai S, Ohizumi Y. 2006. Anti-obesity effect of *Nelumbo nucifera* leaves extract in mice and rats. J. Ethnopharmacol. 106(2), 238–244.
4. Ohkoshi E, Miyazaki H, Shindo K, Watanabe H, Yoshida A, Yajima H. 2007. Constituents from the leaves of *Nelumbo nucifera* stimulate lipolysis in the white adipose tissue of mice. Planta Medica. 73(12), 1255–1259, 2007.
5. Yamasaki K, Hashimoto A, Kokusenya Y, Miyamoto T, Sato T. 1994. Electrochemical method for estimating the antioxidative effects of methanol extracts of crude drugs. Chem. Pharm. Bull. l(42), 1663-1665.

Anti-cancer effect of *Etlingera pavieana* rhizome extracts

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ABSTRACT

The ethanolic extract of *E. pavieana* rhizome and its fractions were evaluated for their anti-cancer activity in four different cancer cell types (HepG2, HCT116, MCF-7, MDA-MB-231) and two non-cancerous cell lines (293T and HaCaT). The MTT assay showed that the ethanolic extract preferentially reduced the proliferation of cancer cells in a dose- and time- dependent manner rather than the non-cancerous ones. Among three fractions, the ethyl acetate fraction exhibited strong inhibition against cancer cell growth and it was thus separated by column chromatography. The obtaining F1 to F5 subs-fractions were subsequently tested for their cytotoxicity. The results indicated the anti-proliferative capability of all five subs-fractions, but only the F1 sub-fraction showed specifically targeted effect against cancer cells. Treatment cancer cells with F1 led to cell morphological change and reduced in colony formation. The bioactive compounds in the F1 sub-fraction are under identifying and the anti-cancer activity of F1- isolated pure compounds will be further investigated.

Keywords: anti-cancer activity, *Etlingera pavieana* rhizome

INTRODUCTION

Chemotherapeutic drug resistance of cancer cells is a *major* cause of treatment failure leading to increase the mortality rate of cancer patients. Attempts to search for the new bioactive phytochemical compounds as alternative anti-cancer agents are challenging. Thailand is a tropical country with a high biodiversity of plants. *Etlingera pavieana* (Pierre ex Gagnep.) R.M.Sm. or Reaw-Horm, belonging to Zingiberaceae family, is one of plants generally cultivated in eastern region of Thailand. Traditionally, its rhizome has been used as an ingredient in Moo lieng noodle, a signature dish of Chantaburi province and also used as folk medicine in treating diuresis, fever and flatulence [1]. The extracts of *E. pavieana* rhizome have been shown to exert anti-inflammatory and antioxidant activities [2]. Although the extracts and isolated compounds from many plants in Zingiberaceae family have been reported for their potential to inhibit tumor growth both in *vitro* and *in vivo* such as *Curcuma longa* and *Zingiber officinale* [3-4], nonetheless, there is no report for that of *E. pavieana*. This prompts us to study the anti-proliferative effect of *E. pavieana* rhizome on various types of cancer cell lines and compare to the non-cancerous ones.

MATERIALS AND METHODS

Preparation of plant extracts

E. pavieana rhizomes were collected from Khung district, Chantaburi province, Thailand and authenticated by Dr. Benchawan Chewprecha, a plant taxonomist at Department of Biology, Faculty of Science, Burapha University. After washing with tap water, the fresh rhizomes were cut into small pieces, dried in a hot-air oven at 50°C and homogenized by a blender. Fine powder of rhizomes was extracted with 95% ethanol by maceration and then filtered by a filter paper.

The solvent was removed by rotary evaporation at 42 °C. The crude extract was subsequently partitioned with hexane and ethyl acetate, respectively. The ethyl acetate fraction was then subjected to fractionation by column chromatography using dichloromethane/methanol mixture as a mobile phase. Based on TLC spot pattern analysis, five sub-fractions, F1 to F5, were obtained. DMSO was used as a solvent to dissolve all extracts with the final concentration in the treatment of 0.2% (v/v).

Cell culture

Four human cancer cell lines (hepatoma HepG2, *colorectal carcinoma* HCT116, and breast *adenocarcinoma* MCF-7 and MDA-MB-231), as well as two non-cancerous cell lines (*embryonic kidney* 293T and keratinocyte HaCaT) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and antibiotics at 37 °C in humidified air containing 5% CO_2.

Cell viability assay

Cell viability was determined by MTT assay. Cells were seeded in 96-well plates and treated with various concentrations of extracts as indicated and incubated for 24, 48 or 72 h. MTT assay was performed as previously described [5]. The absorbance at 540 nm was measured and converted to percentage of cell viability of which cells treated with 0.2% DMSO at 24 h was set to 100%. Each experiment was performed in triplicate.

Statistics

The Student's *t* test was used to determine the significant difference between treated and untreated cells of each cell type at $p < 0.05$ and symbolized by an asterisk (*). If the treated cancer cell was significant inhibited, it was then consequently compared to the noncancerous cells at the same treated concentration and symbolized by a sharp sign (#).

RESULTS

Cytotoxic effect of *E. pavieana* rhizome crude extract

The effect of ethanolic extract of *E. pavieana* rhizome on cell proliferation was determined by MTT assay. As shown in Figure 1, the extract could suppress cell proliferation of all four cancer cell lines in a dose- and time- dependent manner comparing to cells treated with 0.2% (v/v) DMSO (the black solid line), indicating the anti-tumor activity of *E. pavieana* rhizome extract. As a much lesser effect on 293T and HaCaT cells, the cancer cells are preferentially targeted by the extract for inhibiting growth. The anti-cancer effect was obviously observed when at least 200 µg/mL of extract was used to treat cells for at least 24 h. With 400 µg/mL of extract and 24h of incubation, approximately 50% of each cancer cells died while 80% of non-cancerous cells were alive. However, the cytotoxicity of 400 µg/mL extract towards 293T cells after 72h incubation should be concerned.

Anti-proliferative effect of ethyl acetate fraction and its sub-fractions

The various concentrations of hexane, ethyl acetate and aqueous fractions were examined for their cytotoxicity by using MTT assay at 24h of incubation. Comparing to untreated cells and non-cancerous cells, the dramatic reduction in cell viability was observed in cancer cells exposed to ethyl acetate fraction (except MCF-7) rather than the hexane fraction. In contrast, no significant inhibition in cell proliferation was observed in cancer cells treated with aqueous fraction when comparing to the noncancerous ones (Figure 2).

Due to the strongest activity, the ethyl acetate fraction was consequently subjected to be further sub-fractionated by column chromatography. After TLC analysis, the collected fractions with the same TLC spot pattern were grouped and finally gave the five sub-fractions namely F1, F2, F3, F4 and F5, respectively. All sub-fractions were then assayed for their cytotoxicity by MTT method. Although all five sub-fractions exerted their anti-proliferative effect on cancer cells, four of them, except F1, either generate toxic to HaCaT cells (Figure 3). Therefore, the F1 sub-fraction was selected for further isolation of pure compounds. In addition, the cell morphology of F1-treated cells was also observed under inverted microscope. As shown in Figure 4, the morphology of HCT116 cells treated with F1 sub-fraction for 72h was clearly changed and the cell number was decreased sharply comparing to untreated cells. A dose-dependent reduction in colony formation of F1-treated HCT116 cells was also revealed (data not shown). To date, the identification and isolation of pure compounds of F1 sub-fraction are ongoing in our laboratory and the anti-cancer activity of F1-isolated pure compound will be further investigated.

CONCLUSIONS

The anti-cancer activity of *E. pavieana* rhizome was here reported for the first time. All the findings indicated the crude extract of *E. pavieana* rhizome and its containing compounds are the promising agents for cancer therapy.

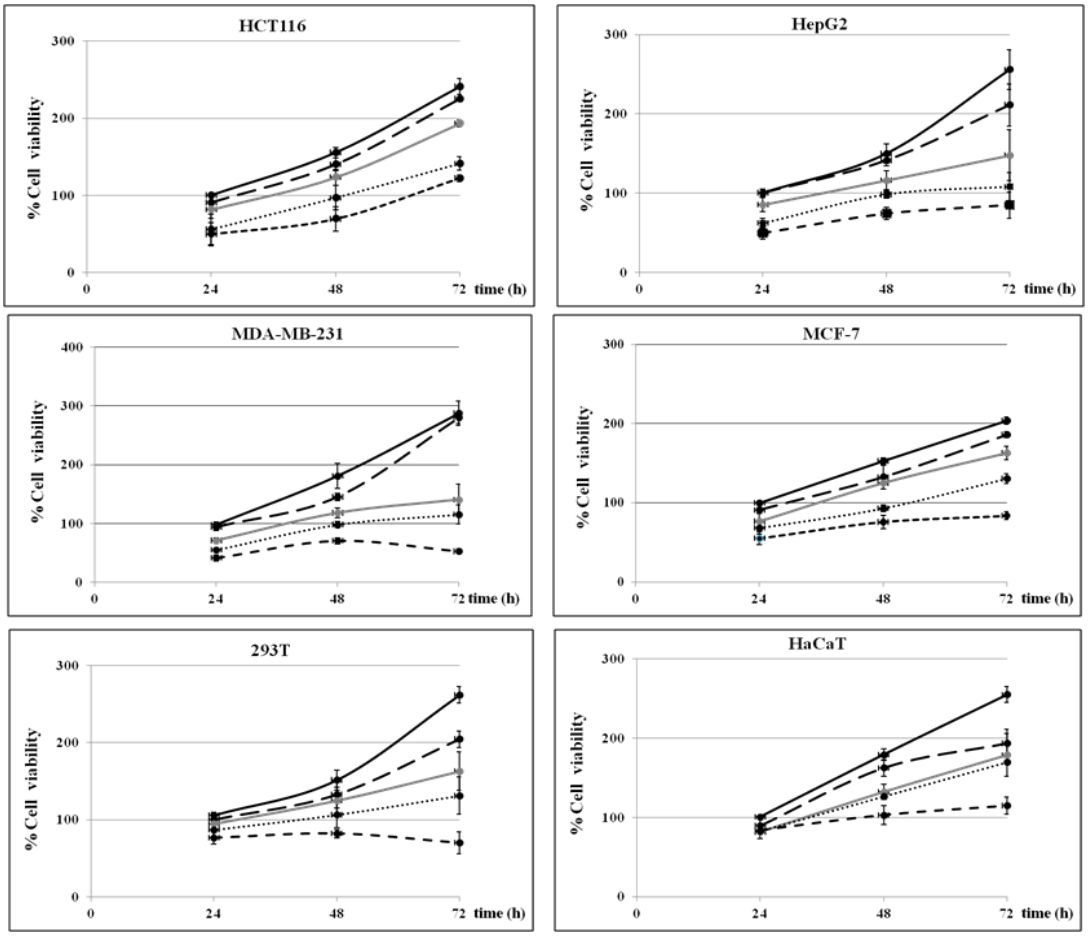

Figure 1. Cytotoxicity of *E. pavieana* rhizome crude extract. The absorbance of converted MTT dye was measured at 540 nm and presented as % cell viability of which 24h-incubated cells was set as 100%. Each line represents the various concentrations of extract;

——— 0.2% DMSO,
– – – 50 μg/mL,
——— 100 μg/mL,
····· 200 μg/mL,
- - - 400 μg/mL.

Data are shown as means ± S.D. of two independent experiments, each performed in triplicate.

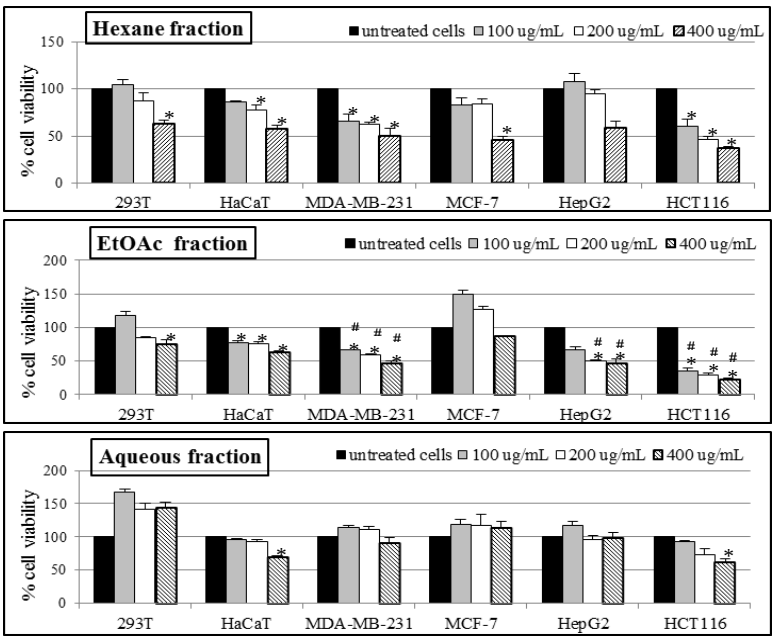

Figure 2. Cell viability under treatment of hexane, ethyl acetate, and aqueous fractions by MTT assay. Cells were exposed to various concentrations of each fraction and incubated for 24 h. The OD_{540} of untreated cells (0.2% DMSO) was set as 100% cell viability. Data are shown as means ± S.D. of two independent experiments, each performed in triplicate. * represents significant growth inhibition of treated cells compared to untreated cells ($p < 0.05$) when # represents significant growth inhibition of cancer cells compared to non-cancerous ones at the same extract concentration ($p < 0.05$).

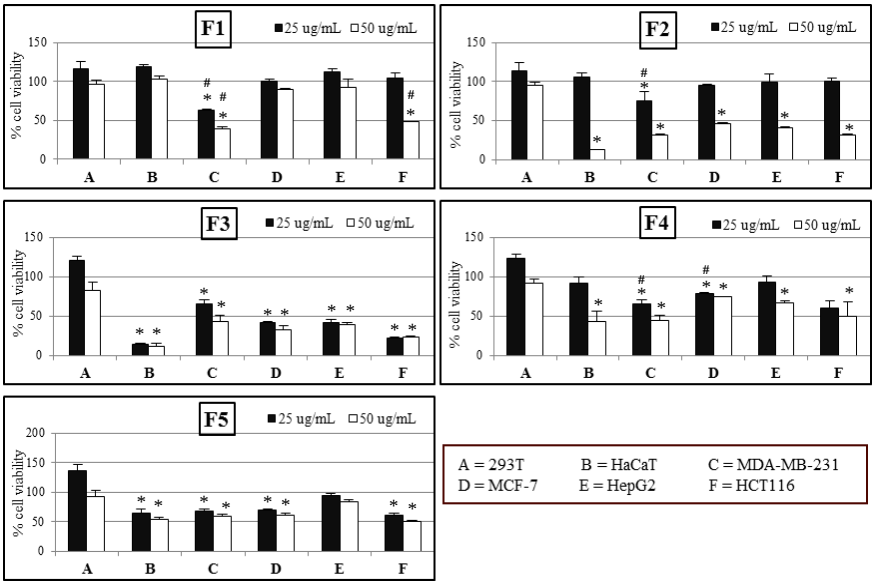

Figure 3. Cytotoxic effect of sub-fractions F1 to F5 by MTT assay. Cells were treated with 25 and 50 µg/mL of each sub-fraction for 24 h. Data are shown as means ± S.D. of two independent experiments, each performed in triplicate. * represents the significant difference between treated and untreated cells ($p < 0.05$) when # represents the significant difference between non-cancerous and cancerous cell lines at the same concentration of extract ($p < 0.05$).

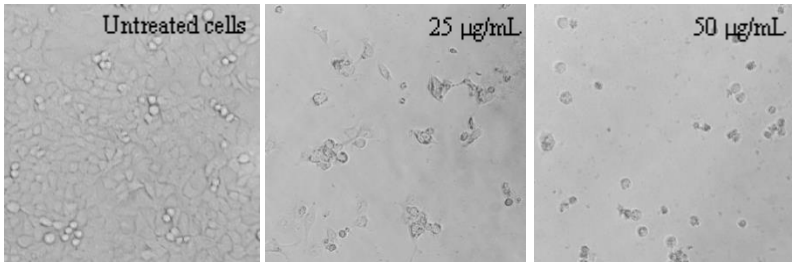

Figure 4. Cell morphology of F1-treated HCT116 cells compared to untreated cells. Cells were treated with 25 and 50 µg/mL F1 sub-fraction for 72 h and observed cell morphological change under inverted microscope. Pictures were taken at total 200X *magnification*.

ACKNOWLEDGEMENTS

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REFERENCES

1. Tachai S, Wangkarn S, Nuntawong N. 2014. Chemical constituents of the rhizome oils of *Etlingera pavieana* (Pierre ex Gagnep.) R.M.Sm. Biochem. Syst. Ecol. 57, 410–415.
2. Srisook E, Palachot M, Srisook K. 2012. *In vitro* anti-inflammatory effect of *Etlingera pavieana* rhizomes and its compounds in lipopolysaccharide-induced macrophages. Proceedings of the 4th international Conference on Natural Products for Health and Beauty. 28-30 November 2012, Chiang Mai, Thailand.
3. Lin YG, Kunnumakkara AB, Nair A, Merritt WM, Han LY, Armaiz-Pena GN, Kamat AA, Spannuth WA, Gershenson DM, Lutgendorf SK, Aggarwal BB, Sood AK. 2007. Curcumin inhibits tumor growth and angiogenesis in ovarian carcinoma by targeting the nuclear factor-kappaB pathway. Clin. Cancer Res. 13(11), 3423-3430.
4. Katiyar SK, Agarwal R, Mukhtar H. 1996. Inhibition of Tumor Promotion in SENCAR Mouse Skin by Ethanol Extract of *Zingiber officinale* Rhizome. Cancer Res. 56, 1023-1030.
5. Wanichwatanadecha P, Sirisrimangkorn S, Kaewprag J, Ponglikitmongkol M. 2012. Transactivation activity of human papillomavirus type 16 E6*I on aldo-keto reductase genes enhances chemoresistance in cervical cancer cells. J. Gen.Virol. 93(Pt 5), 1081-1092.

Phenolic compounds and alpha-glucosidase inhibitory activities of ungerminated and germinated rices

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ABSTRACT

The aim of this study was to investigate phenolic compounds in ungerminated and germinated rice and their ability to inhibit the alpha-glucosidase activity. Seven of ungerminated rices; white jasmine rice (WR), brown jasmine rice (BR), red brown jasmine rice (RR), rice berry rice (RB), lebnok rice (LN), sinlek rice (SL), fresh brown rice (FR) and four germinated rices; germinated rough rice (GH), germinated brown jasmine rice (GBR),germinated red brown jasmine rice (GRR) and germinated rice berry rice (GRB) were extracted with 80% ethanol. Determination of ferulic acid and caffeic acid contents by Folin-Ciocalteau method was carried out to evaluate the phenolic compounds in extracts. It was found that the RR extract had the highest ferulic acid and caffeic acid contents as 206.71 mgFAE/100g dry matter and 212.86 mgCAE/100g dry matter, respectively. The extracts were further tested for alpha-glucosidase inhibitory activities. Base on the results, all extracts showed the potential of anti-alpha-glucosidase activities. Among ungerminated rice extracts, RR extract gave the highest activity than others with IC_{50} about 0.14 mg/mL. Meanwhile, germinated rough rice (GH) extract showed the highest efficient with IC_{50} value as 0.89 mg/ml. In addition the results showed the moderate correlation between alpha-glucosidase inhibitory activity and phonolic contents.

Keywords: phenolic compound, alpha-glucosidase activity, ungerminated rice, germinated rice

INTRODUCTION

Worldwide, type 2 diabetes is one of the major health problems which the most serious and characterized by high blood glucose levels. To postpone absorption of glucose in human through inhibition of alpha-glucosidase enzyme was used for type 2 diabetes treatment. Several plants have been explored alpha-glucosidase inhibitors [1,2]. Which rice is important for consumed worldwide and rice grains are mainly composed of carbohydrates, phenolic compound, flavoniod compound, γ-aminobutyric acid [3]. Phenolic compounds are potent antioxidants previous study reported the anti-diabetic property of phenolic compounds in grape [4]. The present study aimed to investigate *in vitro* alpha-glucosidase inhibition and phenolic compounds in rice extracts.

MATERIALS AND METHODS

Preparetion of rice extracts

Eleven of rice types including seven of ungerminated rices; white jasmine rice (WR), brown jasmine rice (BR), red brown jasmine rice (RR), rice berry rice (RB), lebnok rice (LN), sinlek rice (SL), fresh brown rice (FR) and four germinated rices; germinated rough rice (GH), germinated brown jasmine rice (GBR), germinated red brown jasmine rice (GRR) and germinated rice berry rice (GRB) were studied. The rice powder (2 g) was extracted with 20 mL of 80% ethanol for 2 hour at room temperature. The mixture solution was then centrifuged at 4500 rpm for 20 minutes. The supernatant was kept to test in the next step.

Determination of total phenolic content by Folin-Ciocalteau method

0.1 mL of rice extract was mixed with 0.1 mL of 50% Folin-Ciocalteau reagent and the reaction continued for 3 minutes. The reaction was treated with 2 % Na_2CO_3. The mixture was incubated at room temperature for 30 minutes.

Then, the mixture was measured at 750 nm using UV-Vis spectrophotometer. The data were expressed as mg ferulic acid equivalents (FAE)/ 100g dry matter and mg caffeic acid equivalents (CAE)/ 100g dry matter [5].

Alpha-glucosidase inhibitory assay

50 µl of 0.2 M phosphate buffer (pH 6.8) was mixed with 50 µl alpha-glucosidase (0.5 U/ml) and 50 µl of each extract at various concentrations. The solution was pre-incubated at 37°C for 15 minutes. Then, 100 µl of 0.3 mM 4-nitrophenyl-α-D-glucopyranoside (pNPG) was added. The enzymatic reaction was allowed to proceed at 37°C for 20 min. The reaction was stopped by adding 750 µl of 0.1 M Na_2CO_3. Then, the mixture was measured at 405 nm using UV-Vis spectrophotometer.

The percent inhibition of alpha-glucosidase was calculated as

$$\text{(\%) inhibition of alpha-glucosidase} = \left[1-\left[\frac{A_{sample}-A_{blank}}{A_{control}}\right]\times 100\right]$$

Solution without sample was used as a control. Solution without substrate was used as a blank [6].

Statistical analysis

Analysis of the variance and significance of differences among samples were analyzed using One way analysis of variance (ANOVA) and the Tukey's Honestly Significant Difference Test were used to determine the differences among treatment means.

RESULTS

Total phenolic content by Folin-Ciocalteau method

Free radicals are involved in the development of diabetes complications and cardiovascular [7]. Phenolic compound was potent antioxidant. In this study we investigated the total phenolic compound of the ethanolic rice extacts by using ferulic acid and caffeic acid as standards. The results are presented in Table 1.

Ungerminated red brown jasmine rice (RR) showed the highest total phenolic compound of 206.71 mgFAE/100g dry matter and 212.86 mgCAE/100g dry matter. In addition RR extract also had higher total phenolic compound when compared with germinated red brown jasmine rice (GRR). The germination process may reduce the phenolic compound such as flavonoids and anthocyanins, which are pigment in rice according to the soaking in germination process [8]. Conversely, in non-pigment rice GBR extract showed higher phenolic compound than in BR as value 78.97 mgFAE/100g dry metter and 66.53 mgCAE/100g dry matter. In this case, the germination process may induce changes in phenolic compound content in rice by causing significant increase of phenolic content as reported that the levels of ferulic acid increased significantly in germinated rice [9].

Alpha-glucosidase inhibitory assay

Alpha-glucosidase inhibitors are effective in delaying glucose absorption and preventing elevation of the postprandial blood glucose level and therefore, they play a significant role as chemotherapeutic agents for type 2 diabetes [2]. This study was to investigate the inhibitory activities of rice extracts against alpha- glucosidase. It was found that all rice extracts had the effect on alpha-glucodidase activities as shown in Table 2.

In pigment rices, the RR extract showed the highest alpha-glucosidase inhibition with IC_{50} of 0.14 mg/ml followed by RB with IC_{50} of 0.81 mg/ml and showed the moderate correlation between alpha-glucosidase inhibitory activity and phonolic contents. In addition, GH which was non-pigment rice showed the highest alpha-glucosidase inhibitory activity with IC_{50} of 0.89 mg/ml. SL, LN and FR had a moderate effect on alpha-glucosidase activity with IC_{50} of 4.60, 4.53 and 4.85 mg/ml, respectively.

Table 1. Total phenolic acids in germinated and ungerminated rice extracts.

Rice	Total phenolic acids (mgFAE/100g dry matter)	Total phenolic acids (mgCAE/100g dry matter)
Germinated rice		
Germinated brown jasmine rice	78.97 ± 1.19^{c}	66.53 ± 2.19^{d}
Germinated red brown jasmine rice	101.62 ± 0.79^{b}	98.93 ± 1.22^{b}
Germinated rice berry rice	60.77 ± 1.33^{de}	41.80 ± 0.44^{ef}
Germinated rough rice	63.05 ± 0.48^{d}	43.89 ± 0.48^{e}
Ungerminated rice		
Brown rice	56.85 ± 3.09^{f}	66.11 ± 0.01^{d}
Red brown jasmine rice	206.71 ± 0.06^{a}	212.86 ± 2.07^{a}
Rice berry rice	103.07 ± 0.02^{b}	72.31 ± 0.00^{c}
Lebnok rice	57.69 ± 0.77^{ef}	44.11 ± 0.44^{e}
Sinlek rice	57.27 ± 0.00^{ef}	38.18 ± 0.00^{g}
Fresh brown rice	53.08 ± 1.33^{g}	38.72 ± 0.88^{fg}
Jasmine rice	8.20 ± 0.44^{h}	2.31 ± 1.32^{h}

Values in a column with different letters are significantly different at $p<0.05$.

Table 2. Alpha-glucosidase inhibitory activity of rice extracts.

Rice	IC_{50} (mg/ml)
Germinated rice	
Germinated brown jasmine rice	3.62 ± 1.14^{b}
Germinated red brown jasmine rice	1.42 ± 0.47^{a}
Germinated rice berry rice	1.64 ± 0.83^{a}
Germinated rough rice	0.89 ± 0.08^{a}
Ungerminated rice	
Brown rice	6.11 ± 0.10^{d}
Red brown jasmine rice	0.14 ± 0.01^{a}
Rice berry rice	0.81 ± 0.58^{a}
Lebnok rice	4.53 ± 0.99^{bc}
Sinlek rice	4.60 ± 0.08^{bc}
Fresh brown rice	4.85 ± 1.98^{bc}
Jasmine rice	9.47 ± 1.15^{e}

Values in a column with different letters are significantly different at $p<0.05$.

CONCLUSIONS

The phenolic content and alpha-glucosidase inhibitory activities of eleven rice extracts were investigated. The RR extract showed the highest activity of phenolic content and the best potent of alpha-glucosidase inhibitory activity. This suggested that the rice extract is helpful to control the rapid increasing of blood glucose level.

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REFERENCES

1. Chen HH, Chang HC, Chen YK, Hung CL, Lin SY, Chen YS. 2016. An improved process for high nutrition of germinated broew rice production: Low-pressure plasma. Food Chem. 191, 120-127.
2. Indrianingsih A. W, Tachibana S, Itoh K. 2015. In vitro evaluation of antioxidant and ∝-glucosidase inhibitory assay of several tropical and subtropical plants. Procedia Environ. Sci. 28, 639-648.
3. Kim JS, Hyun TK, Kim MJ. 2011. The inhibitory effects of ethanol extracts from sorghum, foxtail millet and proso millet on ∝-glucosidase and ∝-amylase activities. Food Chem. 2014, 1647-1651.
4. Montagut G, Onnockx S, Vaque M, Blay M, Feznandez-Larrea J, Pujadas G, Salvado M. J, Arola L, Pirson I, Ardevol A, Pinent M. 2010. Oligomers of grape-seed procyanidin extract activate the insulin receptor and key targets of the insulin signaling pathway differently from insulin. J. Nutr. Biochem. 21, 476-481.
5. Moongngarm A, Saetung N. 2010. Comparison of chemical compositions and bioactive compounds of germinated rough rice and brown rice. Food Chem. 122, 782-788.
6. Panesar PS, Kaur S. 2016. Rice: Types and composition. Encyclopedia of Food and Health. 646-652.
7. Premakumara GAS, Abeysekera WKSM, Ratnasooriya WD, Chandrasekharan NV, Bentota AP. 2013. Antioxidant, anti-amylase and anti-glycation potential of brans of some Sri lankan traditional and improved rice (*Oryza sativa L.*) varieties. J. Cereal Sci. 58, 451-456.
8. Rattanasene P, Bussaman P. 2013. Antioxidant activities, total phenolic compounds and γ-aminobutyric acid contents of extracts derived from thai pre-germinated brown rice and pre-germinated rough rice. SWU Sci. J. 29, 95-109.
9. Shodehinde SA, Ademiluyi AO, Oboh G, Akindahunsi AA. 2015. Contribution of *Musa paradisiacal* in the inhibition of ∝-amylase, ∝-glucosidase and Angiotensin-I converting enzyme in streptozotocin induced rats. Life Sci. 133, 8-14.

Apoptosis study, antiproliferative effects and antioxidant activity of *Urobotrya siamensis* Hiepko

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ABSTRACT

This study was aimed to examine the antioxidant activities, antiproliferative effects and cytotoxic activity of *Urobotrya siamensis* Hiepko. This plant was extracted by solvent extraction using soxhlet apparatus. The antioxidant activities of the extracts were determined using 2,2-diphenyl-1-picryhydrazyl radical scavenging (DPPH) assay, ferric reducing antioxidant power (FRAP) assay, and nitric oxide (NO) assay. The antiproliferative effects of the extracts were determined in human epithelial cancerous cell lines (Colon HCT116, hepatocellular carcinoma HepG2, leukemic Jurkat) and normal African green monkey kidney epithelial (Vero) cell lines by using neutral red assay. Apoptosis inducing effect was performed in HCT116 cell lines using DAPI staining assay. The extracts of *U.siamensis* exhibited moderate to relatively high antioxidant activities in DPPH (IC_{50}=1.03-12.54 μg/mL) and FRAP (IC_{50}=194-9,813 μg/mL) assays. The 50% cell viability or IC_{50} values of the ethyl acetate extract was 86.24±4.87μg/mL in Colon cell lines in comparison with cisplatin (166.62±4.00μg/mL). The methanolic extract inhibited Jurkat cell proliferation (115.80±6.64 μg/mL) without toxicity to Vero cells. The hexane and methanolic extracts also induced apoptotic cell death in HCT116 cell lines. The result is the first report about antiproliferative effects of *U.siamensis* Hiepko extract. It also suggests for potential research of the natural compounds derived for anticancers.

Keywords: *Urobotrya siamensis* Hiepko, Antioxidant activity, Antiproliferative effect, Apoptosis

INTRODUCTION

Urobotrya siamensis Hiepko or pak-wan mao is a wild plant, belonging to Opiliaceae family. It is a deciduous tree native to Southeast Asia including Thailand. There is less information about chemical components of pak-wan mao and only few reports mention about its toxicity. However, it is still controversy. One study reported that the toxicity was due to eating raw paw-wan or foodborne disease [1]. Twenty-five years later the other study reported that clinical symptoms and sudden deaths were observed at the western area of Cambodia due to *Urobotrya* poisoning [2]. Thai traditional medicine reported that drinking of boiling water of leaves and fruits can reduce the toxicity in the intestine, but could be poisonous at high dose [3]. Preliminary phytochemical screening founded that the methanolic extract contained alkaloid, coumarin, tannins and carbohydrates while the other extracts mainly contained alkaloids [4]. This research was explored more about the biological activities.

MATERIALS AND METHODS

Plants and sample preparation

Pak-wan mao was obtained from Kanchanaburi province in Thailand. The plant was examined according to its botanical characteristics to identify the right specie [3]. The fresh leaves and stems were dried in a hot air oven at a temperature of 50-60 ^{O}C for 6-24 hours, then were ground and weighed. Each 500 mL of three solvent systems were used consecutively in the extraction process: hexane, ethyl acetate, and methanol, using soxhlet apparatus. The crude

extracts were filtered and dried *in vacuo*. The samples were then boiled in 500 mL of distilled water for 3 hours, left to cool down, and filtered. The water extracts were freeze dried using a lyophilizer.

Antioxidant activities

DPPH radical scavenging assay [5]***:*** DPPH solution was prepared in a concentration of $2x10^{-4}$ M in ethanol. The pak-wan extracts were prepared in distilled water or 10%DMSO in 10 mg/ml and diluted to 0.05-5 mg/ml (n=3). Both 100 μL of the sample solution and 100 μL of the DPPH solution was added into a 96-well plate and mixed well. After leaving in the dark and at room temperature for 30 minutes, absorbance was measured at the 517 nm wavelength using a Biorad microplate reader. Gallic acid in the concentration range of 0-200 μg/mL was used as the standard curve. The assessment of the antioxidant activity was expressed in milligram gallic acid equivalent (GE) per 100 gram of dry weight extract and percentage inhibition was calculated.

FRAP assay [6]***:*** FRAP reagent was prepared in 20 mM concentration from the mixture of TPTZ, $FeCl_3.6H_2O$ and sodium acetate buffer. The 30 μL aliquot of the sample solution (n=3) was added to the 96-well plate and mixed with 270 μL of FRAP reagent. After 30 minutes of dark light at room temperature, the reduction was monitored by the change in absorption at 595 nm wavelength. Trolox in the concentration of 0-250 μg/mL was used as the standard, and the assessment of the antioxidant activity was expressed in milligram trolox equivalent (TE) per 100 gram of dry weight extract.

Determination of total phenolic content: Total phenolic content of pak-wan pa was determined by Folin-Ciocalteuassay [7]. A 12.5 μL aliquot of the sample solution (n=3) was diluted with 50 μL distilled water in a 96-well plate, and 12.5 μL of Folin-Ciocalteu solution was then added and mixed well. The plate was kept in the dark at room temperature for 6 minutes. Following this, 125 μL of 7% w/v Na_2CO_3 solution was added and mixed, then another 100 μL of distilled water was added immediately. After they were kept at room temperature with no light for 90 minutes, the absorbance was measured at 760 nm wavelength. Gallic acid in the concentration range of 0-200 μg/mL was used as the standard, and the assessment of the antioxidant activity was expressed in milligram gallic acid equivalent (GE) per 100 gram of dry weight extract.

Determination of flavonoid content: The flavonoid content of pak-wan pa was determined by spectrophotometric method in a 96-well plate [8]. The 25 μL aliquot of sample solution (n=3) was diluted with 125 μL distilled water; and 10 μL of 5% sodium nitrite solution was then added and mixed well. The plate was kept in the dark at room temperature for 6 minutes. Then 15 μL of 10% w/v aluminium chloride was added and the plates were left in the dark at room temperature for 90 minutes. After 50 μL of 1 M sodium hydroxide was added and mixed, the absorbance was measured at 510 nm wavelength. Catechin in the concentration range of 0-200 μg/mL was used as a standard, and the assessment of the antioxidant activity was expressed in milligram catechin equivalent (CE) per 100 gram of dry weight extract.

Nitric oxide assay [9]***:*** All pak-wan pa extracts were prepared in concentration of 1-10 mg/mL (n=3). All extracts were dissolved in water, apart from the hexane and ethyl acetate extracts which were dissolved in 5% dimethyl sulfoxide. After 100 μL of the sample solution and 100 μL of 5 mM sodium nitroprusside in phosphate buffer were added into the 96-well plates, they were left in the dark at room temperature for 3 hours. Then 50 μL of Griess reagent (1% sulphanilamide in ultrapure water, 0.1% naphtylethylenediamine dihydrochloride, 3% phosphoric acid in water) was added, and the absorbance was measured at 546 nm wavelength. The 50 percent inhibition concentration (IC_{50}) of nitric oxide was calculated from the graph of percent scavenging activity and concentration of each sample. Ascorbic acid in the concentration of 0-100 μg/mL was used as the standard.

Antiproliferative effects

Cell culture: Human epithelial cancerous cell lines (Colon HCT 116 and Liver HepG2) and normal African green monkey kidney epithelial (Vero) cell lines were cultured in the same medium [10]. The medium was Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 μg/ml streptomycin. The leukemia cells (Jurkat) were cultured using RPMI medium with the components of FBS and penicillin as above. The cells were cultured at 37°C under a humidified atmosphere containing 5% CO_2.

Cytotoxic activity test: The crude extracts were dissolved in dimethyl sulfoxide (DMSO) as stock solutions then diluted with fresh media to the desired concentrations ranging from 20 to 1,000 μg/ml. The cancerous cell line and normal Vero cell line were used as cell models. Cytotoxicity testing was performed with a neutral red (NR) method. Standard anticancer drug, Cisplatin was used for comparison with the crude extracts. Briefly, the cells were seeded in 96-well plates (100 μl/well at a density of 3×10^5 cells/ml for epithelial cells and 5×10^5 cells/ml for leukemia cell) and treated with various concentrations of the samples at 24 hours exposure. Then cells were washed with 1× PBS and the supernatant was discarded. A total of 100 μl NR solutions (50 μg/ml) was added to each well and incubated at 37°C for 2

hours. The excessive NR solutions were washed by adding 100 µl 1× PBS and the supernatant was discarded. The viable cell that accumulated NR was then lysed by 100 µl of 0.33% HCl in isopropanol. Absorbance of NR dye was detected by a dual-wavelength UV spectrometer at 537 nm with a 650 nm as a reference wavelength. A plot of % cell viability versus sample concentrations was used to calculate the concentration which showed 50% cell viability (IC_{50}). The percentages of cell viability compared to the untreated cell were determined from [100 × Absorbance of treated group] / [Absorbance of untreated group]

Apoptosis study: The human epithelial cancerous cells (HCT116) were cultured in growth medium on a chamber slide. After treatment with the extract, the culture medium was discarded and the cells were fixed by methanol. The fixed solution was removed and the cells were allowed to dry at room temperature. Then, the cells were stained with 4, 6 diamino-2-phenylindole (DAPI) stain solution. In the following step, stain solution was removed and the cells were washed. Then mounting solution was dropped on the cells before covered by a cover slide. Stained nuclei were observed under an inverted fluorescence microscope. Apoptotic nuclei were observed from punctuated staining pattern or nuclei swelling.

RESULTS

The dry weight of 385.78 g of pak-wan mao was extracted and yielded total 89.96 g (41.33%) of the crude extract. Each solvent extraction obtained 5.87 g (2.70%), 3.05 g (1.40%), 66.75 g (30.44%), 14.79 g (6.79%) for hexane, ethyl acetate, methnol and water, respectively. In this study, the ethyl acetate extracts of pak-wan mao exhibited the highest IC_{50} in DPPH and FRAP assays but not in nitric oxide assay (Table 1). However, the methanolic extract exhibited the highest values of DPPH radical scavenging activity and in the FRAP assay as well as the highest contents of total phenolic and flavonoid compounds.

