ASNP
The Asian Society of Natural Products
www.webofasnp.org

ISSN 2635-6643

NATPRO 8

The 8th International Conference on Natural Products
July 25(Tue) - 27(Thu)
Bangkok, Thailand

Natural perspectives for Health

The Asian Society of Natural Products

NATPRO: NATPRO8
On occasion of NATPRO8 organized by ASNP

Edited by Hyang-Ryeol Lee and Jaehong Han
Design and Printed by Phetkasem Printing Group Co., Ltd., Thailand
ISSN 2635-6643

The 8th International Conference on Natural Products

Natural Prospectives for Health

July 25 – 27, 2023

Bangkok, Thailand

Organized by

Faculty of Medicine, Thammasat University

The Asian Society of Natural Products

Hosted by

ASNP Thailand Chapter

Sponsored by

Message from Rector of Thammasat University

Associate Professor Gasinee Witoonchart
Rector of Thammasat University

The 8th International Conference on Natural Products (NATPRO8) under the theme "Natural Prospectives for Health" is a major conference organized with the objective of featuring progress in the research and development of nature-based products, Thai traditional medicine, and health care.

Organized by the Faculty of Medicine of Thammasat University at the Asawin Grand Convention Hotel, Bangkok, Thailand, during 25-27 July 2023, the conference is a result of the collaboration between Thammasat University and the Asian Society of Natural Products (ASNP). The conference provides scientists a stage for presenting their research works, particularly integrated ones, that help to further our state of knowledge and innovation – an important mechanism for functioning efficiently in the ASEAN and international communities. It also creates a strong academic network through academic dialogues between lecturers, researchers, students, and the general public.

As Thammasat University Rector, I would like to extend my profound gratitude to the organizing committee the Faculty of Medicine of Thammasat University, and the ASNP, including all the parties involved in organizing this conference, for their devotion to making this conference achieve its objectives. I wish you all happiness, prosperity, and personal professional success, and truly hope that you will gain fresh and illuminating insights from this conference that you can apply to advancing your country afterwards.

Associate Professor Gasinee Witoonchart, MBA

Rector of Thammasat University

Message from Dean of Faculty of Medicine

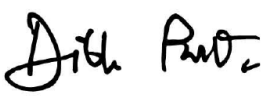
Associate Professor Dilok Piyayotai MD.
Dean of the Faculty of Medicine
Thammasat University

Distinguished speakers, guests of honor, representatives from international affiliates, presenters, participants, clinical nutrition and traditional medicine colleagues, ladies and gentlemen. On behalf of the Faculty of Medicine, Thammasat University, it is my great pleasure to welcome you to the 8th International Conference on Natural Products (NATPRO8), held at the Asawin Grand Convention Hotel, Bangkok, Thailand, during July 25-27, 2023.

I would like to acknowledge the many people who have contributed to the great success of this conference, namely the organizing committee members, distinguished speakers, presenters, and participants from across the world, including lecturers and researchers, students, and the general public, all of whom deserve our recognition and thanks.

Under this year's conference theme "Natural Prospectives for Health", we will learn from a wide variety of studies showcasing the making of nature-based products and new dimensions of integrated research along with its roles in advancing the body of knowledge and innovation in clinical nutrition and traditional medicine. The conference will also serve as a platform for scholarly exchanges and academic collaboration on both local and international levels, thereby strengthening our discipline now and in the years to come.

With the variety of topics discussed and the many local and international scholars presenting and participating in the conference, I truly hope that it will prove a fruitful one and achieve its objectives. Have an enjoyable time!



Associate Professor Dilok Piyayotai, MD
Dean of the Faculty of Medicine
Thammasat University

Message from Chairperson of Organizing Committee

Professor Arunporn Itharat
Chairperson of Organizing Committee
Thammasat University

We are thrilled to extend a warm welcome to all attendees of the 8th International Conference on Natural Products (NATPRO), taking place at the Asawin Grand Convention Hotel in Bangkok, Thailand, from July 25th to 27th, 2023. On behalf of the organizing committee, we would like to express our gratitude to the Asian Society of Natural Products (ASNP) board for granting us this incredible opportunity to manage an international conference. This conference marks the second NATPRO event organized by ASNP and the first time it is being hosted in Thailand, courtesy of the Faculty of Medicine and the Center of Excellence in Applied Thai Traditional Medicine Research (CEATMR) at Thammasat University.

We consider it a great honor that our country is the principal organizer of this esteemed conference. The Faculty of Medicine at Thammasat University serves as the principal organizing committee, with valuable support from ASNP, CEATMR at Thammasat University, and the Department of Thai Traditional and Alternative Medicine under the Ministry of Public Health, who have generously subsidized the conference budget.

Drawing from our experience hosting the 20th World Congress on Clinical Nutrition (WCCN) in 2016, we aim to further disseminate research locally and internationally while fostering collaboration with international organizations in the realms of research, education, and business. Recognizing the significance of natural product research for health, the Faculty of Medicine at Thammasat University has joined hands with ASNP to host NATPRO8 in 2023, bringing together participants from academia, research, students, business, and the general public, all of whom have been actively involved in natural product research and are passionate about sharing their knowledge with the world.

This year's conference features a novel addition, the Innovation Competition on Natural Products for Health, marking the first time ASNP has included in an academic competition in Thailand. This competition aims to showcase innovative natural products developed by Thai and foreign researchers, attracting potential business partners interested in further development and commercialization of these products in both local and international markets. Additionally, the conference provides a platform for local and international business representatives involved in natural product production to exchange

ideas and explore opportunities for collaboration. We are excited to introduce this business matching aspect for the first time at an academic conference, hoping to facilitate meaningful exchanges and inspire new strategies for marketing these natural products.

Under the theme "Natural Prospective for Health," this major conference endeavors to highlight advancements in nature-based product research, Thai traditional medicine, and healthcare. We have also included discussions on cannabis research, an engaging and topical subject. Our plenary and invited speakers hail from 10 countries, enriching the conference with diverse perspectives. This gathering of practitioners, researchers, and educators from around the globe underscores their shared commitment to natural product research and its impact on human health and wellness.

We have devoted our utmost efforts to ensure the conference's success, both academically and socially. Alongside the informative sessions, we have organized two exciting events, the Welcome Party and the Gala Dinner, the latter of which will take place aboard a Riverside Cruise on the Choawpraya River. We would like to express our sincere appreciation to all the sponsors from both the public and private sectors, as their generous contributions have played a pivotal role in making this conference possible. We are also immensely grateful to the organizing committee, including ASNP directors, colleagues from the Faculty of Medicine at Thammasat University, and particularly the dedicated staff in the Department of Applied Thai Traditional Medicine, as well as the master's and doctoral students in CEATMR, who have made invaluable contributions to every aspect of this conference.

In closing, we sincerely hope that each attendee thoroughly enjoys all the academic, business, and entertainment programs offered during the 8th NATPRO conference and has an unforgettable experience in Thailand.

Professor Arunporn Itharat, PhD
Chairperson of Organizing Committee
Head of Center of Excellence on Applied Thai Traditional Medicine Research (CEATMR)
Faculty of Medicine
Thammasat University

Message from the President of the Asian Society of Natural Products

Jaehong Han

I would like to welcome the ASNP members and the distinguished invited speakers to NATPRO8. The 8th international Conference on Natural Products in Bangkok would not have been possible without compassion from Arunporn. I wish to express my most sincere gratitude and appreciation to Aj. Arunporn and her staff. Also, I would like to thank the Faculty of Medicine at Thammasat University for hosting NATPRO8. The NATPRO8 is the first conference held other than Korea under the umbrella of ASNP since its establishment in 2016. Under the new NATPRO conference organizing system, the NATPRO8 organizer and her committee, ASNP main office and ASNP national chapters devoted themselves to making NATPRO8 successful. I would like to praise the efforts of these numerous supporters. I also deeply appreciate financial support from the sponsors in Thailand and Korea.

The only reason that ASNP exists is to support the members. I wish all the ASNP members to be benefited academically by building international networks during NATPRO8.

Professor Jaehong Han, PhD in Chemistry
Chung-Ang University
The 2nd president of ASNP

Manual for ASNP members at NATPRO8

For the NATPRO conference, the registration and abstract submission are handled by ASNP. If you have any inquiry on these issues, please contact ASNP staff during the conference. The ASNP membership is processed by each ASNP national chapter, so the issue on the membership can be address by each national chapter secretary.

Thailand	Suda Chakthong	Prince of Songkla University
Philippines	Elena S. Catap	University of Philippines Diliman
Korea & Others	Jongkeun Choi	Chungwoon University

Briefing on the academic activity

As ASNP publishes NATPRO abstract book as a serial publication (ISSN: 2635-6643), ASNP adopted a few new systems for NATPRO8 abstracts. While all the submitted abstracts can be published in NATRPO, the submitter can choose whether the abstract will be reviewed or not. No review submission only accepts abstract and it is published in the book without DOI (digital object identifier) number. The abstract applied for no review submission will still go through the grammar and format checks. The review submission accepts the abstract and proceeding paper which will be published with doi number after acceptance. You can find the DOI number on the upper right side of some abstracts in this book. Such abstract submission system will continue in the future NATPRO conferences. ASNP wants to watch the members at the international standard. This unique system will also boost newly launched NATPRO Journal (ISSN 2983-0877).

Briefing on the ASNP member activity

General Meeting will be held on the last day of NATPRO8, July 27, to deliberate and vote over the following matters of the SOCIETY, including the operation and settlement of account of the current fiscal year, operation plan and budget of the next fiscal year, approval of election of the President-elect, Vice-Presidents, elected members of Board of Directors, and the Auditors, amendments to the Constitution, matters referred by the Board of Directors, and other matters deemed to be important or set forth in the ASNP Constitution. New regular members and existing members with valid membership will have the right to act in the General Meeting.

Briefing on the ASNP officer activity

Several meetings, including ASNP BOD meeting, NATPRO9 steering committee meeting and workshop for ASNP officers will be held during NATPRO8. Notes for the convention will be delivered personally.

NATPRO8
The International Conference on Natural Products

The 8th International Conference on Natural Products

July 25 – 27, 2023

Bangkok, Thailand

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Committees

Conference Chair

Arunporn Itharat	Thammasat University	Thailand

Executive Organizing Committee

Jaehong Han	Chung-Ang University	Korea
Jongkeun Choi	Chungwoon University	Korea
Hyang-Yeol Lee	Korea National University of Transportation	Korea

Academic Committee

Hyang-Yeol Lee	Korea National University of Transportation	Korea
Neal S. Davies	University of Alberta	Canada
Omboon Vallisuta	Thammasat University	Thailand
Sirithon Siriamornpun	Mahasarakham University	Thailand
Buncha Ooraikul	University of Alberta	Canada
Siriwat Jinsiriwanich	Chiang Mai University	Thailand

International Organizing Committee of ASNP

Jongkeun Choi	Chungwoon University	Korea
Suda Chakthong	Prince of Songkla University	Thailand
Elena S. Catap	University of the Philippines, Diliman	Philippines
Neal S. Davies	University of Alberta	Canada

Local Organizing Committee

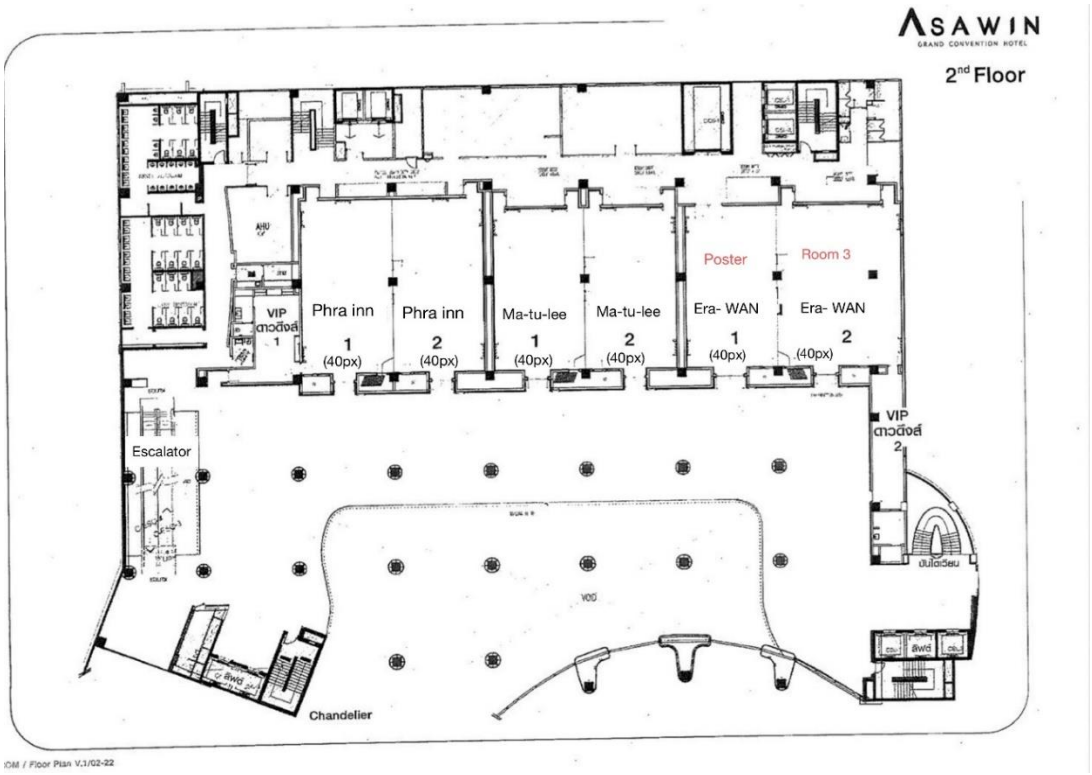
Puritat Kanokkangsadal	Thammasat University	Thailand
Saovapak Poomirat	Thammasat University	Thailand
Onmanee Prajuabjinda	Thammasat University	Thailand
Nuanjan Jaiarree	Thammasat University	Thailand
Sumalee Panthong	Thammasat University	Thailand
Pakakrong Thongdeeying	Thammasat University	Thailand
Srisopa Ruangnoo	Thammasat University	Thailand
Nichamon Mukkasombut	Thammasat University	Thailand
Pannawat Chaiyawatthananthn	Thammasat University	Thailand
Jurairat Boonruab	Thammasat University	Thailand
Katanchalee Houngiam	Thammasat University	Thailand
Intouch Sakpakdeejaroen	Thammasat University	Thailand

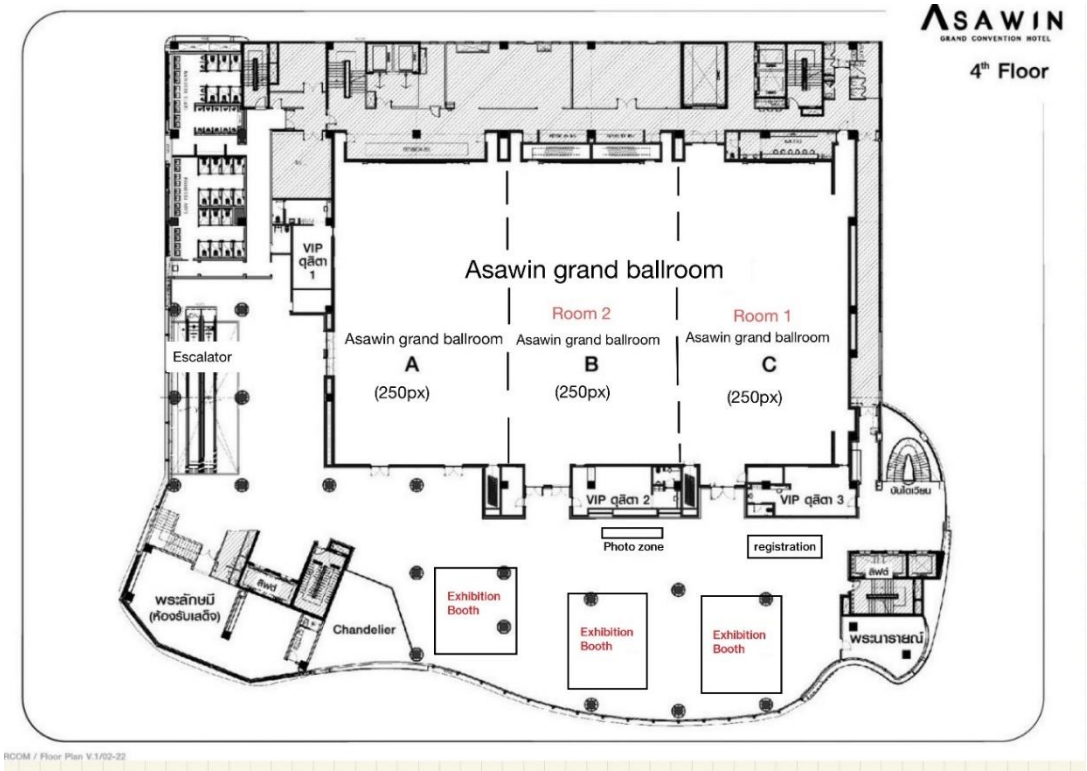
Outline of NATPRO8

Title	The 8th Intnerational Conference on Natural Products	
Theme	Natural Prospectives for Health	
Date	July 25 – 27, 2023	
Venue	Asawin Grand Convention Hotel	
Organizers	Faculty of Medicine, Thammasat University	
	The Asian Society of Natural Products	
Host	ASNP Thailand Chapter	
Keywords	PL	Plenary lecture
	IS	Invited lecture
	OP	Oral presentation
	GS	Graduate student presentation
	IN	Innovation competition
	P	Poster presentation

Venue and Floor Plan

Asawin Grand Convention Hotel, Bangkok





Daily Program

Day 1 (July 25, Tuesday)