Table 1. Inhibition concentration (IC_{50}) and anti-oxidant activities of pak-wan mao extracts

U. siamensis Extracts Assays	Hexane	Ethyl acetate	Methanol	Water
DPPH[1] (µg/mL)	12.54 ± 0.36	1.03 ± 0.04	4.51 ± 0.19	7.35 ± 1.22
FRAP[2] (µg/mL)	9,813 ± 1380	194 ± 10	198 ± 10	325 ± 3
Nitric oxide[3] (µg/mL)	3,377 ± 34	20,830 ± 76	6,709 ± 41	6,055 ± 25
DPPH (mg GE/100 g sample)	0.94 ± 0.03	6.74 ± 0.17	29.52 ± 1.21	27.18 ± 0.50
FRAP (mg TE/100 g sample)	2.07 ± 0.29	30.00 ± 0.83	185.59 ± 6.66	79.17 ± 3.47
Phenolic content (mg GE/100 g sample)	ND	39.88 ± 5.90	715.21 ± 13.11	87.60 ± 17.35
Flavonoid content (mg CE/100 g sample)	ND	4.42 ± 0.36	58.98 ± 3.72	13.63 ± 0.54

The values are mean±SD (n=3) ND = not determined

[1] *Gallic acid*, IC_{50}=2.80 ± 0.01 µg/mL [2] *Trolox*, IC_{50}=0.94 ± 0.22 µg/mL [3] *Ascorbic acid*, IC_{50}=3.37 ± 0.15 µg/mL

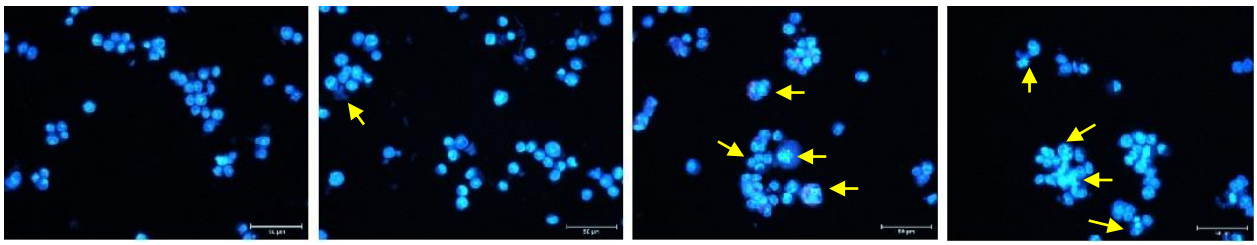
Pak-wan mao extracts were tested in four cell lines; Colon, HepG2, Jurkat and Vero cell lines (Table 2). Most of pak-wan mao extracts were inactive in vero cell lines except for the hexane extract. In contrast, most of the extracts inhibited colon cancer cell lines with relatively high potency (86-163 µg/ml) in comparison with cisplatin (166 µg/ml) and the highest inhibition was shown in the ethyl acetate extract but inactive in water extract. The hexane and ethyl acetate extracts of pak-wan mao inhibited HepG2 cell lines with 9-fold lower potency in comparison with cisplatin. The methanolic and hexane extracts of pak-wan mao inhibited Jurkat cell lines with 3- to 4-fold lower potency in comparison with cisplatin.

Table 2. Antiproliferative effects of pak-wan mao extracts

Extracts / Cell lines	IC_{50} (µg/ml)			
	VERO	Colon (HCT116)	HepG2	Jurkat
Hexane	296.35 ± 16.28	107.42 ± 3.60	359.24 ± 24.10	153.94 ± 65.64
Ethyl acetate	Inactive[1]	86.24 ± 4.87	343.87 ± 41.17	Inactive[1]
Methanol	Inactive[1]	163.28 ± 7.76	Inactive[1]	115.80 ± 6.64
Water	Inactive[1]	Inactive[1]	Inactive[1]	Inactive[1]
Cisplatin	ND	166.62 ± 4.00	36.83 ± 3.54	36.83 ± 3.54

The values are mean±SD (n=3) ND = not determined [1] IC_{50} >100 µg/ml is considered to be inactive.

The nuclei morphological change based on DAPI staining was conducted to identify the cell undergoing apoptotic death mode against the HCT116 cell lines. The successful anticancer treatment was via apoptosis induction because this death mode is not harmful to neighboring cells [11]. Figure 1 showed that nuclei morphologic changes of apoptotic cells as observed from punctuated staining pattern or nuclei swelling (arrow). Result showed that the hexane and methanolic extracts of pak-wan mao caused apoptotic cell death in comparison with cisplatin at the same concentration (320µg/ml).



A **B** **C** **D**

Figure 1. Apoptotic cells were observed in HCT116 cell lines
A. Untreated HCT116 cell lines
B. Treated with cisplatin
C. Treated with pak-wan mao hexane extract
D. Treated with pak-wan mao methanolic extract

CONCLUSIONS

The ethyl acetate extracts exhibited the highest inhibition in DPPH and FRAP assays, but the hexane extract exhibited the highest inhibition in the nitric oxide assay. The hexane extracts showed no selectivity in the antiproliferative effects of colon, HepG2 and Jurkat cell lines in comparison with Vero cell lines. The ethyl acetate extracts showed more selective inhibition of HCT116 and HepG2 cell lines, while the methanolic extracts showed more selective inhibition of HCT116 and Jurkat cell lines. This is the first report about *in vitro* cytotoxic studies of *U. siamensis* Hiepko. The results suggest the potential use as chemopreventive and also imply the anticancer potential of these plant-derived natural compounds.

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REFERENCES

1. Sawasdeemonkol K. 1980. Pak-wan and toxicity in consuming. Bull. Depart. Med. Sci. 22(1), 47-51.
2. Tourdjman M, Srihawong R, Soy TK, Touch S, Hul S, Janssens B. 2009. Plant poisoning outbreak in the western area of Cambodia 2005. Trans. R Soc. Trop. Med. Hyg. 103(9), 949-951.
3. Hiepko P. 1987. Opiliaceae. Flora of Thailand. In: Smitinand T. Larsen K. Eds. Flora of Thailand. Volume Five Part One. Bangkok: The Chutima Press, 93-103.
4. Charoenchai L, Settharaksa S, Songsak T, Ruangrungsri N and Kraisintu K. 2013. Phytochemical screening tests of *Melientha suavis* Pierre and *Urobotrya siamensis* Hiepko. BHST. 11(2), 13-20.
5. Brand-Williams W, Cuvelier ME, Berset C. 1995. Use of a free radical method to evaluate antioxidant activity. *LWT* Food Sci. Tech. 28(1), 25-30.
6. Benzie FFI, Strain JJ. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "Antioxidant Power": The FRAP assay. Anal. Biochem. 239(1), 70-76.
7. Slinkard K, Singleton VL. 1997. Total phenol analysis: automation and comparison with manual methods. AmerJ. Enol. Viticul.28, 49-55.
8. Chen JJ, Li XG. 2007. Hypolipidemiceffect of flavonoids from mulberry leaves in triton WR-1339 inducehyperlipidemic mice. Asia Pac. J. Clin. Nutr. 1, 290-294.
9. Sun J, Zhang X, Broderick M, Fein H. 2003. Measurement of nitric oxide production in biological systems by using griess reaction assay. Sensors. 3, 276-284.
10. Weerapreeyakul N, Nonpunya A, Barusrux S, Thitimetharoch T, Sripanidkulchai B. 2012. Evaluation of the anticancer potential of six herbs against a hepatoma cell line. Chinese Medicine. 7(15), 1-7.
11. Rebecca SY Wong.2011. Apoptosis in cancer: from pathogenesis to treatment. J Exp & Clin Cancer Res. 30, 87.

Screening for antibacterial and antioxidant activities of *Oroxylum indicum* fruit extracts

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ABSTRACT

Oroxylum indicum which is called in Thai as Pheka is a Thai traditional plant in Bignoniaceae family with various ethnomedical uses such as astringent, anti-inflammatory, anti-bronchitic, anti-helminthic and anti-microbial agents. The young fruits of this plant have also been consumed as vegetables. However, there is no report concerning antibacterial activity, especially the activity on the clinical isolated pathogenic bacteria and the *in vitro* antioxidant effects of this plant. Therefore, the extracts from *O. indicum* fruits and seeds collected from different provinces in Thailand were prepared by decoction and maceration with ethanol and determined for *in vitro* antibacterial effects to two clinical isolated bacteria which are *Streptococcus suis* and *Staphylococcus intermedius* by disc diffusion assay. Ethanol extracts from *O. indicum* fruits collected from Nakorn Pathom province at the concentration of 1,000 mg/ml exhibited intermediate antibacterial activity against *S. intermedius* with the inhibition zone of 15.11 mm. Moreover it promoted moderate inhibitory effect to *S. suis* with the inhibition zone of 14.39 mm. The extracts prepared by maceration with ethanol promoted higher antibacterial activities than water extract. The ethanol extract from the seeds of this plant purchased from Bangkok showed stronger *in vitro* antioxidant activities than other extracts with EC_{50} value of 68.85µg/ml. From the results, extracts from *O. indicum* fruits have *in vitro* antioxidant effect with antibacterial potential to clinical pathologic bacteria which could be developed for medicinal and pharmaceutical purposes in the future.

Keywords: antibacterial, antioxidant, *Oroxylum indicum*, *Streptococcus suis*, *Staphylococcus intermedius*, DPPH, Disc diffusion

INTRODUCTION

Ethnoveterinary medicine (EVM) is the traditional practices of using natural products mainly plant materials to protect, treat or support animal health [1]. Natural products are sources of new chemical diversities and also pharmaceutical components. There are the future antimicrobial candidates, which could provide more effective and less toxic antimicrobial compounds [2]. *Oroxylum indicum* (L.) Vent. is a medium size, deciduous tree with various ethnomedical uses. Mature Fruits are acrid and sweet which promote anthelmintic and stomachic effects [3]. The seeds have been used as purgative while the seed paste is applied to the throat for quick relief of tonsil pain [4-5]. Many flavonoids such as baicalein and biochanin A were previously reported in the pods, seeds and root barks of this plant [6-11]. Therefore, the extracts from *O. indicum* fruits and seeds collected from different provinces in Thailand were prepared by decoction and maceration with ethanol and determined for *in vitro* antibacterial effects to two clinical isolated bacteria which were *Streptococcus suis* and *Staphylococcus intermedius* by disc diffusion assay and determined for *in vitro* antioxidant effect using DPPH scavenging method.

MATERIALS AND METHODS

Preparation of *Oroxylum indicum* fruits extracts

Plant material preparation

The fruits of *Oroxylum indicum* were separately collected from Chiang Rai and Nakhon Pathom provinces while the seeds were purchased from traditional herbal shop in Bangkok in 2015. Plant samples were cleaned and dried in a hot air oven at 60 °C then ground using an electric mill (20 mesh sieve).

Plant extract preparations

Each *O. indicum* fruit and seed powder was separately extracted by maceration using 95% ethanol and decoction with distilled water (plant:solvent raito 1:20 w/v). Each extraction process was repeated for three times. The extraction solutions were then combined, filtered and evaporated using water bath to yield the dried extracts.

Determination of *in vitro* antibacterial activity of *O. indicum* extracts by disc diffusion assay [12]

The agar disc diffusion method was used to determine the diameter of the inhibition zone of each extracts from the fruits and seeds of *O. indicum* against 2 clinical pathogenic bacteria (*Streptococcus suis* and *Staphylococcus intermedius*). Each extract was dissolved in 95% ethanol or sterile water at different concentrations (1.5625, 6.25, 25, 100, 125, 250, 500 and 1,000 mg/ml). Then each solution was impregnated onto a small disc of filter paper and placed on the top of blood agar containing 100µl of bacterial solution at the concentration of $1.5x10^8$ CFU/ml per plate (adjusted by comparing to 0.5 McFarland). The plate was incubated at 37 °C for 24 hours and the zone of inhibition was recorded. Amoxicillin/clavulanic acid 30 µg (AMC30), gentamicin 10 µg (CN10) and sulfamethoxazole-trimethoprim 25 µg (SXT25) were used as positive controls. All determination was done in triplicate and the average zone of inhibition was calculated with the standard deviation.

Determination of *in vitro* antioxidant activity of *O. indicum* extracts by DPPH scavenging method [13]

2,2-diphenyl-1-picrylhydrazyl (DPPH) was dissolved in methanol to prepare the DPPH solution at the concentration of 207 µM. DPPH solution (100µl) was added to each plant extract solution with various concentrations ranged from 5 - 640 µg/ml in the same volume (100µl). The mixture was mixed and kept in the dark for 15 minutes. The absorbance of each reaction solution was determined at the wavelength of 515 nm using microplate reader. The percentage of inhibition of each reaction was then calculated, and EC_{50} values (µg/ml) were calculated from the linear equation from the curve between percentage of inhibition and the solution concentration. Each experiment was conducted in triplicate. The EC_{50} value of each extract was expressed as mean ± SD.

RESULTS

***In vitro* antibacterial activity of *O. indicum* extracts by disc diffusion assay**

The antimicrobial activity of the extracts from the fruits and seeds of *O. indicum* were studied against two clinical pathogenic bacterial strains (*Staphylococcus intermedius* and *Streptococcus suis*) by disc diffusion assay. As shown in Table 1, the fruit extracts of *O. indicum* prepared by both decoction and maceration methods promoted intermediate inhibiting effects to *S. intermedius* with the inhibition zone ranged from 11-15 mm. at the concentration of 1,000 mg/ml. These extracts also promoted moderate inhibiting activity to *S. suis* with the zone of inhibition ranged from 10-14 mm. at the concentration of 1,000 mg/ml. The antibacterial activities of the extracts increased in dose dependent manner to the concentration of extracts. However, the seed water and ethanol extracts of this plant promoted low inhibitory effects to both bacteria.

Table 1. Antibacterial activity of *Oroxylum indicum* fruit and seed extracts against *Staphylococcus intermedius* and *Streptococcus suis* determined by disc diffusion method

Bacterial strain	Concentration (mg/ml)	Zone of inhibition (mm.)								
		OPCD	OPCE	OPND	OPNE	OSBD	OSBE	AMC 30 µg	CN 10 µg	SXT 25 µg
Staphylococcus intermedius	1.5625	0	0	0	0	0	0	24.44 ± 0.73	15.00 ± 0.50	0
	6.25	0	0	0	0	0	6.78 ± 1.54			
	25	0	0	0	0	0	7.34 ± 1.87			
	100	0	8.33 ± 0.71	0	8.56 ± 0.73	7.83 ± 0.98	7.89 ± 4.89			
	125	0	9.28 ± 0.36	0	10.22 ± 0.83	-	-			
	250	6.22 ± 0.44	10.33 ± 0.43	6.72 ± 0.51	11.33 ± 0.83	-	-			
	500	8.72 ± 0.44	11.56 ± 0.46	8.67 ± 0.50	13.50 ± 1.32	-	-			
	1,000	11.17 ± 0.35	12.67 ± 0.35	11.17 ± 0.61	15.11 ± 2.10	-	-			
Streptococcus suis	1.5625	0	0	0	0	0	8.22 ± 0.97	32.56 ± 0.53	0	0
	6.25	0	0	0	0	0	8.56 ± 1.34			
	25	0	0	0	0	0	8.78 ± 1.86			
	100	0	0	0	0	0	7.56 ± 2.69			
	125	0	0	0	0	-	-			
	250	0	7.28 ± 0.44	0	8.06 ± 0.39	-	-			
	500	7.00 ± 1.00	8.67 ± 1.00	7.72 ± 0.75	11.33 ± 1.00	-	-			
	1,000	10.78 ± 0.67	10.61 ± 0.99	10.28 ± 0.91	14.39 ± 2.47	-	-			

OPCD = water extract from *O. indicum* fruits collected from Chiang Rai province, OPCE = ethanol extract from *O. indicum* fruits collected from Chiang Rai province, OPND = water extract from *O. indicum* fruits collected from Nakorn Pathom province, OPNE = ethanol extract from *O. indicum* fruits collected from Nakorn Pathom province, OSBD = water extract from *O. indicum* seeds purchased from Bangkok, OSBE = ethanol extract from *O. indicum* seeds purchased from Bangkok, AMC 30 µg = Amoxicillin/clavulanic acid 30 µg, CN 10 µg = gentamicin 10 µg and SXT 25 µg sulfamethoxazole-trimethoprim 25 µg

***In vitro* antioxidant activity of *O. indicum* extracts by DPPH scavenging method**

As shown in Table 2 the ethanol and water extract from *O. indicum* seeds purchased from Bangkok (OSBE and OSBD) exhibited high antioxidant activities determined by DPPH scavenging assay with EC_{50} values of 68.85 and 86.03 µg/ml, respectively. However, the water and ethanol extracts from the fruits of *O. indicum* promoted lower antioxidant effects with EC_{50} values ranged from 160 – 378 µg/ml.

Table 2. Antioxidant activity of *O. indicum* fruit extracts determined by DPPH scavenging assay.

Extracts	EC_{50} (µg/ml)
OPCD	378.30±0.97
OPCE	160.23±0.61
OPND	504.32±0.87
OPNE	184.92±0.31
OSBD	86.03±0.69
OSBE	68.85±0.73
Ascorbic acid	8.45±0.44

OPCD = water extract from *O. indicum* fruits collected from Chiang Rai province, OPCE = ethanol extract from *O. indicum* fruits collected from Chiang Rai province, OPND = water extract from *O. indicum* fruits collected from Nakorn Pathom province, OPNE = ethanol extract from *O. indicum* fruits collected from Nakorn Pathom province, OSBD = water extract from *O. indicum* seeds purchased from Bangkok, OSBE = ethanol extract from *O. indicum* seeds purchased from Bangkok

CONCLUSIONS

In the present study, the water and ethanol extracts from *Oroxylum indicum* fruits and seeds were tested to their *in vitro* antibacterial and antioxidant activities. The extracts from *O. indicum* fruits promoted moderate to intermediate antibacterial activities against both clinical isolated bacteria; *Streptococcus suis* and *Staphylococcus intermedius*. The ethanol and water extracts from the seeds of *O. indicum* showed high *in vitro* antioxidant effects. Phytochemical study and quantitative analysis of active compounds in *O. indicum* fruit and seed extracts should be further investigated in the future.

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REFERENCES

1. McGaw LJ, Van der MD, Eloff JN. 2007. In vitro anthelmintic, antibacterial and cytotoxic effects of extracts from plants used in South African ethnoveterinary medicine. Vet. J.173, 366–372.
2. Saleem M, Nazir M, Ali SM, Hussain H, Lee SY, Riaz N, Jabbar A. 2010. Antimicrobial natural products: an update on future antibiotic drug candidates. Royal Soc. Chem. 27, 238-254.
3. Harminder, Singh V, Chaudhary A.K. 2011. A review on the taxonomy, ethnobotany, chemistry and pharmacolody of *Oroxylum indicum* Vent. Indian J. Pharm. Sci. 73(5), 483-490
4. Warrier PK, Nambiar VPK, Ramankutty C. 1993. Indian Medicinal Plant: A compendium of 500 species (4). Orient Longman. 186-190.
5. Chauhan SN. 1999. Medicinal and Aromatic plants of Himachal Pradesh. Indus publishing. 296-298.
6. Subramaniam SS, Nair AG. 1972. Flavonoids of the stem bark of *Oroxylum indicum*. Curr. Sci. 41, 62-63.
7. Jayaram K, Prasad MN. 2008. Genetic diversity in *Oroxylum indicum* (L.) Vent. (Bignoniaceae), a vulnerable medicinal plant by random amplified polymorphic DNA marker. Afr. J. Biotech. 7, 254-262.
8. Bhattacharje AK, Das AK. 1969. Phytochemical screening of some Indian plants. QJ Crude Drug. Res. 9, 1408.
9. Chen LJ, Games DE, Jones J. 2003. Isolation and identification of four flavonoid constituents from the seeds of *Oroxylum indicum* by high-speed counter-current chromatography. J Chromatogr A. 988(1), 95-105.
10. Chen LJ, Song H, Lan XQ, Games DE, Sutherland IA. 2005. Comparison of high-speed counter-current chromatography instruments for the separation of the extracts of the seeds of *Oroxylum indicum*. J Chromatogr A. 1063(1-2), 241-245.
11. Zaveri M, Khandhar A, Jain S. 2008. Quantification of Baicalein, Chrysin, Biochanin-A and Ellagic acid in root bark of *Oroxylum indicum* by RP-HPLC with UV detection. Eurasian J Anal Chem. 3, 245-257.
12. Ali M.R, Houghton P.J, Raman A, Hoult J.R.S. 1998. Antimicrobial and anti-inflammatory activities of extracts and constituents of *Oroxylum indicum* (L.) Vent. Phytomedicine. 5(5), 375-381.
13. Sithisarn P, Jarikasem S. 2009. Antioxidant Activity of *Acanthopanax trifoliatus*. Medicinal Principle and practices. 18, 393-398.

Effects of *Rhodomyrtus tomentosa* extract on the killing activity of human neutrophils against enterohaemorrhagic *Escherichia coli* O157:H7

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ABSTRACT

Enterohaemorrhagic *Escherichia coli* O157:H7 is a major Gram-negative foodborne pathogen producing Shiga-like toxins and the most corporate cause of haemorrhagic colitis in human. Use of antimicrobial drugs against the pathogen might contribute to an increased risk of uraemic syndrome, particularly in children and elderly patients. One alternative strategy to the antibiotic therapy is the incorporation of plant resources as immunostimulant against the pathogenic bacteria. The aim of this study was to evaluate the effects of *Rhodomyrtus tomentosa* leaf extract on the killing activity of human neutrophils against enterohaemorrhagic *E. coli*. Neutrophils were coincubated with the extract at 62.5, 125, and 250 µgmL^{-1}and exposed to *E. coli* O157:H7 and ATCC 25922 for 30, 45, and 60 min. Within 45 min, neutrophils in the presence of the extract at 125-250 µgmL^{-1} significantly reduced percentage survival of *E. coli* O157:H7 and *E. coli* ATCC 25922 up to 58.48-50.28% and 69.13-35.35%, respectively. After 60 min of coincubation with the same concentrations of the extract, neutrophils showed reduction in percentage survival of *E. coli* O157:H7 and *E. coli* ATCC 25922 up to 50.34-40.15% and 58.46-47.02%, respectively, compared with the control ($p< 0.05$). The results indicated that *Rhodomyrtus tomentosa* extract could enhance the killing activity of human neutrophils against *E. coli* infections.

Keywords: *Rhodomyrtus tomentosa*; human neutrophils; *Escherichia coli* O157:H7; immunostimulant

INTRODUCTION

Enterohaemorrhagic *Escherichia coli* O157:H7 is an important Gram-negative foodborne pathogen, which produces Shiga-like toxin that causes diarrhoea and haemorrhagic colitis in human. One of therapeutic approach for treatment of the disease is antibiotic administration. However, the use of the antibiotic can lead to severe infections and life-threatening complications such as haemolytic uraemic syndrome and thrombotic thrombocytopenic purpura, especially in children and elderly person [1−4]. Additionally, there is no specific antibiotic for treating of the organism and its toxins nowadays [5]. Therefore, many attempts to find alternative ways for control of *E. coli* O157:H7 infections are urgently needed. Plant extract is one of interesting alternative strategy and could be used as an immunostimulant to enhance the activity of the host cellular immunity against the infections. *Eucalyptus* oil extract [6], a polysaccharide fraction from *Solanum nigrum* Linn [7], and *Coffea arabica* L. seed extract [8] have been studied and well-demonstrated that the plant extracts can activate and strengthen the host immune response.

Rhodomyrtus tomentosa (Aiton) Hassk. is a medicinal plant belonging to Myrtaceae family. The plant has been utilized for remedy of diarrhoea, gynaecopathy, urinary tract infections, and wound healing [9–12]. Moreover, the leaf extract of this plant species demonstrated antibacterial activities, antioxidant, and anti-inflammatory properties [13–17]. This study was aimed to investigate the effects of *Rhodomyrtus tomentosa* extract to enhance the killing activity of human neutrophils against *E. coli* O157:H7.

MATERIAL AND METHODS

Pathogens used and culture conditions

Enterohemorrhagic *Escherichia coli* O157:H7 RIMD 05091078 and *Escherichia coli* ATCC 25922 were used as target strains. The strains were cultured in tryptic soy broth (TSB, Difco, USA) at 37 ^{o}C for 18 h. After incubation, the cells were pelleted at 2,000xg for 7 min, washed twice with phosphate buffer saline (PBS) (pH 7.4). The cell pellet was suspended in Roswell Park Memorial Institute 1640 medium (RPMI-1640, Sigma-Aldrich, USA) and used for further experiments.

Serum preparation and opsonization

Fresh serum was obtained from normal AB blood group donors. The cell suspensions in the RPMI-1640 medium were incubated with 10% AB serum for 30 min at 37 ^{o}C. After incubation, the suspensions were centrifuged at 2,000xg for 10 min and washed twice with PBS. The cells were diluted to reach a concentration of 10^{8} cfumL^{-1} using RPMI 1640 medium. The numbers of opsonized cells were counted using a haemocytometer.

Isolation of human neutrophils

The buffy coat of healthy volunteers was obtained from the blood bank of Songklanagarind hospital, and 6 % dextran was added (Fluka, Switzerland). The mixture was gently shaken and allowed to stand at room temperature for 1 h to sediment erythrocytes. Leukocytes from the upper layer were transferred to a sterile falcon tube and neutrophils were isolated through centrifugation through the Histopaque 1.077 (Sigma-Aldrich, USA) density gradient (Strasser, et al., 1998). The viability and purity of the neutrophils were determined by the trypan blue (Difco) exclusion assay.

Preparation of *Rhodomyrtus tomentosa* leaf extract

Rhodomyrtus tomentosa leaves were collected from Sadao District, Songkhla Province in the southern part of Thailand. The collected samples were dried, grinded into a powder, and then soaked with 95% ethanol in the dark chamber for seven days. The ethanol layer was collected and evaporated till completely dried using a rotary evaporator. The ethanolic extract was dissolved in 100% dimethyl sulfoxide (DMSO, Merck, Germany) before used.

Cell survival assay

One hundred microlitres of neutrophils with 10^{6}cellsmL^{-1} were challenged by adding 100 µL of *E. coli* suspension and transferred to sterile 1.5 mL eppendorf tubes. *R. tomentosa* extract with concentration ranging from 62.5 to 250 µgmL^{-1} was separately added into each tube. The mixture of neutrophils and *E. coli* without the extract was used as a control. The tubes were incubated for 30, 45, and 60 min at 37 ^{o}C in incubator shaker. An aliquot of 10 µL of the sample was plated on TSA and incubated at 37 ^{o}C, overnight. Number of colony forming units per milliliter (cfumL^{-1}) was determined.

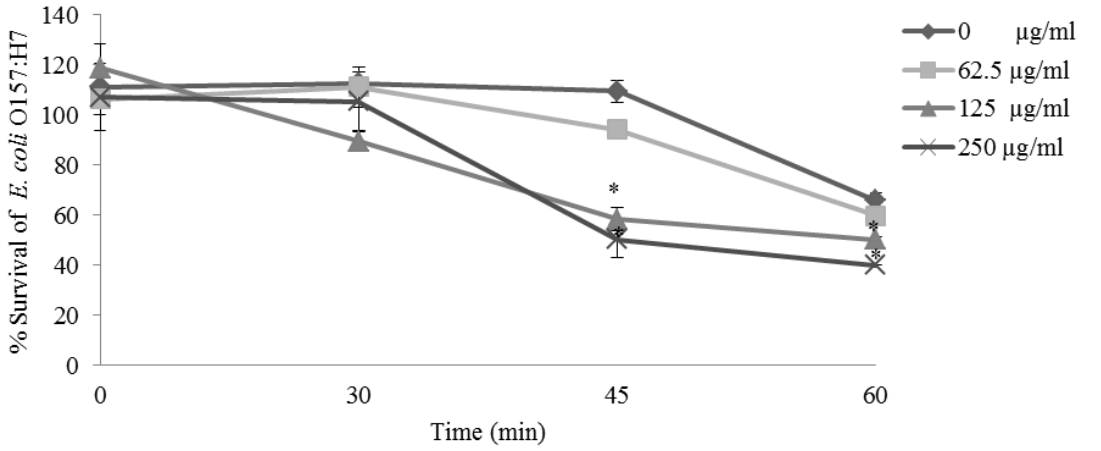
Statistical analysis

Statistical significance was calculated by analysis of variance (Anova). Comparisons between means were carried out according to the Dunnett test. *P* values lower than 0.05 were considered to be statistically significant.

RESULTS

In this study, we have carried out a preliminary screening to detect effects of *Rhodomyrtus tomentosa* extract on the killing activity of human neutrophils against *Escherichia coli* O157:H7 RIMD 05091078 and *Escherichia coli* ATCC 25922. The percent survival of *E. coli* from each condition was determined at various time intervals (Figure 1a, and 1b). At 30 min, the extract present at values of 62.5-250 µgmL^{-1} showed a slight to moderate enhancing on the killing activity of neutrophils for both *E. coli* strains. However, a significant increase in the activity of neutrophils was observed in the presence of the same concentrations of the extract after 45 and 60 min ($p< 0.05$). Within 45 min, the survival numbers of *E. coli* O157:H7 and *E. coli* ATCC 25922 in the cell culture were 94.15%, 58.49%, 50.28%, and 73.15%, 69.13%, and 33.35%, respectively, in the presence of 62.5, 125, and 250 µgmL^{-1} of the extract. The percentage survival of *E. coli* O157:H7 and *E. coli* ATCC 25922 after treated with neutrophils in the presence of the same concentrations of the extract at 60 min was 59.91%, 50.34%, 40.15% and 78.79%, 58.46%, and 47.02%, respectively.

(a)



(b)

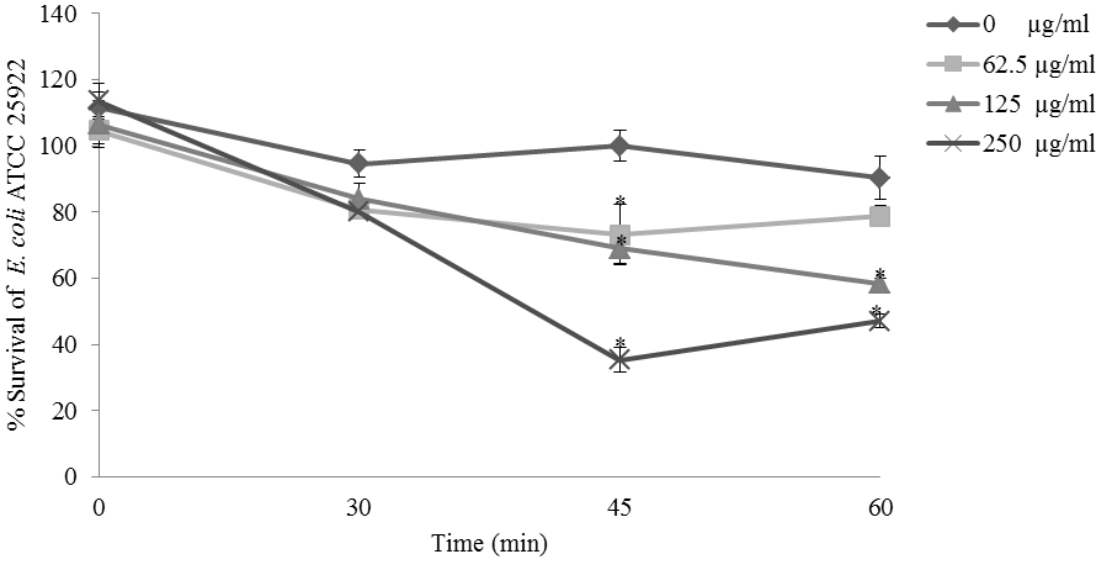


Figure 1. Effects of *Rhodomyrtus tomentosa* extract on the killing activity of human neutrophils against *E. coli* O157:H7 RIMD 05091078 (a) and *E. coli* ATCC 25922 (b). The percentage survival of *E. coli* after exposure to the extract (0-250 µgmL^{-1}) at 0, 30, 45, and 60 min was determined. Data are expressed the mean values ± standard error from two independent experiments. The experiment was performed in triplicate and * Significance at $p< 0.05$.

CONCLUSIONS

This is the first *in vitro* study to demonstrate the ability of a plant extract to enhance the activity of neutrophils against *E. coli*. Further studies are required to establish the phytochemical constituents from *Rhodomyrtus tomentosa* extract that induce this activity.

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REFERENCES

1. Serna AT, Boedeker EC. 2008. Pathogenesis and treatment of Shiga toxin-producing *Escherichia coli* infections. Curr. Opin. Gastroenterol. 24, 38-47.
2. Wong CS, Mooney JC, Brandt JR, Staples AO, Jelacic S, Boster DR, et al. 2012. Risk factors for the hemolytic uremic syndrome in children infected with *Escherichia coli* O157:H7: a multivariable analysis. Clin. Infect. Dis. 55, 33-41.
3. Smith KE, Wilker PR, Reiter PL, Hedican EB, Hedican Eb, Bender JB, et al. 2012. Antibiotic treatment of *Escherichia coli* O157 infection and the risk of hemolytic uremic syndrome, Minnesota. Pediatr. Infect Dis. J. 31, 37-41.
4. Nguyen Y, Sperandio V. 2012. Enterohemorrhagic *E. coli* (EHEC) pathogenesis. Front. Cell Infect. Microbiol. 2, 1-7.
5. Lim JY, Yoon J, Hovde CJ. 2010. A brief overview of *Escherichia coli* O157:H7 and its plasmid O157. J. Microbiol. Biotecnol. 20, 1-10.
6. Serafino A, Vallebona PS, Andreola F, Zonfrillo M, Mercuri L, Federici M, et al. 2008. Stimulatory effect of *Eucalyptus* essential oil on innate cell-mediated immune response. BMC Immunol. 9.
7. Razali FN, Ismail A, Abidin NZ, Shuib AS. 2014. Stimulatory effects of polysaccharide fraction from *Solanum nigrum* on RAW 264.7 murine macrophage cells. PLoS ONE. 9, e108988.
8. Rafiul Haque M, Ansari SH, Rashikh A. 2013. *Coffea arabica* seed extract stimulate the cellular immune function and cyclophosphamide-induced immunosuppression in mice. Iran J. Pharm. Res. 12, 101-108.
9. Ong HC, Nordiana M. 1999. Malay ethno-medico botany in Machang, Kelantan, Malaysia. Fitoterapia. 70, 502-513.
10. Geetha KM, Sridhar C, Murugan V. 2010 Antioxidant and healing effect of aqueous alcoholic extract of *Rhodomyrtus tomentosa* (Ait.) Hassk on chronic gastric ulcers in rats. J. Pharm. Res. 3, 2860-2862.
11. Wei, F., 2006a. Manufacture of oral liquid containing traditional Chinese medicine extract for treating gynecopathy (Guangxi Huahong Pharmaceutical Co., Ltd., People's Republic of China; Shanghai Fosun Pharmaceutical (Group) Co., Ltd.), Faming Zhuanli Shenqing Gongkai Shuomingshu. People's Republic of China patent CN1846715.
12. Wei, F., 2006b. Manufacture of traditional Chinese medicine composition for treating urinary tract infection (Gungxi Huahong Pharmaceutical Co., Ltd., People's Republic of China; Shanghai Fosun Pharmaceutical (Group) Co., Ltd.), Faming Zhuanli Shenqing Gongkai Shuomingshu. People's Republic of China patent CN1853687.
13. Limsuwan S, Trip EN, Kouwen TR, Piersma S, Hiranrat A, Mahabusarakam W, et al. 2009. rhodomyrtone: a new candidate as natural antibacterial drug from *Rhodomyrtus tomentosa*. Phytomedicine. 16, 645-651.
14. Geetha KM, Sridhar C, Murugan V. 2010. Antioxidant and gastroprotective activities of *Rhodomyrtus tomentosa* (Ait.) Hassk. Int. J. Pharmatech Res. 2, 283-291
15. Voravuthikunchai SP, Dolah S, Charernjiratrakul W. 2010. Control of *Bacillus cereus* in foods by *Rhodomyrtus tomentosa* (Ait.) Hassk. Leaf extract and its purified compound. J Food Prot. 73, 1907-1912.
16. Saising J, Ongsakul M, Voravuthikunchai SP. 2011. *Rhodomyrtus tomentosa* (Aiton) Hassk. ethanol extract and rhodomyrtone: a potential strategy for the treatment of biofilm-forming staphylococci. J. Med. Microbiol. 60, 1793-1800.
17. Lavanya G, Voravuthikunchai SP, Towatana NH. 2012. Acetone extract from *Rhodomyrtus tomentosa*: a potent natural antioxidant. Evid. Based Compl. Altern. Med. 2012, 535479.

In vitro-In silico of alpha- beta- gamma-mangostin from *Garcinia mangostana* pericarp extract

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ABSTRACT

The purpose of experiment was to predict pharmacokinetics properties and pharmacodynamic properties of alpha- beta- gamma -mangostin extracted from *Garcinia mangostana L.* pericarp. The study on pharmacokinetics properties was conducted via ADMET Predictor™ software. The software was used as a predictive modeling of absorption, distribution, metabolism, elimination, and toxicity (ADMET) of chemical substances from molecular structures. ADMET risk rule set, S+Absn_Risk (risk of low absorption from oral dose), TOX_Risk (overall toxicity risk) and CYP_Risk (metabolic risk) plus two additional rules, high plasma protein binding and high steady-state volume of distribution were used to determine the comparative relation between efficacy and safety. From overall of ADMET risk rule set, the results showed that from 24 criteria, the scores of 2.95, 2.65 and 2.17 were for alpha- beta- and gamma-mangostin respectively. In general, the scores of 0-24 indicates the number of potential ADMET problem. These findings may be valuable to explain pharmacokinetics data of these compounds. For pharmacodynamic study, the cytotoxicity on human MCF7 breast carcinoma cells were tested. The average value of half maximal inhibitory concentration (IC50) of alpha- beta- gamma-mangostin and 5-FU to MCF7 cells were 34.52±4.38, 83.54±0.87, 92.69±0.35 and 64.00±1.52 respectively. According, alpha-mangostin is the most cytotoxic to MCF7 cells. However, alpha-mangostin has highest ADMET risk score. Thus, further study of mangostins should focus on the optimization of their pharmacokinetic and pharmacodynamic properties.

Keywords: anticancer activity, *Garcinia mangostana*, alpha- beta- gamma-mangostin

INTRODUCTION

In silico Biopharmaceutics study has found widespread effectiveness as vital tool in drug discovery and development [1]. The ADMET Predictor™ software was used as a predictive modeling of absorption, distribution, metabolism, elimination, and toxicity (ADMET) of chemical substances from molecular structures. Mangosteen (*Garcinia mangostana* L.), a tropical evergreen tree with purple-red fruit, its thick rinds have been used as traditional medicine treatment for skin wounds, diarrhea and infection [2]. The pericarp crude extract can be isolated to fifty xanthone compounds, alpha-, beta- and gamma-mangostins, gartanin etc [3]. The main isolated xanthone, yellow color from pericarp mangostin was name as mangostin [4.]. Several studies have been designed to investigate mechanism of inducing cell death via mangostins treatment in cancer cells line. [5-7]. Breast cancer is the most frequently diagnosed cancer among women in worldwide [8].Progression of breast cancer is a multistep process, which involves hormones and genes. Estrogen is a hormone which activates growing of breast cancer [9]. MCF7 cell line is a general studied model for hormone-dependant human breast cancer. These cells contain functional estrogen receptors and show a pleotropic response to estrogen. Estrogen stimulates proliferation of these cells in vitro[10]. Therefore the purposes of this study

are to predict pharmacokinetics properties of mangostins via in silico and to investigate effect of mangostins on MCF7 breast carcinoma cells.

MATERIALS AND METHODS

Prediction pharmacokinetic porperties of mangosteen pericarp extract

MedChem Designer™ sofeware version 2.0.0.34, Simulations Plus, Inc. was used to create illustration of xanthones's IUPAC chemical structure in specific file type for ADMET Predictor™. ADMET Predictor™ software version 6.0.0007, Simulations Plus, Inc. was used to predict pharmacokinetic properties of mangostins shown in Table 1**.**

Table 1. Summary of ADMET risk 24 scores via ADMET Predictor™ modules

ADMET risk	Criteria
S+Absn_Risk (risk of low absorption from oral dose)	
1. Too large molecule(Sz)	MWt. > 500, Number atom > 35
2. Too flexible molecule(Rb)	Number of free lotate bond > 10
3. Too many H-bond donors(HD)	H-bond Donor Protons > 4
4. Too many H-bond acceptors(HA)	H-bond acceptors > 9
5. Excessive partial atomic charge(ch)	NPA partial atomic charges > 21
6. Too lipophilic(ow)	logP > 4.5 or logD >3.5
7. Low permeability(Pf)	jejunal < 0.25 or MDCK<30
8. Low solubility(Sw)	Solubility in water <= 0.010
TOX_Risk (overall toxicity risk)	TOX_hERG > 6
9. hERG liability(hE)	TOX_RAT < 320
10. Acute toxicity in rats(ra)	TOX_BRM_Rat
11. Carcinogenicity in chronic rat studies(Xr)	TOX_BRM_Mouse < 25
12. Carcinogenicity in chronic mouse studies(Xm)	
13. Human liver hepatotoxicity(Hp)	
14. SGOT and SGPT elevation in blood (severe hepatic injury)(SG)	TOX_MUT_Risk > 2
15. Mutagenicity (following five strains of Salmonella typhimurium, each with and without microsomal activation)	
CYP_Risk (metabolic risk)	
16. High metabolic clearance by human CYP P450 1A2(1A)	Is substrate and CLint in each CYP > 30
17. High metabolic clearance by human CYP P450 2C19(19)	
18. High metabolic clearance by human CYP P450 2C9(C9)	
19. High metabolic clearance by human CYP P450 2D6(D6)	
20. High metabolic clearance by human CYP P450 3A4(3A)	
21. Inhibition of human CYPP450 3A4 with midazolam as substrate(mi)	
22. Inhibition of human CYPP450 3A4 with testosterone as substrate(ti)	
23. Plasma protein binding(fu)	PrUnbnd < 3.5%
24. Steady-state volume of distribution(Vd)	Vd > 5.5

Primary cell culture

Human MCF7 breast carcinoma cells were grown in T-25 flask at 37 °C in an atmosphere of 5% CO_2. The adherent cells were cultured in Dulbecco's Modified Eagle media(DMEM) with 10% Fetal bovine serum and 1% Pen strep. The medium was replaced regularly three times a week until the flask reach 80% confluence. The cells were moved from the flasks by incubating the monolayers with 0.5% trypsin for 2-3 min at 37 °C. The cells were collected into centrifuge tubes, and then centrifuged at 1,000 rpm for 4 minutes and the pellets were resuspended in DMEM. Cells used for this study were in third passage in late subconfluency.

Cytotoxicity effect of mangostin to MCF7 cell

In vitro cytotoxicity studies Cytotoxicity of alpha- beta- and gamma-mangostin was evaluated for MCF7 cells using the MTT assay. MCF7 cells were cultured in 96-well plates for 24 h at a seeding density of 1.0×10^4 cells/well before the addition of test samples. Mangostin were diluted with medium as culture medium to different concentrations.

Control test samples were medium, 5-FU in medium (positive control) and medium (negative control). Experiments were initiated by replacing the culture medium in each well with 100 µl of sample solutions at 37 ˚C in the CO_2 incubator. After 24 h of incubation, 10 µl of MTT reagent (1 mg/ml) in the phosphate buffer saline was added to each well. The plates were then incubated at 37 ◦C for another 24 h. At the end of the incubation period, the intracellular formazan was solubilized with 50 µl DMSO and quantified by reading the absorbance at 590 nm on a micro-plate multi-detection instrument, SpectraMax M2. Percentage of cell viability was calculated based on the absorbance measured relative to the absorbance of cells exposed to the negative control. The percentage of cell viability was calculated as follow: % cell viability=(OD of treated cells)/(OD of untreated cells)×100. Half maximal inhibitory concentration (IC50) was calculated via Graphpad prism version 6.

Statistical analysis

Statistical data analysis was done using statistical package for GraphPad Instat version 6. The values are expressed as mean. Hypothesis testing method included one-way analysis of variance (ANOVA) followed by post hoc testing performed with least significant difference test. For all comparisons, differences were considered statistically significant at $p<0.01$.

RESULTS

These results were characterized to ADMET risk score, the scores of 2.95, 2.65 and 2.17 were for alpha- beta- and gamma-mangostin, respectively. In general, the scores of 0-24 indicate the number of potential ADMET problem. These findings may be valuable to explain pharmacokinetics data of these compounds. Lower score can determine to low ADMET problem and has high possibility to be developed to new drug. All mangostins had closely ADMET risk score and were too lipophlilic(LogP>4.5 or LogD>3.5). Gamma-mangostin had lowest score(2.17). Nevertheless, gamma-mangostin had ADMET problem in low permeability (jejunal < 0.25 or MDCK<30) and high plasma protein binding (PrUnbnd < 3.5%). ADMET risk score and potential ADMET problems of mangostins shown in Table 2.

Table 2. ADMET risk score and ADMET_Code of alpha-beta-gamma-mangostin via ADMET Predictor™ software.

Name	alpha-mangostin	beta-mangostin	gamma-mangostin	Criteria
ADMET Risk	**2.95**	**2.65**	**2.17**	
ADMET_Code	**ow,Sw,fu,ra,C9**	**ow,Sw,fu**	**ow,Pf,fu**	
ow: Logarithm of partition coefficient	4.497	4.811	4.213	> 4.5
Logarithm of distribution coefficient	4.377	4.772	4.089	> 3.5
Sw: Native water solubility	0.01	0.004	0.015	<= 0.010
Pf: Apparent MDCK COS permeability	40.814	89.332	22.456	< 30
Human jejunal effective permeability	4.801	6.812	3.165	< 0.25
ra: Acute toxicity in rats	304.327	334.06	331.008	< 320
fu: Percentage of Plasma protein non-binding	1.139	1.274	1.357	< 3.5%
C9: CLint by human CYP P450 2C9	99.146	nonsubstrate	nonsubstrate	> 30

The cytotoxicity of mangostins in MCF7 cells was evaluated by MTT assay. Cells were treated with various doses of mangostins (0.1µM to 100µM) for 24h. The average value of half maximal inhibitory concentration(IC50) of alpha- beta- gamma-mangostin and 5-FU to MCF7 cells were 38.42±4.38, 83.54±0.87, 92.69±0.35 and 64.00±1.52 respectively. The significant values represent mean ± SD of three separate experiments(n=3). 5-FU was significant difference with alpha- beta- and gamma-mangostin($p<0.01$). According, alpha-mangostin is the most cytotoxic to MCF7 cells that shown in Figure 1.

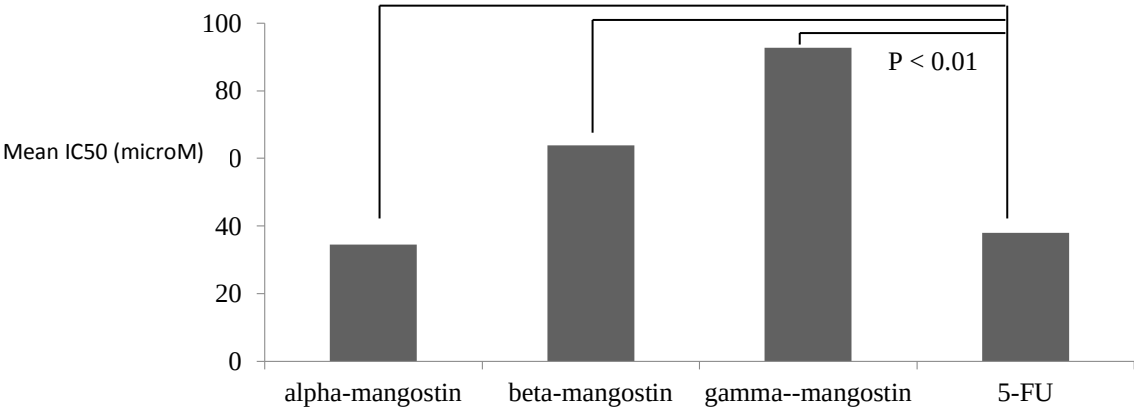

Figure 1. Cytotoxicity effect of various concentrations of alpha- beta- gamma-mangostin and 5-FU on MCF7 cells.

CONCLUSIONS

From the result alpha-mangostin is the most cytotoxic to MCF7 cells. However, alpha-mangostin has highest ADMET risk score. Thus, further study of mangostins should focus on the optimization of their pharmacokinetic and pharmacodynamic properties and should be more studied via in vivo or other in vitro models

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REFERENCES

1. Tseme Y, Mudie DM, Langguth P, Amidon GE, Amidon GL. 2014. The Biopharmaceutics classification system: subclasses for in vivo predictive dissolution (IPD) methodology and IVIVC. Eur. J. Hosp. Pharm. Sci. Pract. 57, 152-163.
2. Chen LG, Yang LL, Wang CC. 2008. Anti-inflammatory activity of mangostins from Garcinia mangostana. Food Chem. Toxicol. 46(2), 688–693.
3. Chairungsrilerd N, Takeuchi K, Ohizumi Y. 1996. Mangostanol, a prenyl xanthone from Garcinia mangostana. Phytochem. 43(5), 1099-1102.
4. Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, et al. 2004.Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell. 117(7), 927–939.
5. Won YS, Lee HY, Kwon SJ, Kim J, Park H, Lee MK, et al 2014. α-Mangostin-induce apoptosis is meadiated by estrogen receptor α in human breast cancer cells. Food Chem. Toxicol. 66, 158-165.
6. Yoo JH, Kang K, Jho EH, Chin YW, Kim J, Nho CW. 2011. Alpha- and gamma-Mangostin inhibit the proliferation of colon cancer cells via beta-catenin gene regulation in Wnt/cGMP signalling. Food Chem. 129(4), 1559-1566.
7. Syam S, Bustamam A, Abdullah R, Sukari MA, Hashim NM, Ghaderian M, et al. 2014. Beta-Mangostin induces p53-dependent G2/M cell cycle arrest and apoptosis through ROS mediated mitochondrial pathway and NfkB suppression in MCF-7 cells. J. Funct. Foods. 6(0), 290-304.
8. GLOBOCAN 2012. 2012. Estimated cancer incidence, mortality and prevalence worldwide in 2012. International Agency for Research on Cancer (IARC).
9. Pedraza-Chaverri J, Cardenas-Rodriguez N, Orozco-Ibarra M, et al. 2008. Medicinal properties of mangosteen (*Garcinia mangostana*). Food Chem. Toxicol. 46, 3227-3239.
10. Katzenellenbogen BS, Kendra KL, Norman MJ, Berthois Y. 1987. Proliferation, hormonal responsiveness,and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long term absence of estrogens. Cancer Res. 47(16), 4355–4360.

Anti-oxidant efficiency of mangosteen pericarp and centella leaf mixture formulation

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ABSTRACT

Mangosteen and centella are widely known as herbal plants since mangosteen pericarp (MP) and centella leaf (CL) have medical therapeutic effects. Both of them have been investigated for their pharmacological activities. It was found that MP had anti-oxidant, anti-bacterial and anti-inflammatory activities, while CL possesses anti-oxidant, anti-bacterial, anti-viral and wound healing activities. Although the anti-oxidant activities and total phenolic content (TPC), expressed as gallic acid equivalent (GAE) of MP and CL have been numerously reported, but the formulation of MP and CL have not been performed and investigated. Hence, this study aimed to compare the anti-oxidant activities of combined MP and CL formulations with uncombined MP and CL. Five formulations of MP and CL (GC1 to GC5) were prepared and characterized for anti-oxidant activity using DPPH scavenging assay comparing with standard ascorbic acid. It was found that GC1 showed the highest anti-oxidant activity with 17.1 µg/mL of IC_{50} and 70.0% of radical scavenging among five formulations. But this result was also found to be lower than that of MP extract alone (14.8 µg/mL of IC_{50} and 75.9% of radical scavenging). However, CL exhibited the lowest antioxidant property (61.7 µg/mL of IC_{50} and 31.8% of radical scavenging). From TPC assay, the results confirmed that MP was more effective than CL, which TCP of MP and CL were found to be 5,179.64 and 2,418.81 mg GAE/g extract, respectively. These results had confirmed the results from DPPH assay that MP was more effective in anti-oxidation than CL. This study will lead to further investigation for the other activities of MP-CL formulations, such as wound healing and anti-inflammation.

Keywords: mangosteen pericarp, centella leaf, antioxidant activity

INTRODUCTION

Mangosteen (*Garcinia mangostana*) is an herbal plant that native to Southeast Asia. It has been widely used as in traditional medicine for wounds, skin infections and acne treatments. Recently, it has been scientifically reported that mangosteen fruit rind extract (MP) shown an effective pharmacological activities such as anti-oxidant, anti-inflammatory and anti-allergic activities [1]. Especially, it is widely used as an anti-microbial agent against *Propionibacterium acnes*, which could induce acne inflammation [2]. This extract was found that contained high amount of phenolic compounds such as xanthones, tannins and flavonoids, mangostin. The major active compound in this extract was found to be α-mangostin, a xanthone compound [3]. Pennywort (*Centella asiatica*) or centella is an herbal plant that has been used in Ayurvedic traditional medicine. The active compounds of the centella leaf extract (CL) were found to be madecassosides and asiaticosides. For wound healing activity, Shukla *et al*. (1999) was revealed that asiaticoside could promote wound healing in both normal healing and delayed-healing models. It assisted cell proliferation and collagen synthesis in the wound [4,5].

Combination of MP and CL might increase the efficiency of acne treatment product, which could inhibit acne causing bacteria and enhance wound healing. Reactive oxygen species (ROS), free radical molecules, are very harmful molecule which can cause oxidative stress, cell damage, DNA damage, delayed-wound healing and inflammation. Antioxidant agents could reduce excessive ROS [6-8]. Preliminary, determination of antioxidant activity could be used to investigate the synergistic effect between MP and CL. Thus, this study aimed to investigate synergistic effect between MP and CL on the anti-oxidant activity of MP and CL.

MATERIALS AND METHODS

Herbal extractions

Mangosteen pericarp and centella leaf were separately dried at 45°C for 72 h and then ground into powder. For mangosteen pericarp extraction, maceration extraction was performed by soaking the powder (5 g) in 95% ethanol (30 mL) at room temperature for 2 days. After that, it was filtered through Whatman No.1 filter paper to collect the filtrate, and the remaining residue was macerated again with the same condition for 7 days. The filtrate of both macerations were combined before drying using rotary evaporator under 100 mbar at 45°C until obtain the viscose crude extract. The extract was then dried in hot air oven at 45°C until obtain a constant dry weight. For centella leaf extraction, the powder (30 g) contained in cellulose thimble was extracted by using Soxhlet extraction apparatus with 80% ethanol (300 mL). The extractions were done for 30 cycles at boiling temperature of the solvent. The crude extract was filtered through Whatman No.1 filter paper before drying as the processes described above.

Formulation preparations

The extracts from mangosteen pericarp and centella leaf were dissolved in absolute ethanol to obtain 1 mg/mL stock solution. It was coded as MP and CL, respectively. The extract stock solutions were mixed together with various ratios to prepare 5 formulations namely GC1, GC2, GC3, GC4 and GC5 (Table 1).

DPPH assay

The formulations were characterized the anti-oxidant activity by using modified DPPH assay [9]. Before doing the reactions, 100 µL of the samples, which were MP, GC1, GC2, GC3, GC4, GC5 and CL, were 2-fold serial diluted in 96-well plates from the 1st well to the 11th well to prepare the concentrations as 500, 250, 125, 62.5, 31.3, 15.6, 7.8, 3.9, 2.0, 0.1 and 0.05 µg/mL. For the 12th well, it was reserved for the control, no extract added. After that, 100 µL of 0.2 mM DPPH stock solution was added into each well to begin the reaction. The plates were incubated for 30 min in the dark before measured the absorbance at 520 nm by using microplate reader instrument (BioTek, PowerWave XS2, USA). Each sample was done in triplicate and standard ascorbic acid (AA) was used as the standard antioxidant. The percentage of radical scavenging inhibition was calculated by using Equation (1). Subsequently, the radical scavenging values of each sample were collected to calculate the inhibitory concentration 50% (IC_{50}) using Standard Curves Analysis tool in SigmaPlot program. In addition, the radical scavenging values of the formulations, at 31.3 µg/mL, were statistically compared to determine the difference of each formulation. Radical scavenging values were averaged and calculated the standard deviation (S.D.). The statistical difference was analyzed by the One-Way ANOVA tool on SPSS for windows with the Tukey's HSD test. The significant difference was noted at 95% confidence interval.

$$\text{Radical scavenging (\%)} = 100\,(C - S) / C \qquad (1)$$

Where C is the absorbance of DPPH without sample and S is the absorbance of DPPH with sample.

Total phenolic assay

The herbal extracts, MP and CL, were determined the total phenolic contents (TPC) using Folin-Ciocalteu method. Before doing the reaction, Folin-Ciocalteu (FC) reagent and sodium carbonate (Na_2CO_3) solution were diluted with deionized water into 10 and 7 %w/v, respectively. 30 µL of the extract stock solutions were pipetted into the 1st well of row A and E, diluted with 120 µL of absolute ethanol and then serially diluted two fold from the 1st to the 10th well to obtain concentrations as 100, 50, 25, 12.5, 6.3, 3.1, 1.6, 0.8, 0.4 and 0.2 µg/mL, respectively. Subsequently, 150 µL of FC reagent was added and mixed. After leaving avoid light for five minutes, it was then added by sodium carbonate solution. The reaction mixtures were incubated at room temperature in the dark for 30 min. The reactions were done in triplicate. The absorbance of the reaction was measured at 765 nm using micro-plate reader. For standard gallic acid (GA), it was prepared with the same processes performed above and the reactions were done with the same conditions. The calibration curve was plotted to get the slope for gallic acid equivalent (GAE) calculation and TPC values were expressed as mg GAE/g extract.

RESULTS

Anti-oxidant activities of GC1 to GC5 were shown in Table 1 and Figure 1. From the inhibitory concentration 50% (IC_{50}) and radical scavenging values, the efficiency of the formulations and the extracts on anti-oxidant activities were ordered to be MP, GC1, GC2, GC3, CG4, GC5 and CL. The increase of IC_{50} and radical scavenging followed the increase in MP content. When compare only the extracts, it was found that MP was more effective in anti-oxidation than CL. Among 5 herbal formulations, it was investigated that GC1 was the most effective formulation (17.1 µg/mL of IC_{50} and 70.0±4.57 % of radical scavenging). For TPC determination, the data as expressed in Table 1 shown that MP (5,179.64±542.66 mg GAE/g extract) contained phenolic compounds more than CL (2,418.81±123.10 mg GAE/g extract). These results had confirmed the results from DPPH assay that MP was more effective in anti-oxidation than CL. Thus, this formulation had compromised that might possessed high anti-oxidant activity with some wound healing activity due to the wound healing activity of centella leaf extract [4].

Table 1. Inhibitory concentration 50% (IC_{50}) of herbal formulations

Codes	Mangosteen pericarp extract (% w/w)	Centella leaf extract (% w/w)	Inhibitory Concentration 50% (IC_{50}, µg/mL)	Radical Scavenging at 31.3 µg/mL (%)	Total Phenolic Content at 5 µg/mL (mg GAE/g extract)
MP	100	0	14.8	75.9±2.16 $^{\alpha}$	5,179.64±542.66
GC1	80	20	17.1	69.9±.67 $^{\alpha, \beta}$	-
GC2	60	40	20.0	62.3±.14 $^{\alpha, \beta}$	-
GC3	50	50	22.7	58.9±5.59 $^{\alpha, \beta}$	-
GC4	40	60	27.1	52.8±.83 $^{\alpha, \beta}$	-
GC5	20	80	36.8	41.5±.26 $^{\beta, \gamma}$	-
CL	0	100	61.7	31.7±0.54 $^{\gamma}$	2,418.81±123.10
AA	-	-	3.0	-	-

(Different symbols including α, β and γ represent significant difference at 95% confidence interval)

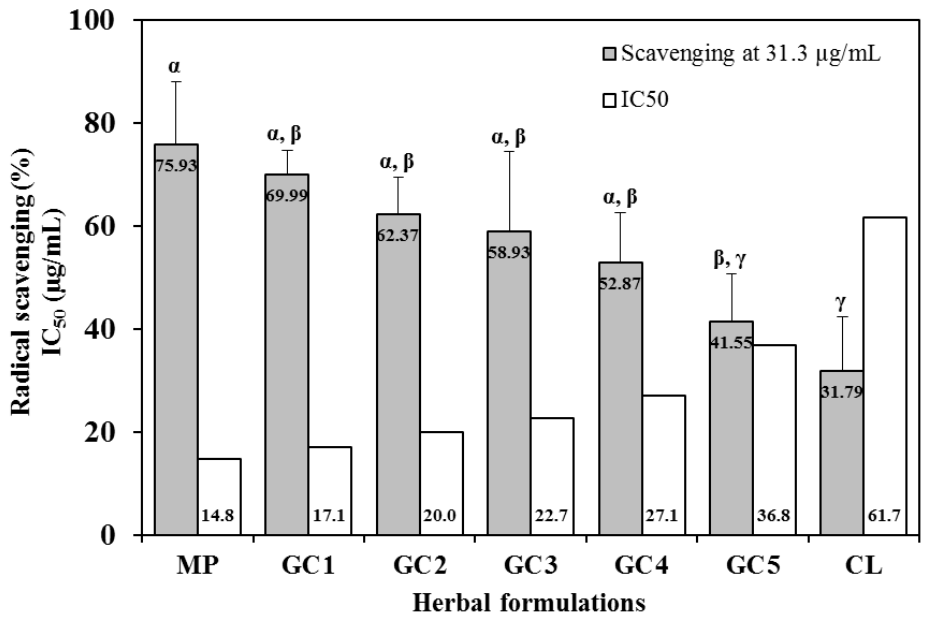

Figure 1. The IC_{50} and Radical Scavenging at 31.3µg/mL of Herbal Formulations (Different symbols including α, β and γ represent significant difference)

CONCLUSIONS

In this study, it could be concluded as MP was more effective in anti-oxidation than CL. For the herbal formulations, it was found that MP content had influenced on the anti-oxidant activities of the herbal formulations, which GC1 that contained by 90% of mangosteen pericarp extract and 10% of centella leaf extract expressed the highest anti-oxidant activity among all of the herbal formulations. Thus, this formulation had compromised that might possessed high anti-oxidant activity with some wound healing activity due to the wound healing activity of centella leaf extract. This study will lead to further investigation for the other activities of MP-CL formulations, such as wound healing and anti-inflammation.

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REFERENCES

1. Pedraza-Chaverri J, Cárdenas-Rodríguez N, Orozco-Ibarra M, Pérez-Rojas JM. 2008. Medicinal properties of mangosteen (Garcinia mangostana). Food Chem. Toxicol. 46(10), 3227-3239.
2. Pothitirat W, Chomnawang MT, Gritsanapan W. 2010. Anti-acne-inducing bacterial activity of mangosteen fruit rind extracts. Med. Princ. Pract. 19(4), 281-286.
3. Ibrahim MY, Hashim NM, Mariod AA, Mohan S, Abdulla MA, Abdelwahab SI, Arbabe IA. α-mangostin from Garcinia mangostana Linn: an updated review of its pharmacological properties. Arabian J. Chem.
4. Shukla A, Rasik AM, Jain GK, Shankar R, Kulshrestha DK, Dhawan BN. 1999. In vitro and in vivo wound healing activity of asiaticoside isolated from Centella asiatica. J. Ethnopharmacol. 65(1), 1-11.
5. Gohil KJ, Patel JA, Gajjar AK. 2010. Pharmacological review on Centella asiatica: a potential herbal cure-all. Indian J. Pharm. Sci. 72(5), 546-556.
6. Shetty BS. Wound healing and indigenous drugs: role as antioxidants: a review. Res Rev J Med. Health Sci. 2(2), 5-16.
7. Masaki H. 2004.Role of antioxidants in the skin: Anti-aging effects. J. Dermatol. Sci.58(2):85-90.
8. Apel K, Hirt H. Reactive oxigen species: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Physiol. 55, 373-99.
9. Sharma OP, Bhat TK. 2009. DPPH antioxidant assay revisited. Food Chem. 113(4), 1202-1205.

Pharmacological activities of Triphala recipe with Thai herbal extract additions

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ABSTRACT

Oxidative related stress causes the generation of excess reactive oxygen species (ROS) which is harmful to organisms, and internal and external antioxidant agents helps overcome oxidative stress damages. Tripala recipe is well-known for various medicinal benefits. It is extensively utilized in Ayurvedic traditional medicine and Thai traditional medicine. This recipe contains 3 fruits, *Terminalia chebula* Retz. (TC), *Terminalia bellirica* (TB) and *Phyllanthus emblica* (PE). In this study, Thai herbal plants including *Tinospora cordifolia* stem and *Caesalpinia sappan* heartwood were formulated in Triphala recipe to improve their antioxidant activities. These extract were randomly abbreviated as HB1 and HB2. The plants were extracted using 95% ethanol. After that, dried extracts were dissolved in absolute ethanol and mixed together before determination of antioxidant activity using DPPH assay. From DPPH assay, the IC_{50} values revealed that HB2 possessed the strongest antioxidant activity (3.34 µg/mL). However, it was still lower than standard ascorbic acid (AA) and standard gallic acid (GA), which were respective 2.95 and 0.57 µg/mL. Surprisingly, when HB2 was combined to PE, it significantly synergized the antioxidant activity by increasing radical scavenging from 61.46% and 56.30%, respectively, into 71.57%. Subsequently, we had tried to optimize the suitable ratio between HB2 and PE that exhibited the highest radical scavenging. It was observed that PE+HB2-1, composed of PE for 70% (w/w), exhibited the strongest antioxidant activity (IC_{50} = 0.93 µg/mL). For Triphala recipe (TRI), the results revealed that addition of HB2 (TRI+HB2) significantly improved their antioxidant activity. However, it is still lower than PE+HB2-1 significantly.

Keywords: Thai traditional medicine, Triphala recipe, *Tinospora cordifolia*, *Caesalpinia sappan*, antioxidant activity

INTRODUCTION

Reactive oxygen species (ROS) are free radical molecules which are generated naturally in the presence of oxygen and catalyst. ROS are very harmful molecule which can cause oxidative stress, cell damage, DNA damage and disrupt metabolism system balance under stress environment condition. Internal feedback mechanism respond system such as expression of antioxidant proteins and external antioxidant agents counter excess ROS and return the balance. [1, 2]

Our nature is blessed with ubiquitous plants which contains rich antioxidant compounds. Triphala, literally means three fruits in Hindi, is well-known for various medicinal benefits which was extensively utilized in traditional Ayurvedic medicine. The three fruits of Triphala recipe are *Terminalia chebula* Retz. *Phyllanthus emblica* and *Terminalia bellirica*. Triphala recipe is found to be rich in phenolic compounds and tannins [3]. In addition, its pharmacological activities had been widely investigated, which was found that possesses immunomodulatory activity, anti-diabetic activity, antioxidant activity, anti-mutagenic activity, anti-inflammatory and others [4, 5].

The synergistic antioxidant activity of green tea with some herbs had been investigated and found that combination of the herbs could improve antioxidant activity. Antioxidant activity of this herbal combination was comparable to standard ascorbic acid [6]. *Tinospora cordifolia* and *Caesalpinia sappan* is widely known as highly effective Thai traditional medicine, which numerous pharmacological activities of them were observed [7, 8]. In this study, *T.*

cardifolia, *C. sappan* extracted and combined into HB1 and HB2 formulations. The formulations were improved by adding HB1 and HB2 into Triphala recipe to improve the synergy of its various antioxidant agent, especially Ascorbic acid and gallic acid compounds to scavenge free radicals more effectively.

MATERIALS AND METHODS

Materials

Herbal plants were purchased from local market (Pathum Thani, Thailand) and local Chinese traditional herbal store (Saraburi, Thailand). 95% ethanol we used is an industrial grade. Absolute ethanol and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich Co. LLC. as analytical grade chemicals.

Herbal extraction

Terminalia chebula Retz. Fruit (TC), *Terminalia bellirica* fruit (TB), *Phyllanthus emblica* fruit (PE), *Tinospora cordifolia* stem and *Caesalpinia sappan* heartwood were dried and then ground to obtain a fine powder. *T. cordifolia* stem and *C. sappan* heartwood were randomly coded as HB1 and HB2. For the extraction of TC, TB, PE, and TC, 2 cycles of the maceration extractions were performed with 95% ethanol for 3 days to obtain liquid crude extracts. To extract *C. sappan* using 95% ethanol, Soxhlet extraction apparatus was used. Finally, liquid crude extracts were dried in hot air oven at 45ºC until the constant dry weight is observed, and crude extracts were obtained.

Herbal recipe preparation

The crude extracts were dissolved in absolute ethanol to prepare 1,000 µg/mL stock solutions. The stock solution of each extract was mixed together following the ratios shown in Table 1. Before performing DPPH assay, the concentrations were adjusted to be 2.50 µg/mL and kept avoid light exposure. Ascorbic acid (AA) and gallic acid (GA) were as the standard antioxidant agent.

Antioxidant activity determination

Antioxidant activity of each extract was conducted in micro-plate by modified DPPH scavenging method [9]. 0.1 mM DPPH in absolute ethanol was prepared from a stock solution before determination. 100 µL of 0.1 mM DPPH was mixed with 100 µL of the extracts that prepared by 2-fold serial dilution to obtain various concentrations (3.13 - 50.0 µg/mL). Thus, the final concentration of DPPH and the extract were 0.05 mM and 1.5 - 25.0 µg/mL, respectively. The reaction was done by incubating at room temperature for 30 min under dark condition. Subsequently, the absorbance at 520 nm was measured using micro-plate reader (BioTek, PowerWave XS2, USA). To obtain the inhibitory concentration 50% (IC_{50}) of the extract, the absorbance values were used to calculate radical scavenging (%) following Equation 1. Then, it was analyzed IC_{50} via Standard Curves Analysis tool on SigmaPlot software. For the comparison of antioxidant activity of the herbal recipes, the radical scavenging was calculated from the selected concentration (1.25 µg/mL). After it was reacted with the same concentration of DPPH and incubation condition, it was measured the absorbance at 520 nm using spectrophotometer and 10 mm path length cuvette (Biochrom Libra S21 Visible Spectrophotometer, United Kingdom).

$$\text{Radical scavenging (\%)} = 100\ (C - S) / C$$

Equation 1. Radical scavenging calculation; C means the absorbance of DPPH without sample and S means the absorbance of DPPH with sample.

Statistical analysis

Radical scavenging values and IC_{50} values were averaged and calculated the standard deviation (S.D.). To determine the statistical difference, the radical scavenging was analyzed via the One-Way ANOVA tool on SPSS for windows. The comparisons were done by using the Tukey's HSD test which is significant difference was noted at 95% confidence interval.

RESULTS

From the determination of IC_{50}, it was revealed that HB2 exhibited the strongest antioxidant activity among TC, TB, PE and HB1 (Table 1 and Figure 1). It could inhibit 50% of DPPH radical at the concentration of 3.34 µg/mL while TC, TB, PE and HB1 inhibited at 13.20, 11.20, 4.08 and 6.61 µg/mL, respectively. However, the antioxidant activity of HB2 was still lower than standard agents including ascorbic acid (IC_{50} = 2.95 µg/mL) and gallic acid (IC_{50} = 0.57 µg/mL). When each extract was factorially mixed together to determine the synergistic effect, it was found the significant synergistic effect in

PE+HB2, which is the combination of PE and HB2 as shown in Table 1. Moreover, this formulation exhibited the highest radical scavenging among another 9 formulations that was contained by 2 extracts. This synergistic effect increased the radical scavenging from 56.30% (PE) and 61.46% (HB2) to be 71.57% (PE+HB2). Subsequently, we had tried to optimize the suitable ratio between PE and HB2 that could provide the highest radical scavenging. This optimization revealed that PE+HB2-1 exhibited the strongest antioxidant activity as exhibited in Table 1 and Figure 1. Furthermore, the IC_{50} was found to be 0.93 µg/mL which was greater than the IC_{50} of standard ascorbic acid (2.95 µg/mL). For the antioxidant activity of Triphala recipe (TRI) with Thai herbal extract additions, the results revealed that HB2 addition could significantly improve the antioxidant activity of TRI from 27.76% into 39.93%. (Table 1 and Figure 1) However, the efficiency of TRI+HB2 was still lower than PE+HB2-1 significantly. In the addition of HB1, it did not increase the antioxidant activity of TRI but decrease the activity significantly.

Table 1. The compositions of herbal formulations and its antioxidant activity.

Codes	Compositions (% w/w)						Inhibitory Concentration 50% (IC_{50}, µg/mL)	Radical scavenging at 1.25 µg/mL (%)
	Triphala recipe			Thai herbs	Standard agents			
	T. chebula (TC)	*T. bellirica* (TB)	*P. emblica* (PE)	*T. cordifolia*/ *C. sappan*	Ascorbic acid (AA)	Gallic acid (GA)		
AA	-	-	-	-	100	-	2.95	53.78 ± 2.66
GA	-	-	-	-	-	100	0.57	91.01 ± 0.78
TC	100	-	-	-	-	-	13.20	36.22 ± 3.84
TB	-	100	-	-	-	-	11.20	40.47 ± 3.04
PE	-	-	100	-	-	-	4.08	**56.30 ± 2.55** $^{\alpha 1}$
HB1	-	-	-	100	-	-	6.61	38.39 ± 3.94
HB2	-	-	-	100	-	-	3.34	**61.46 ± 2.53** $^{\alpha 1}$
TC+TB	50	50		-	-	-	-	42.89 ± 16.57
TC+PE	50	-	50	-	-	-	-	64.04 ± 1.19
TC+HB1	50	-	-	50	-	-	-	43.80 ± 4.20
TC+HB2	50	-	-	50	-	-	-	52.81 ± 6.53
TB+PE	-	50	50	-	-	-	-	65.60 ± 2.64
TB+HB1	-	50	-	50	-	-	-	35.61 ± 22.01
TB+HB2	-	50	-	50	-	-	-	63.73 ± 2.65
PE+HB1	-	-	50	50	-	-	-	64.09 ± 2.20
PE+HB2	-	-	50	50	-	-	-	**71.57 ± 2.36** $^{\beta 1}$
HB1+HB2	-	-	-	50 + 50	-	-	-	59.23 ± 4.86
PE+HB2-1	-	-	70	30	-	-	**0.93**	**47.06 ± 0.99** $^{\beta 2}$
PE+HB2-2	-	-	50	50	-	-	-	37.11 ± 2.25 $^{\alpha 2}$
PE+HB2-3	-	-	30	70	-	-	-	40.17 ± 2.52 $^{\alpha 2}$
AA+HB2-1	-	-	-	30	70	-	-	**50.24 ± 2.75** $^{\beta 3}$
AA+HB2-2	-	-	-	50	50	-	-	43.76 ± 3.02 $^{\alpha 3}$
AA+HB2-3	-	-	-	70	30	-	-	44.48 ± 2.28
TRI	33.33	33.33	33.33	-	-	-	-	27.76 ± 1.77 $^{\beta 4}$
TRI+HB1	16.67	16.67	16.67	50	-	-	-	16.49 ± 1.36 $^{\gamma 4}$
TRI+HB2	16.67	16.67	16.67	50	-	-	-	39.93 ± 0.90 $^{\alpha 2, \alpha 4}$

Notes: $^{\alpha}$, $^{\beta}$ and $^{\gamma}$ are significantly different at 95% confidence interval.

CONCLUSIONS

HB2 possessed the strongest antioxidant activity among 5 other herbal extracts. The synergistic effect on antioxidant activity was only observed from the formulations containing PE or HB2, and the combined mixture of PE and HB2 exhibited the highest antioxidant activity. After optimizing the suitable ratio between HB2 and PE, it was observed that PE+HB2-1 exhibited the most sensitive 50% inhibition concentration (IC_{50} = 0.93 µg/mL). For Triphala recipe (TRI), the results revealed that addition of HB2 (TRI+HB2) significantly improved their antioxidant activity but significantly lower

than PE+HB2-1. This finding could be concluded as PE+HB2-1 was the most effective formulation for using as active ingredients in antioxidant products.