Time	Room 1 (Ballroom C)	Room 2 (Ballroom B)	Room 3 (Erawan 2)
08:00 – 09:00	Registration (Hall 4th floor)		
09:00 – 09:30	Opening ceremony		
09:30 – 12:00	Plenary lecture 1 - 4		Innovation Exhibition
12:00 – 13:00	Lunch		
13:00 – 14:30	Poster presentation (Erawan 1)		
14:30 – 15:00	GS 1, 2	GS 3, 4	Innovation Competition
15:00 – 15:15	Break		
15:15 – 16:15	OP 1 – 3	OP 4 – 6	Innovation Competition
16:15 – 17:00	GS 5 – 7	GS 8 – 10	
17:00 – 20:30	Welcome party		

Day 2 (July 26, Wednesday)

Time	Room 1 (Ballroom C)	Room 2 (Ballroom B)	Room 3 (Erawan 2)
08:00 – 09:00	Registration (Hall 4th floor)		
09:00 – 10:00	Invited 1, 3	Business Matching Session	Invited 2, 4
10:00 – 10:15	Break		
10:15 – 11:15	Invited 5, 7	Business Matching Session	Invited 6, 8
11:15 – 12:15	OP 7 – 9		OP 10, 11, GS 11
12:15 – 13:15	Lunch		
13:15 – 14:45	Poster presentation (Erawan 1)		
14:45 – 15:00	Break		
15:00 – 20:30	Dinner in cruise @ Chaopraya River		

Day 3 (July 27, Thursday)

Time	Room 1 (Ballroom C)	Room 2 (Ballroom B)
08:30 – 09:30	Registration (Hall 4th floor)	
09:30 – 10:15	Plenary 5	
10:15 – 10.30	Break	
10:30 – 11:30	Invited 9-10	
12:00 – 13:00	Lunch	
13:00 – 14:00	ASNP General Meeting	
14:00 – 14:15	Break	
14:15 – 15:00	ASNP AWARD and public relation	

Note: Exhibition Booth at the hall 4th floor

Academic Program

July 25, 09:30 – 12:00 ‖ **Room 1 & 2, Ballroom B + C**

Plenary lecture	Chair: ASNP President-elect, Supayang Voravuthikunchai

PL – 1 09:30 – 10:15

Cannabis: from traditional to forbidden and now medicinal plant

Raimar Loebenberg

Faculty of Pharmacy and Pharmaceutical Sciences Katz Group-Rexall Centre for Pharmacy & Health Research University of Alberta, Canada

PL – 2 10:15 – 11:00

The importance of gut microbiota for the activity of herbal medicine

Rudolf Bauer

Institute of Pharmaceutical Sciences, Pharmacognosy, University of Graz, 8010 Graz, Austria

PL – 3 11:00 – 11:30

Symptom management in patients with advanced stage cancer at Arokhayasala Kham-pramong temple

Phrapraponpat Jirathammo

Arokhayasala Khampramong Temple, Pannanikom, Sakonnakorn, Thailand

PL – 4 11:30 – 12:00

Health innovation program for the detoxification of amphetamine derivatives addicted patients

Chalermkiat Srivorakan[1*], Sunisa Ratanasribuathong[2], Uaekan Worapaitoon[2] , Supaluck Thudsri[2], Puritat Kanokungsadal[2,3], Nichamon Mukasombat[2,3], Pranporn Kuroprakornpong[2,3], Kulyarat Pumlek[2,3], Sunita Makchuchit[2,3], Jedsada Wongsua[4], Chanuntachai Pruksukarn[5] and Arunporn Itharat[2,3]

[1]*Government House of Thailand. 1, Nakhon-Pathom Rd., Dusit, Bangkok, 10300, Thailand*

[2]*Center of Excellence in Applied Thai Traditional Medicine Research (CEATMR), Thammasat University, Klongluang, Pathumthani,12120, Thailand*

[3]*Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Klongluang, Pathumthani, 12120, Thailand*

[4]*Royal Police Cadet Academy, Sam Phran, Nakorn Prathom, 73110, Thailand*

[5]*Nakhon Pathom Provincial Administrative Organization, Nakhon Pathom, 73110, Thailand*

July 25, 14:30 – 15:00 ‖ **Room 1, Ballroom C**

Graduate student presentation	Chair: NATPRO8 organizer, Arunporn Itharat

GS – 1 14:30 – 14:45

HPLC development of *Thunbergia laurifolia* leaf extract capsule using design of experiments for a stability indicating method

Thanapat Onsawang[1], Pongtip Sithisarn[2], Chutima Phechkrajang[1] and Piyanuch Rojsanga[1*]

[1]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand*

[2]*Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand*

GS - 2 14:45 - 15:00

Cell-free biotransformation of aryl allyl/methyl ethers by human gut bacterium *Blautia* sp. MRG-PMF1

Santipap Chaiyasarn and Jaehong Han*

Metalloenzyme Research Group and Department of Plant Science and Technology, Chung-Ang University, 4726 Seodong-daero, Anseong 17546, Republic of Korea

July 25, 15:15 - 16:15 **‖ Room 1, Ballroom C**

Oral presentation	Chair: NATPRO8 organizer, Arunporn Itharat

OP - 1 15:15 - 15:35

Longan (*Dimocaepus longan* Lour.): economic fruit with high value for phytonutrients and phytocosmetics

Surapol Natakankitkul[1*], Charin Techapun[2], Atthaphon Khamwangpruk[3], Lucas Huber[4] and Günther Bonn[5]

[1]*Faculty of Pharmacy, Chiang Mai University, Muang, Chiang Mai, 50200, Thailand*

[2]*Faculty of Agro-Industry, Chiang Mai University, Muang, Chiang Mai, 50200, Thailand*

[3]*PM 80 Ltd. 88 Moo 13, A. Pasang, Lamphun 51120, Thailand*

[4]*Institute of Cell Biology, Medical University of Innsbruck, Innsbruck, Austria,* [5]*The Austrian Drug Screening Institute (ADSI), Innsbruck, Austria*

OP - 2 15:35 - 15:55

In-vitro, in-vivo, and in-silico antioxidant and antihypertensive activities of *Nauclea subdita* (Korth.) Steud. stem bark aqueous extract

Finna Setiawan[1*], Erfan Tri Prasongko[2], Nikmatul Ikhrom Eka Jayani[1], Dini Kesuma[3], Christina Avanti[4] and Kartini Kartini[1]

[1]*Department of Pharmaceutical Biology, Faculty of Pharmacy, University of Surabaya, Surabaya, Indonesia*

[2]*Master of Pharmaceutical Science, Faculty of Pharmacy, University of Surabaya, Surabaya, Indonesia*

[3]*Department of Pharmaceutical Chemistry, Faculty of Pharmacy, University of Surabaya, Surabaya, Indonesia*

[4]*Department of Pharmaceutics, Faculty of Pharmacy, University of Surabaya, Surabaya, Indonesia*

OP - 3 15:55 - 16:15

Phytochemical and biological examination of *Aconitum heterophyllum*

Md. Saiful Alam[1], Satyajit Roy Rony[2], Farhana Afroz[2], Abdullah Al-Mansur[3], Suriya Sharmin[2], Fatema Moni[2], Shammi Akhter[2], Md. Hossain Sohrab[2] and Mohammad Musarraf Hussain*[1]

[1]*Department of Pharmacy, Faculty of Life and Earth Sciences, Jagannath University, Dhaka-1100, Bangladesh*

[2]*Pharmaceutical Sciences Research Division, Bangladesh Council of Scientific and Industrial Research (BCSIR), Dr. Qudrat-I-Khuda Road, Dhaka 1205, Bangladesh*

[3]*Institute of National Analytical Research and Services, Bangladesh Council of Scientific and Industrial Research (BCSIR), Dr. Qudrat-I-Khuda Road, Dha-ka 1205, Bangladesh*

July 25, 16:15 – 17:00 ‖ **Room 1, Ballroom C**

Graduate student presentation	Chair: ASNP director, Sirithon Siriamornpun

GS – 5 16:15 – 16:30

Glycosidic C-O bond cleavage by DgpA

Heji Kim[1], Joong-Hoon Ahn[2], Jong Suk Lee[3] and Jaehong Han[1]*

[1]*Metalloenzyme Research Group and Department of Plant Science and Technology, Chung-Ang University, 4726 Seodong-daero, Anseong 17546, Republic of Korea*

[2]*Department of Integrative Bioscience and Biotechnology, Bio/Molecular Informatics Center, Konkuk University, Seoul 05029, Republic of Korea*

[3]*Biocenter, Gyeonggido Business & Science Accelerator (GBSA), Suwon, Gyeonngi-do 16229, Republic of Korea*

GS – 6 16:30 – 16:45

Effects of extraction methods on composition and functional properties of silkworm pupae protein

Sasithorn Boonchimplee, Wiriya Onsaard and Ekasit Onsaard*

Indigenous Food Research and Industrial Development Center, Department of Agro-Industry, Faculty of Agriculture, Ubon Ratchathani University, 85 Sathonlamark Road, Warin Chamrap District, Ubon Ratchathani, 34190 Thailand

GS – 7 16:45 – 17:00

Antioxidant and anti-tyrosinase potentials of giant granadilla (*Passiflora quadrangularis* L.) fruit extracts for skin care products

Nareekan Yanasan[1], Surapol Natakankitkul[1]*, Kanokwan Kiattisin[1], Natthanan Phupaisan[1] and Worrapon Wangkananon[2]

[1]*Department of Pharmaceutical Science, Faculty of Pharmacy, Chiang Mai University, Muang, Chiang Mai, 50200, Thailand*

[2]*TNK Beauty Ltd, 15/4 Soi Phra Mae Maha Karun 25, Ban Mai, Pak Kred District, Nonthaburi, Thailand*

July 25, 14:30 – 15:00 ‖ **Room 2, Ballroom B**

Graduate student presentation	Chair: ASNP director, Jong Suk Lee

GS – 3 14:30 – 14:45

New source of bioactive compounds from rice plant at different growth stages

Chainarong Chuayjum[1], Sinsupa Phukhamorn[1], Songkran Disa-nguan[1], Wiriya Onsaard[1,2] and Ekasit Onsaard[1,2]*

[1]*Department of Agro-Industry, Faculty of Agriculture, Ubon Ratchathani University, Maung Srikai sub-district, Warin Chamrap district, 34190, Thailand*

[2]*Indigenous Food Research and Industrial Development Center, Ubon Ratchathani University, Warinchumrap, Ubon Ratchathani, 34190, Thailand*

GS – 4 14:45 – 15:00

In silico screening and molecular docking shows cannabicitran isolated from *Cannabis sativa* as potential HIV-1 integrase and protease inhibitor

Celynros Victoria Caudal[1], Rod Vincent Borromeo[1]*, Jeffrey Clement Bungar[1], Sheriah Laine de Paz-Silava[2] and Ahmad Reza Mazahery[1]

[1]*Institute of Biology, National Science Complex, University of the Philippines Diliman, Quezon City, Philippines*

[2]*College of Public Health, University of the Philippines Manila, Manila, Philippines*

July 25, 15:15 – 17:00 ‖ **Room 2, Ballroom B**

Oral presentation	Chair: ASNP director, Sonia D. Jacinto

OP – 4 15:15 – 15:35

Role of probiotics in non-communicable diseases

Chaiyavat Chaiyasut[1]*, Periyanaina Kesika[1,2] and Sundaram Sivamaruthi[1,2]

[1]*Innovation Center for Holistic Health, Nutraceuticals, and Cosmeceuticals, Faculty of Pharmacy, Chiang Mai University, Chiang Mai 50200, Thailand*

[2]*Office of Research Administration, Chiang Mai University, Chiang Mai 50200, Thailand*

OP – 5 15:35 – 15:55

Determination of total phenolics, total flavonoids, and antioxidant activity of chewable gummy tablets of moringa leaf extract during the storage period

Nikmatul Ikhrom Eka Jayani[1*], Karina Citra Rani[2], Ni Putu Ardhiani Kencana Putri[3], Angeline Thereciany[3], Finna Setiawan[1] and Kartini Kartini[1]

[1]*Department of Pharmaceutical Biology, Faculty of Pharmacy, University of Surabaya, Indonesia*

[2]*Department of Pharmaceutics, Faculty of Pharmacy, University of Surabaya, Indonesia*

[3]*Faculty of Pharmacy, University of Surabaya, Indonesia*

OP – 6 15:55 – 16:15

Antioxidant activity and β-carotene production of extracts from *Bacillus infantis*

Bang Hong Lam, Bui Thanh Phu, Nguyen Le Thanh Đat, Huynh Nguyen Nhu Ngoc, Truong De and Nguyen Minh Chon*

Food and Biotechnology Institute, Cantho University, Vietnam

July 25, 16:15 – 17:00 ‖ **Room 2, Ballroom B**

Graduate student presentation	Chair: ASNP director, Sun Chul Kang

GS – 8 16:15 – 16:30

Authentication by using chemical method, cytotoxic test, and anti-nitric oxide production test of different nutmeg crude drugs sold in Thailand

Suthiwat Khamnuan[1], Pornsiri Pitchakan[2], Chuda Chittasupho[1], Mingkwan Na Takuathung[3], Ampai Phrutivorapongkul[1] and Aekkhaluck Intharuksa[1*]

[1]*Department of Pharmaceutical Science, Faculty of Pharmacy, Chiang Mai University, Suthep, Meung Chiang Mai, Chiang Mai, 50200, Thailand*

[2]*Department of Biochemistry, Faculty of Medicine, Chiang Mai University Suthep sub-district, Meung Chiang Mai, Chiang Mai, 50200, Thailand*

[3]*Department of Pharmacology, Faculty of Medicine, Chiang Mai University Suthep sub-district, Meung Chiang Mai, Chiang Mai, 50200, Thailand*

GS – 9 16:30 – 16:45

A novel development and optimization of a hydrogel patch containing *Crinum asiaticum* for osteoarthritis and its stability test

Chonthicha Kongkwamcharoen[1], Arunporn Itharat[2,3,*], Wichan Ketjinda[4], Hyang-Yeol Lee[5], Gi-Seong Moon[5] and Neal M. Davies[6]

[1]*Graduate School on Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Pathumthani 12120, Thailand*

[2]*Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Pathumthani 12120, Thailand*

[3]*Center of Excellence in Applied Thai Traditional Medicine Research (CEATMR), Thammasat University, Pathumthani 12120, Thailand*

[4]*Faculty of Pharmaceutical Sciences, Prince of Songkla University, Thailand*

[5]*Department of Biotechnology, Korea National University of Transportation, 61 Daehak-ro, Jeungpyeong-gun, Chungbuk, 27909, Republic of Korea*

[6]*Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Canada*

GS – 10 16:45 – 17:00

Metabolite profiling and bioactivity screening of novel Streptomycete *Streptomyces coriariae* isolated from the Actinorhiza *Coriaria intermedia*

Keith Sigfred Ancheta[1], Jessica Simbahan[1], Fede Berckx[2], Katharina Pawlowski[3] and Cyndi Mae Bandong[4]

[1]*Institute of Biology, College of Science, University of the Philippines, Diliman, Quezon City, Philippines*

[2]*Department of Crop Production Ecology, Swedish University of Agricultural Sciences, Uppsala, Sweden*

[3]*Department of Ecology, Environment and Plant Sciences, Stockholm University, 106 91 Stockholm, Sweden*

[4]*Department of Bio-engineering Sciences, Faculty of Sciences and Bioengineering Sciences, Vrije Universiteit Brussel, Brussels, Belgium*

July 26, 09:00 – 10:00 ‖ **Room 1, Ballroom C**

Invited lecture	Chair: Raimar Loebenberg

IS – 1 09:00 – 09:30

Development of *Plantago major* extract for diabetic wound healing

Kartini Kartini[1*], Ridho Islamie[2], and Endang Wahyu Fitriani[3]

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IS – 3 09:30 – 10:00

Research and development of cannabis health products

Kornkanok Ingkaninan[1*], Prapapan Temkitthawon[1], Vorawut Wongumpornpinit[1], Sujittra Paenkaew[1], Tongchai Saesong[1], Natthareen Chaiwangrach[1], and Neti Waranuch[2]

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July 26, 10:15 – 11:15 **‖ Room 1, Ballroom C**

Invited lecture	Chair: Neal M. Davies

IS – 5 10:15 – 10:45

Using natural substances with biological properties in cosmetic applications

Hyang-Yeol Lee* and Jun-Sub Kim

Department of Biotechnology, Korea National University of Transportation, Jeungpyeong, Republic of Korea

IS – 7 10:45 – 11:15

A transition from natural product research to industry: a successful story on *Quercus infectoria*

Supayang P. Voravuthikunchai

Center of Antimicrobial Biomaterial Innovation-Southeast Asia, Prince of Songkla University, Hat Yai, Songkhla, Thailand

July 26, 11:15 – 12:15 **‖ Room 1, Ballroom C**

Oral presentation	Chair: ASNP director, Elena S. Catap

OP – 7 11:15 – 11:35

Safety, tolerability and pharmacokinetics of *Boesenbergia rotunda* (L.) Mansf. extract in healthy human volunteers: a clinical trial phase I

Monthaka Teerachaisakul[1,*], Thanwa Buamahakul[2], Sommai Jugul[2], Natamon Aramchot[2], Rossukon Klinhom[3], Kanthika Lumpool[3], Nitchanan Boonsuk[1], Kamonwan Banchuen[3] and Phisit Khemawoot[4]

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OP – 8 11:35 – 11:55

Development of gluten-free banana cake substituted sugar with yacon flour

Sirinda Kusump*, Papatsorn Kitisutatam and Natnicha Charoenwong

Division of Food Science and Technology, Thammasat University, Rangsit Campus, Thailand

OP – 9 11:55 – 12:15

Development of plant-based recombinant protein production using a biofoundry system

Sunmee Choi, Jin Hwa Kim, Hye-In Kim, Soo-Yun Kim, Kyoung Yeon Heo, Hyo Hyun Seo, Young Soon Kim, Jeong Hun Lee and Sang Hyun Moh*

Plant Cell Research Institute, BIO-FD&C Co., Ltd., Incheon, 21990, South Korea

July 26, 09:00 – 10:00 ‖ **Room 3, Erawan 2**

Invited lecture	Chair: ASNP director, Kanit Vichitphan

IS – 2 09:00 – 09:30

The colorful world of microorganisms and carotenoids

Nguyen Minh Chon*

Food and Biotechnology Institute, Cantho University, Vietnam

IS – 4 09:30 – 10:00

Pharmaceutical excipients: an alternative green solvent for herbal extraction and product development