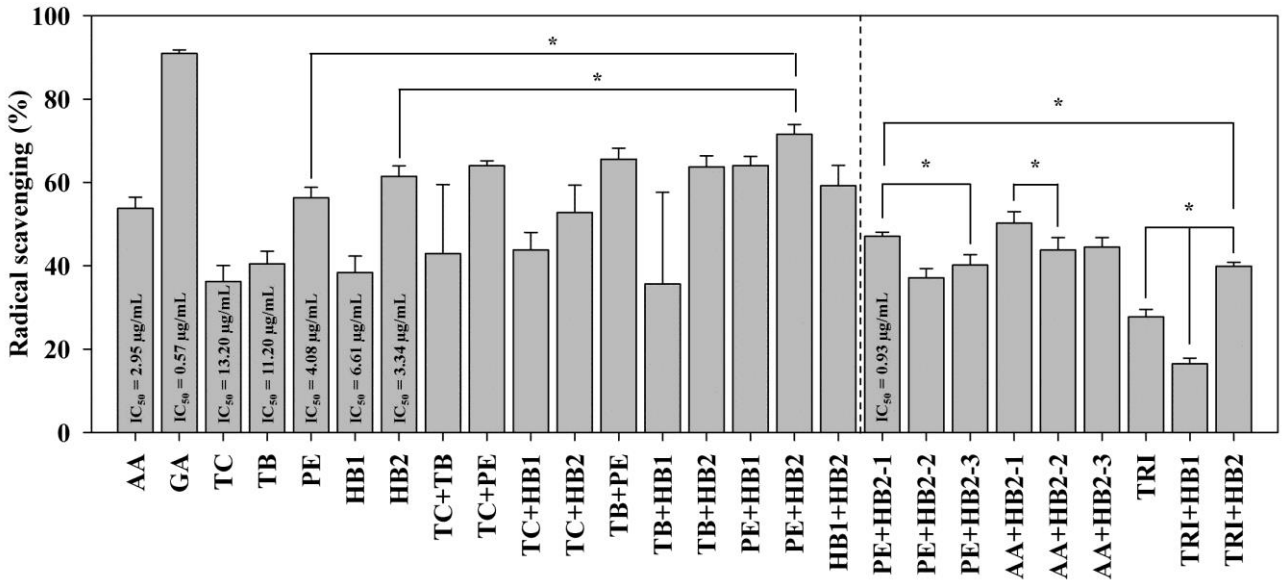

Figure 1. Inhibitory concentration 50% (IC_{50}) and radical scavenging of the herbal formulations. (* means significant difference at 95% confidence interval.)

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REFERENCES

1. Masaki H. 2010. Role of antioxidants in the skin: anti-aging effects. J. Dermatol. Sci.58(2):85-90.
2. Apel K, Hirt H. 2004. Reactive oxygen spicies: metabolism, oxidative stress, and signal transduction. Annu. Rev. Plant Biol. 55, 373-399.
3. Naik GH, Priyadarsini KI, Mohan H. 2006. Free radical scavenging reactions and phytochemical analysis of triphala, an ayurvedic formulation. Curr. Sci. 90(8), 1100-1105.
4. Belapurkar P, Goyal P, Tiwari-Barua P. 2014. Immunomodulatory effects of Triphala and its individual constituents: a review. Indian J. Pharm. Sci. 76(6), 467-475.
5. Kamali SH, khalaj AR, Shirin HR, Esfehani MM, Kamalinejad M, Larijani B. 2013.A systematic review of the antioxidant, anti-diabetic, and anti-obesity effects and safety of triphala herbal formulation. J. Med. Plant Res. 7(14), 831-844.
6. Jain DP, Pancholi SS, Patel R. 2011. Synergistic antioxidant activity of green tea with some herbs. J. Adv. Pharm. Technol. Res. 2(3) 177-183.
7. Upadhyay AK, Kumar K, Kumar A, Mishra HS. 2010. Tinospora cordifolia (Willd.) Hook. f. and Thoms. (Guduchi) – validation of the Ayurvedic pharmacology through experimental and clinical studies. Int. J. Ayurveda Res. 1(2), 112-121.
8. Hu J, Yan X, Wang W, Wu H, Hua L, Du L. 2008. Antioxidant activity In vitro of three constituents from Caesalpinia sappan L. Tsinghua Sci. Technol. 13(4), 474-479.
9. Sharma OP, Bhat TK. 2009. DPPH antioxidant assay revisited. Food Chem. 113(4), 1202-1205.

Health and Beauty Product Development and Innovation (HBP)
Full Paper

Formulation of cosmetic emulsion containing biocomposite sunscreen

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ABSTRACT

Biocomposite, the blending of polymer which results in new materials with improved physicochemical properties, has received considerable attention in several fields. Recently, we have reported the preparation of novel biocomposite delivery system from *Ananas comosus* carboxymethylcellulose and carboxymethylchitosan. In this work, cosmetic formulation containing sunscreen trapped in biocomposite which help to protect the sunscreen from degradation was prepared in the form of oil-in-water emulsion. Standard sunscreen agent, phenyl benzimidazole sulfonic acid and titanium dioxide, were varied at ratio of 2 : 4, 4 : 6, 6 : 8 and 8 : 10%*w*. The pH values increase when standard sunscreen was increased. The emulsion presented light texture but the one at high ratio of sunscreen agent presented white opaque characteristic when used. The biocomposite sunscreen was varied at 1, 3 and 5%*w* in the formulation. They showed light beige color with characteristic odor and the pH increased when ratio of biocomposite sunscreen increased. *In vitro* sun protection factor (SPF) value was determined using SPF analyzer. Emulsion base showed SPF of 0.99 and increase to 11.67 in formulation with standard sunscreen agent at ratio of 2 : 4%*w*. While that of 5%*w* biocomposite sunscreen showed SPF of 6.97. Octylmethoxycinnamate (4%*w*), primarily used in sunscreen products to absorb UV-B, was added in both emulsions and the SPF increased from 11.67 to 16.67 and 6.97 to 21.98 for standard sunscreen and biocomposite sunscreen emulsion, respectively. Product containing biocomposite sunscreen exhibited synergistic effect with octylmethoxycinnamate which give higher SPF than that of standard sunscreen agent.

Keywords: sunscreen emulsion, *Ananas comosus*, carboxymethylcellulose, chitosan, biocomposite

INTRODUCTION

Excessive exposure to solar UV radiation is the cause of the vast majority of skin cancers in humans, as well as of photoageing and sunburn. The use of sunscreen preparations became necessary after the harmful effects of sunrays were realized [1]. Phenylbenzimidazole sulphonic acid (PBSA) is an authorized hydrophilic organic UV filters relatively photostable UV-B filter but it was reported to generate under solar simulated radiation a variety of free radicals and active oxygen species that cause photo-induced damage to DNA *in vitro*. Thus, the inclusion of PBSA in sunscreen formulations and its overall sunscreen performance, particularly sustained delivery may be enhanced by incorporation in an appropriate delivery system. As most organic UV filters are lipophilic compounds, solid lipid microcapsule and nanoparticles have been widely investigated as delivery systems. However, approved UV filters include hydrophilic agents may need alternative matrix materials to enhance drug incorporation [2]. Biocomposite, blending of polymers to improve their chemical and physical properties, has received extensive attention in the past several decades. The development of biocomposite can be achieved by mixing polymers together with a crosslinking agent. New materials will be obtained with new physical and chemical properties, for example, glutaraldehyde blended with epoxyaminated chitosan to create the adsorbent for the removal Cu(II) [3] and genipin blended with casein protein for used as drug delivery [4]. Recently, we have prepared the biocomposite from carboxymethylchitosan and *Ananas comosus* cellulose. Ferulic acid was used as biocompatible crosslinker [5]. It showed soft light yellow powder and presented absorption peak at 280-350 nm which indicate that not only it can be used as delivery system, but also has sun protection properties cover both UVA and UVB.

To use this biocomposite as sunscreen delivery system, hydrophilic sunscreen agents, i.e., phenylbenzimidazole sulphonic acid and titanium dioxide were added in the system. In this work sunscreen formulation was developed using biocomposite sunscreen as active ingredient. Evaluation of sunscreen formulation including physicochemical, SPF and sensory properties were assessed.

MATERIALS AND METHODS

Materials

The standard sunscreen agent, phenylbenzimidazole sulphonic acid (PBSA) was from DSM (The Netherlands) and titanium dioxide (TiO_2) was supplied by Shanghai Creation Technology&Trade Co, Ltd., China. Biocomposite sunscreen was prepared according to previous report [5]. Octylmethoxycinnamate (OMC) was purchased from Chemspec Chemicals Pvt, Ltd., India. Xanthan gum and veegum were from FMC International Wallingstown, Little Island Co. Cork., Ireland. Propylene glycol was supplied by Lyondell South Asia Pte. Ltd., Singapore. Dimethicone was from Dow Corning Singapore Pte. Ltd., Singapore. Cetyl alcohol was from Godrej Industries Ltd., India. Jojoba oil was from Henry Lamotte Oils GMBH (Germany). Glyceryl stearate was from INOLEX® Chemical company (USA). Beeswax was from Poth Hille & Co. Ltd., England. Sorbitan monooleate and polyethylene sorbitol ester were from NOF corporation (Japan). Methylparaben and propylparaben were from SHARON Laboratories Ltd., Israel. All ingredients for cosmetic emulsion formulation are cosmetic grade.

Formulation preparation

The formulation were prepared via emulsification process. Oil phase consisted of dimethicone (0.5%), cetyl alcohol (1.0%), jojoba oil (5.0%), glyceryl stearate (2.0%), beeswax (3.0%), sorbitan monooleate (3.0%), polyethylene sorbitol ester (2.0%), propylparaben (0.2%) and octylmethoxycinnamate (4.0%). Water phase consisted of xanthan gum (0.3%), veegum (0.2%), propylene glycol (5%), methylparaben (0.2%) and distilled water (up to the 100%). Base formulation was first developed. Both phase were separately heated at 70±2^{0}C. After that, water phase was added into the oil phase with homogenizer (IKA®, T25D) at 5000 RPM for 5 minutes. The percentage of sunscreen agents were varied to study the properties of products (Table 1).

Evaluation of sunscreen formulation

Physicochemical evaluation

pH of formulation was determined by using pH meter electrode for emulsion (Mettler Toledo, InLab® easy). Electrode was dipped in 50 g sunscreen formulation and pH value was record at ambient condition.

Viscosity was determined by viscometer (Brookfield, RVDV2T extra) using number 4 spindles with 60 rpm. Spindle was dipped in 100 g sunscreen formulation and viscosity was record at ambient condition.

SPF evaluation

In vitro sun protection factor, SPF, was measured using SPF analyzer (Optometrics LLC/SPF 290F, United States). Sunscreen product of 0.1 µl/cm^2 was applied on transpore tape and investigated at wavelength between 290-400 nm.

Sensory evaluation

Sensory assessment (5-point hedonic scales) was investigated by 10 volunteers, the product was evaluated in color, odor, texture, spreadability, stickiness, oiliness and its overall properties.

RESULTS

Sunscreen formulation

The sunscreen formulation was prepared in the form of oil-in-water emulsion base to have white creamy color with light texture (Figure 1, F1) and possesses pH 6.10 with viscosity of 2527 cps. Phenylbenzimidazole sulphonic acid (PBSA) and titanium dioxide (TiO_2) were used as active sunscreen in biocomposite carrier. Thus, they were used as sunscreen agent in standard formulas so as to compare with biocomposite formulas. The standard sunscreens were varied in F2 - F5 formulas at ratio of 2 : 4, 4 : 6, 6 : 8 and 8 : 10%*w*, (Table 1). The color of emulsions was relatively the same as that of base formulation (Figure 1). All products possessed light texture but the one at high ratio of sunscreen agent presented white opaque characteristic when used. The pH of emulsion proportionally increased with the increased amount of standard

sunscreens, e.g., from 6.10 in emulsion base to 7.25 in formula with highest PBSA : TiO_2 at 8 : 10 %w (Table 1). The change of pH values is due to the alkaline agent required to facilitate the solubility of PBSA before adding into the formulation [6]. Considering the viscosity of the products, it can be seen that the values increased when increasing the sunscreens, e.g., from 1599 cps (base) to 3160 cps in F5 (Table 1) and this may due to the addition of TiO_2 which is in the powder form thus raise the viscosity of the products. The biocomposite sunscreen was varied at 1, 3 and 5%*w* in the formulation. The prepared biocomposite sunscreen is in a powder form with light yellow color and it has pH of 5.02 when dissolved in water (1.00%). However, the dispersibility is increased when the pH of solution is at 7 which also raise its sun protection ability. Thus, the alkaline was used to help dispersion of biocomposite before adding into the formulation and it resulted in the increase of pH of the formulation as can be seen in Table 2. Moreover, the addition of biocomposite sunscreen also resulted in the change of white color of base to light beige with characteristic odor. The viscosity values increased with increasing concentration of biocomposite sunscreens which may be from adding biocomposite that is in the powder form thus increase the viscosity of the product. In addition, octylmethoxycinnamate (OMC) which is generally used as UV-B absorber in cosmetics, was added in the formulations and it decreased the pH and viscosity values of the products, see F9 and F10 in Table 2. The decrease in viscosity may affect a long term stability of the products [7]. In contrast, the use of biocomposite sunscreen results in the higher of viscosity which in turn may help increase a stability of emulsions. However, it also affects the color and pH of the products, thus the information given in this work is useful in further use of biocomposite sunscreen in cosmetics.

Figure 1. Oil-in-water emulsion sunscreen.

Ingredients	%*w*/*w*									
	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**
PBSA	-	2	4	6	8	-	-	-	2	-
TiO_2	-	4	6	8	10	-	-	-	4	-
Biocomposite sunscreen	-	-	-	-	-	1	3	5	-	5
OMC	-	-	-	-	-	-	-	-	4	4
O/W base	100	94	90	86	82	99	97	95	90	91

Table 1. The sunscreen formulation of standard sunscreen and biocomposite sunscreen.

SPF evaluation

In vitro sun protection factor (SPF) value was determined using SPF analyzer and emulsion base showed SPF of 0.99 and increase to 11.67 in formulation with standard sunscreen agent at ratio of 2 : 4%*w* (Table 2). While that of 5%*w* biocomposite sunscreen showed SPF of 6.97. When OMC (4%*w*), was added in the formulations, SPF increased from 11.67 to 16.67 (F9) and 6.97 to 21.98 (F10). Product containing biocomposite sunscreen exhibited synergistic effect with OMC which give higher SPF than that of standard sunscreen agent. The synergistic effect with OMC may occur from the biocomposite composed of two polymer which help evenly distribute the OMC by forming a film layer when applied.

Table 2. The evaluation parameter of sunscreen formulation

Evaluation Parameter	Formulation									
	F1	**F2**	**F3**	**F4**	**F5**	**F6**	**F7**	**F8**	**F9**	**F10**
SPF	0.99	11.67	14.06	19.15	21.8	2.33	4.53	6.97	16.67	21.98
pH	6.10	6.50	6.50	6.55	7.25	6.34	6.70	6.87	6.40	6.60
Viscosity (cps)	1599	1950	2027	2200	3160	1633	2667	2817	1443	1847

Sensory evaluation

The sensory test was performed on 10 volunteers to determine the consumer's acceptance on the formulation containing biocomposite sunscreen (F10). The test uses 5 point hedonic scales of satisfaction (scales 1 = very dissatisfied, 2 = somewhat dissatisfied, 3 = no opinion, 4 = somewhat satisfied and 5 = very satisfied)). The customer who satisfied the product in each parameter at scale of 4 and 5 will be assessed to satisfy or accept with this parameter (1 customer = 10%). The customer was satisfied with color, texture and spreadability of product which each cited by 90%, 90% and 80% of the customer, respectively (Figure 2). However, the product still need improvement in the odor, stickiness and oiliness which each cited by 70% of the customer, but also considered in high rating. The overall satisfaction of product was acceptance from customer.

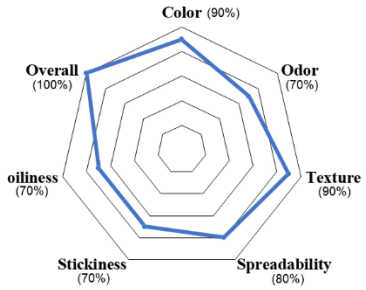

Figure 2. Sensory parameter of formulation containing biocomposite sunscreen.

CONCLUSIONS

Cosmetic emulsion was prepared using biocomposite sunscreen as active ingredient. They showed light beige color with characteristic odor and the addition of biocomposite sunscreen resulted in a increase in pH value. The formulation of 5%*w* biocomposite sunscreen showed SPF of 6.97 and the SPF increase to 21.98 when 4%*w* OMC was added. Product containing biocomposite sunscreen exhibited synergistic effect with OMC which gave higher SPF than that of standard sunscreen agent. The overall satisfaction of product was acceptance from customer.

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REFERENCES

1. Serpone N, Dondi D, Albini. 2006. Inorganic and organic UV filters: Their role and efficacy in sunscreens and suncare products. Inorganica. Chimica. Acta. 360, 794-802.
2. Gomaa YA, El-Khordagui1LK, Boraei NA, Darwish IA. 2010. Chitosan microparticles incorporating a hydrophilic sunscreen agent. Carbohyd Polym. 81, 234–242.
3. Anirudhan TS, Rijith S. 2009. Glutaraldehyde cross-linked epoxyaminated chitosan as an adsorbent for the removal and recovery of copper (II) from aqueous media. Colloid. Surface. A. 351, 52-59.
4. Song F, Zhang LM, Yang C, Yan L. 2009. Genipin-crosslinked casein hydrogels for controlled drug delivery. Int. J. Pharm. 373, 41-47.
5. Wongkom L, Jimtaisong A, Saewan N. 2015. Preparation and characterization of biocomposite of carboxymethyl chitosan and carboxymethylcellulose from *Ananas comosus*. Proceedings PACCON. 5, 859-863.
6. Shen L. 2015. Photophysical and photosensitizing characters of 2-phenylbenzimidazole-5-sulfonic acid. A theoretical study. Spectrochim. Acta. A. Mol. Biomol. Spectrosc. 150, 187-189.
7. Jiao J, Burgess DJ. 2003. Rheology and stability of water-in-oil-in-water multiple emulsions containing span 83 and tween 80. Pharm. Sci. 5(1), 1-12 (Article 7)

Development of Quercetin-loaded liposomes

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ABSTRACT

Quercetin is a bioactive flavonoid compounds mostly found in vegetable and fruits [1,2]. However it properties such as poorly skin penetration [9], moisture sensitive, and poorly soluble in water, make it difficult to develop into pharmaceutical formulations [7,8]. Liposome is a spherical vesicle, mostly composed of phospholipids. Liposome could be used as a delivery tool for quercetin [19][20]. The aim of this study was to develop quercetin-loaded liposomes (QL). Soy bean lecithin and cholesterol in the ratio of 90:10, 80:20, and 70:30 (w:w) were used to prepare liposomes by using hydration of thin film method, therefore L90:10, L80:20, and L70:30 were obtained [17]. Size, size distribution, zeta potential, as well as appearance of the prepared liposomes were evaluated. The best formulation was then loaded with quercetin. The amount and antioxidant activity of quercetin-loaded liposome was evaluated by using UV-spectrophotometry and DPPH assay, respectively. Liposomes composed of soybean lecithin: cholesterol in the ratio of 90:10, 80:20, and 70:30 have shown the size of 459.33 ±4.24 nm, 486.33 ±4.74 nm and 363.40 ±3.14 nm, respectively. Moreover, zeta potential was -17.17 ±0.90 mV (L90:10), -19.27 ±1.10 mV (L80:20) and -14.17 ±0.35 mV (L70:30). L70:30 was chosen for loading quercetin, thus QL70:30 was obtained. Entrapment efficiency of QL70:30 was 45%. Moreover, antioxidant activity of QL70:30 is 27%, which is identical to those of the original quercetin. In conclusion, liposome composed of soybean lecithin and cholesterol prepared by hydration of thin film method can encapsulate quercetin, and can be able to maintain antioxidant property of quercetin.

Keywords: Quercetin, liposomes, Quercetin-loaded liposomes, Antioxidants

INTRODUCTION

Quercetin is a bioactive flavonoid compounds and commonly found in vegetable and fruits [14] such as apple, onion, red grapes, tomato, and strawberry [15]. Quercetin presents in anti–inflammation, anti-viral infection, and antioxidants activities [11,12]. However, quercetin's poorly soluble in water, poorly capability to permeate through skin, and moisture sensitive [1], make it difficult to develop to pharmaceutical formulations [7,8].

A liposome is a nano-vesicle, which has a spherical shape of vesicle [4,16]. It composed of natural phospholipid such as yolk egg or soybean and combination with cholesterol for the strongest and stable of a liposome wall [10,13]. The advantages of liposomes are the ability to entrap lipophilic and hydrophilic drug [3,18]. It is also the stability enhancer. The purpose of this study is development of Quercetin-loaded liposomes.

MATERIALS AND METHODS

Materials

Soybean lecithin (L-α-Phosphatidylcholine, Type IV-S, 30%, SIGMA life science®, USA). Cholesterol (CARLO ERBA reagent®, France), Quercetin (Sigma Life Science, India), Chloroform AR. AR1027E-G2.5L (RCI Labscan, Thailand), Methanol AR. (RCI Labscan, Thailand), KCl (Ajax Finechem Pty Ltd, New Zealand), NaCl (Ajax Finechem Pty Ltd, New Zealand), KH_2PO_4 (Carlo Erba Reagent, France), $Na_2HPO4{\cdot}2H_2O$ (RCI Labscan, Thailand)

Preparation of liposomes by the hydration of thin film method

Liposomes were manufactured by the hydration of thin film method [5,6,17]. Three liposomes formations were prepared by soybean lecithin: cholesterol in the ratio of 90:10 (L1), 80:20 (L2) and 70:30 (L3) (w:w) [17] as shown in Table 1. Soybean lecithin and cholesterol were dissolved by 10 mL solution of chloroform and kept it evaporation (Rotary evaporator (Rotavapor R-114, BUCHI, Switzerland), Water bath (Water bath B-480, BUCHI, Switzerland), Vacuum controller (Vacuum controller B-721, BUCHI, Switzerland), Cooling bath (CRAI, Thailand)) until film formed on the wall of the round bottom flask. Then, the dried film was hydrated with phosphate buffer (PBS) pH 7.4 for hour in water bath at 55°C, and then, liposomal was kept at 4°C overnight. Size reduction was performed by using ultrasonic sonicator bath (Power Sonic 410, Hwashin Technology, Korea) for 60 minutes [21]. Finally size (Z-AV), and size distribution (PdI), and zeta potential of the obtaining liposome were evaluated by zetasizer (Nano series, MALVERN Instruments, United Kingdom).

Preparation of Quercetin-loaded liposomes using the hydration of thin film method

Quercetin-loaded liposomes were prepared using the method described above. One mL of Quercetin solution (20 mg/mL in methanol) was loaded in liposomes in the solvent evaporation step. Quercetin-loaded liposomes composed of soybean lecithin: cholesterol: quercetin solution in the ratio of 90:10:1 (QL1), 80:20:1 (QL2), and 70:30:1 (QL3) (w:w:v) were obtained [17]. Then, morphology, size (Z-AV), and size distribution (PdI), zeta potential, %entrapment efficiency (%EE), and antioxidant activity (%In) of the liposomes were evaluated. UV-visible spectrophotometer (UV-1601, SHIMADZU, Japan) under wavelength 378 nm was used for determination %entrapment efficiency, and DPPH assay was used for characterization antioxidant activity that was carried out by microplate reader (200 RT, AnthosZenyth, Austria) under wavelength 517 nm. QL3 were viewed under transmission electron microscope (TEM) (JEOL, JEM-2200FS, United States of America) to study shape and surface of the vesicles.

RESULTS

The size (Z-AV), size distribution (PdI), zeta potential of liposomes (L1-3) and quercetin-loaded liposomes (QL1-3) were evaluated and the results are given in table 1 and 2, respectively. The size of liposomes were 350 – 490 nm, while quercetin-loaded liposomes were 380 – 500 nm. Nevertheless, size distribution of both liposomes amd quercetin-loaded liposomes were wide (PdI 0.410 – 0.560). TEM pictures of quercelin-loaded liposome revealed the rounded-shape vesicle with rough surface. Antioxidant activity of QL1-3 were 20%, 24%, and 27%, respectively, while the antioxidant activity of the non-encapsulated quercetin were 27%.

Table 1. The size (Z-AV), size distribution (PdI), and zeta potential of liposomes

Formulation	Soybean lecithin: Cholesterol (w:w)	Z-AV (Size) (nm)	Polydispersity index	Zeta-Potential (mV)
L1	90: 10	459.33 ±4.24	0.554 ±0.04	-17.17 ±0.90
L2	80: 20	486.33 ±4.74	0.508 ±0.02	-19.27 ±1.10
L3	70: 30	363.40 ±3.14	0.479 ±0.02	-14.17 ±0.35

Table 2. The size (Z-AV), size distribution (PdI), zeta potential, %entrapment efficiency, and antioxidant activity of quercetin-loaded liposomes

Formulation	Soybean lecithin: Cholesterol: Quercetin (w:w:v)	Z-AV (Size) (nm)	Polydispersity index	Zeta-Potential (mV)	%EE	Antioxidant activity (%In)
QL1	90: 10: 1	482.90 ±5.33	0.499 ±0.01	-19.80 ±0.85	16 ±1.00	20 ±0.38
QL2	80: 20: 1	485.13 ±1.07	0.424 ±0.01	-19.77 ±1.00	28 ±2.08	24 ±1.09
QL3	70: 30: 1	385.60 ±1.57	0.431 ±0.01	-16.93 ±0.25	45 ±1.50	27 ±0.50

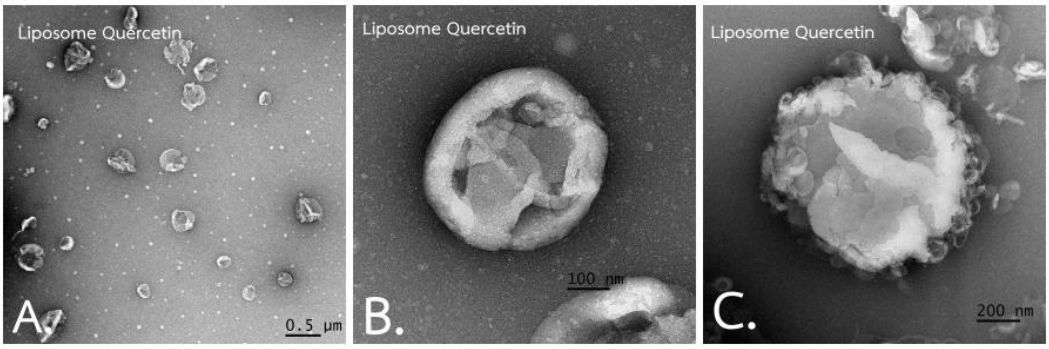

Figure 1. The surface and shape of quercetin-loaded liposomes (QL3)

CONCLUSIONS

Liposomes and quercetin-loaded liposome prepared by using hydration of thin film method were negative charge. Liposomes composed of soybean lecithin: cholesterol in the ratio of 70:30 (L3) (w:w) was the smallest (363.40 ±3.14) and quercetin-loaded liposome in the ratio of 70:30:1 (QL3) (w:w:v) shown the highest encapsulation efficiency (%EE) (45%). The antioxidant activity of QL3 was 27%, which are same as the non-encapsulated quercetin (27%). Therefore liposome composed of soyben lecithin: cholesterol 70:30 (w:w) may be the best formulation for the further development. Moreover, QL3 have a round shape and rough surface when viewed under TEM. In conclusion liposome prepared by hydration of thin film method produced rounded-shaped vesicle and can be able encapsulate quercetin and also preserve its antioxidant activity.

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REFERENCES

1. Alessi P, Cortesi A, De Zordi N, Gamese T, Kikic I, Moneghini M, Solinas D. 2012. Supercritical atisolvent precipitation of quercetin system. Chem. Biochem. Eng. Q. 26 (4), 391-389.
2. Antonella S, Antonio T, Domenico T, Maria LP, Beatrice T, Chiara M, et al. 2003. '*In vivo*' antioxidant and photoprotective properties and interaction with model membranes of three new quercetin esters. Eur. J. Pharm. Biopharm. 56, 167-174.
3. Ajazuddin, Saraf S. 2010. Application of novel drug delivery system for herbal formation. Fitoterapia. 81, 680-689.
4. Dua J.S, Rana A.C, Bhandari A.K. 2012. Review Article; Liposome: Methods of preparation and applications. Int. J. Adv. in Pharm. Res. 3 (2), 14-20.
5. Elhissi AMA, O'Neill MAA, Roberts SA, Taylor KMG. 2006. A calorimetric study of dimyristoyl phosphatidyl-choline phase transitions and steroid-liposome interactions for liposomes prepared by thin film and proliposome methods. Int. J. Pharm. 320, 124-130.
6. Eskandar M, Somayeh H. 2013. Liposomes as drug delivery systems: properties and applications. Res. J. Pharm. Biol. Chem. Sci. 4, 169-185.
7. Eva UG, Joey W, Sike M, Anne-Kathrin R, Bernhard U, Bernd D, et al. 2001. Pharmacokinetics and bioavailability of quercetin glycoside in human. J. Clin. Pharmacol. 41, 492-499.
8. Gabrielska J, Socozynska-Kordala M, Przestalski S. 2004. Quercetin reduces prooxidant action of organometallic compounds on liposome membranes irradiated with UV. J. Environmental studies. 13 (5), 509-514.

9. Chen-yu G, Chun-fen Y, Qi-lu L, Qi T, Yan-wei X, Wei-na L, Guang-xi Z. 2012. Development of Quercetin-loaded nanostructure lipid carrier formulation for topical delivery. Int. J. Pharm. 430, 292-298.
10. Himanshu A, Sitasharan P, Singhai AK. 2001. Liposome- as drug carrier. International Journal of Pharmacy & Life Sciences. 2 (7), 945-951.
11. Jessy Shaji, Sheha Iyer. 2012. Novel double loaded Quercetin liposomes: evidance of superior therapeutic potency against CCL4 induced hepatotoxicity a comparative study. Asian Journal of Pharmaceutical and Clinical Research. 5 (2), 104-108.
12. Shaji J, Iyer S. 2012. Preparation, optimization and In vivo hepatoprotective evaluation of Quercetin liposomes. Int. J. Curr. Pharm. Res. 4 (2), 24-32.
13. Kant S, Kumar S, Prashar B. 2012. A complete review on: Liposomes. Int. Res. J. Pharmacy. 3 (7), 10-16.
14. Maura C, Carla C, Donatella V, Maria M, Chiara S, Anna M. F. 2011. Effect of penetration enhancer containing vesicles on the percutaneous delivery of quercetin through new born pig skin. Pharmaceutics. 3, 497-509.
15. Nathiya S, Durga M, Devasena T. 2014. Quercetin, encapsulated quercetin and it's application - A review. Int. J. Pharm. Pharm. Sci. 6 (10), 20-26.
16. Olga P, Jana S, Zoran K, Vesna R. 2013. Review article; an overview: Methods for preparation and characterization of liposomes as drug delivery systems. Int. J. Pharm. Phytopharm. Res. 3 (3). 182-185.
17. Ranjit S, Sankar C. 2012. Formulation of herbal liposomes containing green tea and GAULTHERIA PROCUMBENS for anti acne activity. Int. J. Adv. Pharm. Phytopharm Res. 3 (10), 1211-1216.
18. Varshneya A, Padmini R. 2014. Liposomes as carries in skin ageing. International Journal of Current Pharmaceutical Research. 6 (3), 1-7.
19. Aroonsri P, Jintanaporn W, Saegrawee S, Wathita P, Suppaporn M. 2008. Anxiety and cognitive effect of Quercetin liposomes in rats. Nanomedicine: Nanotechnology Biology, and Medicine. 4, 70-78.
20. Terdthai Tong-un, Muchimapura S, Phachonpai W, Wattanathorn J. 2010. Effect of quercetin encapsulated liposomes via nasal administration: A novel cognitive enhancer. Am. J. Applied Sci. 7 (7), 906-913.
21. Aranya M, and Jiradaj M. 2550, Liposomes for cosmetic, O.S. Printing House, THAILAND.

Development of tamarind *(Tamarindus indica* L.) seed extracts loaded wax-incorporated alginate-based emulsion gel beads using a modified ionotropic gelation

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ABSTRACT

The objective of this study was to investigate the effect of types and concentrations of waxes on the percentage of encapsulation efficiency and percentage of active ingredient release of wax-incorporated alginate-based emulsion gel beads containing tamarind *(Tamarindus indica* L.) seed extract. The wax-incorporated alginate-based emulsion gel beads were prepared using a modified ionotropic gelation technique. Tamarind seed extract was used for the active ingredient in all formulations. A concentration of tamarind seed extract was used at 1% (w/w). The waxes in alginate containing tamarind seed extract were also melted, homogenized and then extruded into calcium chloride solution. The beads formed were separated, washed with distilled water and dried in hot air oven. The effect of types and concentrations of waxes on encapsulation efficiency and percentage of active release of alginate gel beads was also investigated. The results demonstrated that the incorporation of waxes into the gel beads had an effect on the change in % encapsulation efficiency and % active ingredient release. Furthermore, the addition of water insoluble waxes (i.e. carnauba wax & bee wax) significantly retarded the active release. The different waxes showed the slight effect on the drug release behavior. Nevertheless, the increase in amounts of incorporated waxes in all formulations could sustain the percentage of active ingredient release. In conclusion, the wax-incorporated emulsion gel beads using a modified ionotropic gelation technique could be applied as intragastric floating delivery and controlled delivery.

Keywords: Ionotropic gelation technique, Tamarind seed extract, Emulsion gel beads, Encapsulation

INTRODUCTION

Tamarind or *Tamarindus indica* L., which is widely growth in Thailand, is one of the ancient herbal medicine plants **[1-2].** It has long been used as the food nutrition sources and active ingredients in cosmetics products. In Thailand, the flower, fruit and leaf are consumed in food materials. There were several attempts for utilization of tamarind seed which was waste from the consumption. The biological activities assessment of tamarind seed was also reported such as anti-oxidant, reducing lipid peroxidation, anti-tyrosinase, simulating collagen, anti-microbial activities and anti-inflammatory potential **[3-4]**. Moreover, the development of tamarind seed extract has been attempted to increase the innovation of food, pharmaceutical and cosmetic products such as gel beads or spheres.

The gel beads or spheres could be prepared from a natural biopolymer such as sodium alginate, chitosan and pectin. Sodium alginate is a non-toxic, biodegradable and naturally occurring polysaccharide derived from marine brown algae which contained the bacteria species **[5-6]**. It is a sodium salt of alginic acid containing a linear polymer composed of 1, 4-linked β-D-Mannuronic acid (M) and α-D-gluronic acid (G) residues which is soluble in water and form a reticulated structure. Alginate gel beads are the result of the cross-links with the divalent or polyvalent cations to form insoluble

meshwork. Calcium and zinc cations have been reported for cross-the linking of acid groups of alginate. It has been used as a controlled release of active ingredients **[5-9]**. The benefits are cheap and abundant sources, excellent biocompatibility and total degradation without hazardous by-products. The encapsulation efficiency, morphology, swelling, floating properties and drug release of gel beads were also studied. However, the development of alginate gel bead could not prolong the gastric retention of active ingredients. New formulations were attempted with different processes such as floating, expansion/plug type, high density or adhesion to mucosa. The research in floating system in particular has been widely conducted. Nevertheless, the floating system had no an effect on the motility of the GI tract. Immediate floating could be obtained when the density was low at the beginning, for example, when provided by the entrapment of air or by the incorporation of low density materials such as oils and foam powders **[8-9]**

Therefore, the objective of the present study was to investigate the effect of types and concentrations of waxes on percentage of encapsulation efficiency and percentage of active release. The wax-incorporated alginate-based emulsion gel beads were prepared using a modified ionotropic gelation technique. Tamarind seed extract was used for the active ingredient in all formulations. A concentration of tamarind crude extract was used at 1% w/w.

MATERIALS AND METHODS

Materials

Sodium alginate and calcium chloride, (Sigma Aldrich,), olive oil, carnauba wax and white wax (Nam Siang Co., Ltd., Bangkok, Thailand) were used as received. Termarind seed extract was harvested from Agricultural and Food Technology department, Thailand institute of Scientific and Technological Research. All other chemicals were of standard pharmaceutical grade.

Preparation of conventional calcium alginate gel beads

Calcium alginate gel beads were prepared by the ionotropic gelation method which was adapted from other reports **[8].** Frist of all, 2% w/w alginate was dispersed in water with agitation, and then the concentration of tamarind seed extract at 1% w/w was dispersed in alginate solution to make a 100-g solution. After that, the dispersion was extruded by a plastic needle into 2% (w/w) calcium chloride and was stirred at room temperature. Next, the gel beads formed were allowed to stand in the solution for 20 min before being separated and washed with distilled water. The beads were dried at 37°C for 12 h.

Preparation of wax-incorporated alginate-based emulsion gel beads

The several amounts of waxes (bee wax & carnauba wax) were melted in water bath at 60 -85 °C, depending on the melting range of the waxes used as adapted from other reports **[5-6, 8]**. The molten wax was dispersed in the homogenized emulsion mixture of alginate, oil and a concentration of tamarind seed extract at 1% w/w which was already heated at the same temperature, and then mixed until the homogenous mixture occurred. The hot-melted mixture was extruded into 2% calcium chloride (cooled at 5°C). The wax-incorporated emulsion gel beads obtained were treated in the same manner as calcium alginate gel beads.

Study of particle size of gel beads

The mean diameter of 50 dried beads was determined by optical microscopy (BH-2, Olympus, Japan). The microscope eyepiece was fitted with a micrometer by which the size of the beads could be determined.

Determination of percentage of encapsulation efficiency (%EE)

To determine the total flavonoid content and % EE of the beads, 500 mg beads were crushed and dispersed in methanol. The dispersion was sonicated for 15 min, left overnight for 24 h and filtered. A 1 ml sample was taken and diluted with methanol, and flavonoid content assay **[10].** The filtrate obtained after bead collection on the filter medium and diluting with phosphate buffer 7.4 was analyzed using a UV-Visible spectrophotometer at 506 nm. Total flavonoid content was determined following a method by Park et al **[10]**. Frist, 0.3 ml of supernatant from gel beads, 3.4 ml of 30% methanol, 0.15 ml of $NaNO_2$ (0.5 M) and 0.15 ml of $AlCl_3*6H_2O$ (0.3 M) were mixed. After 5 min, 1 ml of NaOH (1 M) was added. The solution was mixed well and the absorbance was measured against the reagent blank at 506 nm. The standard curve for total flavonoids was made using rutin standard solution (0 to 100 mg/l) under the same procedure as earlier described. The total flavonoids were expressed as milligrams of rutin equivalents per g of dried fraction. EE (%) = Active content in beads / Theoretical active content x 100

Determination of active ingredient release

The in vitro release of tamarind seed extract from the different formulations was examined using a USP dissolution apparatus 1 (Erweka, Germany) with 1000 ml of SGF (pH 1.2) and the basket rotation at 100 rpm. The temperature was controlled at 37 ± 0.1°C. Samples were taken at appropriate time intervals and assayed spectrophotometrically (UV-Visible spectrophotometer, Hitachi U-2000, Japan) for the total flavonoid content as described in Park *et al* **[10].** All dissolution runs were performed in triplicate.

RESULTS

The emulsion gel beads were prepared by the ionotropic gelation method and were described in other reports **[5, 8].** Sodium alginate could emulsify the mixture of aqueous tamarind seed extract solution, resulting in the surface active ability of sodium alginate which could reduce the interfacial tension between oil phase and water phase or steric and mechanical stabilization mechanisms. The emulsion-gel beads were prepared by ionotropic gelation method and were gelled by the action of calcium chloride. The molten wax was dispersed in the homogenized emulsion mixture of alginate. In addition, wax and tamarind seed extract were heated at the same temperature, and then mixed until the homogenous mixture was obtained. Finally, the hot-melted mixture was extruded into calcium chloride solution. The spherical beads of wax-incorporated emulsion gel were obtained by hot melt extrusion and ionotropic gelation as illustrated in **Fig.1**.

The mean diameter of the active-loaded gel beads and wax-incorporated emulsion gel beads was shown in Table 1. The mean diameter of the tamarind seed extracts loaded gel beads ranges between 2.2 and 2.5 mm. The mean size of the beads insignificantly changed when the amount of wax was changed. This agreed with the previous reporting that which the type of additives used (e.g. waxes or polymers) insignificantly influenced the mean diameter of the emulsion gel beads **[5]**.

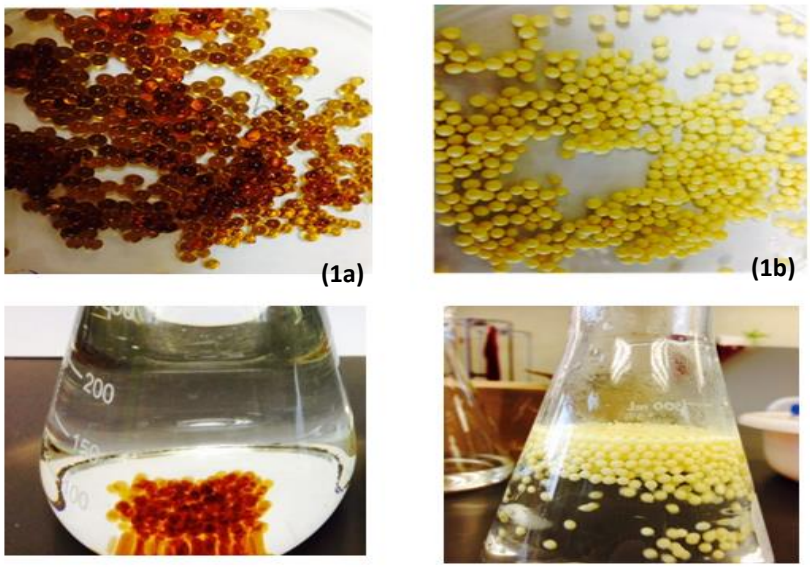

Fig 1 Photo images showing the floating of conventional calcium alginate gel beads (1a) and emulsion gel beads (1b).

The % EE of active ingredients from tamarind seed extract was increased with the incorporation of waxes in the formulation **(Fig 2)**. The % EE in all formulations was 68.56 ± 1.67 %, 85.43 ± 2.54% and 80.12 ± 1.54 % for without wax, bee wax and carnauba wax, respectively. The addition of both waxes into gel beads showed the significant ($P < 0.05$) change in % EE of emulsion gel beads, in comparison with gel bead without the addition of wax. The maximum of 85.43 ± 2.54 % active ingredients of % EE was obtained in emulsion gel beads containing bee wax, because, both waxes were natural complex lipid materials, consisting of different amounts of primarily acid esters i.e., free acids, fatty alcohols, and hydrocarbons **[8, 11]**. In addition, the results were in agreements with the decrease in mean diameter of emulsion gel beads, because, the addition of both types of wax into gel bead resulted in the reduction of mean diameter of gel beads. The more decrease in mean diameter of emulsion gel beads, the increase in % EE of the beads was obtained. According

to Sriamornsak **[8, 11]**, white bee wax contains a higher percentage of free fatty acid, resulting in a lower percentage of fatty esters than carnauba wax**.**

Table 1 Effect of types and concentrations of waxes on mean diameter of alginate gel beads.

Gel bead with different types and concentrations of wax	Mean diameter (mm)
Gel bead (No wax)	2.47 ± 0.16
Gel bead + Bee wax 1%	2.26 ± 0.08
Gel bead + Bee wax 2%	2.23 ± 0.09
Gel bead + Bee wax 2%	2.20 ± 0.12
Gel bead + Carnauba wax 1%	2.37 ± 0.05
Gel bead + Carnauba wax 2%	2.33 ± 0.07
Gel bead + Carnauba wax 2%	2.31 ± 0.16

Fig. 3 showed the effect of amounts of both waxes on % EE of emulsion gel beads. The results indicated that the increase in amounts of both waxes gave the significant ($P < 0.05$) increase % EE when compared with the conventional gel beads. The increase in amount of wax contributed to the decrease in diameter of emulsion gel beads, resulting in the increase in % EE of gel beads. The %EE was not significantly changed when the amount of waxes was increased from 1% to 3%. The % EE of gel beads were increased from 85.43± 2.54%, 89.45 ± 7.98 % and 92.06± 5.98 % for 1%, 2% and 3% of white wax while the EE of gel beads were 80.12 ± 1.54%, 82.67 ± 6.67 % and 85.32 ± 3.56 % for 1%, 2% and 3% of carnauba wax, respectively. Between both waxes, bee wax of all concentrations showed the % EE higher than carnauba wax, due to the different structures of both waxes and the effect of diameter of this wax. The higher amount of wax caused the higher compact structure of gel bead, and it could be potentially loaded the active ingredients from tamarind seed extract into the structure of gel beads.

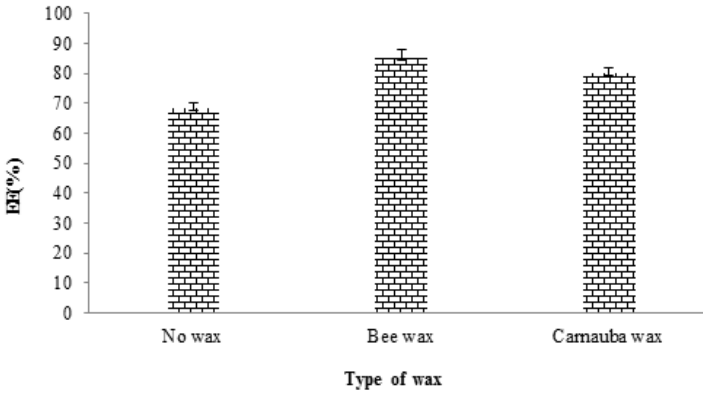

Fig. 2 Effect of different types of waxes on percentage of the percentage of encapsulation efficacy of alginate gel beads.

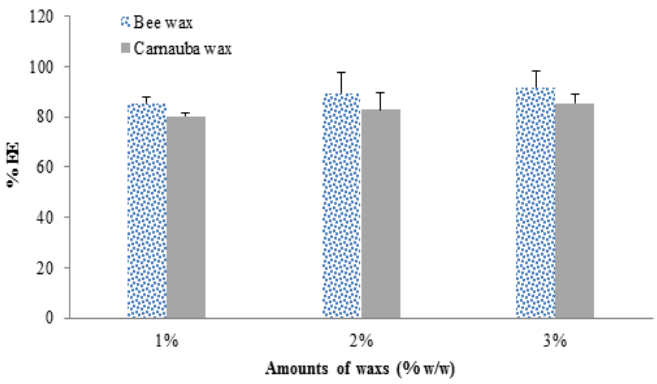

Fig. 3 Effect of amount of waxes on the percentage of encapsulation efficacy of alginate gel beads.

The evaluations of active release of the beads floated in SGF were performed, in order to determine the appropriate beads for intragastric floating delivery system. **Fig. 4** showed the effect of various types of waxes on the active ingredient release profiles of emulsion gel beads. The results indicated that the profile of active release was also reported for both emulsion gel beads that contained white wax and carnauba wax in SGF in comparison with conventional gel beads. The results were due to the hydrophobic nature of waxes in order to dispersion in the structure of emulsion gel beads. The matrix with more hydrophobicity that consequently delayed the diffusion of drug from the beads occurred. However, the different types of hydrophobic wax had the slight effect on the drug release due to similar structure of bee wax and carnauba wax. From other reports, both waxes were natural complex lipid materials, consisting of different amounts of primarily acid esters, free acids, fatty alcohols, and hydrocarbons **[8, 11].** However, the active release from formulation containing carnauba wax was slower than containing bee wax, due to the higher percentage of free fatty acid and the lower percentage of fatty esters of white wax, in comparison with the carnauba wax. The effect of amount of waxes on

the active ingredient release profiles of emulsion gel beads was displayed in **Fig. 5**. The results demonstrated that the increase in amount of waxes would show the active ingredient of tamarind seed extracts release from emulsion gel beads. The increased amounts of carnauba wax were slower release than the increased amount of bee wax due to the increase in water resistance of wax. The similar influence of the increase in amount of the wax on active ingredient release from gel based on the difference waxes.

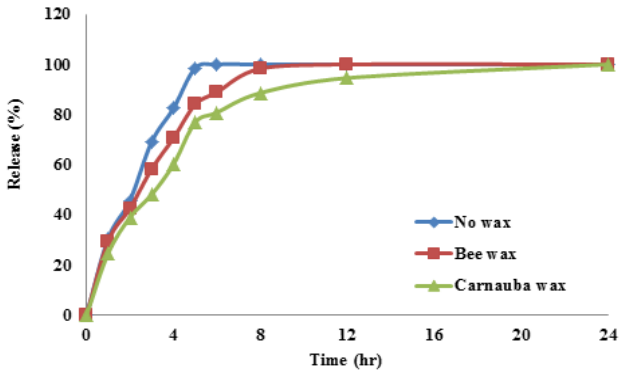

Fig. 4 Effect of different waxes on the percentage of active release from alginate gel beads.

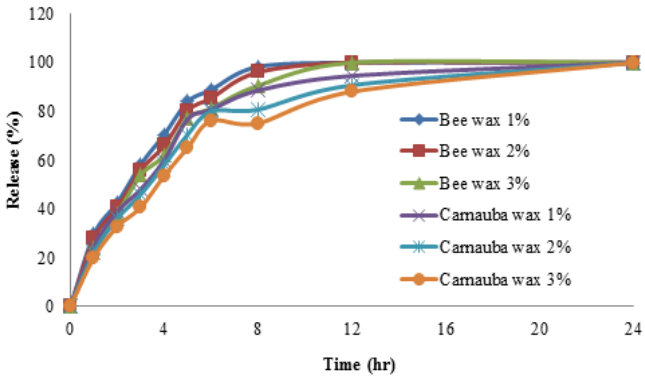

Fig. 5 Effect of amounts of waxes on the percentage of active release from alginate gel beads.

CONCLUSIONS

In conclusion, tamarind (*Tamarindus indica* L.) seed extract could be loaded into emulsion gel beads for the floating drug delivery system which was a practical approach to control the active ingredient release over extended period of time. Intragastric floating drug delivery system was prepared by incorporating low density materials i.e., oils and/or waxes. The incorporation of wax into the beads had an influence on the percentage of encapsulation efficiency and percentage of active release, resulting from the structure of wax and diameter of gel beads. The maximum of 85.43 ± 2.54 % active ingredients of EE was obtained in emulsion gel beads containing bee wax, and the percentage of active release from the formulation containing carnauba wax was slower than containing white bee wax. In addition, the increased amount of waxes could slowed the active ingredient of tamarind seed extracts released from emulsion gel beads. Hence, the wax-incorporated emulsion gel beads using a modified ionotropic gelation technique could be applied as intragastric floating delivery and controlled delivery for food supplement and food nutrition.

ACKNOWLEDGEMENTS

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REFERENCES

1. Siddhuraju P. 2007. Antioxidant activity of polyphenolic compounds extracts from defatted raw and dry heated tamarind us indica seed coat. LWT. 40, 982-990.
2. Luengthanaphol S, Mongkholkhajornsilp D, Douglas S, Douglas PL, Pengsopa L, Pongamphai S. 2004. Extraction of antioxidant from sweet Thai tamarind seed coat – preliminary experiments. J. Food Eng. 63, 247-252.
3. Tsuda T, Watanabe M, Ohshima K, Yamamoto A, Kawakishi S, Osawa T. 1994. Antioxidative components isolated from the seed of tamarind (*Tamarindus indica* L.). J. Agric. Food Chem. 42, 2671-2674.
4. de M, Krishna DA, Baneerjee AB. 1999. Antimicrobial screening of some Indian spices. Phytother. Res. 3, 616-618.

5. Sriamornsak P, Thirawong N, Puttipipatkhachorn S, 2005. Emulsion gel beads of calcium pectinate capable of floating on the gastric fluid: effect of some additives, hardening agent or coating on release behavior of metronidazole. Eur J Pharm Sci. 24, 363–373.
6. Ganesh NS, Bharathi G, Jayanthi C, Hanumanthachar J. 2014. Comparative evaluation of wax incorporated alginate and pectinate gel beads of Metformin. Der Pharmacia Lettre. 6 (2), 10-16.
7. Shaik AA, Khair U, Praneetha Pb. 2011. Preparation and in vitro evaluation of Chitosan-Carrageenan,Chitosan-Alginate beads for controlled release of Nateglinide. Der Pharmacia Sinica. 2 (2), 375-384.
8. Sriamornsak P, Asavapichayont P, Nunthanid J, Luangtana-anan M, Limmatvapirat S, Piriyaprasarth S. 2008. Wax-incorporated Emulsion Gel Beads of Calcium Pectinate for Intragastric Floating Drug Delivery. AAPS PharmSciTech. 9 (2), 571 – 576.
9. Sriamornsak P, Nunthanid J. 1998. Calcium pectinate gel beads for controlled release drug delivery: I. Preparation and in-vitro release studies. Int J Pharm. 160, 207–212.
10. Park Y-S, Jung S-T, Kang S-G, Heo BK, Arancibia-Avila P, Toledo F, Drzewiecki J, Namiesnik J, Gorinstein S. 2008. Antioxidants and proteins in ethylene-treated kiwifruits. Food Chem. 107, 640–648.
11. Rodriguez L, Passerinim N, Cavallari C. Cini M, Sancin P, Fini A. 1999. Description and preliminary evaluation of a new ultrasonic atomizer for spray-congealing processes. Int J Pharm. 183, 133–143

Rhodopseudomonas palustris CH12 producing polyhydroxyalkanoate (PHA): screening, condition optimization and primer design and amplification of PHA synthase gene

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ABSTRACT

There were 58 starins of purple non-sulfur bacteria isolated from 108 samples of chicken faeces. The bioplastic granules inside the cells of the bacteria were investigated under Confocal Laser Scanning Microscope (CLSM) at λ_{ex} 530 nm and λ_{em} 605 nm. Gas chromatography (GC) was carried out for quantitative analysis of polyhydroxybutyrate (PHB). The results showed that CH12 could accumulate PHB the maximum of 5% (w/w) of the cell dry weight (CDW) in GM medium without glutamic acid. The CH12 strain was identified as *Rps. palustris* NCIB8288 based on its taxonomic characteristics and 16S rDNA sequence analysis. The CH12 could accumulate the highest amount of PHB, 15% (w/w) of CDW, after 3 day-cultivation under micro-aerobic condition with light (2,000-2,500 Lux) in GM medium in the presence of butyrate instead of glutamic acid, pH 9.0 at 35°C. The activity of PHA synthase of *Rps. palustris* CH72 was 24.57 unit/mL. Primer was designed based on the conserved region of PHA synthase gene (classI) among various strains of *Rps. palustris*. The results showed that the primer could bind the template of *Rps. palustris* CH72 chromosomal DNA with the same length of 538 bp.

Keywords: PHA synthase, *Rhodopseudomonas palustris*, primer design, polyhydroxyalkanoate, purple non-sulfur bacteria

INTRODUCTION

Plastics synthesized by chemical reactions cause problems to the environment due to the long-term degradation. Among the candidates for biodegradable plastics, polyhydroxyalkanoates (PHAs), polylactides, aliphatic polyesters, polysaccharides, and their composites, PHAs have attracted much interest because of their similar material properties to conventional plastics and their complete biodegradability [1]. PHAs are polyesters of various hydroxyalkanoate monomers, which function as an energy and carbon reserve in microorganisms [2] under aerobic condition [3]. Polyhydroxybutyrate is a member of polyhydroxyalkanoate family accumulated not only in prokaryotic, but also in eukaryotic cells, and can form complexes with other macromolecules [4-5]. Purple non-sulfur bacteria is from the group of purple bacteria. Their predominant mode of growth under anaerobic conditions is photoheterotrophy using various organic substrates, e.g., acetate, pyruvate and dicarboxylic acids. Some species of purple non-sulfur bacteria are able to use hydrogen or sulfide as electron donors, and carbon dioxide as the sole of carbon source [6]. In addition, purple non-sulfur bacteria can survive in environments with various levels of oxygen. Most of them are capable of growing under micro-aerobic to aerobic conditions with light or without light. They also grow well in polluted environments [7]. There have been reports on wastewater treatments using purple non-sulfur bacteria. These treatment include various kinds of microbial industry wastes, food industry wastes, agricultural effluents and waste effluents from starch and wool industries [8]. Thus, the PHBs in purple non-sulfur bacteria are interesting and could be considered an expedient by-product of the treatments. In this study, we describe screening of purple non-sulfur bacteria producing biopolymer from

chicken faeces. We provide data on the effects of dark incubation, micro-aerobic condition, glutamic acid, yeast extract, incubation time, light intensity, pH and other carbon sources on PHB accumulation in the bacterial cell. In addition, primer design and amplification of PHA synthase gene were investigated.

MATERIALS AND METHODS

Materials and type culture strain

All purchased media and chemicals were commercial grades. Type culture strain of *Azotobacter* sp. TISTR 1094 was obtained from the Thailand Institute of Scientific and Technological Research, Bangkok, for comparison of PHB production with that of the purple non-sulfur bacteria.

Isolation

The strains used for screening of purple non-sulfur bacteria producing PHB were isolated from 108 chicken faeces samples in the chicken-egg farm of Department of Animal Science, Faculty of Natural Resources, Prince of Songkla University, Hat Yai.

Cultivation of purple non-sulfur bacteria

Five percent (v/v) of purple non-sulfur bacteria were inoculated in a 15-mL screw-cap tube containing 12.5 mL of GM medium and incubated under micro-aerobic condition with tungsten light intensity of 3,000 Lux at 35°C for 48 hr. The culture broth was centrifuged at 12,000 rpm for 5 min. The cell pellet was washed with 0.85% saline solution and freeze-dried for detection of PHB by gas chromatography analysis.

In vivo detection of biopolymer granules by fluorescence microscopy

The bacterial cells cultivated under micro-aerobic condition under light (3,000 Lux) at 35°C for 5 days were smeared and heat fixed on a glass slide. They were stained with 1.0% aqueous solution of Nile blue A at 55°C for 10 min. The slide was washed with tap water and then with 8% aqueous acetic acid for 1 min. The stained smear was then washed and blotted dry, remoistened with distilled water and covered with glass cover slip. The slide was observed by Confocal Laser Scanning Microscope (CLSM) with excitation and emission wavelength of 530 and 605 nm, respectively.

Esterification of PHB for gas chromatography (GC) analysis

The freeze-dried cells were used for esterification of PHB [9]. Five milligram of freeze dried bacterial cell mass was weighed in 5-mL screw-cap vial. Five hundred microlitre of 1, 2-dichloroethane (DCE), 500 μL of n-propanol containing hydrochloric acid (HCl) (1 volume of concentrated HCl mixed with 4 volumes of n-propanol) and 50 μL of internal standard (2 g of benzoic acid in 50 mL of n-propanol) were added. The mixture was incubated for 4 hr in a water bath at 85° C and shaken intermittently. It was then cooled to room temperature. One millilitre of water was added. The mixture was shaken for 20-30 sec. The heavier DCE-Propanol phase was collected and injected directly into the gas chromatography machine. PHB content was defined as the ratio of PHB to cell dry weight.

Gas chromatography conditions

HP-1 (HP 19091Z-413E, Crosslinked Methyl Siloxane, Hewlett Packard, USA) capillary column, 30 m in length with 0.32 mm inner diameter was used. The temperature of the injection port and the flame ionization detector (FID) port was 250°C. The temperature profile used was 5 min at 80°C, followed by 7°C/min rise to reach the final 200°C. The flow rate of nitrogen gas was 5 mL/min. Injection volume was 1.0 μL by splitless mode. A computer controlling gas chromatography (HP 6850) equipped with double FID was used.

Calibration

Two hundred milligram of PHB was weighed and dissolved by heating in a small volume of DCE. After cooling to room temperature, the solution was made up to 10 mL. Concentrations of 200, 400, 600, 800 and 1000 μg/mL of this solution were esterified as described above and subjected to GC machine.

Effect of GM medium without added yeast extract or glutamic acid on PHB accumulation

The isolated purple non-sulfur bacteria were cultivated in a 15-mL screw-cap tube containing 12.5 mL of GM medium without added yeast extract or glutamic acid under micro-aerobic condition with light (3,000 Lux) at 35°C for 5 days. *In vivo* detection of PHA granules by fluorescence microscopy was carried out. PHB content was analyzed as described above.

Taxonomic characteristics of CH12

Some taxonomic characteristics of CH12 strain were identified according to the Bergey's Manual of Systematic Bacteriology. They were Gram staining, cell shape, flagellatin, photoautotrophic growth, and carbon sources and photosynthetic electron donors.

16S rDNA sequence analysis

CH12 strain was cultivated in GM medium under micro-aerobic condition with light (2,500 Lux) at 35°C for 12 hr. Genomic DNA was extracted by using the standard method and then amplified by GeneAmp PCR System 9600. Universal primers were used; position 170 for forward and 705 for reverse. The PCR product was 537 bp. The amplified DNA was sequenced by using API 377 DNA sequencer.

Effect of dark incubation, incubation time, light intensity, pH and carbon source on PHB accumulation

Dark incubation: To study the impact of dark incubation on PHB accumulation, the CH12 strain was inoculated and incubated under micro-aerobic condition in darkness in GM medium, and GM medium without added glutamic acid at 35°C for 5 days. The growth was observed at 660 nm using a spectrophotometer and PHB was analysed by the GC as mentioned above.

Incubation time: The CH12 strain was cultivated in GM medium without added glutamic acid in a 500 ml-screwed bottle under micro-aerobic conditions with light at 35°C. The PHB accumulation was analysed on the 1-, 2-, 3-,...,6-day cultivation.

Light intensity: The amount of PHB was determined after cultivating CH12 strain in a 15-mL screw-cap tube containing 12.5 mL of GM medium without added glutamic acid under micro-aerobic condition with the following tungsten light intensities: 2,000, 2,500, 3,000 and 3,500 Lux, at 35°C for 3 days.

pH: The CH12 strain was inoculated in a 15-mL screw-cap tube containing 12.5 mL of GM medium without added glutamic acid at a pH of 5, 6, 7, 8 and 9 under micro-aerobic condition with light at 2,000 Lux, and at 35°C for 3 days. PHB accumulation was analysed.

Carbon source: Acetate, propionate and butyrate, 3.8 g/L each were used separately instead of glutamic acid in GM medium. After cultivating CH12 strain in a 15-mL screw-cap tube containing 12.5 mL of the media at pH 9 under micro-aerobic condition with light at 2,000 Lux, and at 35°C for 3 days, the accumulation of PHB was analysed.

Primer design

The nucleotide sequences of PHA synthase gene were aligned and the conserved region was selected using Clustal X program. The primer was then designed from the conserved region. The values of Tm, %GC and primer dimer were determined by Vector NTI program.

Amplification of PHA synthase gene

To amplify the PHA synthase gene, the step of annealing of PCR at different temperatures: 50, 52.5, 55 and 60°C for 30 sec were investigated. The amplified gene was detected by agarose gel electrophoresis.

RESULTS

The total of 58 strains of purple non-sulfur bacteria could be isolated from 108 samples of chicken faeces. They were cultivated under micro-aerobic condition under light (3,000 Lux) at 35°C for 5 days. There was no PHB observed by fluorescence microscopy and GC analysis. In contrast, there were 5 strains: CH12, CH52, CH72, CH90 and CH92 showed PHA granules under fluorescence microscopy after cultivating in GM medium without added glutamic acid. CH12 gave the

highest PHB, 5% (w/w) of the CDW (Figure 1). The CH12 strain was selected as the potent strain for further study. The 16S rDNA sequence analysis of CH12 strain showed a high homology of 100% correlation to that of *Rhodopseudomonas palustris* strain NCIB8288. Regarding the taxonomic characteristics and 16S rDNA sequence analysis, CH12 strain was identified as *Rps. palustris* NCIB8288. The CH12 could accumulate the highest amount of PHB, 15% (w/w) of CDW, after 3 day-cultivation under micro-aerobic condition with light (2,000-2,500 Lux) in GM medium in the presence of butyrate instead of glutamic acid, pH 9.0 at 35°C. The activity of PHA synthase of *Rps. palustris* CH72 was 24.57 unit/mL. Forward and reverse primers were designed based on the conserved region of PHA synthase gene (classI) among various strains of *Rps. palustris*. The optimal temperature of annealing step was 52.5°C. The PCR product was detected on agarose gel electrophoresis corresponded to the defined length of 538 bp (Figure 2).

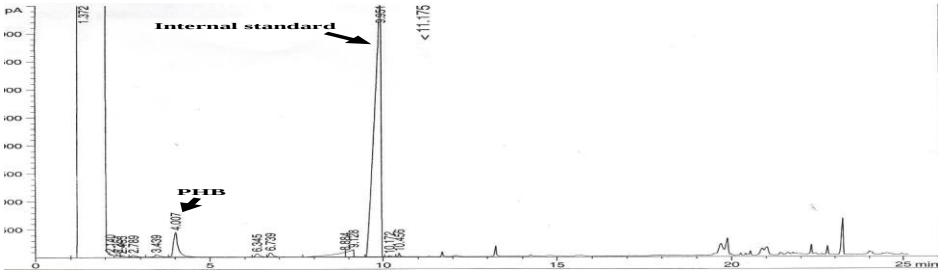

Figure 1. Polyhydroxybutyric acid propyl ester of freeze-dried CH12 cells by GC analysis

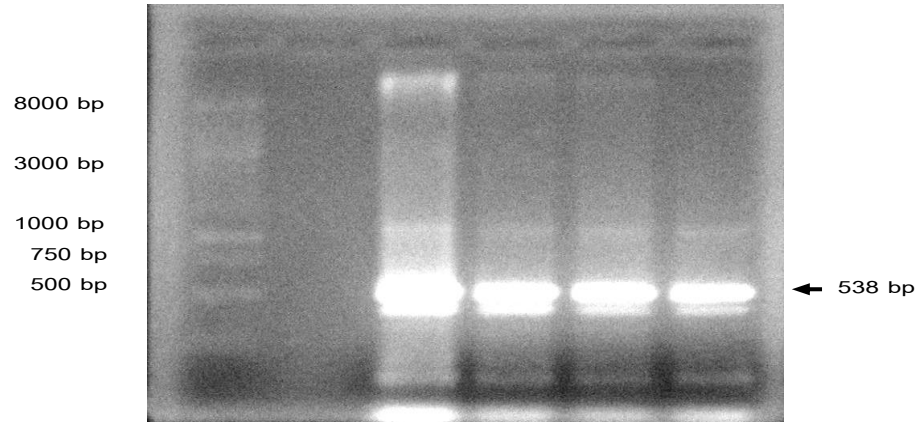

Figure 2. *phaC* gene amplified by PCR

CONCLUSIONS

The CH12 could accumulate the highest amount of PHB, 15% (w/w) of CDW, after 3 day-cultivation under micro-aerobic condition with light (2,000-2,500 Lux) in GM medium in the presence of butyrate instead of glutamic acid, pH 9.0 at 35°C. To increase the expression of PHA synthase gene, the gene cloning should be further studied.

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REFERENCES

1. Steinbuchel A. 1992. Biodegradable plastics. Curr. Opin. Biotechnol. 3, 291-297.
2. Salehizadeh H, Loosdrecht Van MCM. 2004. Production of polyhydroxyalkanoates by mixed culture: recent trends and biotechnological importance. Biotechnol. Adv. 22(3), 261-279.
3. Gobi K, Vadivelu VM. 2014. Aerobic dynamic feeding as a strategy for in situ accumulation of polyhydroxyalkanoate in aerobic granules. Bioresour. Technol. 161, 441-445.
4. Reush RN, Shabalin O, Crumbaugh A, Wagner R, Schröder O, Wurm R. 2002. Posttranslational modification of *E. coli* histone-like protein H-NS and bovine histones by short-chain poly-(R)-3-hydroxybutyrate (cPHB). FEBS Lett. 527, 319-322.
5. Reush RN, Sadoff, HL. 1983. D-(-)Poly-β=hydroxybutyrate in membranes of genetically competent bacteria. J Bacteriol. 156, 778-788.
6. Pfennig N, Truper HG. 1989. Anoxygenic phototrophic bacteria. In: Staley JT, Bryant MP, Pfennig N (eds), Bergey's Manual of Systematic Bacteriology, Vol. 3, Williams and Wilkins, Baltimore, MD, pp 1635-1637.
7. Kobayashi M, Katsura E, Kondo T. 1978. *Chromatium* sulfite reductase. I. Characterization of thisulfate-forming activity at the cell extract level. J. Biochem. 84, 1209-1215.
8. Kobayashi M, Kobayashi M. 1995. Waste Remediation and treatment using anoxygenic phototrophic bacteria. In: Blankenship RE, Madigan MT and Bauer CE (eds), Anoxygenic Photosynthetic Bacteria, Vol. 2., Kluwer Academic Publishers, London, pp 1269-1282.
9. Riis V, Mai W. 1988. Gas chromatographic determination of poly-β-hydroxybutyric acid in microbial biomass after hydrochloric acid propanolysis. J. Chromatogr. 445, 285-289.

Microemulsion and biological activity of Indian gooseberry extract

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ABSTRACT

The purpose of this study was to develop a new formulation for microemulsion delivery of the most yielded variety (56.61%) of *Phyllanthus emblica* fruit extracts which was antioxidant and antityrosinase effective and exhibited ellagic acid as the RP-HPLC prominent peak. Formulations were prepared by HLB 10 and HLB 12 phase diagram (HLB, Hydrophilic lipophilic balance). The amount of mixed surfactants (Tween80 and Span80), oils (mineral oil, coconut oil, grape seed oil, sunflower oil and jojoba oil) and water were varied to select suitable proportions for microemulsion preparation. The resulted microemulsions were clear and golden-yellow. The appearance, color, particle size and % entrapment efficiency of the selected *P. emblica* extract loaded micro emulsion was evaluated. The particle size of microemulsion exhibited in range of 150-200 nm, the smallest was sunflower oil No. 81 HLB 12 at 154 ± 0.01 nm. The % entrapment efficiency of microemulsion was in the range of 85-96%, and grape seed oil No.81 HLB 12 gave the maximum entrapment efficiency of 95.17 % ± 0.01%. All of them were stable under heating-cooling test at 4°C 24 hrs and 45°C 24 hrs for 6 cycles.

Keywords: Microemulsion, Hydrophobic lipophilic balance, *Phyllanthus emblica*, antioxidant, antityrosinase, RP- HPLC

INTRODUCTION

Microemulsion is thermodynamically stable transparent system dispersions of oil and water stabilized by interfacial film of surfactant frequently in combination with co-surfactant. The droplet size of microemulsion is usually in 20-200 nm. Microemulsion is easy to prepare without energy consumption during preparation. Microemulsion formation is reversible, it may become unstable at low or high temperature but when the temperature returns to the range microemulsion reforms. Microemulsion is used as a delivery system to improve the efficacy of a drug, thus the total dose could be minimized together with the size effects [1, 2].

Phyllanthus emblica L. (EUPHORBIACEAE), Indian gooseberry or Ma-khampom (in Thai), is a tree of small to moderate size found in tropical Southeast Asia and throughout Malaysia and East Timor. The fruit is commonly used in Asian traditional medicine as an alternative treatment of diarrhea, jaundice, skin disorders, inflammations, premature graying and the burning sensation of the body. The fruit contains vitamin C, gibberellins, lupeol, kaempferol, quercetin, emblicanin A and B, punigluconin, pedunculaginn, phyllanthin, zeatin, amlaic acid, corilagin, ellagic acid, putranjivain A, digalic acid, phyllemblic acid, emblicol and galactaric acid [3]. *P. emblica* fruit extract is anti-tyrosinase and antioxidant effective which is beneficial as an agent for skin-lightening and skin protection against UV induced free radicals [4].

This study was to develop a formulation for microemulsion delivery of *P. emblica* fruit extract by HLB 10 and HLB 12 phase diagram (HLB, Hydrophilic lipophilic balance) with varying amounts of mixed surfactants (Tween80 and Span80), oils (mineral oil, coconut oil, grape seed oil, sunflower oil and jojoba oil) and water. The appearance, color, particle size and % entrapment efficiency of the stable microemulsion was evaluated. Comparative chemical and biological properties of 5 varieties of *P. emblica* were also reported.

MATERIALS AND METHODS

Plant materials

Fruits powder of *P. emblica* was provided by the Agricultural Technology Department of Thailand Institute of Scientific and Technological Research (TISTR).

Preparation of the extracts

Five varieties of *P. emblica* fruit powder, 500 g each, was separately macerated with ethanol-water for 3 nights, filtered, rinsed and evaporated at 45 °C under reduced pressure using a rotary evaporator.

RP-HPLC of the extract

RP-HPLC at 285 nm of the most yielded *P. emblica* extract was carried out on a X-terra RP18 (3.9 mm x 150 mm, 5 µ) column, using 0.2% formic acid (A) and acetonitrile (B) as mobile phase at 0.6 ml/ min, 30˚C.

Total flavonoid content assay

Total flavonoid content was measured by Aluminium chloride colorimetric assay. One milliliter of sample or standard solutions of rutin was added to a 10 mL volumetric flask containing 4 mL of distilled water. At T_0 (start time), 0.3 mL of 5%NaOH was added and after 5 min (T_5) add 0.3 mL of 10%$AlCl_3$. At 6 min (T_6), 2 mL of 1M NaOH was added, and then added water to make 10 mL and mixed well. The absorbance was measured at 510 nm versus a blank.

Total phenols and tannin assay

Total phenol and tannin contents were determined by Folin-Ciocalteau assay using gallic acid and ellagic acid as the standard solution respectively. Sample or standard solution 0.1 mL was added to a 25 mL test tube containing 8.4 mL of distilled water. Folin-Ciocalteau reagent 0.5 mL was added to the mixture and shaken for 5 min before adding 1 mL of 20%Na_2CO_3 solution. The mixture was shaken before incubated in the dark for 1 hour at room temperature. The absorbance was measured against the reagent blank at 760 nm.

Antioxidant assay

Antioxidant activity of *P. emblica* extracts were determined by DPPH radical scavenging micro-plate method as described by Potduang *et al.* [5]. Mixed 0.1 mL each of absolute ethanol solutions of the extract and 0.06 mM DPPH (2, 2-diphenyl-1-picrylhydrazyl) in a micro-plate for 30 min. Measured the 517 nm absorbance using a micro-plate reader. All samples were run in triplicate compared to BHA. Calculated from the equation:

$$\% \text{ Scavenging} = 100 \times [C-(A-B)] / C$$

The absorbance are represented as A = the reaction mixture, B = blank (ethanol) and C = control (DPPH in ethanol). Calculated the EC_{50} using the resulted linear equation of %scavenging *vs.* log concentration.

Antityrosinase assay

Antityrosinase activity of *P. emblica* extracts were determinded by the Dopachrome micro-plate method as described by Potduang *et al.* [6]. The solutions of 50 µL extract in 20% ethanol, 50 µL of 314.8U/mL mushroom tyrosinase enzyme in buffer and 100 µL of 0.02 M sodium phosphate buffer (pH 6.8) were mixed. After 10 min, added 50 µL of 0.34 mM L-Dopa (Sigma) in buffer, mixed well and measured the absorbance at 492 nm by a micro-plate reader. Re-measured the absorbance after 2 min. All samples were run in triplicate compared to Kojic acid. Calculated from the equation:

$$\% \text{ Tyrosinase inhibition} = [(A-B) - (C-D)] \times 100 / (A-B)$$

The absorbance differences are represented as A = control (L-Dopa + enzyme), B = blank 1 (L-Dopa), C = reaction mixture (enzyme + sample + L-Dopa) and D = blank 2 (sample + L-Dopa). Calculated the IC_{50} using the resulted linear equation of %tyrosinase inhibition *vs.* log concentration.

Preparation of *P. emblica* extract loaded microemulsion

The O/W microemulsions were prepared using pseudo-ternary phase diagram at room temperature. HLB 10 and HLB 12 were used to calculate the condition of microemulsion form. Different oils (mineral oil, coconut oil, grape seed oil, sunflower oil and jojoba oil) and surfactants (tween 80, span 80) were used. 0.5 g *P. emblica* extract were mixed with 10 g water, and then made to 100 g without heating by mixing with various mixtures constituted by oil, surfactant and water in varying constant percentages from 0 to 100.

Evaluation of microemulsion

The organoleptic of microemulsion was observed including color, odor, and cloudiness.

Stability assessment

The stability of microemulsion was assessed under extreme by heating at 60˚C for 5 hours in an oven for separation check [7], and by heating-cooling assay at 4˚C 24 hrs and 45˚C 24 hrs for 6 cycles.

Droplet size determination

The droplet size of microemulsion was investigated in triplicate, using Mastersizer 2000 which measures particles in wide range from 0.02-2,000 μm. The measurements were conducted in distilled water with 10% laser obscuration.

Entrapment efficiency assessment

The 1.5 g of microemulsion was dissolved in 5 ml methanol, vortexed for 2 min and centrifuged at 6,000 rpm for 10 min at 10 °C. Discarded the clear solution, and then added 5 mL methanol and sonicated for 15 min. The absorbance at 282 nm was measured and used to calculate from the equation [8, 9]:

$$\% \text{ Entrapment efficiency} = [\text{Amount of drug encapsulated} / \text{Total loading amount}] \times 100$$

Data analysis

All experimental measurements were triplicate performed. Result values were expressed as mean value ± standard deviation. Statistical significance in this study was examined using analysis of variance (ANOVA). The value of $p < 0.05$ was considered statistical significant.