Pharkphoom Panichayupakaranant[1,2,*]

[1]*Department of Pharmacognosy and Pharmaceutical Botany*

[2]*Phytomedicine and Pharmaceutical Biotechnology Excellence Center, Faculty of Pharmaceutical Sciences, Prince of Son gkla University, Hat-Yai 90112, Thailand*

July 26, 10:15 – 11:15 ‖ **Room 3, Erawan 2**

Invited lecture	Chair: ASNP director, Sang Hyun Moh

IS – 6 10:15 – 10:45

Estrogenic activity of traditional plants

Kaoru Umehara

Faculty of Pharmaceutical Sciences, Yokohama University of Pharmacy, Japan

IS – 8 10:45 – 11:15

Phytochemical analysis by LC-MS and cardioprotective activity of *Imperata cylindrica* L. and *Eleuisine indica* L. methanolic extracts on doxorubicin-induced zebrafish model

Diadem Cruz[1], Jong Suk Lee[2], and Norielyn N. Abalos[1*]

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July 26, 11:15 – 12:10 ‖ **Room 3, Erawan 2**

Oral & graduate student presentation	Chair: ASNP director, Suda Chakthong

OP – 10 11:15 – 11:35

Preparation and evaluation of liposome-containing Pra-Sa-Mang-Khud extract as an anti-acne topical product to reduce skin irritation and in-crease the transdermal flux

Kalyarut Phumlek[1*], Arunporn Itharat[1], Wichan Ketjinda[2], Padcha Pongcharoen[3] and Panlop Chakkavittumrong[3]

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[3]*Department of Internal Medicine, Thammasat University, Klongluang, Pathumthani, 12120, Thailand*

OP - 11 11:35 - 11:55

Neuro-protective evaluation of natural-derived compounds on AchE activity against neurotransmitters abnormalities in SHSY5Y neuroblastoma cells

Farah J. Hashim[1], Sukanda Vichitphan[2,3] and Kanit Vichitphan[2,3]*

[1]*Department of chemistry, College of Science, University of Baghdad, Baghdad 10071, Iraq*

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[3]*Fermentation Research Center for Value Added Agricultural Products (FerVAAP), Khon Kaen University, Khon Kaen 40002, Thailand*

GS - 11 11:55 - 12:10

Development of a microemul-foam from Thai essential remedy for topical anti-inflammatory treatment

Ninnart Intharit[1], Arunporn Itharat[1]* and Raimar Löbenberg[2]

[1]*Department of Applied Thai Traditional Medicine and Center of Excellence in Applied Thai Traditional Medicine Research (CEATMR), Faculty of Medicine, Thammasat University (Rangsit campus), Khlong Luang, Pathum Thani 12120, Thailand*

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July 27, 09:30 - 10:15 ‖ **Room 1 & 2, Ballroom B + C**

Plenary lecture	Chair: ASNP director, Nguyen Minh Chon

PL - 5 09:30 - 10:15

Up in smoke: investigating medical marijuana and hemp research in Canada

Neal M. Davies[1]*, Conor O'Croinin, Andres Garicia Guerra, Tyson Le, Michael Doschak, Raimar Lobenberg

Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, T6G2N8 Canada

July 27, 10:30 - 11:30 ‖ **Room 1 & 2, Ballroom B + C**

Invited lecture	Chair: ASNP director, Monthaka Teerachaisakul

IS - 9 10:30 - 11:00

Gut metabolism of selected natural products

Huynh Thi Ngoc Mi, Santipap Chaiyasarn, Heji Kim and Jaehong Han*

Metalloenzyme Research Group and Department of Plant Science and Technology, Chung-Ang University, 4726 Seodong-daero, Anseong 17546, Republic of Korea

IS - 10 11:00 - 11:30

Production of safe and compliant extracts and isolates of cannabinoids and other natural products using eco-sustainable technologies

Gerard Rosse

Arrival Discovery LLC, 12396 World Trade Drive, Suite 216, San Diego, CA, USA

Innovation Competition

July 25, 14:30 – 15:00 **‖ Room 3, Erawan 2**

Innovation competition	Chair: ASNP President, Jaehong Han

IN – 1 14:30 – 14:40

Development of thermoreversible hydrogel loaded with *Chrysanthemum indicum* L. extract nanoemulsion for cosmeceutical application

Nichkarn Monphaneewong and Chanan Phonprapai
Department of Biotechnology, Faculty of Science and Technology, Thammasat University, Rangsit Centre, Khlong Nueng, Klong Luang, Pathum Thani 12120, Thailand

IN – 2 14:40 – 14:50

TMR microemul-foam: topical anti-inflammatory painkillers

Ninnart Intharit[1], Boonchana Pongcharoen[2], Padcha Pongcharoen[3], Raimar Löbenberg[4] and Arunporn Itharat[1]*
[1]*Department of Applied Thai Traditional Medicine and Center of Excellence in Applied Thai Traditional Medicine Research (CEATMR), Faculty of Medicine, Thammasat University (Rangsit campus), Khlong Luang, Pathum Thani 12120, Thailand*
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IN – 3 14:50 – 15:00

C-cider, Thai herbal-extract cider for healthy drink

Parichat Phalanisong[1], Kanit Vichitphan[2,3]* and Sukanda Vichitphan[2,3]
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July 25, 15:15 – 17:00 **‖ Room 3, Erawan 2**

Innovation competition	Chair: ASNP President, Jaehong Han

IN – 4 15:15 – 15:25

Hand washing gel containing *C. sappan* Linn. extract

Sumalee Kondo[1], Pornthep Temrangsee[1] and Arunporn Itharat [2]
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[2]*Department of Applied Thai Traditional Medicine, Faculty of Medicine, Thammasat University, Rangsit Campus, Pathumthani, Thailand*

IN - 5 15:25 - 15:35

Reduction of formaldehyde concentration by using herbal oil in embalming fluid

Decha Buranajitpirom[1*], Jitpanu Kanchanakaittikun[2,4] and Arunporn Itharat[3,4]

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[4]*Center of Academic Excellence in Applied Thai Traditional Medicine, Faculty of Medicine Thammasat University, Thailand*

IN - 6 15:35 - 15:45

Anti-acne products derived from a formulated combination of *Garcinia mangostana* and Thai medicinal remedy extract

Kalyarut Phumlek[1*], Arunporn Itharat[1], Pranporn Kuropakornpong[1], Hyang-Yeol Lee[2], Gi-Seong Moon[2] and Wichan Ketjinda[3]

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[3]*Department of Pharmaceutical Technology, Faculty of Pharmaceutical Sciences, Prince of Songkhla University, Songkhla, 90112, Thailand*

IN - 7 15:45 - 15:55

Development of low-cost complementary food innovation for infants and young children from germinated paddy rice

Kanmanee Sukkasem[1], Arunporn Itharat[2,3*], Nattapol Tangsuphoom[4], Pakakrong Thondeeying[2,3], Sunita Makc huchit[2,3], Kitiya Yangthaworn[2] and Nichaputh Kaewmak[4]

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IN - 8 15:55 - 16:05

Electric herb ball and gel

Puritat Kanokkangsadal[1,3*], Tanakorn Sunantachaikul[2] and Arunporn Itharat[1,3]

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IN – 9 16:05 – 16:15

The development of an innovative sunscreen gel derived from the extract of *Acanthus ebracteatus* Vahl holds promise for preventing oxidation and establishing a sustainable community spa

Montrasiri Jerasilp, Supalak Fakkam, Jatupat Anuchon and Narin Kakatum*

Applied Thai Traditional Medicine Program, College of Allied Health Sciences, Suan Sunandha Rajabhat University, Samut Songkhram 75000, Thailand

IN – 10 16:15 – 16:25

Hypoglycemic activity of the combined extract from Satagavata-Matumeho-Tubpikarn antidiabetic herbal formula

Omboon Vallisuta[1]*, Penchom Peungvicha[2], Supachoke Mangmool[2], Thadchawan Sirithamwanich[3] and Ratthabol Siritamvanich[3]

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Evaluation & Award 16:25 – 17:00


AROONPON
THAI HERB

Plenary Lecture

PL-1

Cannabis: from traditional to forbidden and now medicinal plant

Raimar Loebenberg

Faculty of Pharmacy and Pharmaceutical Sciences Katz Group-Rexall Centre for Pharmacy & Health Research University of Alberta, Canada

Cannabis is a plant with a long history of human use, both for recreational purposes and as a medicinal remedy. Many potential modern medical applications for cannabis have been proposed and are currently under investigation. The medical and recreational use of cannabis was legalized in Canada in 2018. The rich chemical content of the plant implies many possible physiological actions. Many scientists and the public are not well informed about phytocannabinoids. THC is the well-known narcotic/psychoactive and pain relieving molecule in cannabis. However, THC is a thermal degradation product of the naturally occurring THC-acid in the plant. The acid form is non psychoactive and has a large safety margin, anti-inflammatory and anticancer properties. As the medicinal use of cannabis has gained significant attention over the past few years, it is very important to understand phytocannabinoid disposition within the human body, and especially their metabolic pathways and potential drug-drug interactions, examples will be given. Finally, the difference between opioids and phytocannabinoids will be highlighted and the future of certain forbidden plants will be discussed.

PL-2

The importance of gut microbiota for the activity of herbal medicine

Rudolf Bauer

Institute of Pharmaceutical Sciences, Pharmacognosy, University of Graz, 8010 Graz, Austria

Despite intensive research the active principles and mechanisms of action of many herbal medicines and medicinal plants are still not known. Moreover, the bioavailability of many plant constituents is rather low, why they are not likely to act systemically. Therefore, alternative approaches to explain their activity have to be considered. Gut microbiota and the human body form a symbiosis which is essential for our health and well-being. Dysbiosis can lead to serious diseases, like inflammation, obesity, asthma, diabetes, and even cancer. Therefore, gut microbiota may be a relevant target for herbal medicinal products, and may help to understand their effects [1]. For example, Faecalibacterium. prausnitzii has been identified as a major actor of human intestinal health [2], the mucin-degrading bacterium *Akkermansia muciniphila* has been linked to obesity and type 2 diabetes (T2D) [3], and members of the genus *Fusobacterium* have been identified as potential causative agents in colorectal carcinomas [4]. In order to study the interaction of medicinal plant extracts with gut microbiota, we have established a research platform, which allows the analysis of metabolization of plant constituents by LC-HRMS, and microbiome shifts by 16S RNA sequencing [5]. We are now going to study also the interaction of plants used for mental health via microbiome-gut-brain axis [6].

Keywords: Gut microbiota, Herbal medicine, Microbiome, Metabolism

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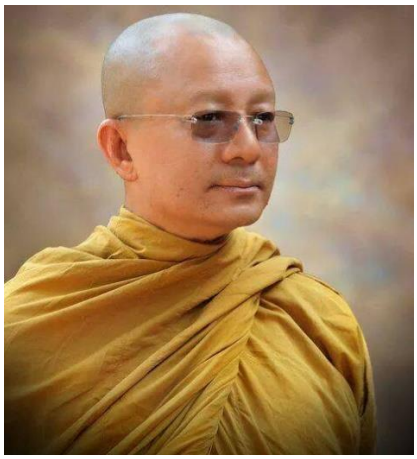
PL-3

Symptom management in patients with advanced stage cancer at Arokhayasala Khampramong temple



Phrapraponpat Jirathammo

Arokhayasala Khampramong Temple, Pannanikom, Sakonnakorn, Thailand

Cancer is a significant global public health issue and is prevalent in Thailand. Many cancer patients are diagnosed at an advanced stage, where the disease has spread and cannot be completely cured. This often leads to severe symptoms and complications due to the involvement of vital organs. These patients require comprehensive care and treatment. Treatment options for advanced stage cancer patients typically involve chemotherapy, radiation therapy, and surgery, all of which can have side effects. Common symptoms experienced by these patients include pain, loss of appetite, weight loss, breathing difficulties, confusion, nausea, fatigue, constipation, bloating, and insomnia. Pain, in particular, is a distressing and frequent symptom in advanced stage cancer patients. Therefore, effective pain management is crucial to improve their comfort and enhance their quality of life during the palliative stages. Arokhayasala Khampramong Temple, also known as Wat Khampramong, offers comprehensive care for cancer patients of all types using an integrated approach that combines Thai traditional medicine with alternative medicine methods.

Under the guidance of Luang Ta Phraponpatchara Jirathammo, patients receive treatment free of charge. Established in 2004, Arokhayasala Wat Khampramong has become a specialized hospital in Thai traditional medicine and integrative medicine in Sakonnakhon Province. Currently, over 6,500 patients are receiving treatment at the facility. The primary goal of Luang Ta Phraponpat Jirathammo, the temple's Abbot, is to alleviate patients' mental suffering. He believes that without relieving the patients' mental distress, their chances of survival are diminished. The guiding principle is to help patients live comfortably, pass away peacefully, and avoid financial burdens. The cancer treatment approach at Arokhayasala Khampramong Temple incorporates high-quality and standardized herbal remedies supported by research and certifications. Complementary treatments are also provided alongside the main treatment, focusing on holistic healthcare. Regular activities are organized for patients and caregivers to learn self-care practices. Effective pain management involves a combination of medication and non-pharmacological approaches. Non-pharmacological methods offered at Wat Khampramong include Meditation Pyramid Healing Therapy, Art Therapy, Music Therapy, Hug Therapy, Pet Therapy, Hydrotherapy, and Laugh Therapy. Pharmacological management includes herbal medicine, such as the "Yod Ya Wat Khampramong" formula, "Sabaan" herbal formula, "cannabis oil" formula, and "Ya Phok Kao" formula. Research

conducted by Prof. Dr. Arunporn Ittirattana and Dr. Sirisopa Ruengnu (2014) has explored the toxic effects of Sabaan herbal extracts on liver cancer cells and cholangiocarcinoma cells, demonstrating their toxicity against both types of cancer cells. In addition to the herbal formulas used in advanced stage cancer treatment, marijuana (cannabis) has been employed for symptom management in terminally ill cancer patients at Arokhayasala Khampramong Temple. Out of 423 patients, 42 individuals with colorectal cancer received cannabis treatment, with 14 patients using rectal suppositories and 42 patients using sublingual drops. The results revealed reduced odor and inflammation in the perianal area, decreased pain, and improved sleep in colorectal cancer patients. When administering cannabis extracts, it is crucial to start with small doses, such as one drop, and exercise caution as cannabis extracts are not considered a primary treatment for all cancer patients. Usage should be adjusted based on the patients' symptoms and used in combination with herbal formulas for cancer treatment. It should not be solely relied upon as a standalone herbal remedy.

Furthermore, special precautions and close monitoring are necessary for patients with pre-existing conditions such as heart disease, those taking anticoagulant medication, and individuals with kidney disease or diabetes. The primary objective of Arokhayasala Wat Khampramong is not to cure the disease itself, but rather to promote physical, psychological, social, and spiritual well-being for both patients and caregivers. The facility emphasizes the acceptance of death as a natural process and encourages self-care practices.

Keywords: Cancer treatment, Pain management, Thai traditional medicine, Alternative medicine

PL-4

Health innovation program for the detoxification of amphetamine derivatives addicted patients

Chalermkiat Srivorakan[1]*, Sunisa Ratanasribuathong[2], Uaekan Worapaitoon[2], Supaluck Thudsri[2], Puritat Kanokungsadal[2,3], Nichamon Mukasombat[2,3], Pranporn Kuroprakornpong[2,3], Kulyarat Pumlek[2,3], Sunita Makchuchit[2,3], Jedsada Wongsua[4], Chanuntachai Pruksukarn[5] and Arunporn Itharat[2,3]

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Last year, methamphetamine use in Thailand increased by 30 percent due to a surge in illicit drug production in the neighboring country, which flooded the region with increasingly cheaper supplies. Nakhon Phanom, Mukdahan, and Nong Bua Lamphu are provinces in the northwest of Thailand that are geographically close to the neighboring country. News reports frequently highlighted the destructive actions and violence committed by many drug-addicted patients. Therefore, a health innovation program was established as a new approach to treating drug addiction in these three provinces, which are known to have a significant number of drug addicts. The program comprises three components: physical, mental, and social treatment. In the physical treatment phase, herbal medicine based on Thai traditional medicine principles was utilized for detoxification. It was found that the levels of methamphetamine in the urine of 79 patients from the three provinces decreased by more than 85% after four days of receiving herbal medicine and reached 100% reduction after a period of nine days. For the mental treatment component, patients underwent mental therapy guided by spiritual specialists and Dharma teachings. The results indicated that the patients demonstrated increased motivation to quit drugs and displayed a positive attitude towards improving their lifestyle. Regarding social treatment, the patients underwent occupational training and received support from local communities to facilitate their reintegration into society. In conclusion, this study represents a new innovative approach to treating drug addiction. It exemplifies a holistic therapy method for drug addicts, particularly those addicted to amphetamine derivative drugs, based on Thai traditional medicine principles. This approach effectively aids in the rehabilitation of the physical and mental health of drug-addicted patients, enabling them to reintegrate into society successfully.