RESULTS

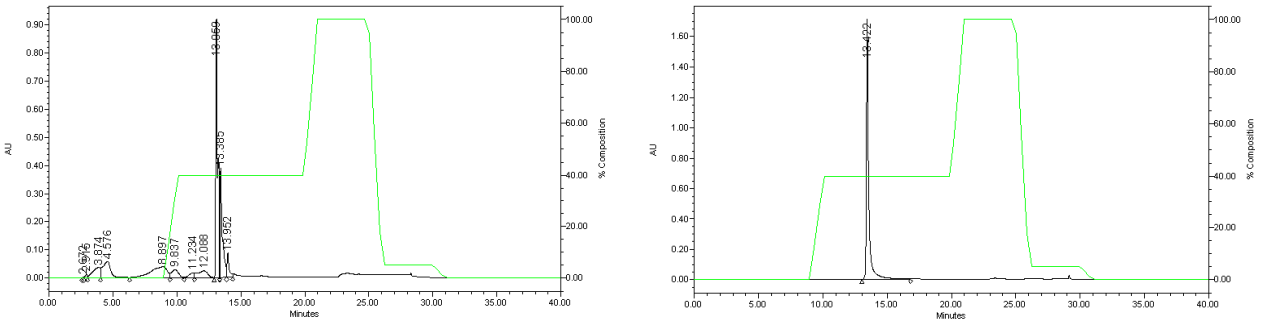
The ethanol-water extract of 5 varieties of *P. emblica* yielded 5.67 ± 0.01 - 56.61 ± 0.07 %. The extract No. 5 gave the maximum yield ($p < 0.05$) of 56.61 ± 0.07 %, in comparison to other *P. emblica* extracts. The extracts showed the antioxidant effects at the EC_{50} range of 2.04 ± 0.01 - 3.22 ± 0.01 μg/mL with the significantly difference of $p < 0.05$. They exhibited antityrosinase activity at the IC_{50} range of 2.78 ± 0.08 - 4.29 ± 0.04 mg/mL, but the IC_{50} of antityrosinase was not significantly different at $p < 0.05$. The range of chemical constituents in the *P. emblica* fruit extracts were total phenolic compounds at 222.82 ± 0.05 -518.56 ± 0.01 mg gallic acid equivalent/g sample, total flavonoids at 86.75 ± 0.05 - 122.10 ± 0.02 mg rutin equivalent/g sample and total tannins at 110.11 ± 0.25 - 928.52 ± 0.02 mg ellagic acid equivalent/g sample as shown in Table 1. The difference in total flavonoids, total phenols and total tannin of extracts were significant at $p < 0.05$.

Table 1. Comparative %yield, biological activities and chemical properties of *P.emblica* extracts

Varieties	% yield	Antioxidant EC_{50} (μg/ml)	Antityrosinase IC_{50} (mg/ml)	Total flavonoids (mg rutin quivalent/ g sample)	Total phenols (mg gallic acid equivalent/ g sample)	Total tannin (mg ellagic acid equivalent/ g sample)
1	33.63 ± 0.73[a]	2.14 ± 0.02[a]	3.48 ± 0.74[a]	122.10 ± 0.02[a]	333.03 ± 0.03[a]	928.52 ± 0.02[a]
2	30.13 ± 0.12[a]	2.04 ± 0.01[a]	2.78 ± 0.08[a]	88.45 ± 0.02[a]	314.68 ± 0.01[a]	796.13 ± 0.44[a]
3	43.33 ± 0.35[a]	2.22 ± 0.01[a]	3.63 ± 0.28[a]	86.75 ± 0.05[a]	337.86 ± 0.01[a]	917.71 ± 0.53[a]
4	5.67 ± 0.01[a]	3.11 ± 0.30[a]	4.29 ± 0.04[a]	101.65 ± 0.02[a]	222.82 ± 0.05[a]	638.43 ± 0.08[a]
5	56.61 ± 0.07[a]	2.19 ± 0.01[a]	3.05 ± 0.37[a]	103.60 ± 0.07[a]	518.56 ± 0.01[a]	110.11 ± 0.25[a]

Different superscript in the same column indicated significant differences ($P < 0.05$)

RP-HPLC chromatogram of *P.emblica* extract showed a prominent peak of compound at 13.059 min which was the same peak as standard ellagic acid (13.422 min) when spiked, as shown in Figure 1.



1 **2**

Figure 1. RP-HPLC chromatograms of *P. emblica* (A) and standard ellagic acid (B)

The 5th variety of *P. emblica* extract which gave the most yielded extract of 56.61 ± 0.07 % was chosen to prepare microemulsions using pseudo-ternary phase diagrams (HLB 10 and HLB 12) at room temperature. The suitable ratio of microemulsions which were non-separated after heating test and 6 cycles of heating-cooling test are shown in Table 2.

Table 2. The suitable ratio of microemulsion

HLB	Type of oil	Sample No.	% oil	% surfactant	% water
10	Grape seed oil	sample No.77	25	65	10
		sample No.78	20	70	10
		sample No.79	15	75	10
	Sunflower oil	sample No.77	25	65	10
		sample No.78	20	70	10
		sample No.79	15	75	10
12	Mineral oil	sample No.75	35	55	10
		sample No.76	30	60	10
		sample No.77	25	65	10
		sample No.78	20	70	10
		sample No.79	15	75	10
		sample No.80	10	80	10
		sample No.81	5	85	10
	Grape seed oil	sample No.76	30	60	10
		sample No.77	25	65	10
		sample No.78	20	70	10
		sample No.79	15	75	10
		sample No.80	10	80	10
		sample No.81	5	85	10
	Sunflower oil	sample No.76	30	60	10
		sample No.77	25	65	10
		sample No.78	20	70	10
		sample No.79	15	75	10
		sample No.80	10	80	10
		sample No.81	5	85	10

Table 3 shows droplet size and percentgae of entrapment efficiency of the stable microemulsions containing *P. emblica* extract with different oil i.e., grape seed oil, sunflower oil and mineral oil. The results indicated that only 6 samples of the microemulsions had the droplet size within the range of 154 ± 0.01 to 199 ± 0.01 nm. Sunflower oil gave the significant ($p < 0.05$) smallest size (154 ± 0.01 nm) microemulsion. Grape seed oil gave the significant ($p < 0.05$) maximum % entrapment efficiency of 95.170 ± 0.01 %.

Table 3. Particle size and entrapment efficiency of the stable microemulsions

Sample	Size (nm)	% Entrapment
Sunflower oil, No. 81 HLB12	154	94.960
Grape seed oil, No.81 HLB12	163	95.171
Mineral oil, No 75 HLB12	184	90.776
Mineral oil, No 76 HLB12	199	91.159
Mineral oil, No 80 HLB12	156	93.366
Mineral oil, No 81 HLB12	155	89.554

CONCLUSIONS

The 5th variety of *P.emblica* extract which gave the most yielded extract of 56.61% was chosen to prepare microemulsions. The extract exhibited antioxidant effects at EC_{50} = 2.44 µg/ml, and antityrosinase activity at IC_{50} = 3.04 mg/ml. Its total phenol was 518.66 mg gallic acid equivalent/g sample, total flavonoids was 103.60 mg rutin equivalent/g sample and total tannins was 110.11 mg ellagic acid equivalent/g sample. Sunflower oil gave the smallest size microemulsion of 154 nm. Grape seed oil gave the microemulsion of maximum entrapment efficiency at 95.17 %

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REFERENCES

1. Azeem A, Rizwan M, Ahmad FJ, Khan ZI, Khar RK, Aqil M, et al. 2008. Emerging role of microemulsions in cosmetics. Recent Pat. Drug Deliv. Formul. 2, 275-289.
2. Singh PK, Iqubal MK, Shukla VK, Shuaib M. 2014. Microemulsion: Current Trends in Novel Drug Delivery System. J. Pharm. Chem. Biol. Sci. 1, 39-51.
3. Phyllanthus emblica L. Available at http://www.globinmed.com/index.php?option=com_content&view=article&id=79255:phyllanthus- emblica-l&catid=718:p&Itemid=150, accessed 2 December 2015.
4. Penning A. 2012. Beauty in layers: Multitasking ingredients. GCI Magazine, 5 pages. Available athttp://www.gcimagazine.com/marketstrends/segments/antiaging/156350405.html?page=3, accessed December 2015.
5. Potduang B, Chongsiriroeg C, Benmart Y, Giwanon R, Supatanakul W, Tanpanich S. 2007. Biological Activities of *Schefflera leucantha*. Afr. J. Trad. CAM. 4(2):157-164.
6. Potduang B, Meeploy M, Giwanon R, Benmart Y, Kaewduang M and Supatanakul W. 2008. Biological activities of *Asparagus racemosus*. Afr. J. Trad. Complement. Altern. Med. 5(3), 230-237.
7. Rukmini A, Raharjo S, Hastuti P, Supriyadi S. 2012. Formulation and stability of water-in-vergin coconut oil microemulsion using ternary food nonionic surfactants. Int. Food Res. J. 19(1), 259-264.
8. Saranyu Songkho, Wiwat Pitchayakorn and Juraithip Hwangsinthaweekul. 2010. Plauoi-extract in microemulsion for treatment of dermatitis. The Thailand Research Fund.
9. Siriphan P, Chutimaworapan S, Chaichantipyut C. 2008. Antioxidant evaluation and formulation development of Zingiber officinale extract loaded in solid lipid nanoparticles for skin delivery. Thesis (M.Sc.) Chulalongkorn University.

Formulation development of self-emulsifying tea seed oil

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ABSTRACT

In this study, tea seed oil (*Camellia Oleifera*) was formulated into self-emulsifying oil formulations (SEOF) for enhancing the aqueous solubility and bioavailability of essential fatty acids. Self-emulsifying tea seed oils were developed by using different concentration of lecithin in combination with linker molecules (Span®80 as the lipophilic linker and Tween®80 as the hydrophilic linker). The developed SEOF were investigated for physicochemical properties including appearance, emulsion droplets size, size distribution and zeta potential. In addition, the chemical compositions of tea seed oil and developed SEOF were compared using gas chromatography-mass spectrometry (GC-MS). It was found that lecithin-linker systems were able to provide clear liquid and stable formulations. The selected SEOF were then diluted with fed-state simulated gastric fluid (FeSSGF) to produced O/W emulsions, the droplet size obtained was close to 200 nm with low PDI values and the zeta potential was negative. GC-MS chromatograms revealed that chemical compositions of developed SEOF were not significantly different from original tea seed oil. Oleic acid (C18:1) was found to be the predominant fatty acid constituent in both of them.

Keywords: Self-emulsifying oil formulations, *Camellia Oleifera*, Lecithin, Linker molecules

INTRODUCTION

Self-emulsifying delivery systems (SEDS) are a vital method that used to improve the solubility and bioavailability of lipophilic bioactive compounds. These systems can form fine oil in water (O/W) emulsions when introduced into an aqueous phase, such as GI fluids, under mild agitation [1,2]. For purposes of oral administration, self-emulsifying oil formulations (SEOF) should be safe for consumption. Lecithin is especially desirable for using as a good surface active agent in food applications because it's GRAS status. However, the use of lecithin tends to form liquid crystal phases that promote the formation of undesirable emulsions. This limitation has been overcome with the introduction of linker molecules in lecithin microemulsion systems. It was found that the linker molecules associate with lecithin leads to a special self-assembly of hydrophilic and lipophilic linkers resulted in more stable emulsions [3].

Tea seed oil (*Camellia Oleifera*) is one of the most important edible oils which are an excellent source of unsaturated fatty acids. It has been reported that tea seed oil provides the great health benefits due to its high oleic acid composition [4,5]. It also contains a variety of bioactive components, such as vitamin E, phytosterols, squalene and flavonoids. Several studies have reported that the consumption of tea seed oil can significantly provide health-promoting effects. Long-term intake is helpful in preventing coronary heart disease, cancer, arteriosclerosis and increase gastrointestinal absorption function [4,6].

In this work, self-emulsifying oil formulations (SEOF) of tea seed oils (*Camellia Oleifera*) were developed with the aim of increasing its solubility to achieve higher bioavailability. The self-emulsifying tea seed oil was formulated with soybean lecithin in combination with linker molecules (Span®80 as the lipophilic linker and Tween®80 as the hydrophilic linker). The appearance, emulsion droplets size and zeta potential of developed formulations were studied. In addition,

the GC-MS analysis was performed to evaluate the chemical composition of developed SEOF compare to original tea seed oil.

MATERIALS AND METHODS

Construction of ternary phase diagram

Ternary phase diagram was constructed according to the preparation of various formulations containing different ratios of tea seed oil, lecithin and linker molecules (Span®80/Tween®80 ratio of 1:1). In our case, we used hydrophilic linker (Tween®80) to exhibit polar characteristics in the system. Each formulation was stirred thoroughly for 5 h (500 rpm) and kept at ambient temperature overnight. Finally, the prepared formulations were observed for their appearance and categorized as isotropic mixtures in phase diagram when they were yellowish clear liquids.

Preparation of self-emulsifying oil formulations (SEOF)

Series of formulations comprising different concentrations of tea seed oil, lecithin and linker molecules (Span®80/Tween®80 ratio of 1:1) were prepared base on the ternary phase diagram. The mixtures were added in clear glass vials and thoroughly stirred at 500 rpm overnight.

Emulsion droplet size and zeta potential measurement

The samples were diluted 1000 folds with fed-state simulated gastric fluid (FeSSGF) pH 6.4 and induced agitation by votex mixer. These mixtures were then analyzed for determined emulsion droplet size, PDI and zeta potential using Malvern zetasizer. All study experiments were conducted in triplicate to ensure reproducibility and the mean values were reported.

Chemical composition analysis

The tea seed oil and developed SEOF were methyl-esterified and analyzed by gas chromatography-mass spectrometry (GC-MS) which equipped with DB-wax column. Fatty acid composition was expressed as percentages of total fatty acids.

Statistical analysis

The experiments were performed in triplicate and values were expressed as mean ± SD. One-way analysis of variance (ANOVA) was used when comparing emulsion droplet size, PDI and zeta potential value of SEOF. The differences between means were evaluated by Tukey's test.

RESULTS AND DISCUSSION

The ternary phase diagram of tea seed oil with different concentrations of lecithin and linker molecules (Span®80/Tween®80 ratio of 1:1) was showed in Figure 1. The grey region represented yellowish clear liquids of tea seed oil when formulated into SEOF. It was observed that the combination of hydrophilic and lipophilic linkers alone cannot provided isotropic liquids when mixed with tea seed oil. This could be explained on the basis that Tween®80 which is a hydrophilic linker (HLB = 15) induced an immiscible oil/linker molecules mixture [7]. On the other hand, the use of an appropriate concentration of lecithin in combination with linker molecule blends could provide yellowish clear liquids. There have been found that linker-based microemulsion formulation with lecithin provided an excellent oil solubilization capacity for a wide range of oils [8-9]. Athas et al. (2014) also reports that unsaturated tails of lecithin and Tween®80 help them to interact synergistically with each other leading to a tightly packing at the surface of the oil [10].

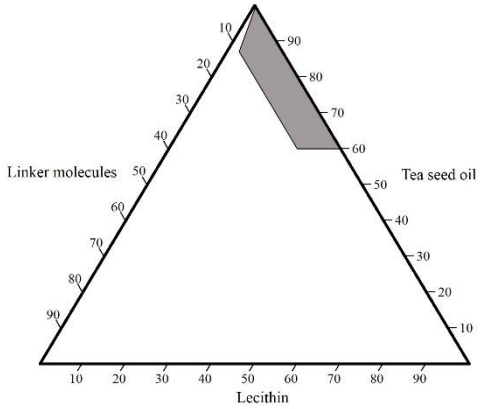

Figure 1. Ternary phase diagram showing the region of clear isotropic liquids for lecithin-linker formulations

The series of developed SEOF were prepared based on the ternary phase diagrams. After aqueous dilution with fed-state simulated gastric fluid (FeSSGF), the droplet size of all developed formulation was close to 200 nm with low PDI values (< 0.3). These results indicated that the system has narrow size distribution which leads to more stability of emulsion. As shown in Table 1, the zeta potential was negative in a range between -6.16 to -20.39 mV due to the presence of free fatty acids in the formulation [1, 11]. The higher loading concentrations of linker molecules (nonionic surfactant) in the formulations cause an increase of their zeta potential close to 0 mV. The appearance of developed SEOF compared with tea seed oil is shown in Figure 2.

Table 1. Emulsion droplet size, polydispersity index (PDI) and zeta potential (ZP) of self-emulsifying oil formulations (SEOF).

Lecithin (%)	linker molecule blends (%)	Droplet size (nm)^{NS}	PDI	ZP (mV)
3	0.1	216.34±16.38	0.271±0.058^{ab}	-17.98 ± 1.63^{a}
	2	208.20±7.03	0.277±0.048^{b}	-7.78 ± 0.73^{cd}
	4	220.42±15.77	0.244±0.029^{ab}	-6.16 ± 0.63^{d}
6	0.1	225.29±29.39	0.264±0.022^{ab}	-18.86 ± 2.57^{a}
	2	209.30±8.49	0.239±0.022^{ab}	-11.03 ± 1.77^{bc}
	4	221.60±20.00	0.195±0.018^{ab}	-7.85 ± 0.71^{cd}
9	0.1	222.74±15.13	0.259±0.022^{ab}	-18.87 ± 1.45^{a}
	2	212.42±8.34	0.204±0.031^{ab}	-11.97 ± 1.30^{bc}
	4	214.84±9.15	0.190±0.020^{ab}	-8.99 ± 0.73^{bcd}
12	0.1	224.91±23.79	0.239±0.031^{ab}	-20.39 ± 2.57^{a}
	2	206.40±3.00	0.175±0.040^{a}	-13.08 ± 1.44^{b}
	4	208.51±10.27	0.197±0.010^{ab}	-10.39 ± 1.35^{bc}

All data are presented as mean ± SD (n= 3).

NS = Not statistically different.

Means in the same column followed by different letters (a-d) were significantly different ($p < 0.05$).

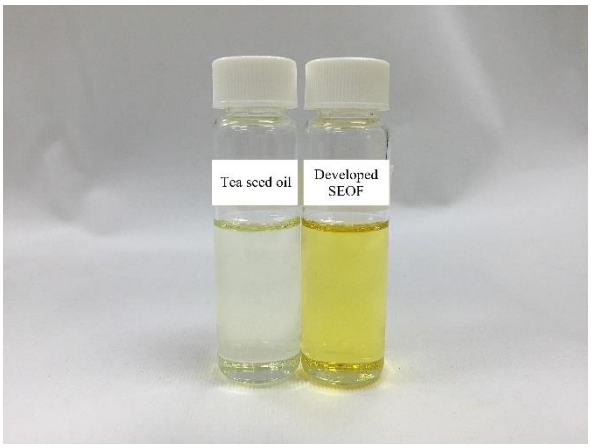

Figure 2. Appearance of tea seed oil (left) and developed self-emulsifying oil formulation (SEOF) (right)

The GC-MS chromatograms of tea seed oil and developed SEOF are shown in Figure 3. It was found that the fatty acid composition profile of developed SEOF was not significantly different from original tea seed oil. The results showed a variety of fatty acids, consisting of palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2).

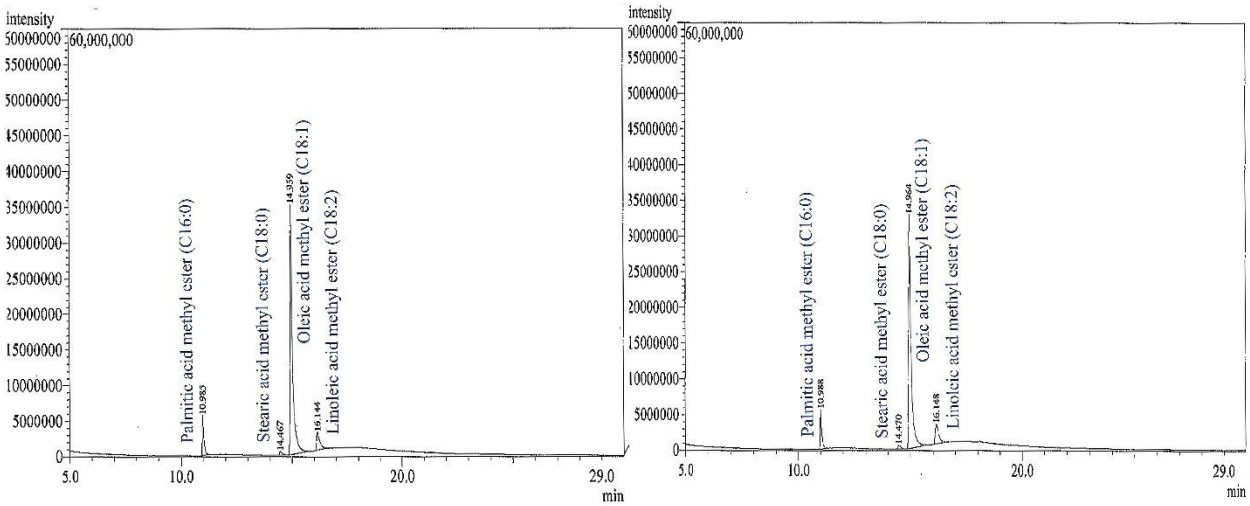

Figure 3. GC-MS chromatograms of tea seed oil (left) and developed self-emulsifying oil formulation (SEOF) (right) after transesterification.

Table 2. Levels (%) of fatty acid composition obtained from tea seed oil and developed self-emulsifying oil formulations (SEOF).

Sample	Palmitic acid (C16:0)	Stearic acid (C18:0)	Oleic acid (C18:1)	linoleic acid (C18:2)
Tea seed oil	8.83±0.13	1.37±0.05	82.17±0.10	7.64±0.13
developed SEOF (Tea seed oil+3% lecithin+0.1% linker molecule blends)	9.01±0.04	1.52±0.07	80.64±0.32	8.83±0.29

Remarkably high levels of unsaturated oleic acid (80.64-82.17%) were found in both of tea seed oil and developed SEOF, followed by palmitic acid (8.83-9.01%), linoleic acid (7.64-8.83%) and stearic acid (1.37-1.52%) (as shown in Table 2). Su et al. (2014) reported that major fatty acid found in tea seed oil (*Camellia* species) was oleic acid with the levels ranged between 41.1- 89.0%, depending on sample collection locations. The presence of high amounts of oleic acid gives tea seed oil a better health-promoting effects and higher storage stability [5].

CONCLUSIONS

In conclusion, self-emulsifying tea seed oil formulations were successfully prepared by using lecithin-linker system. The FeSSGF dilution of developed formulations produced O/W emulsions with droplet size close to 200 nm. The GC-MS analysis demonstrated that oleic acid was the major fatty acid in self-emulsifying tea seed oil formulation.

ACKNOWLEDGEMENTS

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REFERENCES

1. Kumar A, Sharma S, Kamble R. 2010. Self emulsifying drug delivery system (SEDDS): future aspects. Int. J. Pharm. Pharm. Sci. 2(4), 7-13.
2. Shrivastava S, Yadav SK, Verma S. Applications of self emulsifying drug delivery systems in novel drug delivery- a review. 2014. African J. Basic & Appl. Sci. 6(1), 6-14.
3. Chu J, Cheng YL, Rao AV, Nouraei M, Zarate-Muñoz S, Acosta EJ. 2014. Lecithin-linker formulations for self-emulsifying delivery of nutraceuticals. Int. J. Pharm. 471, 92-102.
4. He L, Guo-ying Z, Huai-yun Z, Jun-ang L. 2011. Research progress on the health function of tea oil. J. Med. Plants Res. 5(4), 485-489.
5. Su MH, Shih MC, Lin KH. 2014. Chemical composition of seed oils in native Taiwanese *Camellia* species. Food Chem. 156, 369-373.
6. Lv GP, Aoli M, Zhou B, Zhao J. 2013. Development of a rapid and simple non-derivatization method to determine constituents and antioxidative capacity of camellia oils by HPTLC. Food Nutr. Sci. 4, 204-210.
7. Eid AMM, Baie SH, Arafat OM. 2012. The effect of surfactant blends on the production of a novel *Swietenia macrophylla* oil self-nanoemulsifying system. Int. J. Pharm. Pharm. Sci. 4(4), 481-486.
8. Yuan JS, Ansari M, Samaan M, Acosta EJ. 2008. Linker-based lecithin microemulsions for transdermal delivery of lidocaine. Int. J. Pharm. 349, 130-143.
9. Acosta EJ, Nguyen T, Witthayapanyanon A, Harwell JH, Sabatini DA. 2005. Linker-based bio-compatible microemulsions. Environ. Sci. Technol. 39, 1275-1282.
10. Athas JC, Jun K, McCafferty C, Owoseni O, John VT, Raghavan SR. 2014. An effective dispersant for oil spills based on food-grade amphiphiles. Langmuir. 30(31), 9285-9294.
11. Kohli K, Chopra S, Dhar D, Arora S, Khar RK. 2010. Self-emulsifying drug delivery systems: an approach to enhance oral bioavailability. Drug Discov. Today. 15, 958-965.

The development of *Phyllanthus emblica* and *Zanthoxylum limonella* mouthwash

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ABSTRACT

This research aimed to develop a concentrated mouthwash comprising the patented mix-extract from edible fruits of *Phyllanthus emblica* L. (EUPHORBIACEAE) and *Zanthoxylum limonella* Alston (RUTACEAE), locally known as Ma-khampom and Ma-khwaen respectively. The mix extract is antimicrobial, anti-inflammatory and *in vitro* anti-oxidant (EC_{50} 7.9 mg/ml) effective. The developed mouthwash was an antiseptic solution intended to reduce the microbial load in the oral cavity. It was formulated in 3 formulas with varying amounts of the active extract. They gave similar odor and flavor. The selected formula is stable with acceptable opacity and was active against tested microbial using tissue disk agar diffusion method.

Keywords: Mouthwash, *Phyllanthus emblica*, *Zanthoxylum limonella*

INTRODUCTION

Mouthwash or mouth rinse is an antiseptic solution used to enhance oral hygiene as to kill the bacterial plaque causing cavities, gingivitis and bad breath[1]. Peppermint oil was used in some mouthwashes to counteract the bad breath associated with bacteria growth within the mouth. Alcohol, sodium fluoride, and hydrogen peroxide are also used as effective anti-bacterial ingredients. Although alcohol is an effective bacteria killer, it also dries out the mouth, which can make the problem worse [2].

This research aimed to develop a mouthwash comprising a patented mix-extract from fruits of *Phyllanthus emblica* and *Zanthoxylum limonella* which are anti-microbial and anti-inflammatory effective.

Phyllanthus emblica L. (EUPHORBIACEAE), locally called Ma-khampom, is a tree of small to moderate size found in tropical Southeast Asia and throughout Malaysia and East Timor. The edible fruits are commonly used in Asian traditional medicine to treat diarrehea, jaundice, skin disorders, inflammations, premature graying and the burning sensation of the body. The fruits are rich in vitamin C and contain gibberellins, lupeol, kaempferol, quercetin, emblicanin A and B, punigluconin, pedunculaginn, phyllanthin, zeatin, amlaic acid, corilagin, ellagic acid, putranjivain A, digalic acid, phyllemblic acid, emblicol and galactaric acid [3]. We have found that the ethanol fruit extract of *P. emblica* was anti-microbial (MIC 10-20 mg/mL) effective but not economically enough, and we further found that the water-ethanol extract of the edible *Z. limonella* fruits could enhance the anti-microbial potency of the active extract.

Zanthoxylum limonella Alston (RUTACEAE), locally called Ma-khwaen, is a deciduous tree up to 18 m tall. It is widely distributed in the northern part of Thailand and its edible fruits are traditionally used as spice and in various ailments. The essential oil of the fruits contains sabinene and exhibits the bactericidal and anti-oxidative potential [4,5,6]. We have reported that the water-ethanol fruit extract of *Z. limonella* is effective against tested microbial including *C.*

albicans (MIC 2.5-10 mg/mL). The extract exhibits anti-oxidant activity (EC_{50} 5.94 µg/mL) with total flavonoids of 3.61 mg rutin/g extract [7]. The extract also shows potent anti-tyrosinase (IC_{50} 0.33 mg/mL) activity.

An antiseptic mouthwash was developed from the patented mix-extract fromn fruits of *P. emblica* and *Z. limonella* to benefit from its anti-microbial activity (MIC 4.5 mg/ml) against *P. acnes*, *S. aureus*, *S. epidermidis* and *S. pyogenes*. The mix-extract is also effective as anti-oxidant (EC_{50} 7.9 µg/mL), anti-tyrosinase (IC_{50} 5.52 mg/mL) and anti-inflammatory [8,9]. The assessments of product stability and anti-microbial efficacy were performed.

MATERIALS AND METHODS

Plant material

The dry fruit powder of *P. emblica* and *Z. limonella* were provided by the Agricultural Technology Department, Thailand Institute of Scientific and Technological Research (TISTR).

Preparation of the mix extract

P. emblica extract was prepared by macerated the fruit powder with ethanol-water for 4 nights, filtered, rinsed and evaporated under reduced pressure using a rotary evaporator (Heidolph, Hei-VAP Precision) at 45 °C. *Z. limonella* was extracted with another proportion of ethanol-water for 3 nights, filtered, rinsed and evaporated under reduced pressure. The crude extracts were freshly mixed at a patented appropriate ratio to be effective against tested microbial.

Formulation of the mouthwash

Mouthwash consisting of the *P. emblica* and *Z limonella* mix extract was formulated in 3 formulas with varying amount of ingredients in the master formula as shown in Table 1. The active mix extract was dissolved with a small amount of ethyl alcohol and then added mint essential oil and menthol as odorant and flavoring agent, and thoroughly mixed. Then the co-solvent, surfactant, sweetening agent and salting agent were added respectively and mixed well, before adding the coloring agent and stirred homogeneously.

Table 1. Master formula of the mouthwash

Ingredients	Function	M.F. (%W/W)
Crude extract	Active ingredient	0.1 - 0.5
Ethyl alcohol	Co-solvent	25 - 30
Menthol	Flavoring agent	3.5 - 5.0
Mint essential oil	Odoring agent	3.5 - 5.0
Sodium saccharin	Sweetening agent	4.7 - 5.5
Sodium chloride	Salting agent	1.7 - 2.0
A	Co-solvent, nonionic surfactant	22.1 - 25.0
Color	Coloring agent	0.2 - 0.3
Water q.s.	Vehicle	100

Stability test

The stability of the mouthwash was assessed in accelerated conditions using heating and cooling method at 4 °C for 24 hrs and 45 °C 24 hrs (1 cycle) for 6 cycles. The physical stability was evaluated on turbidity, precipitation and appearance.

***In vitro* anti-microbial assessment of the product:**

A tissue disk agar diffusion assay was performed against *Staphylococcus aureus, S. epidermidis, Streptococcus pyogenes and Candida albicans*. Stock of microorganism was adjusted to the concentration of 0.5 McFarland. The microorganism was distributed evenly over the nutrient agar surface, let dry and then the tissue paper disks were placed on the agar surface. Mouthwash solution (1:1 in water) of 20 µL was pipette onto the tissue disk for 1 min contact before removing the tissue off. Incubated at 37°C for 18-24 hrs, and observed the clear zone of inhibition.

RESULTS

The mouthwash containing the patented mix-extract from fruits of *P. emblica* and *Z limonella* was formulated in 3 formulas with varying amount of the mix extract. The formulas were clear solution and had similar odor and flavor. The color was adjusted giving sky-blue and green in Formula 1 and 2. Formula 1 was selected for their acceptable opacity and stability.

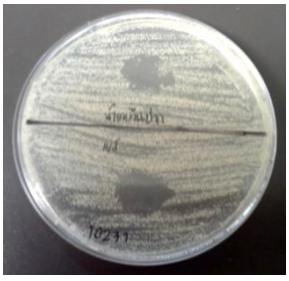
In vitro anti-microbial assessment revealed that the selected mouthwash was active against *Staphylococcus aureus* (DMST 8013), *S. epidermidis* (DMST 12228) and *Candida albicans* (DMST 90028, DMST 10231) and was semi-active against *S. aureus* (DMST 8840) and *Streptococcus pyogenes* (DMST 17020) as shown in Figure 1. The anti-microbial activity was stable after 6 cycles of heating and cooling test (H&C).

Staphylococcus aureus DMST 8013

Staphylococcus aureus DMST 8840

Staphylococcus epidermidis DMST 12228

Streptococcus pyogenes DMST 17020



Candida albicans DMST 10231

Candida albicans DMST 90028

Figure 1. Clear zones of inhibition of the selected *P. emblica* and *Z. limonella* mouthwash (top) compared to the mouthwash base (below).

CONCLUSIONS

The patented mix-extract from fruits of *P. emblica* and *Z. limonella* could be used as an active extract for mouthwash products. The best basic product was Formula 1 which was stable under 6 cycles of heating and cooling test. It gave the mouth fresh and good breath feel after rinsed, and its clarity was acceptable. The product was effective against *Candida albicans* and other tested microbial.

ACKNOWLEDGEMENTS

This work was supported by Thailand Instituted of Scientific and Technological Research (TISTR). We would like to thank Mr. Bundit Fungsin, Director of the Biosciences Department TISTR, for providing microbiology laboratory facilities. We thank all TISTR colleagues who in one way or another help this project to succeed.

REFERENCES

1. Mouthwash. Available at http://en.wikipedia.org/wiki/Mouthwash, accessed November 20, 2015.
2. How effective is mouthwash for bacteria? Available at http://www.wisegeek.com/how-effective-is- mouthwash-for-bacteria.htm, accessed 1 December 2013.
3. *Phyllanthus emblica* L. Available at http://www.globinmed.com/index.php?option=com_content%20&view=article&id=79255:phyllanthus-%20emblica-l&catid=718:p&Itemid=150, accessed November 20, 2015.
4. Mallikarjuna P, Uma Maheswara Rao V, Satyanarayana T. 1999. Antimicrobial activity of *Zanthoxylum limonella*. Indian Drugs. 36(7), 476-478.
5. Tangjitjaroenkun J, Supabphol R, Chavasiri W. 2012. Antioxidant effect of *Zanthoxylum limonella* Alston. J. Med. Plants Res. 6(8), 1407-1414.
6. Charoenying P, Laosinwattana C, Phuwiwat W, Lomratsiri J. 2008. Biological activities of *Zanthoxylum limonella* Alston fruit extracts. KMITL Sci. J. 8, 12-15.
7. Ngamnon Y, Potduang B, Fungsin B, Phasuk S, Takolpuckdee P, Rerk-am U, et al. 2012. Antimicrobial, antioxidant activities and total flavonoids of fruit extracts from Ma-khwaen (*Zanthoxylum limonella*). Thai J. Pharm. Sci. 35, 36.
8. Potduang B, Fungsin B, Keeta I, Ketmanee N, Intarangsie A, Soradech S, et al. 2012. Biological activities of a patented mix extracts from fruits of *Phyllanthus emblica* and *Zanthoxylum limonella*. Thai J. Pharm. Sci. 36, 44-47.
9. Khayungarnnawee A, Soradech S, Potduang B, Sematong T, Laovitthayanggoon S, Keeta I, et al. 2012. Anti-inflammatory activity of fruit extracts from *Phyllanthus emblica* and *Zanthoxylum limonella*. Thai J. Pharm. Sci. 36, 38-40.

The development of *Phyllanthus emblica* and *Zanthoxylum limonella* mouth spray

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ABSTRACT

This research aimed to develop a mouth spray comprising the patented mix-extract from edible fruits of *Phyllanthus emblica* and *Zanthoxylum limonella* to benefit from its antimicrobial, anti-inflammatory and anti-oxidant effects. *P. emblica* and *Z. limonella* are locally known as Ma-khampom and Ma-khwaen respectively. The developed mouth spray was an antiseptic solution to spray into the mouth to make fresh breath and reduce the microbial load in the oral cavity. It was formulated in 7 formulas with varying amounts of ingredients. The selected formula 7 was a clear yellow and sweet liquid solution which gave fresh feeling after applies. The developed mouth spray was stable under heating-cooling test at 4 °C 24 hrs and 45 °C, 24 hrs for 6 cycles. It was active against tested microbial using tissue disk agar diffusion method.

Keywords: Mouth spray, *Phyllanthus emblica*, *Zanthoxylum limonella*, antimicrobial, anti-inflammatory, anti-oxidant

INTRODUCTION

Mouth spray is a solution for spraying into the mouth to produce fresh breath. Some mouth sprays have antibacterial or anti-plaque property. Common flavors used in mouth spray are spearmint and peppermint [1]. This research aimed to develop a mouth spray comprising the patented mix-extract from edible fruits of *P. emblica* and *Z. limonella* which is effective as antimicrobial, anti-inflammatory and *in vitro* anti-oxidant (EC_{50} 7.9 mg/ml). The developed mouth spray is an easy to carry product to refresh up the mouth. The liquid filled bottle is equipped with a product pump spray nozzle, which need no propellant and generate no aerosol. Suitable flavoring agents were used to provide good smell with refresh and relax feeling.

Phyllanthus emblica L. (EUPHORBIACEAE), locally known as Ma-khampom, is an herbal plant commonly used in Asian traditional medicine. Its fresh or dry fruits are used as an alternative treatment of diarrhea, jaundice, skin disorders, inflammations and premature graying. The fruit extract possesses anti-tyrosinase and antioxidant activity. Its phenolic compounds exhibited anti-inflammatory effect [2-4]. Water extract of *P.emblica* has no acute toxicity in rat in terms of behavioral change, mortality or gross appearance of internal organs (LD_{50}>5,000mg/kg). Chronic toxicity by daily oral resulted in slightly significant differences in the body and organ weights [5].

Zanthoxylum limonella Alston (RUTACEAE), locally known as Ma-khwaen, has been extensively used in folk medicines for different medicinal purposes. The fruit has been traditionally used as food flavor in the northern part of Thailand. The essential oil from *Z. limonella* fruits exhibits anti-oxidative potential. The oil contains sabinene which is a potent bactericidal against multi-drug resistant bacteria [6-8].

The patented mix-extract from fruits of *P. emblica* and *Z. limonella* is active as *in vitro* anti-microbial (MIC 4.5 mg/mL) against *S. aureus*, *S. epidermidis* and *S. pyogenes*. It is anti-oxidant (EC_{50} 7.9 µg/mL) and anti-tyrosinase (IC_{50} 5.52 mg/mL). It exhibits potent anti-inflammatory on croton oil-induced rat ear edema better than std. Diclofenac [9-11]. The mix-extract was used to develop a patented antimicrobial mouth refreshing spray.

MATERIALS AND METHODS

Plant materials

The dry fruit powder of *P. emblica* and *Z. limonella* were provided by the Agricultural Technology Department, Thailand Institute of Scientific and Technological Research (TISTR).

Preparation of the mix extract

P. emblica extract was prepared by macerated the fruit powder with ethanol-water, filtered, rinsed and evaporated under reduced pressure using a rotary evaporator at 45 °C. *Z. limonella* was extracted with another proportion of ethanol-water, filtered, rinsed and evaporated under reduced pressure. The crude extracts were freshly mixed when used at a patented appropriate ratio to be effective against tested microbial.

Formulation of the mouth spray

Mouth spray consisting of the *P. emblica* and *Z. limonella* mix extract was formulated in 7 formulas with varying amount of ingredients in the basic formula as shown in Table 1. Sorbitol and glycerin were added to the solution of menthol in ethanol (A). Active ingredients and sweetening agents were dissolved in water (B). Mixed A and B and then added mint oil and a stabilizer.

Table 1 The patented formula of mouth spray

Ingredient	function	%w/w
Mint oil	Flavoring agent	1-2
Ethanol	Solubilizer	40-50
Glycerin	Hemectant	10-20
Sorbitol	Sweetener	5-10
Sodium saccharin	Sweetener	0.1-0.4
DI water	Solubilizer	10-12
Crude extract	Active	0.2
A	Stabilizer	5-10
Menthol	Flavoring agent	0.1-0.4

Stability test

The stability of mouth spray was assessed under heating and cooling test at 4 °C 24 hrs and 45 °C 24 hrs for 6 cycles. The physical stability of samples was evaluated on turbidity, precipitation and appearance.

***In vitro* anti-microbial assessment of the product:**

Agar diffusion assay was performed against *Staphylococcus aureus, S. epidermidis, Streptococcus pyogenes and Candida albicans*. Stock of microorganism was prepared by cultivation on agar, separated to sterile water and adjusted to 0.5 McFarland concentration. Twenty milliliters of nutrient agar was added into a Petri dish, allowed to set, and then the microorganism was evenly distributed over the agar surface, left for a while in aseptic condition before placing 1-cm diam. tissue discs on the agar culture. Pipette sample onto the tissue disc, closed the lid and incubated at 37 °C for 18-24 hrs. The clear zone of inhibition was observed.

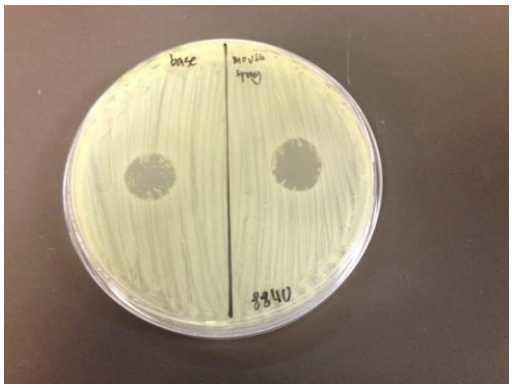
RESULTS

Mouth spray of 7 formulas was formulated. Formula1 was cloudy, but Formula 2 was clearer according to the order of mixing. Formula 3 was clear and less sweet, but different sweetener gave formula 4 with the phase separation. Ethanol was added to Formula 5 to give more freshness. The *P. emblica* and *Z limonella* mix-extract was added in Formula 6 which gave a clear yellow, sweet and fresh product. Menthol was added in Formula 7 for more freshness, and gave stable product which could inhibit the growth of tested microbial as shown in Table 2.

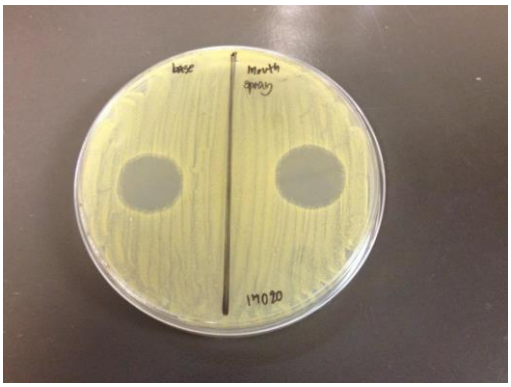
Table 2 Clear zones of inhibitions of mouth spray using agar diffusion method

Microorganisms	Clear zones of inhibition (mm)
Staphylococcus aureus (DMST 8013)	+2.0
Staphylococcus aureus (DMST 8840)	+1.3
Streptococcus pyogenes (DMST 17020)	+2.0
Staphylococcus epidermidis (DMST 12228)	+2.0
Candida albicans (DMST 90028)	+2.0
Candida albicans (DMST 10231)	+1.8

Staphylococcus aureus (DMST 8013)



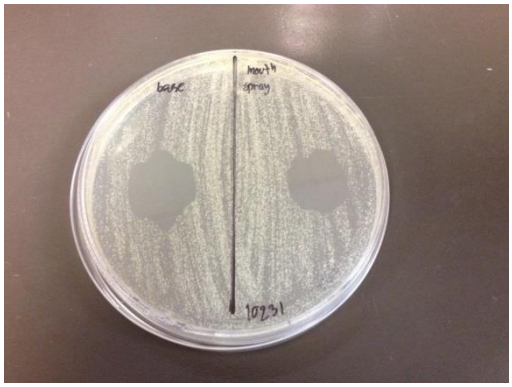
Staphylococcus aureus (DMST 8840)



Streptococcus pyogenes (DMST 17020)

Staphylococcus epidermidis (DMST 12228)

Candida albicans (DMST 10231)



Candida albicans (DMST 90028)

Figure 1. Clear zones of inhibitions of the *P. emblica* and *Z. limonella* mouth spray (right) and mouthwash base (left).

CONCLUSIONS

The patented mix-extract from fruits of *P. emblica* and *Z. limonella* could be used as an active extract in mouth spray product to benefit from its antimicrobial, anti-inflammatory and anti-oxidant effects. The selected formula 7 had appropriate amount of sweetening and flavoring agent. It gave clear yellow liquid solution with sweet and fresh feeling after applied. The mouth spray could inhibit the growth of *Candida albicans* and other tested microbial.

ACKNOWLEDGEMENTS

This work was supported by Thailand Instituted of Scientific and Technological Research (TISTR). We would like to thank Mr. Bundit Fungsin, Director of the Biosciences Department TISTR, for providing microbiology laboratory facilities. We thank all TISTR colleagues who in one way or another help this project to succeed.

REFERENCES

1. Breath spray. Available at http://en.wikipedia.org/wiki/Breath spray , accessed November 20, 2015.
2. *Phyllanthus emblica* L. Available at http://www.globinmed.com/index.php?option=com_content%20&view=article&id=79255:phyllanthus-%20emblica-l&catid=718:p&Itemid=150, accessed November 20, 2015.
3. Liu X, Cui C, Zhao M, Wang J, Luo W, Yang B and Jiang Y. 2008. Identification of phenolics in the fruit of emblica (*Phyllanathus emblica* L.) and their antioxidant activities. Food Chem. 109, 909-915.
4. Muthuraman A, Sood S and Singla SK. 2011. The antiinflammatory potential of phenolic compounds from *Emblica officinalis* L. in rat. Inflammopharmacology. 19(6), 327-334.
5. Jaijoy K, Soonthornchareonnon N, Lertprasertsuke N, Panthong A and Sireeratawong S., 2010. Acute and chronic oral toxicity of standardized water extract from the fruit of *Phylanthus emblica Linn*. Int. J. Appl. Res. Nat. Prod. 3(1), 48-58.
6. Mallikarjuna P, Uma Maheswara Rao V and Satyanarayana T. 1999. Antimicrobial activity of *Zanthoxylum limonella*. Indian Drugs 36 (7): 476-478.
7. Tangjitjaroenkun J, Supabphol R, Chavasiri W. 2012. Antioxidant effect of *Zanthoxylum limonella* Alston. J. Med. Plants Res. 6(8), 1407-1414.
8. Charoenying P, Laosinwattana C, Phuwiwat W, Lomratsiri J. 2008. Biological activities of *Zanthoxylum limonella* Alston fruit extracts. KMITL Sci. J. 8, 12-15.
9. Ngamnon Y, Potduang B, Fungsin B, Phasuk S, Takolpuckdee P, Rerk-am U, et al. 2012. Antimicrobial, antioxidant activities and total flavonoids of fruit extracts from Ma-khwaen (*Zanthoxylum limonella*). Thai J. Pharm. Sci. 35, 36.
10. Potduang B, Fungsin B, Keeta I, Ketmanee N, Intarangsie A, Soradech S, et al. 2012. Biological activities of a patented mix extracts from fruits of *Phyllanthus emblica* and *Zanthoxylum limonella*. Thai J. Pharm. Sci. 36, 44-47.
11. Khayungarnnawee A, Soradech S, Potduang B, Sematong T, Laovitthayanggoon S, Keeta I, et al. 2012. Anti-inflammatory activity of fruit extracts from *Phyllanthus emblica* and *Zanthoxylum limonella*. Thai J. Pharm. Sci. 36, 38-40.

The development of *Phyllanthus emblica* and *Zanthoxylum limonella* toothpaste

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ABSTRACT

This research aimed to develop toothpaste comprising the patented mix-extract from edible fruits of *Phyllanthus emblica* and *Zanthoxylum limonella* which is antimicrobial and anti-inflammatory effective. Toothpaste is a gel or paste which is applied to a toothbrush to clean the teeth by removing food particles and plaque from teeth to prevent bad breath. The toothpaste was developed in 2 formulas which gave similar odor and flavor. The stable formula 1 was selected to add clove oil, and the resulted product is stable under 6 cycles of heating and cooling stability test at 4 °C 24 hrs and 45 °C, 24 hrs. It was active against tested microbial using tissue disk agar diffusion method.

Keywords: Toothpaste, *Phyllanthus emblica*, *Zanthoxylum limonella*, antimicrobial, anti-inflammatory

INTRODUCTION

Toothpaste is a gel or paste applying on a toothbrush to help removing food particles and plaque from teeth to prevent bad breath [1]. Salt and sodium bicarbonate (baking soda) are common ingredients in commercial toothpaste which also contain fluoride in an allowable amount that is not very harmful if accidentally swallowed [2].

This research aimed to develop toothpaste comprising a patented mix-extract from fruits of *Phyllanthus emblica* and *Zanthoxylum limonella* to benefit from its anti-microbial, anti-inflammatory and antioxidant effects.

Phyllanthus emblica L. (EUPHORBIACEAE), locally called Ma-khampom, is a tree of small to moderate size found in tropical Southeast Asia. The fruit is rich in vitamin C, and possesses anti-tyrosinase and antioxidant activity. It is used as an alternative treatment of diarrhea, jaundice, skin disorders, inflammations, premature graying and the burning sensation of the body. Other chemical constituents include gibberellins, lupeol, kaempferol, quercetin, emblicanin A and B, punigluconin, pedunculaginn, phyllanthin, zeatin, amlaic acid, corilagin, ellagic acid, putranjivain A, digalic acid, phyllemblic acid, emblicol and galactaric acid [3]. We have found that the fruit extract of *P.emblica* is anti-microbial (MIC 10-20 mg/mL), anti-oxidant (EC_{50} 0.0187 µg/mL) and anti-tyrosinase (IC_{50} 0.9 mg/mL).

Zanthoxylum limonella Alston (RUTACEAE) locally called Ma-kwaen, is the climbing shrub which was used in Ayuravedic of India for the treatment of respiratory, cardiac disease, tooth infection and stomach infection. It is reported to be antimalarial, antituberculosis and active against *Staphylococcus aureus* [4-6]. We have reported that the fruit extract is active against *C. albicans* and other tested microbial (MIC 2.5-10 mg/mL). It is anti-tyrosinase (IC_{50} 0.33 mg/mL) and anti-oxidant (EC_{50} 5.94 µg/mL), with total flavonoids of 3.61 mg rutin/g extract [7].

The patented mix-extract from fruits of *P. emblica* and *Z. limonella* is anti-microbial (MIC 4.5 mg/mL), anti-oxidant (EC_{50} 7.9 µg/mL), anti-tyrosinase (IC_{50} 5.52 mg/mL) and potent anti-inflammatory on rat ear edema [8, 9]. The mix-extract was used to develop a patented toothpaste. Clove oil was added to adjust the odor and taste. Quality assessments of the product were performed on stability under heating and cooling test; and *in vitro* antimicrobial assay using tissue disc agar diffusion assay.

MATERIALS AND METHODS

Plant material

The dry fruit powder of *P. emblica* and *Z. limonella* were provided by the Agricultural Technology Department, Thailand Institute of Scientific and Technological Research (TISTR).

Preparation of the mix-extract

The extracts of *P. emblica and Z. limonella* fruits were separately macerated with different proportions of ethanol-water, filtered, rinsed and evaporated under reduced pressure using a rotary evaporator at 45 °C. The crude extracts were freshly mixed when used at a patented appropriate ratio to be effective against tested microbial.

Formulation of toothpaste

Toothpaste consisting of the *P. emblica* and *Z limonella* mix-extract was formulated in 2 formulas with varying amount of ingredients, as shown in Table 1. The active mix-extract was dissolved in humectants. Sodium carboxymethyl cellulose were spread in water, stirred, left for 1 hr to swell to be used as gelling agent. Sodium fluoride and sodium saccharin were separately dissolved with small amount of water. The gelling agent, sweetening agent, abrasive agent, flavoring agent, preservative and active ingredient and color were mixed. Then added surfactant and gently stirred homogeneously. Clove oil was added to provide the fresh breath.

Table 1 The patented formula of toothpaste

Ingredients	Function	Amount
		%w/w
Crude extract *	Active ingredient	0.05-0.2
Clove oil	Antibacterial,flavoring agent, anti-inflammation	0.3-0.8
Sodium Carboxymethyl Cellulose	Gelling agent	1-2
Sorbitol 70% solution	Sweetening agent, humectant	19-21
A	Humectant	7-9
Dicalcium phosphate dihydrate	Abrasive agent	30-32
Sodium saccharin	Sweetening agent	0.1-0.2
Menthol	Flavoring agent	1-2
Spearmint oil	Flavoring agent	0.15-0.3
Mint essential oil	Flavoring agent	0.5-0.8
Sodium lauryl sulfate (Food grade)	Anionic Surfactant	2-5
B	Preservative	0.5-1
Sodium fluoride	Anticaries agent	0.1-0.3
Color (brilliant blue FCF)	Coloring agent	0.3-0.5
Water q.s. to	Vehicle	100

Stability test

The stability in accelerated conditions was assessed under heating and cooling test at 4 °C 24 hrs and 45 °C 24 hrs for 6 cycles. The physical stability of toothpaste was evaluated.

***In vitro* anti-microbial assessment of the product:**

Tissue disc agar diffusion assay was performed against *Staphylococcus aureus, S. epidermidis, Streptococcus pyogenes and Candida albicans.* Stock of microorganism was prepared by cultivation on agar, separated to sterile water and adjusted to 0.5 McFarland. Twenty milliliters of nutrient agar was added into a Petri dish, allowed to set, and then the microorganism was evenly distributed over the agar surface and let dry for a while in aseptic condition. Pipette sample solution (1:10 in water) onto the tissue disc on the agar for 1-min contact and then took off the tissue. Closed the lid and incubated at 37 °C for 18-24 hrs. The clear zone of inhibition was observed.

RESULTS

The toothpaste containing the patented mix-extract from fruits of *P. emblica* and *Z limonella* was formulated in 2 formulas with varying amount of the mix-extract. Formula 1 had less extract than formula 2. Both formulas gave similar odor and flavor. Color adjusting gave formula 1 as pale sky-blue cream, and formula 2 as pale green cream. The heating and cooling stability test showed that formula 1 was stable, and it was selected to add clove oil.

The tissue disc agar diffusion assay exhibited that the selected toothpaste formula 1 had was active against *Staphylococcus aureus* (DMST 8013 and DMST 8840), *Candida albicans* (DMST 90028 and DMST 10231), *Streptococcus pyogenase* (DMST 17020) and *Streptococcus epidermidis* (DMST 12228) as shown in Table2.

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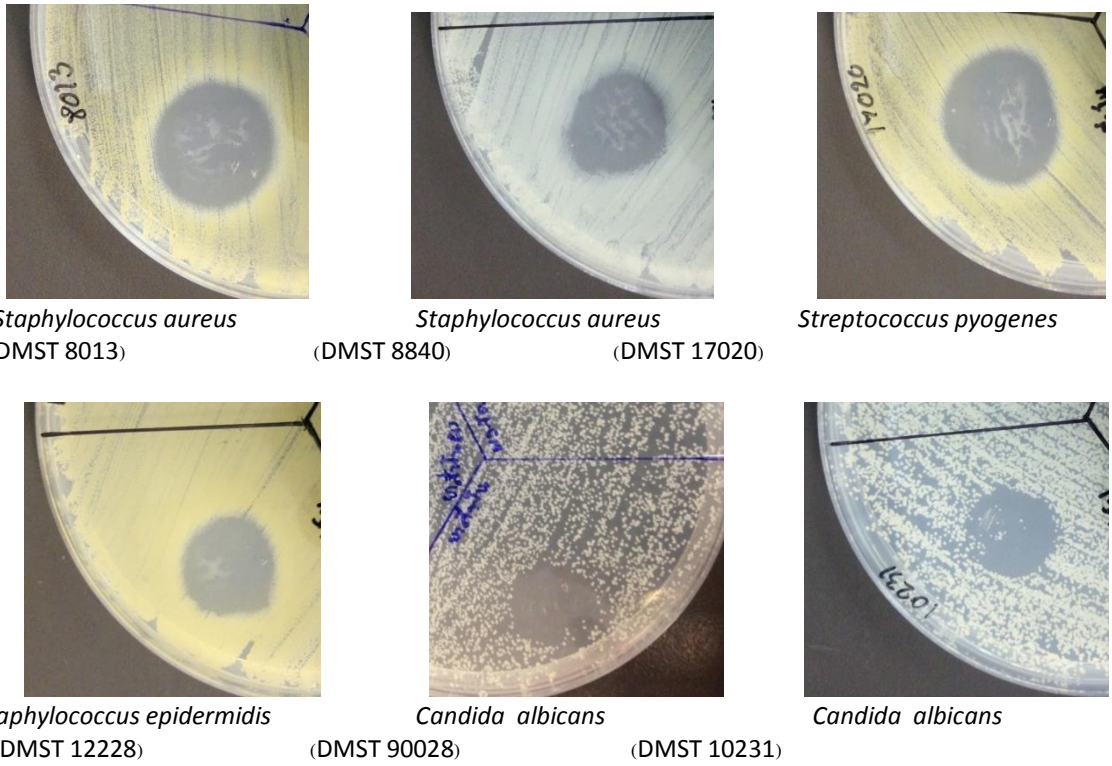
Table 2 Clear zones of inhibitions of the toothpaste using tissue disc agar diffusion method

Microorganisms	Clear zones of inhibition (mm)
Staphylococcus aureus (DMST 8013)	+0.29
Staphylococcus aureus (DMST 8840)	+1.5
Streptococcus pyogenes (DMST 17020)	+1.7
Staphylococcus epidermidis (DMST 12228)	+1.6
Candida albicans (DMST 90028)	+1.7
Candida albicans (DMST 10231)	+2

Note : - no zone of inhibition -/+ no clearly zone of inhibition + clearly zone of inhibition



Staphylococcus aureus (DMST 8013) *Staphylococcus aureus* (DMST 8840) *Streptococcus pyogenes* (DMST 17020)

Staphylococcus epidermidis (DMST 12228) *Candida albicans* (DMST 90028) *Candida albicans* (DMST 10231)

Figure 1. Clear zones of inhibitions of the *P. emblica* and *Z. limonella* toothpaste

CONCLUSIONS

The patented mix-extract from fruits of *P. emblica* and *Z. limonella* was used as an active extract in toothpaste product to benefit from its biological activities as antimicrobial, anti-inflammatory and anti-oxidant. The selected formula 1 was stable under 6 cycles of heating and cooling test. It gave fresh and good breath feel after tooth brushing. The toothpaste could inhibit the growth of *Candida albicans* and other tested microbial.

ACKNOWLEDGEMENTS

This work was supported by Thailand Instituted of Scientific and Technological Research (TISTR). We would like to thank Mr. Bundit Fungsin, Director of the Biosciences Department TISTR, for providing microbiology laboratory facilities. We thank all TISTR colleagues who in one way or another help this project to succeed.

REFERENCES

1. What is Toothpaste?. Available at http://www.medic8.com/cosmetic-dentistry/dental hygiene/toothpaste/what-is-toothpaste.html, accessed November 20, 2015.
2. Toothpaste. Available at http://en.wikipedia.org/wiki/Toothpaste, accessed November 20, 2015.
3. *Phyllanthus emblica* L. Available at http://www.globinmed.com/index.php?option=com_content%20&view=article&id=79255:phyllanthus-%20emblica-l&catid=718:p&Itemid=150, accessed November 20, 2015.
4. Mallikarjuna P, Uma Maheswara Rao V and Satyanarayana T. 1999. Antimicrobial activity of *Zanthoxylum limonella*. Indian Drugs. 36(7), 476-478.
5. Tangjitjaroenkun J, Supabphol R, Chavasiri W. 2012. Antioxidant effect of *Zanthoxylum limonella* Alston. J. Med. Plants Res. 6(8), 1407-1414.
6. Charoenying P, Laosinwattana C, Phuwiwat W, Lomratsiri J. 2008. Biological activities of *Zanthoxylum limonella* Alston fruit extracts. KMITL Sci. J. 8, 12-15.
7. Ngamnon Y, Potduang B, Fungsin B, Phasuk S, Takolpuckdee P, Rerk-am U, et al. 2012. Antimicrobial, antioxidant activities and total flavonoids of fruit extracts from Ma-khwaen (*Zanthoxylum limonella*). Thai J. Pharm. Sci. 35, 36.
8. Potduang B, Fungsin B, Keeta I, Ketmanee N, Intarangsie A, Soradech S, et al. 2012. Biological activities of a patented mix extracts from fruits of *Phyllanthus emblica* and *Zanthoxylum limonella*. Thai J. Pharm. Sci. 36, 44-47.
9. Khayungarnnawee A, Soradech S, Potduang B, Sematong T, Laovitthayanggoon S, Keeta I, et al. 2012. Anti-inflammatory activity of fruit extracts from *Phyllanthus emblica* and *Zanthoxylum limonella*. Thai J. Pharm. Sci. 36, 38-40.

The development of *Phyllanthus emblica* and *Zanthoxylum limonella* toothpowder

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ABSTRACT

This study was to formulate a patented toothpowder from a patented mix-extract of *Phyllanthus emblica* and *Zanthoxylum limonella* fruits which are antimicrobial and anti-inflammatory effective. Clove oil was added to adjust the odor and taste and to enhance the antimicrobial efficacy. The edible, non-toxic and non-mutagenic fruits of *P. emblica* and *Z. limonella* are widely used as anti-oxidant, antimicrobial and anti-inflammatory agent in herbal medicines. The toothpowder was developed in 2 formulas with varied amount of clove oil. The resulted products were white fined powder with the smell of clove oil, and the refreshment, sweetness and foam of the 2 formulas are alike. Formula 1 was selected according to its appropriate smell of clove oil. Stability assessment was performed under 6 cycles of heating and cooling stability test at 4 °C 24 hrs and 45 °C, 24 hrs. The toothpowder was active against tested microbial using tissue disc agar diffusion method.

Keywords: Toothpowder, *Phyllanthus emblica*, *Zanthoxylum limonella*, antimicrobial, anti-inflammatory

INTRODUCTION

Toothpowder is a form of toothpastes which is the oral hygiene routine helping to remove plaque and biofilm (a film of bacteria) from teeth gum or oral cavity [1]. Toothpowder formula contains abrasive, detergent, binding agent, humectants, preservative, fluoride and other active ingredients. The edible, non-toxic and non-mutagenic fruits of Emblica (*Phyllanthus emblica* L., EUPHORBIACEAE) and Ma-khwaen (*Zanthoxylum limonella* Alston, RUTACEAE) are widely used in herbal medicines [2-5]. The fruit contains several active ingredients and is effective as anti-oxidation, antimicrobial and anti-inflammatory [6-11]. We have reported that the mix-extract from fruits of *P. emblica* and *Z. limonella* are anti-oxidation, anti-microbial and anti-inflammatory effective [12, 13, 14].

This study was to formulate a patented toothpowder comprising a patented mix-extract of *P. emblica* and *Z. limonella* fruits to benefits from its antimicrobial and anti-inflammatory activities. Clove oil was added to adjust the odor and taste and to enhance the antimicrobial efficacy.

MATERIALS AND METHODS

Plant material

The dry fruit powder of *P. emblica* and *Z. limonella* were provided by the Agricultural Technology Department, Thailand Institute of Scientific and Technological Research (TISTR).

Preparation of the mix extract

The extracts of *P. emblica and Z. limonella* fruits were separately macerated with different proportions of ethanol-water, filtered, rinsed and evaporated under reduced pressure using a rotary evaporator at 45 °C. The crude extracts were freshly mixed when used at a patented appropriate ratio to be effective against tested microbial.

Formulation of toothpowder

The toothpowder was formulated in 2 formulas with different amount of clove oil. First, the dextrose granule was prepared, and then mixed with the *P. emblica* and *Z. limonella* mix-extract. Added 60% ethanol, and then sieved and dried at 45°C for 12 hrs. Ground calcium carbonate and sodium lauryl sulfate to fined powder in a mortar, and then added the dextrose granules, menthol, sodium saccharin, sodium fluoride, xylitol, dicalcium phosphate dehydrate and clove oil, respectively. The patented formula of toothpowder is shown in Table 1.

Table 1 The patented formula of toothpowder

Ingredients	Function	Amount
		%w/w
Crude extract*	Active ingredient	0.05-0.2
Dicalcium phosphate dihydrate	Abrasive	60-65
Calcium carbonate	Abrasive	18-22
Sodium lauryl sulfate (Food grade)	Foaming agent	2-5
Dextrose	Bulking agent, Sweetener	7-12
Menthol	Flavoring agent	0.5-1
Sodium saccharin	Sweetener	0.1-0.4
Xylitol	Sweetener	0.2-0.6
Clove oil	Antibacterial,flavoring agent,anti-inflammation	0.3-0.8
Sodium fluoride	Anticaries agent Fluoride supplement	0.1-0.3

Stability test

The stability of toothpowder in accelerated conditions was assessed under heating and cooling test at 4 °C 24 hrs and 45 °C 24 hrs for 6 cycles. The appearances (color, odor, texture) of toothpowder were evaluated.

***In vitro* anti-microbial assessment of the product:**

Tissue disc agar diffusion assay was performed against *Staphylococcus aureus, S. epidermidis, Streptococcus pyogenes and Candida albicans.* Stock of microorganism was prepared by cultivation on agar, separated to sterile water and adjusted to 0.5 McFarland. The microorganism was evenly distributed over the nutrient agar surface in a Petri dish and let dry for a while in aseptic condition. Pipette the supernatant solution of sample (1:10 in water) onto the tissue disk on the agar for 1-min contact and then took off the tissue. Closed the lid and incubated at 37 °C for 18-24 hrs. The clear zone of inhibition was observed.

RESULTS

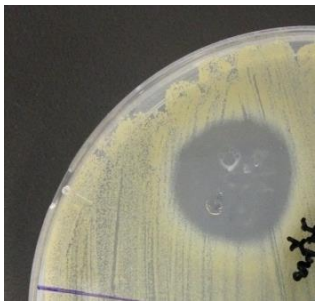
The toothpowder comprising the mix fruit extract of *P. emblica* and *Z. limonella* with clove oil was formulated in 2 formulas of white fined powder which had the same sweetness, foam and refreshment with the smell of clove oil. The selected formula 1 had much less smell of clove oil. Its odor, texture and color were stable under the heating and cooling test at 4 °C 24 hrs and 45 °C 24 hrs for 6 cycles.

The selected toothpowder formula 1 was active against *Staphylococcus aureus* (DMST 8013 and DMST 8840), *Candida albicans* (DMST 90028 and DMST 10231), *Streptococcus pyogenase* (DMST 17020) and *Streptococcus epidermidis* (DMST 12228) using 1-min tissue disk agar diffusion assay as shown in Table2.

Table 2 Clear zones of inhibitions of the toothpaste using tissue disc agar diffusion method

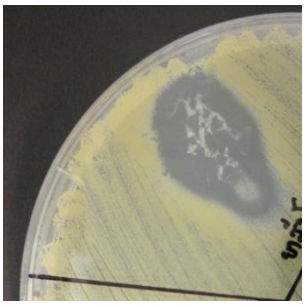
Microorganisms	Clear zones of inhibition (mm)
Staphylococcus aureus (DMST 8013)	+3
Staphylococcus aureus (DMST 8840)	+1
Streptococcus pyogenes (DMST 17020)	+1.7
Staphylococcus epidermidis (DMST 12228)	+1.7
Candida albicans (DMST 90028)	+1.4
Candida albicans (DMST 10231)	+1.9

Note : - no zone of inhibition -/+ no clearly zone of inhibition + clearly zone of inhibition



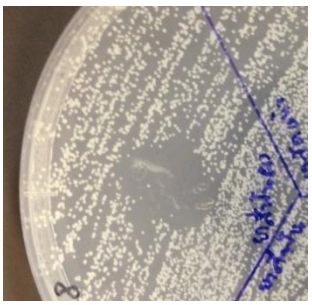
Staphylococcus aureus (DMST 8013)

Staphylococcus aureus (DMST 8840)

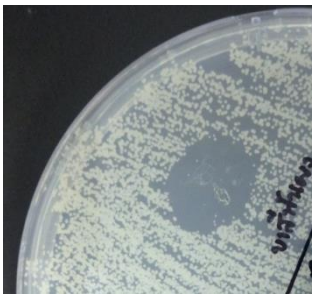


Streptococcus pyogenes (DMST 17020)

Staphylococcus epidermidis (DMST 12228)



Candida albicans (DMST 90028)



Candida albicans (DMST 10231)

Figure 1. Clear zones of inhibitions of the *P. emblica* and *Z. limonella* toothpowder

CONCLUSIONS

The developed formula 1 of toothpowder comprising the patented mix-extract from fruits of *Phyllanthus emblica* and *Zanthoxylum limonella* was chosen according to its appropriate smell of clove oil. The product was stable under 6 cycles of heating and cooling test at 4 °C 24 hrs and 45 °C 24 hrs for 6 cycles. It was active against *Candida albicans* and other tested microbial.

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REFERENCES

1. American Dental Association. Learn more about toothpastes. [internet]. [cited 2014 Oct 17]. Available from : http://www.ada.org/en/science-research/ada-seal-of-acceptance/product-category-information/toothpaste
2. Natural Remedies Pvt. Ltd., Quality Control Dept., Hosur Road, Bangalore. *Emblica officinalis*.
3. Bhowmik D, Samphat Kumar KP, Yadav A, Srivastava S, Paswan S, Dutta AM. 2012.Recent trends in Indian traditional herbs *Syzygium aromaticum* and its health benefits. J. Pharmacog. Phytochem. 1, 13-22.
4. Seidemann J. 2005. World spice plants: Economic usage, Botany, Taxonomy. Springer Science & Business Media.
5. Moazzem Hossen SM, Sarkar R, Mahmud S, Abdul Aziz NM. 2014. Medicinal potential of *Phyllanthus emblica* (Linn.) Fruits Extracts: Biological and Pharmacological activities. BJPR. 4(12), 1486-1499.
6. Dhale DA, Mogle UP. 2011. Phytochemical screening and antibacterial activity of *Phyllanthus emblica* (L.). Sci. Res. Repot. 1(3), 138-142.
7. Arun Kumar KV, Paridhavi M. 2011. Evaluation of physiochemical parameters on the fruit of *Zanthoxylum limonella* Alston (Family - Rutaceae). IJPC. 2, 1-3.
8. Perumal Samy R, Ignacimuthu S, Chow VTK. 2011. Evaluation of antimicrobial studies of traditional medicine. NaturePrceeding. [online]
9. Mallikarjuna P, Uma Maheswara Rao V and Satyanarayana T. 1999. Antimicrobial activity of *Zanthoxylum limonella*. Indian Drugs. 36(7), 476-478.
10. Tangjitjaroenkun J, Supabphol R, Chavasiri W. 2012. Antioxidant effect of *Zanthoxylum limonella* Alston. J. Med. Plants Res. 6(8), 1407-1414.
11. Charoenying P, Laosinwattana C, Phuwiwat W, Lomratsiri J. 2008. Biological activities of *Zanthoxylum limonella* Alston fruit extracts. KMITL Sci. J. 8, 12-15.
12. Ngamnon Y, Potduang B, Fungsin B, Phasuk S, Takolpuckdee P, Rerk-am U, et al. 2012. Antimicrobial, antioxidant activities and total flavonoids of fruit extracts from Ma-khwaen (*Zanthoxylum limonella*). Thai J. Pharm. Sci. 35, 36.
13. Potduang B, Fungsin B, Keeta I, Ketmanee N, Intarangsie A, Soradech S, et al. (2012). Biological activities of a patented mix extracts from fruits of *Phyllanthus emblica* and *Zanthoxylum limonella*. Thai J. Pharm. Sci. 36, 44-47.
14. Khayungarnnawee A, Soradech S, Potduang B, Sematong T, Laovitthayanggoon S, Keeta I, et al. 2012. Anti-inflammatory activity of fruit extracts from *Phyllanthus emblica* and *Zanthoxylum limonella*. Thai J. Pharm. Sci. 36, 38-40.

Safety and Regulations on Natural Products (SRN) Full Paper

Effect of electron beam irradiation on microbiological decontamination and stability of antioxidant activities of *Butea Superba*. Roxb (Kwao Khrua Dang) root extract

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ABSTRACT

Butea Superba. Roxb can be contaminated with microorganisms. To make them suitable for commercialization, the microbial quality is necessary to be achieved. Being a feasible and environment friendly, irradiation is an effective decontamination method for foodstuffs. The objective of this present study was to investigate the effect of electron beam irradiation on microbial load and antioxidant properties of *Butea Superba*. Roxb root extract after irradiation at 0, 5, 10, 15 and 20 kGy. Results indicated that the total bacteria and the total yeast and mold diminished linearly with absorbed dose. Furthermore, the pathogens such as *Salmonella* spp. and *Escherichia coli* were dispelled after irradiation at 5 kGy. Higher dose at 10 kGy was sufficient to eradicate the spore-forming bacteria such as *Clostridium perfringens* and *Bacillus cereus*. The methanolic extract of irradiated and non-irradiated *Butea Superba*. Roxb was determined. The antioxidant properties such as total phenolic content (TPC), ferric reducing antioxidant potential (FRAP) and free radical scavenging activity (DPPH) displayed no significant changes after irradiation, even up to 20 kGy.

Keywords: irradiation, antioxidant activity, microbial decontamination

INTRODUCTION

The world demand for natural products has been increasing, since they are being utilized as medicines, cosmetics and food supplements. *Butea superba* Roxb. (Kwao Khrua Dang) is a local plant found in Thailand as a climbing tree with long tuberous root and red sap [1]. It is abundantly distributed in the Thai deciduous forest and has been popular among Thai males for its supposed effects on rejuvenation and sexual vigor [2]. Compound extracted from *B. superba* is effective in vitro in inhibiting cAMP phosphodiesterase; the mechanism that plays an important role in penile erection. The tuberous roots of *Butea superba* were found to contain flavonoids and flavonoid glycosides as well as sterol compounds, including β-sitosterol, campesterol and stigmasterol [3]. However, Kwao Khrua Dang is often highly contaminated with pathogenic non-sporeforming bacteria. This is one of the most significant public health problems and important cause of human suffering all over the world.

According to the Codex General Standard for Irradiated Food [4] ionizing radiation foreseen for food processing is limited to high energy photons (gamma rays, X-rays and accelerated electron). Electron accelerator is a new irradiation facility of Thailand Institute Nuclear and Technology (Public Organization). The major advantage of using electron is that the source for electrons can be turned off when not in use. Treatment with electron beams can provide higher throughput rates and lower unit costs. However, the major limitation of electron beam is its low penetration depth; therefore it is unsuitable for thick products [5].

Nowadays, food irradiation is increasingly recognized as an effective method for reducing post harvest food losses and decontamination as well as ensuring hygienic quality in medicinal herbs. However, questions concerning loss of

phytochemical constituents, free radicals and radiolytic by-product formation and change of antioxidant properties during irradiation are still being debated [6]. The purpose of this study was to examine the effect of electron beam irradiation at various doses on microbiological decontamination and stability of antioxidant activities of *Butea Superba*. Roxb (Kwao Khrua Dang) root extract.

MATERIALS AND METHODS

Irradiation processing

The *Butea Superba*. Roxb (Kwao Khrua Dang) powder used for this experiment was purchased from retailer and packed in aluminum foil bags. The sample was irradiated with electron beam at room temperature. Irradiation was carried out using an electron beam accelerator (MB 20-10 S/N 021, Mevex Corporation, Canada) with a mean energy of 8 MeV, beam current of 100 mA and dose rate of 20 kGy/pass. The sample was irradiated for a total dose of 0, 5, 10, 15 and 20 kGy.

Sample Analyses

Microbiological determination

The irradiated and non-irradiated samples were analyzed for microbiological qualities such as total bacteria, total yeast and mold, Coliform bacteria, *Escherichia coli*, *Salmonella* sp., *Bacillus cereus* and *Clostridium perfringens*. Microbiological assays were performed using the AOAC standard (1995) protocol [7].

Total phenolic content (TPC)

The content of reduced components (expressed in a form of TPC) was estimated using the Folin-Ciocalteu assay according to a method developed by Velioglu *et al.* (1998) [8]. First, 0.75 ml of 10-fold diluted Folin-Ciocalteu reagent and 100 µl of methanolic extract were added into a test tube. They were mixed and allowed to stand at room temperature for 5 min. Then, 0.75 ml of 6% (w/v) sodium carbonate solution was added. The mixture was homogenized and left at room temperature for 90 min. TPC was determined using a spectrophotometer at 725 nm. The standard calibration curve was plotted using gallic acid at the concentration of 0.02-0.1 mg/ml. The TPC was expressed in terms of gallic acid equivalent (GAE) mg/g.