Keywords: Addiction, Methamphetamine, Herbal medicine, Detoxification

PL-5 10.23177/NATPRO8.2023.06.22.001

Up in smoke: investigating medical marijuana and hemp research in Canada

Neal M. Davies[1]*, Conor O'Croinin, Andres Garicia Guerra, Tyson Le, Michael Doschak and Raimar Lobenberg

Faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta, Edmonton, Alberta, T6G2N8 Canada

Background: With the legalization of recreational marijuana in Canada in 2018, there has been a significant shift in investigation, attitudes, and regulations surrounding cannabis and hemp research. Canada has reported high rates of marijuana use globally. Cannabis is derived from the hemp plant (*Cannabis sativa*) the plant contains various constituents, with tetrahydrocannabinol (THC) being the main psychoactive component, cannabidiol (CBD) as the main non-psychoactive cannabinoid, and cannabinol (CBN) as a by-product of THC. There are over 545 other constituents in the plant, and research is exploring their potential therapeutic significance. Objectives: Our studies aim to investigate non-cannabinoids, including polyphenol flavonoids and stilbenes found in hemp, which contribute to pigmentation and taste while possessing antioxidant, anti-inflammatory, anti-fungal, and anti-bacterial and other properties. The methodology involves the development and validation of liquid chromatography-mass spectrometry (LCMS) methods for characterizing and analyzing cannflavins, hemp-derived stilbenes, and novel polyphenols. In silico modeling has been employed to further characterize these compounds, revealing their therapeutic potential in disease and inflammation. Additionally, these compounds have been formulated into prototype pharmaceutical formulations with topical and oral bioavailability. Results: We have successfully developed and validated LCMS methods for analyzing cannflavins, hemp-derived stilbenes, and novel polyphenols. In silico modeling using ADMET PredictorTM 9.5 and MarvinSketch Chemaxon has further aided in characterizing these compounds. Hemp derived polyphenols have demonstrated therapeutic potential in disease and inflammation in screens of cyclooxygenase, lipoxygenase and SARS COV-2 activity and we have developed prototype topical pharmaceutical formulations using Franz diffusion cells and synthetic membranes and ex-vivo porcine models that simulate transdermal diffusion and prototype capsule formulations using purified hemp extracts and pharmaceutical excipients showing excellent dissolution of the hemp polyphenols using USP2 apparatus.

Keywords: Hemp, Flavonoids, Stilbenes, Cannflavins, COV-19, Anti-inflammatory

Acknowledgement Mitacs, NSERC Graduate Scholarship, Walter H John's Graduate Fellowship, Canurta


Healthcare
Metaverse

Invited Talks

IS-1

Development of *Plantago major* extract for diabetic wound healing

Kartini Kartini[1*], Ridho Islamie[2] and Endang Wahyu Fitriani[3]

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Impaired wound healing is among the serious complications of diabetes that can lead to amputation and even death. Plantago major has been used empirically to improve wound healing. The main bioactive compounds of P. major extracts, ursolic acid (UA) and oleanolic acid (OA), have also been studied for their benefits with non-hyperglycemic wounds. This study was done to examine the in vivo wound healing effects of P. major leaf extracts (PMLE), UA, and OA in hyperglycemic rats, to evaluate their in vitro diabetic wound healing activity, and to observe possible dermal irritation after topical application. Wound closure, duration of epithelialization, and histopathological profiles of healed tissue were observed in the hyperglycemic rats with excision wounds for 21 days. An anti-inflammatory test using the NO inhibitory assay, a fibroblast proliferation assay, and a migration assay with high-glucose medium were done to investigate the mechanism of action of the tested samples in wound healing. The acute dermal irritation test followed the international guidelines. PMLE, UA, and OA increased the percentage of wound closure and accelerated wound healing time. PMLE activities were assessed for the inhibition of NO production in the inflammation phase and enhancement of fibroblast proliferation. UA may contribute to this wound healing process through inhibition of NO production, whereas OA through activation of migration of fibroblast cells. Topical applications of PMLE, UA, and OA did not cause acute dermal irritation. PMLE, UA, and OA have the potential to improve wound healing with diabetes conditions.

Keywords: *Plantago major*, Greater plantain, Ursolic acid, Oleanolic acid, Diabetic wound

IS-2 10.23177/NATPRO8.2023.06.22.003

The colorful world of microorganisms and carotenoids

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Carotenoids are a variety of naturally occurring biomolecules produced by plants, algae, yeasts, fungi, and some bacteria. They range in color from red, and yellow to orange, and belong to the isoprenoid subfamily. Carotenoids are very important supplements for animals and humans as vitamins and very effective antioxidants. The colorful world of microorganisms has been of interest with its potential production of commercially significant carotenoids such as astaxanthin, canthaxanthin, lycopene, carotene, lutein, phytoene, torulene, isorenieratene, arpink red, riboflavin, etc. In this report, the exploitation of microorganisms capable of producing carotenoids will be mentioned. The main contents include the introduction of important carotenoids, commercial value, biological value, and the ability to produce carotenoids from microorganisms such as Bacillus, microalgae, and yeast. The structure of important carotenoids and their biological roles will be elucidated. The collection and isolation of microorganisms as a source of microorganisms directly added to the culture environment or animal feed will be discussed. Isolation and determination of carotenoids will be presented. The achievements of research on microorganisms and special species of microorganisms capable of producing carotenoids in the world will be summarized. This report also demonstrates the potential of carotenoid microbial in the future.

Keywords: *Bacillus*, Bacteria, Carotenoid, Microalgae, Yeast

IS-3

Research and development of cannabis health products

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Cannabis sativa L. or Cannabis have gained global attention due to their potential therapeutic benefit. In Thailand, authorities have taken significant steps to support its medical use and promote it as an economic crop. Recently, cannabis was removed from the narcotic drug act of 2021. However, cannabis extracts containing more than 0.2% tetrahydrocannabinol (THC), the psychoactive compound, remain on the FDA list of controlled drugs. Consequently, the cannabis industry has turned its focus to non-psychoactive cannabinoids like cannabidiol (CBD). CBD offers various therapeutic properties, including anti-anxiety, anti-nausea, anti-arthritic, anti-psychotic, anti-inflammatory, and immunomodulatory effects. CBD isolates and cannabis extracts rich in CBD show promise as ingredients in cosmetics, food supplements, and health products. Producing cannabis extracts with minimal THC content (known as "broad spectrum CBD") and CBD isolates present challenges due to evolving regulations and the supply chain, including raw material availability and quality, extraction and isolation technologies, market demand, and end-user preferences. This lecture will share the experiences of the Center of Excellence in Cannabis Research (NU-CAN) in producing cannabinoid isolates, cannabis extracts and cannabis health products. It will cover extraction technologies and research and development efforts related to herbal products containing cannabis.

Keywords: Cannabis, Cannabidiol, Herbal Product, R&D

IS-4

Pharmaceutical excipients: an alternative green solvent for herbal extraction and product development

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A suitable method for the preparation of herbal extracts plays an important role in the development of herbal products, which are practically ready for commercialization. Various conventional methods for herbal extractions, such as Soxhlet extraction, heat reflux, and maceration, are process-intensive and expensive in terms of energy, time, and required volume of organic solvent. In addition, most organic solvents used for herbal extraction in laboratory research are toxic and therefore not suitable for large scale application in herbal product industries. Therefore, the innovative extraction methods are focused on green technologies that are friendly to the environment and humans, and also reduce the cost of production. Recently, microwave extraction with alternative green solvents has been proposed as a feasible alternative approach for improving both the extraction efficiency and safety profile of herbal products. In this regard, various pharmaceutical excipients, such as the excipients in the formulations of cream, lotion, dermal patch, and pastille, have been successfully used as alternative green solvents for the development of herbal products using a green microwave extraction. For example, the excipients involved in a cream base, namely isopropyl myristate and cetyl alcohol have been used as alternative solvents for extraction of α-mangostin from mangosteen pericarp powders to produce 2.0% w/w α-mangostin extracts, which can be used directly for the formulation of anti-acne creams and oral-ulcer gels without the step of solvent evaporation. The excipients of pastilles, i.e., glycerin and a eutectic mixture of sucrose and citric acid, have been used for the extraction of [6]-gingerol from ginger powders to obtain 1.7% w/w [6]-gingerol extracts for the formulation of anti-emetic pastilles. A plasticizer used in the formulation of a natural-based dermal film, namely propylene glycol, has been used as an alternative solvent for the preparation of 1% w/v phenylbutenoid extract from *Zingiber cassumanar* powders for the development of analgesic and anti-inflammatory dermal patch. These approaches are not only free from toxic organic solvents, but also reduce the cost of production by skipping the step of solvent evaporation in the process of preparing the herbal extracts.

Keywords: Green extraction, Microwave extraction, Pharmaceutical excipient, α-Mangostin, [6]-Gingerol, Phenylbutenoid

IS-5

Using natural substances with biological properties in cosmetic applications

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Natural products and natural product-derived compounds are valuable resources for medicinal, food, and cosmetic applications. We have synthsized beta-carbolines using green chemistry and have demonstrated their antimicrobial and anti-cancer effects. Additionally, we also isolated natural compounds, such as anthocyanins from *Prunus serrulata* L. car. tommentella Nakai. Natural compounds such as flavonoids and stillbenoids with antimicrobial effects against *C. acnes* have been isolated from *Smilax china* L. for use in cosmetic products. Furthermore, cosmetic antiseptics have been achieved through enzymatic synthesis methods using commercially available antiseptics and *E. coli* beta-gal.

Keywords: Natural Products, Cosmetic antiseptics, Cosmetic applications, Antimicrobial effect

Acknowledgement: This study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (No. 2021R1A6A1A03046418).

IS-6

Estrogenic activity of traditional plants

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Phytoestrogens, plant compounds that structually and biologically mimic mammalian estrogen, have been reported to possess diverse pharmacological properties. Recently, much interest has been paid to phytoestrogens for their potential health benefits in counteracting menopausal symptoms and in lowering incidence of hormone dependent diseases. Consumption of a diet rich in soy- containing foods, known to be a rich source of phytoestrogens such as isoflavones, has been proposed as being a chemopreventive factor against breast cancer in Asian populations. In our previous studies, phenolic compounds were isolated from Thai medicinal plants and had their estrogenic and anti-estrogenic activity characterized. There are formulations in Japanese Kampo Medicines that have been designated for women's health. We selected 24 formulations that are employed for menstrual pain, irregular menstruation, hot flashes, and so on. They are consisted of three to twelve herbal medicines. Their estrogenic activity and contribution of each herbal medicine were evaluated using estrogen responsive assay system.

IS-7

A transition from natural product research to industry: a successful story on *Quercus infectoria*

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Our previous studies demonstrated that nutgalls from *Quercus infectoria* Olivier (Fagaceae) exhibited broad spectrum antibacterial, anti-oxidant, as well as anti-inflammatory activities which could make it an interesting candidate compound for curing chronic wounds. Therefore, a clinical study on nutgalls as a topical medication for hard-to-heal chronic diabetic ulcers was further investigated. Subsequently, a commercial prototype was developed, and a clinical assessment of 51 voluntary patients with chronic diabetic ulcers was performed. Within 4 weeks, 30% of the volunteers demonstrated complete wound healing and 80% patients had complete wound healing by 3 months. All subjects presented no signs of irritation or side effects. In addition to curing wound infections, *Q infectoria* enhances wound healing process by promoting cell proliferation, re-epithelialization, and granulation tissue matrix. At present, Qi Care is commercially available in the market. Challenges and difficulties encountered towards the move from academic research to industry will be highlighted during the talk.

Keywords: *Quercus infectoria*, Broad spectrum antimicrobial, Wound, Diabetes

Acknowledgement: National Research Council of Thailand (Grant No. N41A640071) and Higher Education for Industry Program.

IS-8

Phytochemical analysis by LC-MS and cardioprotective activity of *Imperata cylindrica* L. and *Eleuisine indica* L. methanolic extracts on doxorubicin-induced zebrafish model

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Native medicinal grasses, *Imperata cylindrica* and *Eleusine indica*, have known folkloric use against cancer, hypertension, inflammation and helminth parasites. The effects of their methanolic extracts on doxorubicin (Dox)-induced cardiotoxicity were evaluated on zebrafish (*Danio rerio*) embryos. The present study determined the phytochemical profiles of *I. cylindrica* and *E. indica* through LC-MS analysis; investigated their cardioprotective effects in the zebrafish cardiac phenotype; and analyzed the transcriptional levels of the cardiac biomarkers, atrial natriuretic peptide (ANP) and cardiac troponin T (cTnT). The phenotypic screening utilized zebrafish embryos pre-treated with extracts two hours prior to the inducer. The lowest concentration showing a significant result in the phenotype screening was tested for molecular analysis. Furthermore, to establish that Dox activity against cancer cells was not compromised, Dox toxicity against human colorectal carcinoma (HCT116) and hepatocellular carcinoma (HepG2) cell lines were determined by MTT assay. Phytochemical profiling revealed the presence of active secondary metabolites with known antihypertensive, anticancer, and antioxidant properties. The methanolic extracts (10 - 100 µg/mL) significantly ameliorated Dox-induced cardiotoxicity in zebrafish. Pretreatment with 10 µg/mL suppressed the transcription levels of cardiac biomarkers cTnT and ANP. Additionally, the extracts potentiated the cytotoxicity of Dox towards HCT116 and HepG2. Results show that *I. cylindrica* and *E. indica* possess protective activity against Dox-induced cardiotoxicity via downregulation of cTnT and ANP evidenced by recovered cardiac morphology in the in vivo zebrafish model but did not compromise Dox toxicity to cancer cells. These findings revealed that *I. cylindrica* and *E. indica* are safe and inexpensive potential herbal alternative and their several unknown metabolites and cardioprotective mechanism warrant further investigation.

Keywords: Cardiovascular disease, *Imperata cylindrica*, *Eleusine indica*, *Danio rerio*, Doxorubicin, cTnT, ANP

IS-9 10.23177/NATPRO8.2023.06.22.002

Gut metabolism of selected natural products

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Gut metabolism of bioactive natural products is of a great scientific and biotechnological interest due to the unknown metabolic pathways and potential impacts on health-related industry. In our group, gut metabolism of a few selected natural products, including curcuminoids, flavonoids, and coumarins, has been studied to elucidate the new metabolic pathway and biochemical conversions operating in human gut. In this talk, recent progress regarding C-C/C-O bond cleavage reactions, experimental tools required for the study of gut metabolism, and the difficulties encountere during the metabolism study were discussed.

Keywords: Biotransformation, Cleavage, Gut, Mechanism, Metabolism, Flavonoids

Acknowledgement This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2021R1A2C2007712).

IS-10

Production of safe and compliant extracts and isolates of cannabinoids and other natural products using eco-sustainable technologies

Gerard Rosse

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The future of the hemp/cannabis industry is dependent on consistently producing regulation-compliant formulations of cannabis extracts and isolates. An overview of the endocannabinoid system and the health benefits of cannabis compounds such as CBD will be given. The workflow of hemp/cannabis sample processing from biomass drying to final product and the importance of using Supercritical fluid CO2 in the extraction (SFE) and purification (SFC) steps will be discussed. Supercritical fluid chromatography (SFC) is a well establish separation technique extremely well suited for the separation of cannabinoids at research and industrial scale. The mobile phase used in SFC is mainly CO2 which is a non-flammable, non-toxic, FDA approved substance. Recent application of SFC to deliver full spectrum extracts, separate minor cannabinoid isolates at >99.7% purity and isolate terpenes will be exemplified. The presentation will conclude with the development of a technique combining both SFC and SFE in a single instrument to expedite the production of clinical grade ingredients.


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Regular Oral Presentations

OP-1

Longan (*Dimocaepus longan* Lour.): economic fruit with high value for phytonutrients and phytocosmetics

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Longan "*Dimocarpus longan* Lour" is a tropical and subtropical perennial plant widely grown in Asia such as China, India, and Thailand. Chiang Mai University (CMU) together with P80 Factory and ADSI Research Institute developed a new technology for the extraction of fresh longan fruit including pulp, peel, and seed. 100% natural concentrate (P80 Natural Essence) has been verified bioactive substances such as gallic acid, ellagic acid, corilagin, gamma amino butyric acid (GABA) and (-)-epicatechin by HPLC method. Longan extract passed safety test in animals. ADSI institute studied the nutritional phytochemicals of longan extract such as antioxidants, vitamins, amino acids, and polysaccharides. P80 plant has developed a longan extracted health drink. ADSI in collaboration with Innsbruck Medical University studied the efficacy of longan extract nasal spray (P80) against coronavirus disease (COVID-19) by inducing antiviral activity and protecting lung tissues damage after SARS-CoV-2 infection. Faculty of Pharmacy, CMU investigated antioxidant activity and anti-tyrosinase properties for the development of skin whitening serums and face masks from longan extract. ADSI, together with CURA Beauty GmbH, has developed their manufactured cosmetic products through safety testing, and efficacy assessments. Therefore, longan can be used as an easily accessible natural raw material in the food supplement and cosmetic industries.

Keywords: Longan, *Dimocaepus longan* Lour., Economic fruit, Phytonutrients, Phytocosmetics

OP-2

In-vitro, in-vivo, and in-silico antioxidant and antihypertensive activities of *Nauclea subdita* (Korth.) Steud. stem bark aqueous extract

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Nauclea subdita (Korth.) Steud. or Taya is one of the Indonesian popular native plants that is empirically used in traditional psychotherapies for the treatment of hypertension and various other diseases in Kalimantan Island. To verify the activity and possible mechanisms of *N. subdita* in reducing blood pressure, *N. subdita* stem bark aqueous extract was prepared and phytochemical screening was performed. Antioxidant activity was evaluated in vitro by measuring nitric oxide scavenging activity and antihypertensive activity was evaluated by measuring beta adrenergic receptor antagonist activity and diuretic activity of male wistar rats. The effects of *N. subdita* aqueous extract doses 50,75,100, and 125 mg/kg BW on the adrenergic receptors were determined by adrenaline-induced rats animal model. Antioxidant activity was confirmed by the nitric oxide scavenging activity in the concentration of 250 ppm with the inhibition of 31.52 ± 0.771%. The results showed that *N. subdita* stem bark aqueous extract significantly inhibited the escalation of systolic and diastolic blood pressure, mean arterial pressure and heart rate in adrenaline-induced hypertensive rats. In silico study showed that stigmast-4-en-3-one and strictosamide were found to be the responsible compounds of *N. subdita* that have a strong interaction towards -1-adreno receptors. In conclusion, *N. subdita* stem bark aqueous extract has the potential as an antihypertension by inhibiting beta-adrenergic receptor and antioxidant activity.

Keywords: Antioxidant, Antihypertension, Beta Adrenergic, Diuretic, *Nauclea subdita*, Nitric Oxide

Acknowledgement: The authors extend their appreciation to the Indonesian Ministry of Education, Culture, Research and Technology for funding this work through the fundamental research scheme, grant number 011/SP-Lit/LPPM-01/KemendikbudRistek/FF/V/2023.

OP-3 10.23177/NATPRO8.2023.06.22.007

Phytochemical and biological examination of *Aconitum heterophyllum*

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A detailed phytochemical and biological investigation of *Aconitum heterophyllum* was accomplished. The cold methanolic root extract of *A. heterophyllum* was subjected to VLC for fractionation over silica gel, which led to the isolation of two compounds 1,1,8,8-trimethyl-4-methylene-decahydro-1H-cyclopenta-[8]-annulen-1-ol and β-stigmasterol. The compounds were characterized by analysis of their 1D and 2D NMR spectral data. Compound (1,1,8,8-trimethyl-4-methylene-decahydro-1H-cyclopenta-[8]-annulen-ol) is first reported from this plant. The crude extract was screened for antimicrobial activity against several gram-positive and gram-negative bacteria, and fungi using disk diffusion method where significant inhibitory activity was observed. The extract was also tested for cytotoxic property using brine shrimp lethality bioassay, which revealed moderate activity in comparison with the standard anticancer drug, vincristine sulphate. Antioxidant property of the crude methanol extract of *A. heterophyllum* was determined by the scavenging of the DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical and found moderate antioxidant activity (IC_{50} = 53.25 µg/mL) when compared to standard antioxidant. Total phenolic and flavonoid content and polyphenolic compound content of the crude extract were determined by HPLC. The isolated compounds showed dose-dependent antiproliferation activity against the vero and lung A549 cells.

Key words: *Aconitum heterophyllum*, Annulenol, Antiproliferation, Antimicrobial, Antioxidant, Phenolic, Flavonoid

Role of probiotics in non-communicable diseases

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Non-communicable diseases (NCDs) account for 71% of all deaths globally. NCDs can be caused by various factors like genetic, environmental, lifestyle and gut microbial involvement. Enormous research studies revealed that gut microbial dysbiosis promote the development of NCDs like obesity, hypercholesteremia, cancer, cardiovascular diseases (CVDs), arthritis, gastrointestinal disorders and neurological diseases (NDs). The food habits and lifestyle changes greatly influence the health status of humans. Mainly the alterations in the gut microbiota are closely associated with several health complications. The intestinal microbiome plays a very important role in maintaining homeostasis in the human body. Probiotics are live microbes, when taken in sufficient amount that produce health benefit to the hosts by influencing their gut microbiota and immunity. Probiotics are now recognized as an adjuvant and complementary therapeutic agent for the NCDs. Probiotics promote health and ameliorate the physiological and psychological distress. Anti-obese activities of probiotics are exerted by remodeling the energy metabolism, altering the gene expression related to glucose and lipid metabolism, enhancing intestinal permeability, reduce the release of endotoxins and inflammation. Probiotic intervention greatly regulates the intestinal gut microbiota and enables the weight loss in obese people. Studies reveled that probiotic intake can improve the health status of diabetes patients by reducing fasting plasma glucose (FPG), TG, HDL, total cholesterol (TC), insulin, C-reactive protein and inflammatory markers such as TNF-α, IL-6. Probiotic Lactobacillus species produce positive effect on host health. Akkermansia muciniphila has been considered as potential probiotic in treating type 2 diabetes. *Lactobacillus* and *Bifidobacterium* species showed cholesterol lowering effects and improved the lipid profile by reducing LDL-C levels in metabolic syndrome patients. Probiotic interventions improved the cognitive function and mood, reduce anxiety, depression, and stress. By altering host intestinal microbiota probiotics stimulate the growth of beneficial microorganisms and helps maintain the gut-brain axis in balance. Regular consumption of probiotics can alleviate depressive symptoms and restore mood and protect cognitive functions. Probiotics including *Clostridium clusters, Bacteroides uniformis, Akkermansia muciniphila, Faecalibacterium prausnitzii* have been considered as next generation probiotics in promoting human health.

Keywords: Probiotics, Non-communicable diseases, Microbiota, Obesity, Mental health

Acknowledgement This project was supported by Chiangmai University and Faculty of Pharmacy, Chiangmai University.

OP-5

Determination of total phenolics, total flavonoids, and antioxidant activity of chewable gummy tablets of moringa leaf extract during the storage period

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Moringa (*Moringa oleifera* L.) has potent antioxidant activity. It has the potential to be developed into a nutraceutical. Chewable Gummy Tablets (CGT) moringa has been optimized in previous studies with pectin as a gelling agent. This study aims to determine the levels of total phenolics, total flavonoids, and antioxidant activity during the storage period (0, 2, and 4 weeks). The method for testing total phenolic using Folin-ciocalteu reagent and total flavonoid using $AlCl_3$ were measured by a visible spectrophotometer. A microplate reader measured the antioxidant level with ABTS free radicals. The CGT moringa was stored at cool temperatures (8-15°C) with separate packaging. The results showed that the total phenolics content during storage periods were 0.66%GAE±0.0238; 0.60 %GAE±0.0117; 0.54%GAE±0.0048, respectively. The total levels of flavonoids during storage periods were 0.11%QE±0.0032; 0.10%QE± 0.0044; 0.09%QE±0.0049, respectively. The results of the antioxidant activity test showed that the IC_{50} values during the storage periods were 347.80±37.54 ppm, 356.79±33.14 ppm, and 403.15±45.90 ppm, respectively. Statistical tests using one-way ANOVA showed a significant difference in total phenolic (P=0.000) and total flavonoid (P=0.000), while the antioxidant activity showed no significant difference (P=0.138). The conclusion of this study showed that the storage period (0, 2, and 4 weeks) affected the levels of total phenolic and total flavonoids in CGT moringa.

Keywords: Chewable gummy Tablets, Moringa, Phenolics, Flavonoids, Antioxidant, Storage period.

Acknowledgement This work was financially supported by Indonesian Ministry of Education, Culture, Research and Technology under "Fundamental Research" with the contract number: 011/SP-Lit/LPPM-01/Kemendikbud Ristek/FF/V/2023.

Antioxidant activity and β-carotene production of extracts from *Bacillus infantis*

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Carotenoids are the natural pigments in plants, they are also found in the microorganism. Carotenoids play a vital role in protecting cell membranes against high temperatures, intensive UV, and other oxidation agents. *Bacillus* is a big genus of Gram-positive and rod-shaped bacteria. They were known as carotenoid-producing bacteria. In this study, many *Bacillus* samples were collected at the seashore in Kien Giang province of Vietnam and *Bacillus infantis* was used to examine the antioxidant activity and β-carotene production of its extract. DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging assay and reducing power assay (reducing Fe3+ ions) were used. The result of the DPPH assay showed that the carotenoid extract could scavenge DPPH radical. Additionally, the crude carotenoid extract showed antioxidant activity in reducing ferric ions (Fe3+). To identify β-carotene from *Bacillus infantis*, the pigments from *Bacillus biomass* were extracted in the mixture of methanol and chloroform (with a ratio of 1:2 of v/v). The extracted solution was examined by the spectrophotometer to recognize the maximum absorbance wavelength of β-carotene from 400 to 600 nm. The pigment sample was separated by a silica gel glass chromatography column and carotenoids were identified by HPLC-MS. β-carotene is the yellow-orange compound with the maximum absorbance at 454 – 455 nm. The molecular ion [M+H]+ is 537.39, and the ion fragment [M-92] is 444.33. The results showed that Bacillus infantis is a bacterial source for β-carotene and carotenoid production.

Keywords: Antioxidant, Bacillus, β-carotene, Carotenoid, DPPH, Reducing

OP-7

Safety, tolerability and pharmacokinetics of *Boesenbergia rotunda* (L.) Mansf. extract in healthy human volunteers: a clinical trial phase I

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This study aimed to investigate the pharmacokinetics of KraChai extract (KCE). Eleven healthy volunteers received three capsules of KCE per time, which each capsule contained a minimum of 30 mg of Pinostrobin and 9 mg of Panduratin A, three times a day for seven days. Participants were in-patient monitored for 30 hours on Day 1 and Day 7. The study found that the blood level of Panduratin A was lower than that of Pinostrobin. Furthermore, Pinostrobin concentration continuously increased from Day 1-7, indicating its accumulation in the blood. The half-life values (T1/2) of Pinostrobin and Panduratin A were 8.07 ± 2.93 h and 5.51 ± 2.62 h, respectively. The maximum concentration times (Tmax) for Pinostrobin and Panduratin A were approximately 2.67 ± 0.89 h and 3.00 ± 0.95 h, respectively. No adverse events were reported. However, the study observed significant increases in Hematocrit, RBC, BUN, and Total CO2 levels. After discontinuing KCE for three days (Day 10), BUN and Total CO2 levels returned to normal, while Hemoglobin, Hematocrit, and RBC levels remained elevated but within the normal range. Additionally, there was a significant decrease in C-reactive protein levels ($p=0.04$). In conclusion, this study confirmed the safety of consuming KCE capsules, while also validating KCE's anti-inflammatory properties in human subjects.

Keywords: Safety, Pharmacokinetics, Tolerability, *Boesenbergia rotunda* (L.) Mansf., Pinostrobin, Panduratin A

OP-8

Development of gluten-free banana cake substituted sugar with yacon flour

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Yacon (*Smallanthus sonchifolius*), a tuberous herb with sweet taste, contains considerable amount of β-(2-1)-fructoologosaccharide and inulin that can promote gastrointestinal tract health. The objectives of this study were to study the preparation method of yacon flour and to investigate the use of yacon flour in gluten-free banana cake. For yacon flour preparation study, five drying times (0, 30, 60, 90 and 120 minutes) of yacon flour using hot air oven were examined. Water activity and color L*, a*, b* were determined. The result showed that drying time from 60 minutes onwards produced flour with lowest water activity and insignificant color change ($p \leq 0.05$). Therefore, 60-minute drying time was chosen for the next experiment. For gluten-free banana cake formulation, the ratio of sugar substitution with yacon flour was investigated. Four sucrose to yacon flour ratios (100:0, 40:60, 20:80, and 0:100) were used in oat flour-based banana cakes. Physical property and sensorial property were determined. The results found that there was no significant difference in specific volume and cake density among 4 samples ($p>0.05$). Total color change of the cake crust decreased when addition of yacon flour increased ($p>0.05$). Texture profile analysis showed that with increasing ratio of yacon flour, hardness increased whereas springiness and cohesiveness decreased ($p<0.05$). Sensory evaluation revealed that gluten-free banana cake substituted sucrose with yacon flour in the ratio of sucrose to yacon flour of 40 to 60 received higher scores in appearance, texture, taste and overall liking attributes when compared to other substituted formula. The acceptance scores of the cake were in the range of like moderately.

Keywords: Yacon, Celiac disease, Oligo-fructose, FOS, Inulin

OP-9

Development of plant-based recombinant protein production using a biofoundry system

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Plant-based protein expression have gained significant interest in recent years. Plants offer a cost-effective and safe alternative to traditional expression systems like bacteria, yeast, and mammalian cells. Plant cells possess the ability to perform essential physiological activities such as transcription and translation of eukaryotic proteins, including post-translational modifications like phosphorylation and glycosylation. Consequently, plants are well-suited for large-scale production of recombinant proteins. However, the expression of target genes in plant cells is often hindered by cellular stress responses. Overcoming this regulatory bottleneck requires the construction of an appropriate expression system. In this study, we present a novel plant-based platform that addresses this challenge by modulating several key parameters. These parameters include codon optimization, co-expression of a gene silencing suppressor, fusion with a soluble tag, and careful selection of promoters. Particularly, we introduce an inducible system using radiofrequency (RF) treatment to enhance the synthesis of target proteins by altering gene expression profiles in plant cells. By combining a robust promoter with RF optimization, we observed improvements in protein solubility, protein yield, and protein quality. Furthermore, the incorporation of a gene silencing suppressor resulted in enhanced protein expression capabilities within the plant host platforms. To evaluate the efficacy of each parameter, we coupled them with the green fluorescent protein (GFP) gene and assessed their performance in both transient and stable expression systems. The results demonstrate that the radiofrequency inducible system is highly efficient in plant-based protein production. The novel expression systems developed in this study offer precise control over high-level transgene induction in various plant cells. These systems have the potential to be employed in plant-based biofoundries, contributing to sustainable and environmentally friendly protein production.

Keywords: Plant-based protein expression, Radiofrequency, Plant cell, Biofoundry

Acknowledgement: This work was supported by Advanced Technology Center Plus (ATC+, 20017936, Development of growth factors and antibody drugs using plant cell-based platform technology and global sales expansion of fragrance materials.) funded By the Ministry of Trade, Industry and Energy (MOTIE, Korea).

OP-10

Preparation and evaluation of liposome-containing Pra-Sa-Mang-Khud extract as an anti-acne topical product to reduce skin irritation and in-crease the transdermal flux

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Pra-Sa-Mang-Khud (PSM) formula as a mixture of mangosteen pericarp and Ha-Rak remedy was developed for acne treatment. However, the limits of using PSM extract are its potential to be an irritant agent in human skin and also low skin permeation. Therefore, PSM extract was loaded into liposomes (PSM liposomes) made from phosphatidylcholine from soybean, tween 80, and deoxycholic acid using a modified ethanol injection technique with the purpose of reducing irritation and enhancing the transdermal flux. PSM liposomes had a vesicle size of 123.17 nm, a zeta potential of -62.10 mV, a high entrapment efficacy of 90.94%, and a loading capacity of 14.85% were loaded into lotion base and gel base as PSM nanolotion and PSM nanogel, respectively. However, the best matrix containing PSM liposomes was only lotion form due to its stability after storage at 40 ± 2oC with 75 ± 5% RH for 6 months. PSM nanolotion increased release flux by 168% and transdermal flux from 0 to 1.93 μg/cm2/h compared to PSM extract lotion without liposomes. In clinical trial phase I using human closed patch test, none of the volunteers (30 healthy volunteers) had irritation and allergic reactions to PSM nanolotion. Consequently, liposomes are one of the nanotechnologies that can reduce irritation and improve skin permeability for such problematic herbal extracts.

Keywords: Pra-Sa-Mang-Khud, Liposome, Skin irritation, Skin permeation

Acknowledgement: This research was financially supported by the Thailand Science Research and Innovation Fundamental Fund through Grant No. TUFF 28/2565. The authors are grateful to the Center of Excellence in Applied Thai Traditional Medicine Research and the Faculty of Medicine, Thammasat University, for providing laboratory facilities.

OP-11

Neuro-protective evaluation of natural-derived compounds on AchE activity against neurotransmitters abnormalities in SHSY5Y neuroblastoma cells

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Acetylcholinesterase (AchE) inhibition is one of the promising approaches using in the treatment of neurological disorders. Numerous neurological diseases including Alzheimer disease (AD) and Parkinson's disease (PD) mainly relate to abnormal levels of neurotransmitters-controlled enzymes. This study aimed to investigate the neuroprotective effect of edible plants which were *Alpinia galanga* leaves (ALE), *Alpinia galanga* rhizomes (ARE), *Vitis vinifera* seeds (VSE), *Moringa oleifera* leaves (MLE), and *Panax ginseng* rhizomes (PRE) ethanolic extracts on human neuroblastoma (SHSY5Y) cells. AchE activity was determined by Ellman's colorimetric method in both un-differentiated and differentiated SHSY5Y cells while detecting the cytotoxicity was performed by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The results showed that MLE has a potential activity against abnormal levels of AchE in both un-differentiated and differentiated neuron cells due to abundant phenolic compounds. The phytochemical analysis of MLE determined by Liquid chromatography coupled to quadrupole time-of-flight mass spectrometry (LC-QTOF/MS) contained abundance content of phenolic compounds. Natural derived-phenolic compounds in MLE on inhibition of AchE activity could be one mechanism to prevent the neurological disorders.

Keywords: Acetylcholinesterase, Neurological disorders, *Moringa oleifera* leaves, SHSY5Y neuroblastoma cells

Student Oral Presentations

GS-1 10.23177/NATPRO8.2023.06.22.004

HPLC development of *Thunbergia laurifolia* leaf extract capsule using design of experiments for a stability indicating method

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Thunbergia laurifolia Lindl., commonly known as Rang Chuet is a medicinal plant that is widely used in traditional medicine in Thailand. The herbal teas and capsules have been assigned in the National List of Essential Medicines (NLEM) 2019 under the category of herbal medicines for the purpose of use as antipyretics and in the treatments of fever and aphthous ulcers. The aim of this study is to develop a high-performance liquid chromatography (HPLC) method for a stability study of *T. laurifolia* leaf extract capsule using design of experiments (DoE). The optimum separation of analytes was obtained by a RP-C18 column and mobile phase consisting of 0.02% phosphoric acid and 12% methanol in acetonitrile using a gradient elution with a 0.8 mL/min flow rate. The detection wavelength was set at 330 nm. The method was validated for its specificity, linearity, precision, and accuracy. The forced degradation of *T. laurifolia* leaf extract capsule showed that they were stable in acid and oxidative conditions, but highly labile under alkaline hydrolytic conditions. The validated HPLC method was applied to a stability study of *T. laurifolia* leaf extract capsule under accelerated conditions and rosmarinic acid (RA) in *T. laurifolia* leaf extract capsule was found to be stable for 6 months with 97% of initial content.

Keywords: Force degradation study, *Thunbergia laurifolia*, Design of experiments, Rosmarinic acid

Introduction

Thunbergia laurifolia (TL), known as Rang Chuet, is a medicinal plant native to Southeast Asia. In Thai traditional medicine, leaves of TL are used as an antidote for poisons and as treatment for drug addiction [1]. Herbal teas and capsules of this plant are listed in the National List of Essential Medicines (NLEM) 2019 under the category of herbal medicines as antipyretics and treatments of fever and aphthous ulcers [2]. TL has been reported to contain several chemical compounds, including flavonoids and polyphenols, which promote antioxidant and anti-inflammatory properties [1]. Phytochemicals including apigenin and apigenin glucosides, caffeic, gallic, and protocatechuic acids have been determined [1]. Pharmacological activity of the leaf extract from TL has been previously reported to including anti-inflammatory [3], antioxidant [4-6], anti-diabetic [7,8], antimicrobial [9,10], hepatoprotective [11,12], anti-depressant, and anti-dementia activities [13].