Determination of ferric reducing antioxidant potential (FRAP)

FRAP assay was performed according to the method of Benzie and Strain (1996) [9]. The FRAP reagent was prepared by mixing 16.7 mM $FeCl_3 \cdot 6H_2O$ and 8.3 mM 2,4,6-tripyridyl-s-triazine (TPTZ) with 250 mM acetate buffer, pH 3.6. A solution of 75 µl sample and 225 µl of distilled water were added to 2.25 ml of freshly prepared FRAP reagent in a test tube. The mixture was incubated at room temperature throughout the reaction. The absorbance was read after 30 min at 596 nm using UV-vis spectrophotometer during the monitoring period. The antioxidant potential of the samples was determined based on a calibration curve plotted using $FeSO_4 \cdot 7H_2O$ at concentration ranging between 400 and 2000 µM.

Determination of free radical scavenging activity (DPPH)

The DPPH radical scavenging method was performed according to the methodology described by Khattak *et al.* (2008) [10] with a slight modification. It was determined using 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical. The 100 µl of each extract was added to 900 µl of DPPH in methanol solution (150 µM) in a test tube and shaken vigorously. After incubation at room temperature for 15 min in the dark, the absorbance of each solution was determined at 517 nm. The free radical-scavenging activity was expressed in the form of half maximal inhibitory concentration (IC_{50}, mg/ml).

Statistical analysis

Three replicates of each sample were used and all the assays were carried out in triplicate. The results are expressed as mean values and standard deviation (SD). The results were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's HSD Test with $\alpha = 0.05$. This analysis was carried out using SPSS v. 21 programs.

RESULTS AND DISCUSSION

3.1 Effect of electron beam irradiation on the microbiological decontamination

The results regarding the amount of microorganisms in Kwao Khrua Dang powder before and after irradiation was shown in Table 1. Decontamination of the microorganism is the principal purpose of the irradiation. The results of total viable bacterial counts indicated that the non-irradiated samples of Kwao Khrua Dang powder were highly contaminated with bacteria at the level of $3.9x10^6$ cfu/g. The values exceeded the level of $1.0x10^4$ cfu/g determined by WHO (1998) [11] as the maximum permissible total count level. After irradiation at 5 kGy, the total bacterial count decreased to $4.7x10^3$ cfu/g. The total yeasts and molds count of non-irradiated samples was $7.8x10^1$ cfu/g, whereas the counts in irradiated samples at 5, 10, 15 and 20 kGy were below the detection level. Electron beam irradiation at the dose of 10 kGy and 5 kGy were sufficient to practically eliminate the total bacteria and total yeast and mold, respectively. Both microbiological analyses diminished linearly with absorbed doses. These results showed good agreement and similar tendency, with those reported by Nemtanu *et al.* (2006) [12]. Their results also indicated that the electron beam treatment of green coffee beans with the doses 5 kGy led to the desired microbial reduction while those irradiated at 10 kGy showed complete absence of microorganism. The pathogenic microorganism such as Coliform Bacteria, *E. coli* and *Salmonella* spp. were eliminated after irradiation at 5 kGy, while the spore-forming bacteria such as *B. cereus* and *C. perfringens* were purged after irradiation at 10 kGy, due to the fact that spore coating has higher resistance (5-10 fold) to ionizing radiation than vegetative cell [13].

Table 1. Microbiological qualities of Kwao Khrua Dang powder after electron beam irradiation.

Microbiological	0 kGy	5kGy	10kGy	15kGy	20kGy
Total Bacterial Count (CFU/g)	$3.9x10^6$	$4.7x10^3$	<1 Est	<1 Est	<1 Est
Total Yeast &Mold (CFU/g)	$7.8x10^1$	<1 Est	<1 Est	<1 Est	<1 Est
Coliform Bacteria (MPN/g)	460	<0.3	<0.3	<0.3	<0.3
E. coli (MPN/g)	3.6	<0.3	<0.3	<0.3	<0.3
Salmonella spp.	Absent	Absent	Absent	Absent	Absent
B. cereus (CFU/g)	$5.2x10^1$ Est	7 Est	<1 Est	<1 Est	<1 Est
C. perfringens (MPN/g)	20	3.6	<0.3	<0.3	<0.3

3.2 Effect of electron beam irradiation on the stability of antioxidant activities

The effects of electron beam irradiation on total phenolic content, ferric reducing antioxidant potential and free scavenging activity of Kwao Khrua Dang powder were demonstrated in Table 2. The results indicated that the levels of total phenolic content, ferric reducing antioxidant potential and free scavenging activity of irradiated and non-irradiated samples fluctuated between 6.68-7.19 mg GAE/g, 39.04-39.52 µmol FeSO4/g and 2.46-2.70 mg AAE/g, respectively.

Table 2. Antioxidant Activities of Kwao Khrua Dang powder after electron beam irradiation.

dose(kGy)	Total phenolics (mg GAE/g)	DPPH (mg AAE/g)	FRAP (µmol FeSO4/g)
0	7.19±0.021	2.46±0.038	39.52±0.017
5	6.74±0.015	2.69±0.028	38.49±0.025
10	6.90±0.017	2.97±0.039	38.36±0.032
15	6.82±0.017	2.63±0.021	39.04±0.025
20	6.68±0.020	2.7±0.023	39.37±0.015

Values are expressed as mean±SD of triplicate measurement. #No significant difference at p<0.05 within the same column.
The GAE was the abbreviation of gallic acid equivalent.

Additionally, the results in table 2 also displayed that electron beam irradiation did not result in significant effects on antioxidant properties. These results agreed very well with those reported by Murica *et al.* (2004). They studied the effect of gamma irradiation on antioxidant properties of seven desert spices (anis, cinnamon, ginger, licorice, mint, nutmeg and vanilla) and found that the spices irradiated at 1, 3, 5 and 10 kGy did not show significant differences in antioxidant activity compared with non irradiated samples [14]. A report by Mishra *et al.* (2006) also showed that the free radical scavenging activity of tea leaves was not affected by radiation treatment up to 10 kGy [15].

CONCLUSIONS

With increasing total dose, the electron beam irradiation of Kwao Khrua Dang powder led to decreased microbial load. Electron beam irradiation at 10 kGy and 5 kGy was sufficient to practically eliminate the total bacteria and total yeast and mold, respectively. Irradiation at 10 kGy showed complete absence of microorganism. With no significant changes in antioxidant activities such as total phenolic content (TPC), ferric reducing antioxidant potential (FRAP) and free radical scavenging activity (DPPH), electron beam irradiation evidently proves to be a suitable and effective technique for decontamination of Kwao Khrua Dang powder.

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REFERENCES

1. Smitinand T. Plants of Kwao Yai National Park. Bangkok: New Thammada; 1977
2. Suntara A. The remedy pamphlet of Kwao Krua tuber of Luang Anusarnsuntarakromkarnphiset. Chiang Mai, Thailand: Chiang Mai Upatipongsa Press; 1931.
3. Roengsumran S, Petsom A, Ngamrojanavanich N, et al. 2000. Flavanoid and Flavanoid glycoside from *Butea superba* Roxb. and their cAMP phosphodiesterase inhibitory activity. J. Sci. Res. Chulalongkorn University; 25: 169-76.
4. CAC (Codex Alimentarius Commission). 2003. Codex General Standard for Irradiated Foods. CODEX STAN 106-1983. Rev.1-2003.
5. IAEA. 2008. Trends in radiation sterilization of health care products. Vienna, Australia: IAEA.
6. Suhaj M, Racova J, Polovka M and Brezova. V. 2006 Effect of gamma irradiation on antioxidant activity of black pepper (*Piper nigrum*). Food Chem. 97, 696-704.
7. Association of Official Analytical Chemists. 1990. Official Method of Analysis (15th ed.). Washington DC.
8. Velioglu YS, Mazza G, Gao L, Oomah BD. 1998. Antioxidant capacity and total phenolics in selected fruits, vegetables and grain products. J. Agri. Food Chem. 46, 4113-4117.
9. Benzie IFF, Strain JJ. 1996. The ferric reducing ability of plasma (FRAP) as a measurement of "antioxidant power": the FRAP assay. Anal. Biochem. 239, 70-76.
10. Khattak K F, Simpson T J, Ihasnullah. 2008. Effect of gamma irradiation on the extraction yield, total phenolic content and free radical-scavenging activity of *Nigella staiva* seed. Food Chem. 110, 967-972.
11. World Health Organization. 1998. Food irradiation "A technique for preservation and improving the safety of food". World Health Organization of the United Nations,Geneva.
12. Nemtanu MR, Brasoveanu M, Minea R, Grecu MN, Albulescu M, Mitru E. 2006 Microbiological decontamination of *Spirulina platensis* and green coffee using accelerated electron beams. Rom. J. Biophy. 16, 141-148.
13. Moseley BEB. 1989. Ionizing Radiation: Action and Repair. pp. 43-70. In "Mechanisms of Action of Foods Preservation Procedures". G. M. Gould (ed.). Elsevier Science Publishers, Ltd., Essex.
14. Murica M A, Egea I and Romojaro J. 2004. Antioxidant evalution in desert spices compared with common food additives. Influence of irradiation procedure. Journal of Agricultural and food chemistry 52, 1872-1881.
15. Mishra B B, Gautam S and Sharma A. 2006. Microbial decontamination of tea (*Camelia sinensis*) by gamma radiation. J. Food Sci. 71, 151-156.

Effects of gamma irradiation on antioxidant activity, total phenolic content and andrographolide content for improving microbiological safety of *Andrographis paniculata*

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ABSTRACT

Andrographis paniculata (Burm.f.) Nees (Fa ThaLai Chon) is a medicinal plant that has been used to effectively treat several diseases as a good natural source of antioxidant. Microbial contamination of *A. paniculata* can occur during the manufacturing process resulting in several diseases affected final consumer. The purpose of this research was to study the effects of gamma irradiation on microbial decontaminants and to investigate the effects on microbiological quality, antioxidant activity, total phenolic content and andrographolide content of *A. paniculata*. The result indicated that the initial microorganisms of samples were exceed the permissible limits. After gamma irradiation at a dose of 5 kGy, the microbial contamination was reduced to the standard level, complete microbial decontaminations were obtained at 10 kGy irradiation. However, irradiated doses of 5, 10, 15 and 20 kGy did not affect on antioxidant activity, total phenolic content and andrographolide content.

Keywords: *Andrographis paniculata*, gamma irradiation, microbial decontamination

INTRODUCTION

Andrographis paniculata (Burm.f.) Nees (Fa ThaLai Chon) grows abundantly in India, Sri Lanka, Taiwan and was cultivated extensively in China and Thailand [1]. *A. paniculata* is a medicinal plant that has been effectively used in different traditional medicine. It exhibits anti-inflammatory, anti-HIV, antibacterial, antioxidant, antiparasitic, antispasmodic, antidiabetic, anticarcinogenic, antipyretic, hepatoprotective, nematocidal and various other activities. In addition, it is a potent scavenger of a variety of reactive oxygen species (ROS) including superoxide anion, hydroxyl radical, singlet oxygen, peroxynitrite and nitric oxide [2]. Its major constituents are diterpenoids, flavonoids and polyphenols. Among the single compounds extracted from *A. paniculata*, andrographolide is the major diterpenoids in terms of bioactive properties and abundance [3]. In Thailand, this plant was selected by the Ministry of Public Health as one of the medicinal plants to be included in "The national List of Essential Drugs A.D. 1999" (List of Herbal Medicinal Products) [4].

Recently, the requirements of natural products for a medicinal plant have been increasing. However, a serious problem with its microbial contamination affects health and economic impacts. Therefore, decontamination of plant materials is important to increase the safety of medicinal plants. The conventional methods of decontamination were fumigation with gaseous ethylene oxide or methyl bromide, which are now prohibited or being increasingly restricted in most advanced countries for health, environmental or occupational safety reasons [5]. Gamma irradiation has an effective method used for decontamination of microorganisms, its high penetration and can be used for products in final packaging. The Joint Expert Committee on Food Irradiation (JECFI) convened by Food and Agriculture Organization (FAO), World Health Organization (WHO) and International Atomic Energy Agency (IAEA) evaluated available data and concluded that the irradiation of any food commodity up to an overall average dose of 10 kGy presents no toxicological hazard and requires no further testing [6]. Gamma irradiation of *A. paniculata* was also studied at dose of 10 and 25 kGy. It was found that free radicals were induced without affecting the andrographolide content, antioxidant activity, total phenolic content and toxicity [7]. The aim of this research was to use gamma irradiation for microbial decontaminants and to investigate the effects on microbiological quality, antioxidant activity, total phenolic content and andrographolide content of *A. paniculata*.

MATERIALS AND METHODS

Sample irradiation and extraction

A. paniculata powder was purchased from retailer after that it was packed in aluminum foil bags. Samples were irradiated by gamma rays with a fixed dose rate of 6.34 kGy/hr for a total dose of 0, 5, 10, 15 and 20 kGy. Irradiation at each dose was done in triplicate. The samples were soaked in methanol and then sonicated for 60 min. The mixture was centrifuged at 8000 rpm at 25 °C for 5 minutes, its supernatant was used for sample solution.

Microbiological analysis

The irradiated and non-irradiated samples were analyzed for microbiological quality such as total bacteria, total yeast and mold, Coliform bacteria, Escherichia coli, Salmonella sp., Bacillus cereus and Clostridium perfringens. Microbiological assays were performed using the AOAC standard protocol [8].

Determination of Free radical scavenging power (DPPH) and ferric-ion reducing power (FRAP)

Determination of DPPH assay was performed as previously described by Khattate et al [9] with a slight modification. FRAP assay was performed according to the method of Benzie and Strain [10].

Total phenolic content

The total phenolic content was estimates using the Folin-Ciocalteu assay according to a method developed by Velioglu et al [11].

Andrographolide content

Andrographolide content was analysis by HPLC (Jasco) with a uv detector. The separation was carried out using a C_{18}, Phenomenax Licrosphere column. The andrographolide peak was detected at 223 nm. The entire chromatographic separation was performed at an isocratic mobile phase of 60% acetonitrile (pH 2.8 with phosphoric acid) [12].

Statistical analysis

All experiments were carried out in triplicate and data were subjected to one-way analysis of variance (ANOVA) using SPPS version 21. Duncan post hoc test was used to compare the means.

RESULTS

Total elimination of the microorganism is the purpose of irradiation. The results of gamma irradiated on microbial contamination of *A. paniculata* powder was shown in Table 1. The contamination of non-irradiated was largely due to a group of total bacterial count and total yeast & mold at the level of 1.24×10^5 CFU/g and 5.70×10^5 CFU/g, respectively. The level contamination of *A. paniculata* powder was exceed permissible limits of World Health Organization (WHO) [13]. The level of total bacterial count and total yeast & mold were reduced at the dose of 5 kGy. Similarly, another microbial such as Coliform Bacteria, *E. coli*, *B. cereus* and *C. perfringens* were detected in non-irradiated samples which were reduced at the dose of 5 kGy. *Salmonella* spp. was not detected in all samples. Microbial contaminations in *A. paniculata* powder were decreased at the irradiation dose of 5 kGy to standard level, while irradiation dose at 10 kGy were complete microbial decontaminations.

Table 1 Effect of irradiations on microbial contaminations in *A. paniculata* powder

Microbiological	0 kGy	5 kGy	10 kGy	15 kGy	20 kGy
Total bacterial count (CFU/g)	1.24×10^5	3.00×10^2	$<1_{Est}$	$<1_{Est}$	$<1_{Est}$
Total yeast & mold (CFU/g)	5.70×10^5	$<1_{Est}$	$<1_{Est}$	$<1_{Est}$	$<1_{Est}$
Coliform Bacteria (MPN/g)	460	<0.3	<0.3	<0.3	<0.3
E. coli (MPN/g)	3.6	<0.3	<0.3	<0.3	<0.3
Salmonella spp.	Absent	Absent	Absent	Absent	Absent
B. cereus (CFU/g)	2.10×10^5	5_{Est}	$<1_{Est}$	$<1_{Est}$	$<1_{Est}$
C. perfringens (MPN/g)	23	6	<0.3	<0.3	<0.3

$_{Est}$ = Estimated value

The antioxidant activity of *A. paniculata* extracts was investigated using DPPH and FRAP assays. Gamma irradiation did not affect the antioxidant activity. The reduction of DPPH by antioxidants in the *A. paniculata* extracts expressed as ascorbic acid equivalent (AAE) per gram. DPPH activity exhibited ranges from to 6.03 to 6.04 mgAAE/g (Table 2). The irradiated samples were found to be no significant when compared to the non-irradiated samples. In term of the FRAP values, it was determined of change in absorbance at 596 nm owing to the formation of a blue colored Fe^{2+} compound from colorless oxidized Fe^{3+} form by the action of electron donating antioxidants. The FRAP values exhibited ranges from to 78.97 to 82.40 µmol $FeSO_4$/g (Table 2). No significant difference on the FRAP values between non-irradiated samples and irradiated samples.

The total phenolic content (TPC) of *A. paniculata* extracts was carried out using the standard curve of gallic acid and presented as gallic acid equivalents (GAE) per gram. Total phenolic content of non-irradiated and irradiated samples ranges from 8.61 to 9.42 mgGAE/g (Table 2). The results indicated that no significant differences between the phenolic contents of non-irradiated and irradiated samples.

Table 2 Analysis of antioxidant activites by DPPH and FRAP, Total phenolic and andrographolide content of *A. paniculata* extracts at varied irradiation doses.

Irradiation Dose (kGy)	DPPH (mgAAE/g)	FRAP (µmol $FeSO_4$/g)	Total phenolic content (mgGAE/g)	Amount of andrographolide (mg/g sample)
0	6.09 ± 0.08[a]	78.97 ± 9.68[a]	8.61 ± 0.95[a]	35.80 ± 0.42[a]
5	6.13 ± 0.17[a]	82.40 ± 6.42[a]	9.10 ± 0.21[a]	35.27 ± 1.02[a]
10	6.24 ± 0.10[a]	80.64 ± 4.29[a]	9.42 ± 0.21[a]	34.43 ± 0.58[a]
15	6.04 ± 0.14[a]	79.64 ± 2.27[a]	9.22 ± 0.05[a]	34.26 ± 1.86[a]
20	6.03 ± 0.19[a]	79.83 ± 0.93[a]	9.25 ± 0.20[a]	32.96 ± 0.97[a]

Values are expressed as mean ± SD of triplicate measurements. Different letter in the same column indicate significant differences at $p<0.05$

The determination of andrographolide content of *A. paniculata* extracts was performed by using HPLC (High Performance Liquid Chromatography) method. The andrographolide content range between 32.96-35.80 mg/g (Table 2). The results indicated that there was no significant difference between non-irradiated samples and irradiated samples.

CONCLUSIONS

Gamma irradiation was the effective method used for microbial decontamination of *A. paniculata*. Irradiation dose of 5 kGy could reduce microbial contamination to the standard level, while irradiation dose at 10 kGy were completed microbial decontaminations. However, irradiation at doses of 5, 10, 15 and 20 kGy did not affect on antioxidant activity, total phenolic content and andrographolide content.

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REFERENCES

1. Kurzawa M, Filipiak-szok A, Klodzinska E, Szlyk E. 2015. Determindnation of phytochemicals, antioxidant activity and total phenolic content in *Andrographis paniculata* using chromatographic methods. J. Chromatogr. B. 995-996, 101-106.
2. Niranjan A, Tewari SK, Lehri A. 2010. Biological activity of Kalmegh (*Andrographis paniculata* Nees) and its active principles- A review. Indian J. Nat. Prod. Resour. 1(2), 125-135.

3. Wen-Wan Chao and Bi-Fong Lin. Isolation and identification of bioactive compounds in *Andrographis paniculata* (Chuanxinlian). 2010. http://www.cmjournal.org/content/5/1/17.
4. Kanokwan Jarukamjorn and Nobuo Nemoto. 2008. Pharmacological aspects of *Andrographis paniculata* on health and its major diterpenoid constituent andrographolide. Health Sci. 54(4), 370-381.
5. Minea R., Nemtanu M.R., Brasoveanu M. and Oproiu C. Accelerators use for irradiation of fresh medicinal herbs. 2004. Proceedinds of EPAC, Switzerland, 2371-2373.
6. Piyanuch Thongphasuk and Jarunee Thongphasuk. 2012. Effecst of irradiation on active components of medicinal plants: A review. Rangsit J. of Arts Sci. 2, 57-71.
7. Piyanuch Thongphasuk and Jarunee Thongphasuk. 2013. Effects of gamma irradiation on free radical induction, major components and toxicity of ethanolic extract from *Andrographis paniculata* (Burm. f.) Nees leaves. Bulletin of Health, Science and Technology.11, 7-12.
8. Official method of analysis. 1990. Associtation of Official Analytical Chemists, Washington DC, 15th edition.
9. Khattak K.F., Simson T.J. and Ihasnullah. 2008. Effect of gamma irradiation on the extraction yield, total phenolic content and free radical-scavenging activity of Nigella sativa seed. Food Chemistry. 110(4), 967-972.
10. Benzie L.F.F. and Strain J.J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of antioxidant power the FRAP assay Analytical Biochemistry. 239(1), 70-76.
11. Velioglu Y.S., Mazza Gao L., and Oomah B.D. 1998. Antioxidant capacity and total phenolics in selected fruit, vegetable and grain products. Journal of agricultural and food chemistry. 46(10), 4113-4117.
12. G Rao P.R. and Rathod V.K. 2015. Mapping study of an ultrasonic bath for the extraction of andrographolide from *Andrographis paniculata* using ultrasound. Industial Crop and Products. 66, 312-318.
13. World Health Organization. 1998. Food irradiation a technique for preservation and improving the safety of food. World Health Organization of the United Nations, Geneva.

Consumer Research (CR) Full Paper

Factors influencing Thai female consumer purchasing behavior toward Asian cosmetic products

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ABSTRACT

Asian cosmetic products are products that originally produced in Asia. In order to meet Asian consumer needs, Asian cosmetics usually contain whitening agent, collagens, vitamin E, and all sources of skin whitening agent. This research paper aimed to investigate factors influencing Thai female consumer purchasing behavior toward Asian cosmetic products. The investigated factors were Asian cosmetic product characteristics, cultures, and product samplings offered. To achieve the objectives, survey method was employed. From the sample size of 395 Thai females, researcher handed out 395 questionnaires to Thai females in financial district in Bangkok (Bang- Rak, Pathumwan), to investigate the factors influencing their purchasing behavior. The results indicated that product characteristics had negative relationship with Thai female consumer purchasing behavior. However, product samplings and cultures had had strong influences on Thai female consumers when these consumers made their purchasing decisions.

Keywords: brand characteristic, Asian cosmetic, product sampling, culture, purchase decision

INTRODUCTION

For centuries, to enhance beauty, and to promote good health are women best friend, women spent times and money on beauty and health to improve themselves. Since then, cosmetics have become necessities for women. Cosmetics are including make-up products, and skincare products [1]. Makeup products comprise of face, eye makeup, lipstick, and nail products [2]. Skincare products are products comprise of face, body lotion. There are many types and brands of cosmetic products in the market. With the increasing of globalization of fashion and trends, it enables consumers to access and to explore variety of new brands and types of cosmetics from other countries. Some female consumers buy products because of the brand, some buy because of the packaging, some buy because of friends' recommendations, others buy because of its' characteristics and promotions. Asia is an emerging region where people lifestyle has been changed to be more and more modern. People started to concern about their appearances especially, Asian women. They consider spending more money on cosmetic products which will reflect their personalities [3]. In 2013, RNCOS research center reported that Asia is the World's biggest cosmetic market with 34 percent of the World market shares and will continue growing in the future [4]. Due to differences of skin tone, skin types, and interest of Asian women, Asian cosmetic products are mostly different from western cosmetic products. Asian people are more interested in a lighter skin tone and more natural looks than western people. Asian cosmetics are focused more on whitening and brighten up the skin tone. The common ingredients in Asian cosmetic products are whitening and collagen which western product does not typically contain.

Cosmetic market is a very innovative fast paced industry that required innovative keys to success [2]. Thai cosmetic market has been growing continually during the past few years [5]. There are wide ranges selections of products and brands for Thai consumers. Previous research shown, that Thai consumers are more likely to buy foreign cosmetic brands than domestics'. It's also shown that Thai people are quality oriented and they perceived foreign imported brand as higher quality than domestics [6]. Therefore, this research paper aimed to investigate the factors influencing Thai female consumer purchasing behavior toward Asian cosmetic products. The investigated factors were Asian cosmetic product characteristics, cultures, and product samplings offered.

According to Guthrie, M. and Jung, J. research on women's perceptions of brand personality to women's facial image and cosmetic usage, they stated that brand personality can be used to differentiate a brand within a product category and provide product characteristic that can be used to market a brand [3]. They had found that brand

personality was the most important across the entire brand. They conducted an online survey on measuring facial image, cosmetic usage, brand attitude, and brand personality with the sample of 225 female participates in the USA by examined the relationship between consumer perception and brand personality, companies can pinpoint the characteristic that customers look for in a product, which will increase their customer satisfaction and enhance their brand image [3,7].

Consumer behavior is defined as the behavior consumers' exchange something of value for a product or service that satisfies their need [8]. The study focused on individual consumer decisions on how they spend their resources on consumption related items. Therefore, consumer behavior not only consists of purchasing behavior but also consists of thoughts and feeling consumers experience and the actions they perform [4]. They found that the environments such as advertisement, comments of a friend, product appearances and packaging were the factors influenced these behaviors. Also the study of cross-cultural consumer behavior from De Mooij, M. and Hofstede, G. shows those consumers are culture-bonded. There are cultural relationships with the self, personality and attitude, which are the basis of consumer behavior models and branding and advertising strategies. Culture and the environment around them largely affect consumer behaviors [9].

Consumer decision-making process is consisting of five steps. These steps including in the model are; problem recognition, information search, evaluation of alternatives, purchase decision, and post-purchase behavior process. However, not all purchase decision requires these entire steps [10]. According to Hawkins et al., there are more aspects that influence consumers than only decision making process, which are internal factors and external factors. Internal factors are motivation, attention, perception, and attitude, while external factors are influences from social class and reference groups [11]. Dissatisfied consumers may take action or not to take action on their dissatisfaction. Dissatisfied consumers may take action by; complain to the store or manufacturer, stop buying that brand or at that store, notify friends and acquaintances, the most dangerous action is to complain on the internet, which will affect brand image the most [11].

Culture is the most basic factor that has an effect on consumers purchase behavior. Culture can be defined as a set of traditions, values, attitudes, perceptions, wants, behaviors from the family and society that one has been contacted with since childhood to growing up [12]. Consumers are influenced by their interaction with their own society. It's strongly influenced how people eat, drinks, wear, and shop.

Free-product samples are gifts given in the stores to their target consumers, give them chances to try product before making purchase decision [13]. A study on in-store sampling has shown that 92 percent of consumers preferred free-product samples offer rather than free-coupons. And another research from Lindstedt, found that over 70 percent of consumers will try in-store free-product samples and over half of consumers who tried will purchase the real products [14]. Regarding to this study, it had proved that free-product samples could increase sales of sampled product by over 37-50 percent. This is the reason why free-product samples have been widely used as an effective promotional tool in generating product trial and making real purchase [15], this usually uses as a tool in introducing new product to consumers.

MATERIALS AND METHODS

2.1 Group Sampling

Using Yamane formula of sample size with an error of 5 percentages, and a confidence coefficient 95 percent [16], and according to Department Of Provincial Administration Thailand, 2011, reported that the number of female who is 18-60 years old and officially registered in Bang Rak district is 14,228, and female who officially registered in Pathumwan district is 18,377. Therefore, after using Yamane's formula the sample size of this survey is 395 [16].

2.2 Tools to Be Employed

Covariance Analysis of Log-linear Multiple Regression Model using Quantitative and Dummy Independent Variables from Ramanathan in 2002 [17]:

a) The model is estimated by:

$$100\left(\frac{\hat{Y}_1}{\hat{Y}_0}-1\right)=100\{exp[\hat{\beta}_3-\frac{1}{2}\widehat{Var}(\hat{\beta}_3)]-1\}, \qquad (1)$$

Where $\hat{Y}_0$ and $\hat{Y}_1$ are the dependent variables when D_1 (dummy) = 1 and D_0 = 0 respectively; *exp* is exponential function; and $\widehat{Var}$ is the estimated variance. If the model has an interactive term so that it becomes:

$$ln(Y) = \beta_1 + \beta_2 X + \beta_3 D + \beta_4 DX + u \qquad (2)$$

The corresponding expression is much more complicated. Verify that, in this case, it is:

$$100\left(\frac{\hat{Y}_1}{\hat{Y}_0}-1\right)=100\{exp[\hat{\beta}_3-\frac{1}{2}\widehat{Var}(\hat{\beta}_3+\hat{\beta}_3X)]-1\} \quad (3)$$

b) The variance expression depends on the value of *X* and also involves a linear combination of random variables. As for marginal effects of each determinant, the marginal effect of quantitative variable is $\frac{\delta ln(\hat{Y})}{\delta X}=\hat{\beta}_3+\hat{\beta}_3 D$. Thus, using logarithmic differentiation property, it becomes:

$$100\frac{\Delta Y}{Y}=100(\beta_2+\beta_4 D)\Delta X \quad (4)$$

It follows that $100\hat{\beta}_2$ is the approximate percent of change in *Y* for a unit of change in *X* when *D = 0* and that $100(\hat{\beta}_2+\hat{\beta}_4)$ is the approximate percent of change in *Y* for a unit change in *X* when *D = 1*.

2.3 Methodology

Cross-sectioned data presented below was collected from 395 respondents in Bangkok metropolitan area. All the data used was transformed into dummy variables using number 1 and 0 as notations.

Qualitative Variables (Dummy): Age, Marital status, Level of education, Occupation, Income level, Length of Cosmetic Usage (Leng), Amount Cosmetic Used per day (Usage) and Cosmetic Buying Frequency (Bfre)

Quantitative Variables: Brand Characteristic (BrCha), Culture (Cul) and Product Sampling Offer (Samp)

a) Using Gretl software, ln(MATERIALISM) was regressed against a constant, AGEa, AGEb, AGEc, AGEd, AGEe, AGEf, SING, MAR, WID, DIVOR, SEPAR, HIGHS, DIP, BACH, MAST, PHD, OTHERED, STUD, BUSOWN, EMPL, CIVIC, FARM, UNEMP, HOME, INCa, INCb, INCc, INCd, INCe, INCf, INCg, INCh, BrCha, Cul, Samp.
b) The next step was to regress the variables with ln(BrCha) and omitted variables with insignificant coefficients**,** a few at a time, until all coefficients were significant at 10 percent. (The results are shown in Results section below)
c) Finally, the complete model was estimated with all square and interaction terms.
d) The same process was repeated but the dependent variables were replaced by CUL and SAMP at a time.

RESULTS AND DISCUSSION

Table 1: Model 4: OLS, using observations 1-400, Dependent variable: l_BrCha

	Coefficient	*Std. Error*	*t-ratio*	*p-value*	
const	2.83802	0.046623	60.8717	<0.00001	***
AGEe	-0.0406176	0.0183732	-2.2107	0.02763	**
GOV	0.0570801	0.0231776	2.4627	0.01421	**
Cul	0.0161967	0.00221682	7.3062	<0.00001	***
Samp	0.0110385	0.00239323	4.6124	<0.00001	***

Table 2: Model 5: OLS, using observations 1-400, Dependent variable: l_Cul

	Coefficient	*Std. Error*	*t-ratio*	*p-value*	
const	2.0211	0.0766245	26.3767	<0.00001	***
WID	0.215583	0.114374	1.8849	0.06018	*
HIGHS	0.0875611	0.049532	1.7678	0.07788	*
UNEMP	-0.141388	0.0578767	-2.4429	0.01501	**
Usage	-0.0244048	0.0111584	-2.1871	0.02932	**
BrCha	0.0177937	0.00238059	7.4745	<0.00001	***
Samp	0.0182895	0.00316905	5.7713	<0.00001	***

Table 3: Model 6: OLS, using observations 1-400 (n = 399), missing or incomplete observations dropped: 1 Dependent variable: l_Samp

	Coefficient	*Std. Error*	*t-ratio*	*p-value*	
const	2.44521	0.0508358	48.1001	<0.00001	***
AGEc	-0.0256522	0.0148935	-1.7224	0.08579	*
MAR	0.0662693	0.0127301	5.2057	<0.00001	***
WID	-0.146193	0.0855527	-1.7088	0.08828	*
NOJOB	-0.121814	0.0414576	-2.9383	0.00350	***
INCd	-0.0366003	0.0169598	-2.1581	0.03153	**
BrCha	0.00845717	0.00186052	4.5456	<0.00001	***
Cul	0.0141304	0.00231475	6.1045	<0.00001	***

Table 1, 2, and 3 indicate the statistic test on all the suspected variables with BrCha, Cul, and Samp respectively. P-values of all the selected variables demonstrate that all coefficients in the model are significant at 10 percent or lower level. As a result, the logarithm of BrCha, Cul, and Samp were well explained by the constructed model, and hence, clearly signifying the superiority of this model.

Model 4 (Table 1)

Thai women aged between 50 and 59 years old demonstrate negative relationship with Brand Characteristic. As age increases (within the stated range) Brand Characteristic decreases. However, significant relationship with Brand Characteristic does not exist in any other ages. Considering the occupation, only government officer shows significantly positive relationship with Brand Characteristic while the rests demonstrate insignificant relationship with Brand Characteristic. As for Culture, there is a significantly strong relationship with Brand Characteristic. Additionally, it is also appeared that Product Sampling offer displays positively strong relationship with Brand Characteristic, while the rest of the variables have not shown any positive relationship with Brand Characteristic.

Model 5 (Table 2)

It can be obviously seen that only women with unemployment has negative relationship with culture in term of cultural influence on cosmetic buying. The negative relationship is also found in the category of Length of Cosmetic Usage as both factors increase, Thai women tend to be more reluctant to cultural influence. As for marital status, only when people become widow, their behavior toward cultural lessons. Considering the level of education, only high school women demonstrate positive relationship with culture. However, the rest shows the opposite. Lastly, Thai women have positively superior relationship between culture and brand characteristic. Moreover, the same superior positive relationship also applied toward culture and product sampling offer.

Model 6 (Table 3)

According to the table 3, age demonstrates negative relationship with the product-sampling offer. As age increases from 30 to 39, the influence of product sampling offer falls with decreasing rates. As for marital status, only the widow shows significant negative relationship with the product-sampling offer while the married women displays highly positive relationship. Surprisingly, as for occupation, only Thai women with no jobs show significantly negative relationship with their product-sampling offer. As expected, the income level demonstrates negative relationship with the product-sampling offer. As people's income increases (within the range of 15,001 to 20,000), the product sampling offer decline considerably at decreasing rates. Predictably, the superiorities of brand characteristic and culture demonstrate the highly positive relationship with product sampling offer.

CONCLUSIONS

Thailand's cosmetic market could provide a good example of imported cosmetic products market, the market in which an increasing number of foreign cosmetic products are exploded every year. Due to a wide range choice of imported cosmetic products, may shape Thai female decision-making to be more complex and particular. Therefore, it has been assumed that Thai female consumers adopt certain particular decision-making style when interacting with the imported cosmetic. Cosmetic market is one of the most fast growing market sections, which have been competitive and grown continually during the past few years. This research is aimed to investigate the relationship between Asian

Cosmetic brand characteristics, cultures, and product samplings offered to Thai female consumer purchasing behavior living in Bangkok. From this study 395 respondents were participated; respondents' age between 20-29 years old is the majority of this study with 36 percent. 80 percent of respondents own a Bachelor degree followed by a master degree which accounted for 14 percent. Half of the respondents are currently employed. In term of monthly income, the majority of them earn less than 35,000THB accounting for 90 percent. The research was conducted in the limitation of the sample group that might not be able to represent the whole population totally. The further study may use random sample with larger population in different locations to increase the reliability of the result. This research focuses only on Asian product characteristic, culture, and product sampling offer as the indicator that it would affect consumer decision making. It was discovered that there was a weak relationship between product characteristic and consumer purchasing behavior. However, in term of product sampling and culture, they had strong influences to consumers when making purchasing decision. Therefore, researcher would like to suggest that future research should be included some other factors such as reference group, or place of the products and other variables such as attitude, motivation and perception in the study.

REFERENCES

1. Oxford Dictionary. 2012. Oxford Dictionary online. Available from http://www.oxforddictionaries.com/definition/english/cosmetic
2. Kumar V, Pilai PR. 2007. A Study on Purchase Pattern of Cosmetics among Consumers in Kerala. International Marketing Conference of Marketing & Society.
3. Guthrie M, Kim HS, Jung J. 2008. The effects of facial image and cosmetic usage on perceptions of brand personality. JFMM. 12 (2), 164-181.
4. Cosmetic History. 2014. The history of cosmetics from ancient times. Available from: http://www.cosmeticsinfo.org/Ancient-history-cosmetics [Accessed on 10 September 2014]
5. U.S. commercial service. 2008. Doing Business In Thailand: A Country Commercial Guide for U.S. Companies. Available from: http://www.buyusa.gov/thailand/en/ccg.html [Accessed on 15 March 2009]
6. Ponbamrungwong A, Chandsawang S. 2009. The impact of brand on Thai female consumers in purchase decision of foreign makeup product. Available from: http://www.diva-portal.org/smash/record.jsf?pid=diva2:236236 [Accessed 15 August 2014]
7. Simons KL. 2011. Product Market Characteristics and the Industry Life Cycle. Available from: http://homepages.rpi.edu/~simonk/pdf/pmcid.pdf [Accessed on 1 September 2014]
8. Ruth W. 2005. A consumer's dictionary of cosmetic ingredients. Three Rivers Press. Available from: http://edissertations.nottingham.ac.uk/947/1/07MSClixnc2.pdf
9. De Mooij M, Hofstede G. 2011. Cross-cultural consumer behavior: A review of research findings. J. Int. Consum. Market. 23, 181-192.
10. Hoyer W, Maclnnis D. 2001. Consumer Behavior. Boston. Houghton Mifflin Company.
11. Hawkins D, Best R, Coney K. 2000. Consumer Behavior Building Marketing Strategy, New York: McGraw-Hill Irwin.
12. Philip K, Veronica W, John S, Gary A. 2005. Principles of Marketing, 4th European edition, Pearson Education Limited.
13. McGuinness D, Bernnan M, Gendall P. 1995. "The effect of product sampling and couponing on purchase behavior: some empirical evidence", Int. J. Advert. 14 (3), 219-230.
14. Lindstedt S. 1999. "Tops supermarkets in western new York entice shoppers with free food samples". High Beam Research. Available from: http://www.highbeam.com/doc/1G1-54716748.html. [Accessed on 11 September 2014]
15. Rossiter J, Percy L. 1987. "Advertising and promotion management"., New York: McGraw-Hill.
16. Yamane T. 1967. Statistics, An Introductory Analysis, 2nd Ed., New York: Harper and Row. Ramanathan R. 2002. Introductory Econometrics with Applications, Fort Worth: Harcourt, 5th Ed.

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