High-performance liquid chromatography (HPLC) is a widely used technique for the analysis of herbal products. However, the optimal conditions for HPLC analysis could vary depending on the specificity of each herbal product and the targeted compounds. To overcome this challenge, the design of experiments (DoE) can be applied to study the factors that influence the analytical outcome by using a proper design. These factors include mobile phase composition, buffer pH and concentration, column temperature, and sample concentration. DoE was successfully applied in the UHPLC-PDA-MS analysis of flavonoids in *Genkwa Flos*. The optimal analytical method was validated and applied to quantify the content of apigenin 7-O-glucuronide, apigenin, and genkwanin in the extract of *Daphne genkwa* flower bud [14].

In our previous studies, the HPLC method for quantitative analysis of chemical markers, caffeic acid (CA), vicenin-II (VI), and rosmarinic acid (RA) in *T. laurifolia* leaf extracts has been developed and validated [15,16]. However, the step gradient elution of the mobile phase was too complicated for routine analysis. Moreover, there is still no analytical method for the stability study of *T. laurifolia* leaf extract capsule. Therefore, in this study, the full factorial design was applied to the optimization of an HPLC method for the analysis of RA in *T. laurifolia* leaf extract capsule. A forced degradation study was performed to show the specificity of the method. This method could be

applied for quantitative analysis of the marker contents in *T. laurifolia* leaf extract capsule stored in accelerated conditions.

Materials and Methods

Plant extract preparation

Leaves of *T. laurifolia* were collected from Sakaeo province, Thailand. The leaves were identified according to their botanical and taxonomical characteristics mentioned in Thai Herbal Pharmacopoeia 2021. The voucher specimens were deposited at the Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand. The leaves were cleaned, dried in a hot air oven (60°C) for 8 h and powdered with an electronic mill (20 mesh sieve). The leaf powder was boiled with distilled water (1:10 w/v) for 2 h, and then filtered. The residue from the filtration was boiled and filtered again with the same procedure. Maltodextrin was added in the concentration of 10% w/v and the sample was then submitted for the spray drying to yield the dried *T. laurifolia* leaf extract.

Capsule formulation

The capsule of *T. laurifolia* leaf extract was formulated by a dry mixing technique. All ingredients were weighted according to the formula as shown in **Table 1**. The extract mixture was mixed with stearic acid in a plastic bag for 10 minutes. The mixture was filled in each capsule using a capsule filling machine at 25 ± 2 °C and 60 ± 5 % of relative humidity (RH). Subsequently, capsules were packed in Aluminum-PVC bag (30 capsules per bag) and stored in glass bottles.

Table 1. Capsule of *T. laurfolia* leaf extract.

Ingredient	Amount per capsule (mg)	Amount per 300 units (mg)
T. laurifolia spray dried powder	300.00	90,000
Glidant	1.50	450
Total	301.50	90,450

Instrumentation and HPLC condition

A Shimadzu i-Series LC-2050C (Kyoto, Japan) was used for HPLC analysis. The system is composed of a photodiode array (PDA) detector, quaternary solvent and sample manager, cooling autosampler, and column oven. The operating software Lab solution® software (Shimadzu). The HPLC condition consisted of a Zorbax® SB-C18 (3.0 x 150 mm, 5 µm, Agilent, USA). The mobile phase was 0.02% phosphoric acid (mobile phase A) and 12% methanol in acetonitrile (mobile phase B) using gradient elution with a 0.8 mL/min flow rate. The detection wavelength was 330 nm and the injection volume was 20 µL.

Statistical analysis

Two-level five factors full factorial design (FFD) was conducted and statistically analyzed using Design-Expert 13® (Stat-Ease, Inc., MN, USA). The statistically significant coefficients ($p<0.05$) per analysis of variance (ANOVA) were used in framing the polynomial equation followed by the evaluation of the fit of the model. Parameters evaluated for appropriate fitting of the model including coefficient of correlation (r), lack of fit, F-value, and P-value are listed, respectively.

Forced degradation studies

T. laurifolia leaf extract capsule solution was prepared at a concentration of 4 mg/mL in distilled water. The HPLC analysis of marker compounds including CA, VI, and RA contents was conducted with sample solution that had been treated with five stress conditions, including acid and basic hydrolysis, oxidative, photolytic, and thermal conditions [18]. For acid hydrolysis, 10 µL of 0.2N HCl was added to 2 mL of sample solution and remained at room temperature for 2 h. For basic hydrolysis, 5 µL of 0.2 N NaOH was added to 2 mL of sample solution at room temperature for 30 min. For oxidative stress, 10 µL of 30% w/w H_2O_2 was added to 2 mL of sample solution and then the sample was incubated at 60°C for 1h. For thermal stress, 2 mL of sample solution in a plastic tube was kept in a heat chamber at 100 °C for 2 h. For photolysis, 2 mL of sample solution in a plastic tube was exposed to sunlight for 4 h. All stress experiments were undertaken in triplicate and % degradation of chemical marker was calculated.

HPLC method validation

Method validation was evaluated in terms of specificity, linearity, accuracy, and precision according to ICH guidelines [18]. Specificity of the method was evaluated by comparing HPLC chromatograms of sample solution (control), sample solution from a forced degradation study, a standard mixture of caffeic acid (CA), standard vicenin-II (VI), rosmarinic acid (RA), and deionized water (blank). The acceptance criteria for specificity were a peak purity of more than 0.9 and a resolution of more than 2. Linearity of the method was performed in a range of 0.8-28 µg/mL of caffeic acid and 1.2-42 µg/mL of vicenin-II and rosmarinic acid. Linear regression and correlation coefficient (r) were calculated using Microsoft Excel®. Accuracy was performed by standard addition with

three different concentrations of standard mixture. Precision was performed by analyzing sample extract solution at 4 mg/mL (n = 6) on the same day for repeatability, and on three different days for intermediate precision (n = 18). The percent relative standard deviation (%RSD) was calculated.

Stability testing

T. laurifolia leaf extract capsule was stored in an aluminum-PVC bag in a glass bottle with a screw cap at 40 °C ± 2°C/75% RH ± 5% for 6 months. The stored samples were sampled at 0, 1st, 3rd, and 6th months. The contents of CA, VI, and RA in the *T. laurifolia* leaf extract capsule were analyzed in duplicate by the developed HPLC method. The initial amount of each marker content (0 month) analyzed by HPLC was defined as 100%, and the subsequent contents of each time point were calculated as the percentages of the initial concentration which the acceptable limit for most pharmaceutical preparations being typically ± 10%, or within the range of 90 % to 110 % of the marker content in the initial products.

Results & Discussion

Optimization of HPLC condition using design of experiment

Two-level five FFD was carried out for finding critical factors including sample concentration (X1), column temperature (X2), flow rate (X3), pH of mobile phase A (X4), percent of methanol in mobile phase B (X5). Since resolution (R_s) of each marker and tailing factor (T) mostly represent the quality of chromatographic separation, they were chosen for FFD which is examined at just 2-levels (low and high). Factors (X_n) that have a significant influence on individual responses (Y_n) were identified by half-normal plot and Pareto charts. Table 2 shows the analysis of variance (ANOVA) for studying the significant effect of main and interactive variables on responses. The influence of the factors is significant when *p*-value < 0.05. The percentage of methanol in mobile phase B showed as a non-influencing factor for all responses (**Table 2**), however a higher percentage of methanol in mobile phase B was preferable for the faster elution of marker compounds. Since the pH of mobile phase A was not significantly influenced in most monitored responses, except tailing factors of VI and RA. It was found that increasing of the pH of mobile phase A increased the tailing factor of HPLC peaks. In addition, decreasing the pH of mobile phase was observed to promote no significant change in the obtained HPLC chromatogram. As a result, the pH of mobile phase and the percentage of methanol in mobile phase B were optimized to be pH 2.57 and 12% methanol, respectively.

HPLC method validation and forced degradation study

A stability indicating HPLC method was developed for quantitative analysis of CA, VI, and RA contents in *T. laurifolia* leaf extract capsules. A forced degradation study was carried out to demonstrate specificity of the purposed method to evaluate the changes in concentration of CA, VI, and RA. To determine the specificity of the method, peak purity analysis was developed by using PDA detector with the software. The HPLC profiles of *T. laurifolia* leaf extract capsule in all forced degradations including control, acid hydrolysis, basic hydrolysis, oxidation, photolytic, thermal condition, standard mixture solution, and blank are shown in **Figure 1**. The specificity of the method was archived as the peak purities of CA, VI and RA peaks were more than 0.9 and the potential degradation products were separated from those peaks. A degradation amount of compound in the range between 5% to 20% is acceptable for the validation of quantitative analysis of chemical marker contents. The stability limit of 10% degradation or 90% of relative amount is acceptable. As shown in **Table 3**, all markers were stable under acid hydrolysis conditions. With basic hydrolysis treatment, CA and RA were found to be highly labile (cannot be detected), while VI remained up to 96.90%. CA, VI, and RA were stable in 30% hydrogen peroxide at 60°C for 60 minutes. In addition, all markers remained more than 90% at 100°C for 2 hours. Under photolytic condition, VI was stable after exposure to sunlight for 4 hours while CA and RA were degraded around 48% and 18%, respectively.

Table 2. ANOVA results for full factorial design

Code factors	Resolution of CA		Resolution of VI		Resolution of RA		$T_{f10\%}$ of CA		$T_{f10\%}$ of VI		$T_{f10\%}$ of RA	
	Coefficient estimate	*p*-value	Coefficient estimate	*p*-value	Coefficient estimate	*p*-value	Coefficient estimate	*p*-value	Coefficient estimate	*p*-value	Coefficient estimate	*p*-value
Model	6.73991	0.0001	9.18287	<0.0001	5.86459	<0.0001	1.30081	<0.0001	1.14950	<0.0001	1.16319	<0.0001
A	-	-	-6.75781	<0.0001	-0.542031	<0.0001	-	-	-	-	-	-
B	-1.32234	0.0002	1.46606	<0.0001	-0.357656	<0.0001	-0.108812	<0.0001	-	-	0.0140625	0.0392
C	-0.728281	0.0258	1.98325	<0.0001	-0.0158438	<0.5086	-0.0944375	<0.0001	0.0079375	0.0009	-0.04825	<0.0001
D	-	-	-	-	-	-	-	-	-0.0094375	0.0001	-0.027	0.0003
E	-	-	-	-	-	-	-	-	-0.0009375	0.6667	-0.0075	0.2578
AB	-	-	-2.19813	<0.0001	-0.161531	<0.0001	-	-	-	-	-	-
AC	-	-	-1.89444	<0.0001	-0.496281	<0.0001	-	-	-	-	-	-
BC	-	-	1.55669	<0.0001	0.300969	<0.0001	0.111187	<0.0001	-	-	-0.023875	0.0010
BD	-	-	-	-	-	-	-	-	-	-	-0.01075	0.1091
BE	-	-	-	-	-	-	-	-	-	-	0.008	0.2282
CD	-	-	-	-	-	-	-	-	0.007625	0.0013	-	-
CE	-	-	-	-	-	-	-	-	-0.005	0.0271	-0.0160625	0.0199
DE	-	-	-	-	-	-	-	-	0.005625	0.0138	-0.0174375	0.0122
ABC	-	-	-1.92625	<0.0001	-0.110406	<0.0001	-	-	-	-	-	-
BDE	-	-	-	-	-	-	-	-	-	-	-0.0185625	0.0081
R^2	0.3995		0.9978		0.9793		0.9722		0.6501		0.8138	
Adj R^2	0.3652		0.9973		0.9745		0.9697		0.5824		0.7448	
Adeq P	7.2363		133.881		40.7163		41.1370		12.3451		15.9077	

A – concentration of sample (mg/mL), B – column temperature (degree celcius), C – flow rate (mL/min), D – pH of mobile phase A, E – percent of methanol in mobile phase B, $T_{f10\%}$ – tailing factor at 10% peak width, Adj – adjusted coefficient of determination, Adeq P – adequate precision. Terms are statistically significant if $p < 0.05$.

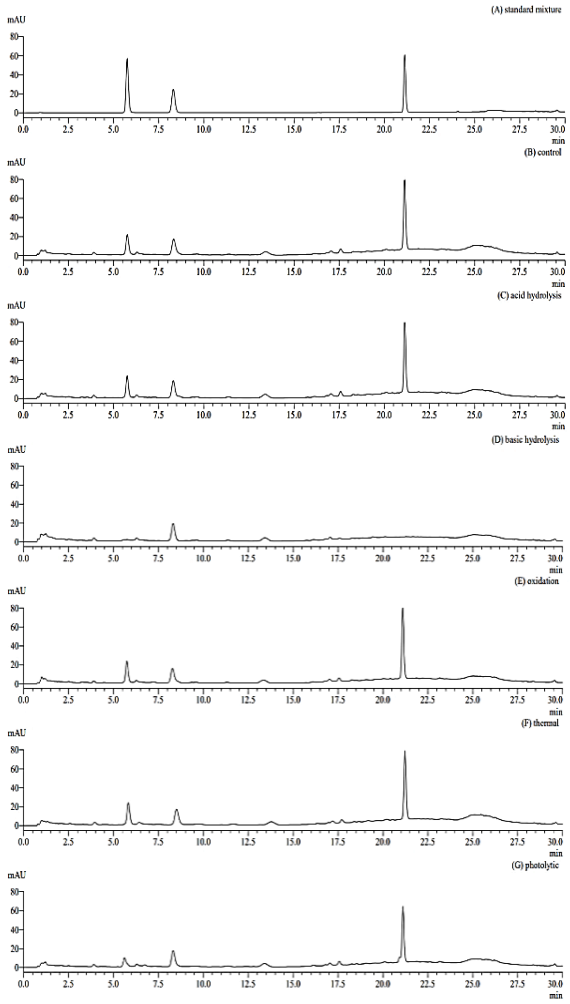

Figure 1. HPLC chromatograms of standard mixture (A), sample solution (control) (B), with acid hydrolysis (C), with basic hydrolysis (D), with oxidation (E), with thermal (F), and under photolytic conditions.

Table 3. Forced degradation study of CA, VI, RA in the *T. laurifolia* leaf extract capsule.

Degradation type	Resolution			Relative amount (%)		
	CA	VI	RA	CA	VI	RA
Control	7.06 ± 0.05	7.64 ± 0.05	41.17 ± 0.25	100	100	100
Acid hydrolysis	7.74 ± 0.12	8.53 ± 0.01	46.13 ± 0.09	99.45 ± 0.33	91.27 ± 0.62	99.53 ± 0.35
Basic hydrolysis	ND	16.05 ± 5.00	ND	ND	96.90 ± 1.16	ND
Oxidation	7.34 ± 0.43	7.44 ± 0.49	40.79 ± 2.31	103.49 ± 3.10	95.88 ± 1.60	102.30 ± 0.35
Thermal	7.36 ± 0.14	7.95 ± 0.17	39.92 ± 0.82	102.20 ± 0.58	100.88 ± 0.67	100.41 ± 1.06
Photolytic	5.75 ± 0.71	7.10 ± 0.87	40.17 ± 2.67	51.99 ± 0.46	98.40 ± 1.26	82.11 ± 3.51

* ND - not detected

Table 4. Validation results of stability indicating HPLC method for the determination of CA, VI, and RA in *T. laurifolia* leaf extract capsule.

Parameter	Acceptance criteria	CA	VI	RA
Linearity	r ≥ 0.99	0.9999	0.9999	0.9999
Range	-	0.8 - 28 µg/mL	1.2 - 42 µg/mL	1.2 - 42 µg/mL
Accuracy	CA; 90 - 108 % VI; 92 - 105 % RA; 92 - 105 %	100 - 101 %	102 - 105 %	99 - 101 %
Repeatability	CA; < 3 % VI; < 2 %	0.3 - 0.5 %	0.6 - 0.8 %	0.2 - 0.5 %
Intermediate precision	RA; < 2 %	3.0 %	0.6 %	1.3 %

The developed HPLC method was found to be linear, accurate, and precise for quantitative analysis of CA, VI, and RA in *T. laurifolia* leaf extract capsules as shown in **Table 4**.

Stability testing

The percent initial content of CA, VI, and RA in *T. laurifolia* leaf extract capsule at each time point under accelerated conditions were summarized in **Table 5**. It was found that RA was the major compound in *T. laurifolia* leaf extract capsule followed by VI and CA respectively. For *T. laurifolia* leaf extract capsule stored at an accelerated condition (40°C and 75%RH), the remaining percentages of all chemical markers were within the acceptable range for 1 month. However, CA significantly decreased at 3 and 6 months when compared to 0 month. Interestingly, VI and RA remained after 6 months at the range of 95.35 - 100.01 % and 90.20 - 99.55 %, respectively. The result suggesting that VI and RA could be suitable to be selected as the quality chemical markers according to excellent stabilities under accelerated conditions for 6 months.

Table 5. Percentage of marker content in *T. laurifolia* leaf extract capsule under accelerated conditions.

Marker	% Remaining			
	0 month	1 month	3 months	6 months
CA	100	101.16 ± 0.48	67.11 ± 0.91	40.99 ± 0.18
VI	100	100.01 ± 0.47	95.37 ± 0.54	99.65 ± 0.95
RA	100	99.55 ± 0.53	93.67 ± 1.30	90.20 ± 0.47

Conclusion

In this study, the HPLC-PDA method for a stability indicating method of CA, VI, and RA in *T. laurifolia* leaf extract capsule was developed. The optimization of the HPLC method was conducted using two-level five factors full factorial design approach. Various variables were screened to obtain a suitable HPLC method condition including the sample concentration of 2 mg/mL, column temperature at 35°C, and flow rate of 0.8 mL/min. The method was simple, accurate, and precise for the stability study of *T. laurifolia* leaf extract capsule. Since RA was a major compound and showed an acceptable percentage remaining after being stored under accelerated conditions for 6 months, RA should be considered as a chemical marker for routine analysis of *T. laurifolia* leaf extract capsule.

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GS-2 10.23177/NATPRO8.2023.06.22.020

Cell-free biotransformation of aryl allyl/methyl ethers by human gut bacterium *Blautia* sp. MRG-PMF1

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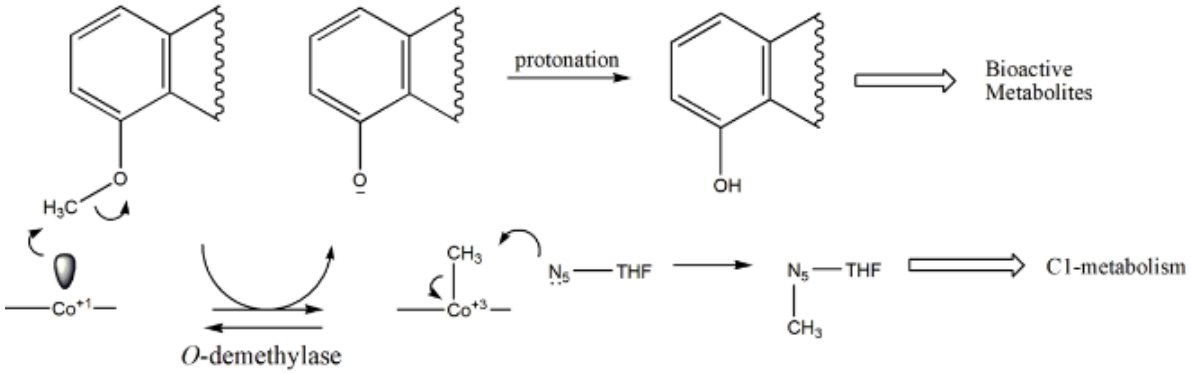
Biotransformation of aryl allyl ethers and aryl methyl ethers is of a great important for greener earth ecology and has a great biotechnological potential related to the biomass valorization. To study the biochemical reaction mechanism of O-demethylase found from human gut bacterium Blautia sp. MRG-PMF1, cell-free biotransformation of various aryl ether compounds has been performed under the anaerobic conditions. The results were compared to the previous finding and the reaction mechanism of O-demethylase was discussed.



Keywords: Biotransformation, C-O bond cleavage, Ether cleavage, Gut metabolism, Mechanism

Acknowledgement: This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2021R1A2C2007712).

GS-3

New source of bioactive compounds from rice plant at different growth stages

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The aim of this study was finding the new source of bioactive compound from rice plant at different growth stages. There parts of rice plant were compared i.e. straw, leave and grains. The rice plant from different parts and different growth stages were dried using freezing drying method to obtain the moisture content raged 3.81 - 7.73 %. The dried powder was then further analyzed for bioactive compounds and antioxidant activities i.e. DPPH and FRAP method. It has found that the water activity (Aw) of dried powder ranged 0.19 - 0.35 ($p>0.05$). Addition, chlorophyll A, chlorophyll B and total chlorophyll content of three parts rice plant at three growth stages ranged 0.26 - 3.02 (mg/g db.), 0.16 - 3.02 (mg/g. db.) and 0.42 - 3.87 (mg/g db.), respectively. The results are similar to those of total phenolic content (TPC) ranged 149.00- 309.00 (mg GAE/100g extract), which the highest content was obtained in the leave of immature growth stage ($p\leq0.05$). The lowest content was obtained in straw at mature stage. The antioxidant activities measurement by DPPH method and FRAP method has presented similar trend as high content ranged from 47.00 – 499.00 (mg TE/100g extract) and 169.00 – 1840.00 (mg FCE/100g extract) respectively, the highest content was found in leaves of immature growth stage ($p\leq0.05$), the lowest content was observed in grains at flowering growth stage. Thus, it can be suggested that the leaves of immature growth stage has strong functional activities and can be used as a functional ingredient in medicine and health food.

Keywords: Rice plant; Growth stage; Bioactive compounds; Antioxidant activity

GS-4

In silico screening and molecular docking shows cannabicitran isolated from *Cannabis sativa* as potential HIV-1 integrase and protease inhibitor

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Human immunodeficiency virus (HIV) remains a global health concern with no developed cure. While antiretroviral therapies are currently utilized for treatment, emerging drug resistance to current regimens poses the need to discover new antivirals with natural products, such as Cannabis sativa (marijuana), as potential sources of novel compounds. Because of regulatory restrictions in its propagation, an initial in silico approach would be essential to circumvent the need for crude plant extraction. This study explores the antiretroviral potential of C. sativa compounds as inhibitors against HIV-1 integrase and protease through in silico analysis. Selection through Lipinski's Rule of 5 and bioactivity, and simulation through molecular docking were conducted to screen compounds for therapeutic activity. In silico results revealed cannabicitran, a non-addictive cannabinoid, to have the best estimated free binding energy of -7.06kcal/mol (positive control: -5.58kcal/mol) and -8.06kcal/mol (positive control: -6.49 kcal/mol) against integrase and protease, respectively. Succeeding molecular dynamics simulation on Cannabicitran and target enzymes will be conducted on succeeding months. This study presents a potential resource for developing new options for antiretroviral therapy and adjunctive treatment for HIV-infected patients. Additionally, it offers opportunities for further clinical research and trials on medical marijuana.

Keywords: Human immunodeficiency virus (HIV), *Cannabis sativa,* Cannabicitran, HIV-integrase, HIV-protease, Adjunct therapy

Acknowledgement: Mammalian Cell Culture Laboratory, Cell In Vitro Applications in Research and Modeling (SCIARM) Laboratory, Insti-tute of Biology of University of the Philippines Diliman, Phil-DIAMOND of Department of Science and Technology, Philip-pine Council for Health Research and Development (DOST PCHRD), Department of Science and Technology-Science Education Institute (DOST-SEI).

10.23177/NATPRO8.2023.06.22.018

Glycosidic C-O bond cleavage by DgpA

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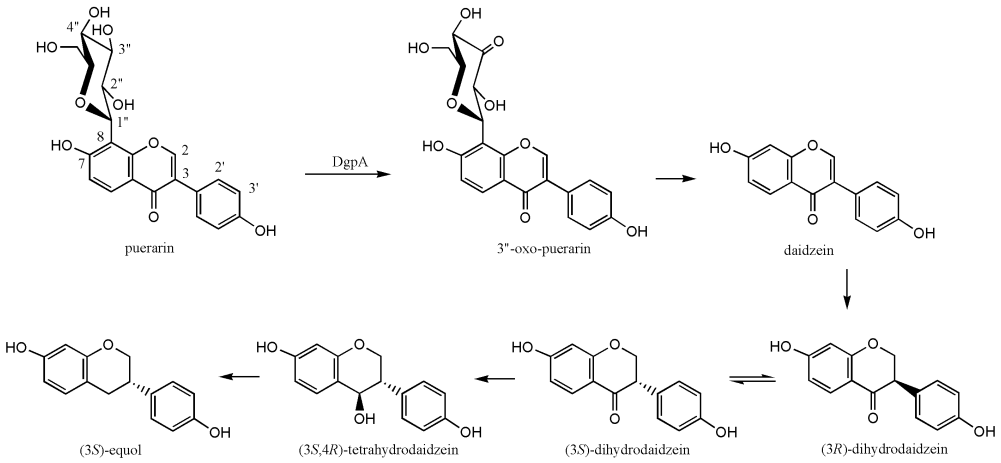
Puerarin, an isoflavone glycoside from *Pueraria lobata*, was known to be converted to *S*-equol in human gut. DgpA was known to catalyze the regioselective oxidation of puerarin to 3"-oxo-puerarin, as the first enzyme during the puerarin metabolism of Dorea sp. MRG-IFC3. To study the biochemical reaction mechanism of DgpA, various C- and O-glycosides were reacted with DgpA under the catalytic conditions. Interestingly, daidzein and genistein were produced from daidzin and genistin, respectively, by DgpA. Therefore, it was found that DgpA also catalyzed the cleavage of the glycosidic C-O bond of isoflavone O-glycosides.



Keywords: C-glycoside, C-O bond cleavage, DgpA, Gut metabolism, Isoflavone, Mechanism

Acknowledgement: This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2021R1A2C2007712).

GS-6

Effects of extraction methods on composition and functional properties of silkworm pupa e protein

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Silkworm pupae (*Bombyzx mori* L.) are one of the main by-products of the silk industry. They are mostly used as animal feed and fertilizer. Silkworm pupae are rich in many nutrients such as protein, fat minerals, and vitamins. There is a lot of interest in the application of silkworm pupae in the food, pharmaceutical and cosmetic industries. The present study used alkali-acid precipitation (AAP), salt precipitation (SP), combined alkali-acid (AAPU), and salt precipitation (SPU) with ultrasonic methods. The effects of four extraction methods on the functional properties (water holding capacity (WHC), fat absorption capacity (FAC), and emulsifying properties) and composition of silkworm pupae protein were compared. The results showed that the AAPU showed the highest yield (62.04%) and highest protein recovery (3.29%), followed by SPU, AAP, and SP. The protein contents of silkworm pupae protein using SPU, AAP, SP and AAPU were 70.79%, 70.08%, 69.77% and 68.06%, respectively. AAP gave the highest WHC (6.10 g of water/g of sample), while the AAPU had the highest FAC (4.09 g of oil/g of sample), than other samples. The highest EAI (32.90 m2/g) of the protein samples was obtained by SPU, while the highest ESI of the samples was produced by SP (60 min). Therefore, the results the silkworm pupae protein extracted by AAPU had high yield excellent some functional properties as a functional food ingredient.

Keywords: Silkworm pupae protein, Extraction method, Functional properties

GS-7

Antioxidant and anti-tyrosinase potentials of giant granadilla (*Passiflora quadrangularis* L.) fruit extracts for skin care products

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The Giant granadilla (*Passiflora quadrangularis* L.) is the largest fruit (1-3 kg) in the Passifloraceae family. It is one of the rare plants in Thailand that has gained attention due to its unique fruit flavor and health benefits. This research studied the bioactive, antioxidant effect, and anti-tyrosinase activity of P. quadrangularis extracts by comparing different parts of the fruit (epicarp, mesocarp, endocarp, and seed) and developed a skincare product containing them. Gallic acid was found to be the chemical marker of the *P. quadrangularis* extract by the HPLC method. The results showed that the seed extract had the highest total flavonoid content and the highest antioxidant activity, While the endocarp extract gave the highest yield and the highest anti-tyrosinase activity. Skincare product containing *P. quadrangularis* fruit extract of the endocarp and seed part was formulated. The formulation had acceptable physical characteristics and good stability. The yellowish color of the product was good texture with a pH of 5.0. Thus, the giant granadilla fruit extract is potent and can be used in cosmetics.

Keywords: Giant Granadilla, *Passiflora quadrangularis* L, Antioxidant, Anti-tyrosinase, Skincare

GS-8

Authentication by using chemical method, cytotoxic test, and anti-nitric oxide production test of different nutmeg crude drugs sold in Thailand

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Nutmeg (*Myristica fragrans* Houtt, Myristicaceae) had been utilized as spice, odorant, and herbal medicine because it contains fixed oil and volatile oil, of which myristicin, sabinene, safrole and 4-terpineol are its major components. Various studies reported that nutmeg showed anti-oxidant, anti-microbial, anti-inflammatory properties. In Thailand, nutmeg known as Chan-thet and its seed and aril crude drugs also known as Luk Chan and Dok Chan respectively. According to our preliminary study, two different shapes of crude drugs sold in Thailand—a global shape and an oval shape—were found so the quality efficacy and safety of nutmeg are inevitable. In this study, we aim to authenticate their botanical origin of 22 crude drugs sold in Thailand by using chemical method and evaluate cytotoxic and anti-nitric oxide production of Luk Chan and Dok Chan sold in Thailand. The results showed that the chromatographic patterns of their extracts obtained by TLC distinguished two different patterns, one was the same with that of the authentic *M. fragrans*, and the other was not the same. According to TLC pattern related with its morphology, thus the crude drugs were divided into two groups: global shape and oval shape. The methanolic extract and volatile oil from seed and aril represented each group were analyzed toxicity test in HaCaT and RAW264.7 cell line to compare toxicity between two groups of nutmeg. According to toxicity test in HaCaT from volatile oil from both groups are harmless and methanolic extract showed higher toxicity than oval shape. On the other hand, the toxicity test of volatile oil and methanolic extract treated in RAW 264.7 of nutmeg having global shape manifested higher toxicity than oval shape, especially in seed crude drugs. Then, RAW 264.7 cell line was tested with the highest dose that still safe of those samples. It revealed that the methanolic extract of aril having global shape showed more effective in reducing inflammation, which had the highest NO production inhibition in RAW 264.7 (37.26%) followed by methanolic extract of aril having oval shape (29.52%). Inconclusion, the seed and aril crude drugs were found that have origin from *M. fragrans* 40% and 50% in respectively. In conclusion, the seed and aril *M. fragrans* or global shape groups had more effective in anti-inflammatory properties but had higher toxicity compare oval shape group.

Keywords: Nutmeg, *Myristica fragrans* Houtt., Cytotoxic, Nitric oxide production inhibition

GS-9

A novel development and optimization of a hydrogel patch containing *Crinum asiaticum* for osteoarthritis and its stability test

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Crinum asiaticum L. has long been used in Thai traditional medicine to treat osteoarthritis and inflammation by applying it directly to pain areas. Thus, this study aims to formulate a topical anti-inflammatory hydrogel patch containing C. asiaticum extracts (CAE). The hydrogel patches were made from carrageenan, locust bean gum, glycerin and CAE by using a response surface methodology based on Box-Behnken design for design, assessing the effect of independent factors and optimizing the formulation. Anti-inflammatory activity, drug-releasing, skin permeability, physicochemical properties and stability were evaluated. The optimized CAE hydrogel patch demonstrated a good optimal tensile strength and the highest cumulative lycorine release and permeation at 6 hours for 69.38±2.78% and 24 hours for 48.51±0.45%, respectively which fit to Higuchi's kinetic model. Increasing main polymer proportion show low release rates. It exhibited potent anti-inflammatory activity with IC50 values of 21.36±0.78 μg/mL and is stable in accelerated condition at Day0-60 ($p>0.05$ with Day0). Thus, the optimized CAE hydrogel patch was excellently formulated, physicochemical properties and biological activity. It is also stable for 8 months at room temperature without losing anti-inflammatory activity. Thus, it could be an herbal application to relieve pain, inflammation and osteoarthritis. This delivery system's anti-inflammatory effect in vivo should be further investigation.

Keywords: Anti-inflammatory, *Crinum asiaticum*, Hydrogel patch, Response surface methodology, Optimization

Acknowledgement: This research was supported by Thailand Science Research and Innovation (TSRI) through the Royal Golden Jubilee Ph.D. Program (Grant No. PHD/0078/2558) and National Research Council of Thailand, the Thailand Science Research and Innovation Fundamental Fund and Center of Excellence in Applied Thai Traditional Medicine Research (CEATMR), Faculty of Medicine, Thammasat University. The authors are grateful to the Department of Biotechnology, the Korea National University of Transportation for providing the facilities to develop and study the hydrogel patch.

GS-10

Metabolite profiling and bioactivity screening of novel Streptomycete *Streptomyces coriariae* isolated from the Actinorhiza *Coriaria intermedia*

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Streptomyces species are microorganisms often found in nature, either as free-living or in direct association with other organisms, known for their ability to produce bioactive compounds that have promising applications in biopharmaceuticals, agriculture, and even manufacturing. Recently, a novel streptomycete – *Streptomyces* coriariae – was accidentally discovered from the root nodule of *Coriaria intermedia* harvested from Benguet, Philippines. Annotation of the novel species' genome showed that it has genes encoding for proteins and enzymes responsible for synthesizing metabolites with antimicrobial and plant growth-promoting activities. This present work explored if the initial whole genome analysis results translate into in vitro bioactivity. Untargeted metabolite profiling of *S. coriariae* extracts was performed using Ultra-High-Performace Liquid Chromatography and Mass Spectrometry, revealing hundreds of putative chemical compounds. Cross-checking each compound to ChemSpider, PubChem, and mzCloud for their reported bioactivity and application showed that the majority of the putative compounds detected have unknown or no reported bioactivity. Nonetheless, a significant number of compounds have reported industrial applications, while some have inhibitory, antimicrobial, and cytotoxic activities. *S. coriariae* exhibited positive enzymatic activity when tested for α-amylase, urease, pectinase, protease, asparaginase, and tyrosinase. *In vitro* antibacterial activity testing of the extracts against human pathogens yielded negative results. Based on plant growth-promoting activity testing, *S. coriariae* can also fix nitrogen and solubilize phosphate. Overall, the results of this present work showcase the diverse bioactivity of *S. coriariae* and its metabolites, and highlight the potential of *S. coriariae* as a novel source of bioactive compounds that have various applications, such as in medicine, agriculture, and manufacturing.

Keywords: Streptomycetes, Metabolic profiling, Crude extract, Antimicrobials, Enzymes, Plant growth promoting activities, Uplc-ms

Acknowledgment: This work was funded by the Philippine Department of Science and Technology – Science Education Institute and supported by the Microbial Ecology of Terrestrial and Aquatic Systems Laboratory of UP Diliman Institute of Biology. Some microbial strains were provided by the United States Department of Agriculture NRRL and the Fungal Laboratory of UP Diliman Institute of Biology. Parts of the laboratory work were conducted at the Functional Bioactivity Screening Laboratory of UP Diliman Institute of Biology. Several professional services were outsourced from the Mammalian Cell Culture Laboratory of UP Diliman Institute of Biology and the Philippine Genome Center – Protein, Proteomics, and Metabolomics Facility.

GS-11

Development of a microemul-foam from Thai essential remedy for topical anti-inflammatory treatment

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A Thai medicinal remedy from the Thailand National List of Herbal Medicines (NLHM) was used to formulate a microemul-foam to achieve a localized topical anti-inflammatory treatment. In addition, the formulation was then investigated for in vitro anti-inflammatory activity, skin cell toxicity, and stability studies. The remedy was extracted with 95% ethanol, which gave a 10.8 percent yield. The best emulsifying vehicles were chosen based on drug solubility and pseudo-ternary phase diagrams. The optimized formulation contained Capryol®90, Smix (Labrasol®: Transcutol®P), DI water, and NaOH. Physicochemical characterization showed a globule nanosized range of less than 100 nm, a pH of 5.50, 98% transmittance, and a viscosity of 160 cP. The in vitro anti-inflammatory efficacy revealed enhanced NO and PGE2 efficacy with IC_{50} values of 8.19±0.49 and 19.66±2.19 µg/ml, respectively, and was not toxic to skin cells. Furthermore, it was declared physically stable following five freeze-thaw cycles, and six months of storage under accelerated conditions disclosed the complete existence of the main active ingredient, piperine. Based on the findings, our optimized formulation possessed good physicochemical properties. It was not harmful to skin cells, exhibited a potent anti-inflammatory effect, and was thermally stable. This product has the potential to be a topical anti-inflammatory therapeutic approach, and clinical research needs to be investigated.

Keywords: Thai essential remedy, Microemul-foam, Topical drug, Anti-inflammatory

Acknowledgement: This work was supported by the Center of Excellence on Applied Thai Traditional Medicine Research, Faculty of Medicine, Thammasat University, faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta and the Thailand Science Research and Innovation Fundamental Fund provided research funding [TUFF 28/2565].

กรมการแพทย์แผนไทยและการแพทย์ทางเลือก
Department of Thai Traditional and Alternative Medicine

Innovation Competition

IN-1

Development of thermoreversible hydrogel loaded with *Chrysanthemum indicum* L. extract nanoemulsion for cosmeceutical application

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Chrysanthemum extract has been found to be rich in antioxidant properties, which is suitable for cosmeceutical products. Nowadays, in beauty arena, many cosmeceutical products have been developed in the form of hydrogels to retain water and add moisture to the skin. This research has developed the hydrogel that can be transformed into a gel at facial skin temperature (34°C). According to the study, antioxidant activity of Chrysanthemum extract, which was extracted by supercritical carbon dioxide extraction and maceration revealed that the maceration method using ethanol infusion at 50% did give the yield of 13.8985% extraction and also gave a high antioxidant activity (IC_{50}) of 22.57 μg/mL, which was higher than that of supercritical carbon dioxide extraction, which yielded of 1.3665% and, had the antioxidant activity of 60.20 μg/mL. In hydrogel development, the process of mixing with nano-emulsion, soaking with the ethanol, the solvent could affect the health of the extractor and also affects to the environment, as a result, the method used of supercritical fluid was then selected. Supercritical fluid extraction has been part of a green technology that could reduce the use of harmful organic solvents and then the products have been found to be safe and can be applied to the skin. Chrysanthemum extract, with a pressure of 230 bar and a temperature of 40°C, was used as an ingredient in hydrogel development being mixed with nano-emulsion. This research has been used the response surface methodology (RSM) to study the components suitable for nano-emulsion preparation by using the Box-Behnken design with a variation in the amount of surfactant and SFT 80 (Tween 80; T80), Span 80 (Span 80; S80), and Glycerine (Glycerine; GLY) from the emulsion stability analysis, T80 and GLY on the upper phase fraction of nano-emulsion to show the stability of emulsion. From the experiment, it was found that upper phase fraction will be increased with higher SFT levels. However, the increase in concentration of T80 will give the results in decreasing of the separation of the emulsion layer of the initial preparation and will gradually increase. The optimal proportion of nano-emulsion used, consisted of 19.99% w/w SFT, 69.99% w/w T80, and 68.66% w/w GLY. The experiments were then performed by selecting the highest concentration of SFT and GLY, and then varying the concentration of T80, the results showed that the most suitable T80 concentration was 75% w/w. The hydrogel used in this study was prepared with poloxamer 407 (P407), poloxamer 188 (P188) and sodium alginate (sodium alginate; SA) as the gelling agent, the results revealed that as the concentration of P407 increased, the sol-gel transition temperature (Tsol-gel) was decrease, and also made an increase in apparent viscosity (AV) of the hydrogel. The increase of P188 concentration could cause an increase in Tsol-gel. However, it has not been found any significantly affect to AV. The concentration of SA was not show any change to Tsol-gel and AV, but it has allowed the hydrogel to make crosslink well. Therefore, the optimum condition used for hydrogel preparation was determined from the concentration ratio of P407 and P188, which gave Tsol-gel formation close to the human skin temperature of 34°C. It consisted of 15% w/w P407 and 1.5% w/w SA. In conclusion, the experiments revealed that nano-emulsion, which was added to the hydrogel, could cause the increasing in Tsol-gel, but it had no effect to the AV significantly ($P<0.05$), and low temperatures should be the most suitable condition for keeping the hydrogel with nano-emulsion.

Keywords: Antioxidant activity, Alpha-glucosidase inhibitory activity, Herbal extract, Hyperglycemia

IN-2

TMR microemul-foam: topical anti-inflammatory painkillers

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An essential Thai medicinal remedy enriched with spices was used to formulate a microemulsion to achieve a localized topical anti-inflammatory treatment option. Although traditional oral administration seems to have a significant anti-inflammatory impact, a topical formulation was created since oral treatment can cause adverse effects such as stomach discomfort for more than 40 percent of patients. In our study, microemulsions were chosen to optimize a topical drug due to their stable monodisperse system and ability to rapidly deliver the drug through the skin. In addition, in vitro anti-inflammatory activity, skin cell toxicity, stability tests, and clinical trials of the product were explored. The optimized formulation possessed desirable physicochemical properties. It expressed a globule nanosized range of less than 100 nm, a pH of 5.50, high drug release, a potent anti-inflammatory effect by enhanced NO and PGE2 efficacy with IC_{50} values of 8.19±0.49 and 19.66±2.19 μg/ml, respectively, was non-toxic to skin cells, and was thermodynamically stable for two years without degrading. Moreover, clinical research found no irritation or allergic responses in healthy volunteers and provided localized drug delivery with the equally efficacy as diclofenac microemulsion in treating 84 patients with OA knees for four weeks. This product was a pharmaceutically stable anti-inflammatory formulation with a potential topical anti-inflammatory therapeutic approach. Fifty milliliters of foam containment make our product convenient to use and easy to apply to a precise area. Increased vasodilation by a warmth sensation might improve patient satisfaction. Nowadays, we are in the process of collaborating with the two companies.

Keywords: Thai medicinal remedy, Microemulsion, Topical, anti-inflammatory, Clinical trial, Product

Acknowledgement: This work was supported by the Center of Excellence on Applied Thai Traditional Medicine Research, Faculty of Medicine, Thammasat University, faculty of Pharmacy and Pharmaceutical Sciences, University of Alberta and the Thailand Science Research and Innovation Fundamental Fund provided research funding [TUFF 28/2565].

IN-3

C-cider, Thai herbal-extract cider for healthy drink

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Caesalpinia sappan Linn. is a flowering tree which belongs to family Leguminosae. Commonly, *C. sappan* is distributed in Southeast Asia including Thailand and this tree has been known as 'Fang' in Thai. The heartwood of this plant is orange (Fang Som) to red color (Fang Dang), widely used as natural a coloring agent and also used in Thai traditional medicine as herbal drink. The extract *C. sappan* exerts a number of medical effects, for example, anti-oxidant, anti-microbial, anti-cancer, anti-diabetic, anti-platelet aggregation, anti-inflammatory, neuroprotective, gastroprotective effects. In addition, anti-oxidant, and anti-hyperglycemic and antibacterial activity against gastrointestinal pathogenic bacteria properties have been reported in our previous studies. Cider is a low-alcoholic healthy drink obtained by fermenting substrate with a symbiotic culture of acetic bacteria and yeasts. The purpose of this project was to develop the fermented healthy drink containing the extract of *C. sappan*, and to develop the ready to drink cider using an extreme vertices-mixture design approach. Three ingredients, water (20-50%), honey (0-30%) and fermented extract solution (40-70%) were designed to obtain thirteen formulations using statistical modelling of extreme vertices-mixture design. The healthy drink ingredients were optimized on the basis of chemical and sensory qualities and the experimental data was analyzed by response surface methodology (RSM). The result indicated that trend in antioxidant activities are high with increasing a proportion of fermented extract solution, while amount of honey in the formulation has effects to increase total phenolic contents and overall acceptability. The optimum mixture for healthy drink was 20.5 ml of water, 25 g of honey 54.5 ml of fermented extract solution. The best formulation showed the predictive score for antioxidant activities of 181.15 mgTE/L, total phenolic contents of 625.29 mgGAE/L and score for overall acceptability of 7.03. The best formulation was developed as the commercial ready to drink cider named C-cider. The C-cider is a novel healthy drink containing benefit from Thai plant extract. C-cider was financially supported by National Innovation Agency, Thailand and this product will be launched as commercial product in December 2023.

Keywords: *Caesalpinia sappan*, Cider, Extreme vertices-mixture design, Response surface methodology (RSM)

IN-4

Hand washing gel containing *C. sappan* Linn. extract

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Hand sanitizer gel is a widely used, convenient prophylactic against contagious diseases. It usually contains ethyl or isopropyl alcohol for antimicrobial activity. However, allergic reaction to the alcohol in hand sanitizercan cause skin irritation and dry skin. Furthermore, alcohol is unable to inhibit some resistant bacterial strains such as Methicillinresistant *S. aureus*, *Escherichia coli*, *Acinetobacter braumanii* and *Pseudomonas aeruginosa*. In search of a more effective, gentler active ingredient for hand sanitizer gel, we considered *C. sappan* Linn. extract. It is an inexpensive, natural extract which doesn't irritate the skin, and it has excellent antimicrobial activity against both gram-negative and gram-positive bacteria, especially resistant strains and fungi. We added *C. sappan* Linn. extract to hand sanitizer gel and tested for antimicrobial activity against bacterial strains. The best formula had effective antimicrobial activity against all tested bacterial strains. Our hand sanitizer with *C. sappan* Linn. extract can prevent a rapid transmission of pathogens which cause hospital-acquired infection from carriers such as patients, physicians, and medical personnel. This extract of *C. sappan* Linn. can be further developed for other medical products, such as topical cream for wound infections. We developed a cream containing *C. sappan* Linn. and other herbal extracts with anti-inflammatory activity to enhance effectiveness for patients with chronic diseases. With increasing global resistance to antibiotics, our new product provides an alternative for medical treatment of infectious diseases. It also benefits the economy by employing farmers.

IN-5

Reduction of formaldehyde concentration by using herbal oil in embalming fluid

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Embalming fluid is used to maintain cadaver conditions, primarily with formaldehyde and other solvents. The formaldehyde is used to kill bacteria and prevent decay in the corpse. However, some chemical compounds may be potentially toxic, with potentially life-threatening side effects. Therefore, specific herbal extracts were chosen, based on their history of treating similar conditions. These herbal extracts contain antioxidants combatting decay-causing bacteria and possess anti-enzymatic properties also preventing decay. A formula was prepared by combining extracts from three different herbs: 95% ethanol extract from black cumin seeds (*Nigella sativa* L.), essential oil from pine tree (*Pinus merkusii* Jungh. & de Vriese), and 95% ethanol extract from betel leaf (*Piper betle* L.) in a ratio of 1:1:2, respectively. The objective was to develop an embalming fluid remedy using herbal extract formulations. Two parts were prepared: a base solution consisting of 40% formalin, glycerin, 95% ethanol, water, and 1% phenol, and a herbal extract formulation, to be added to the base solution according to the desired concentration. The two formulations were mixed, and effectiveness of preserving tissue was evaluated with pig tissue samples. Ten experts in basic anatomy observed and recorded parameters, including physical characteristics of tissue and odor, tissue color, tissue physical properties (flexibility and hardness), and muscle and organ tissue tactile properties. In addition, the level of hair loss from the skin was assessed to determine level of skin peeling and hair loss caused by testing. Results were that Formula 2 (20% of 40% formalin, 15% glycerin, 30% of 95% ethanol, 13% water, 2% of 1% phenol, and 20% herbal extract formulations) and Formula 4 (10% of 40% formalin, 15% glycerin, 30% of 95% ethanol, 13% water, 2% of 1% phenol, and 30% herbal extract formulations) reduced formaldehyde amounts by 50% and 70%, respectively. Statistical analysis showed no significant difference ($p<0.05$) between these formulations and the original embalming fluid used to preserve tissue. These findings suggested that herbal extract formulations consist of *N. sativa* (increased skin moisture and permeability and improving drug efficacy), *P. merkusii* (protein fixation), and *P. betle* (high antimicrobial properties) may be effective, suitable alternatives to formaldehyde. Herbal extract formulations cost approximately 1.3 times more than the original formula, but health benefits and safety aspects potentially reduce harmful effects of formaldehyde exposure, facilitating the teaching and learning process for students and teachers. Further studies are needed to evaluate long-term efficacy and safety of herbal extract formulations, including testing on cadavers to assess tissue preservation effectiveness.

Keywords: Embalming fluid, Formaldehyde, Herbal

IN-6

Anti-acne products derived from a formulated combination of *Garcinia mangostana* and Thai medicinal remedy extract

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Garcinia mangostana (GM), simply known as mangosteen, has long been used in Thai traditional medicine because of its reported antibacterial and anti-inflammatory activities for treating skin infections. Ha-Rak remedy is one of the interesting Thai medicinal remedies to treat acne due to its bitter and cold taste that assists in reducing Pit-ta (fire). Moreover, the previous study of GM combined with Ha-Rak remedy extract showed great effectiveness against *Cutibacterium acnes* and *Staphylococcus epidermidis*. Therefore, mangosteen combined with Ha-Rak remedy called Pra-Sa-Mang-Khud (PSM) extract has been developed into an anti-acne hydrogel patch and nanolotion, along with evaluating chemical and biological activities, and investigation of its safety and efficacy in clinical trial studies. PSM hydrogel patch made from carrageenan and locust bean gum powders was intense yellow in color, smooth, durable, flexible, and effective against *C. acnes* and *S. epidermidis*. The active ingredient, α-mangostin, was released and permeated from the PSM hydrogel patch within the first 30 min at 14.88 ± 0.16% and 17.70 ± 1.27%, respectively. In a phase 1 clinical trial using the human closed patch test, the PSM hydrogel patch showed no irritation in 30 healthy volunteers. The next product, PSM nanolotion was light yellow with a pH value of 5.63. PSM nanolotion had a release and transdermal flux of 4.38 ± 0.57 μg/cm2/h and 1.93 ± 0.16 μg/cm2/h, respectively. The PSM nanolotion showed no irritation in 30 healthy volunteers. Moreover, an investigator-blind, randomized controlled trial, split-face study in 35 volunteers with mild to moderate acne vulgaris showed the effectiveness of PSM nanolotion in reducing acne lesions within 12 weeks. PSM nanolotion showed a significant reduction of non-inflammatory lesions from baseline to week 12 with a lesions count of 13.33 to 3 (a reduction rate of 76.11%), and inflammatory lesions significantly decreased from 6 to 2 with a reduction rate of 66.67%. In conclusion, PSM hydrogel patch and PSM nanolotion are expected to be develop further for future commercial trade in the cosmetic or acne products market, which has risen significantly in recent years, as the study strongly supported the use of PSM hydrogel patch and PSM nanolotion as acne treatment products.

Keywords: Mangosteen, Ha-Rak remedy, Anti-acne product, Nanolotion, Hydrogel patch

Acknowledgement: This research was financially supported by the Thailand Science Research and Innovation Fundamental Fund through Grant No. TUFF 28/2565. The authors are grateful to the Center of Excellence in Applied Thai Traditional Medicine Research and the Faculty of Medicine, Thammasat University, for providing laboratory facilities.

IN-7

Development of low-cost complementary food innovation for infants and young children from germinated paddy rice

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Inadequate nutrients during illness in childhood is a major public health problem in developing countries. The commercially available complementary foods are often high-cost and have inadequate nutrients. Germinated paddy rice with high nutrients and bioactivity has long been used for consumption and fever treatment in children. Therefore, this study aimed to develop low-cost complementary food from germinated paddy rice (GPR-CF) that contains adequate nutrients and high bioactivity for infants and young children aged six months to three years. The study was divided into four steps; 1) to calculate the nutritional composition and develop the GPR-CF formulation, 2) to study the most suitable drying technique (oven drying, drum drying, and freeze drying), 3) to evaluate the physical properties and bioactivities of GPR-CF powder compared with the commercially available product, and 4) to evaluate the nutritional composition and microbiological safety of suitable GRR-CF powder. Germinated paddy rice RD47, which exhibited high γ-oryzanol and anti-inflammatory activity, was used as the main source of carbohydrates. The physical properties of GPR-CF powder obtained from the drum drying were better than the oven drying and freeze drying. The anti-inflammatory activity and γ-oryzanol content were high and remained after the drying process. The energy of suitable GPR-CF powder was 93.96 kcal/100 mL (per serving). The caloric distribution was 53.94% carbohydrate, 14.64% protein, and 31.42% fat. The energy density was 0.94 kcal/g. The nutrients were within the targeted values of dietary requirements according to the complementary food guideline. The nutrition composition of GPR-CF powder was higher than the commercially available product. Thus, GRD-CF powder from drum drying which was low-cost but showed high nutrients, high quality, and safety was suitable for consumption in infants and young children aged six months to three years.

Keywords: Complementary food, Germinated paddy rice, γ-Oryzanol, Anti-inflammatory activity

IN-8

Electric herb ball and gel

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The herbal hot compress ball, known as "luk pra kob," is a traditional Thai medicine used to relieve pain and inflammation. It consists of various herbs wrapped in cloth and formed into a ball shape. Traditionally, a hot herbal compress needed to be steamed before use. However, this method often resulted in unstable temperatures, necessitating frequent ball replacements. Moreover, the herbal ingredients couldn't be preserved, limiting its use to the short-term. To address these issues, an electrical herb ball and gel were invented. The electric ball retains the traditional ball shape and is rechargeable, providing an hour of usage per charge. The electric ball originates heat from the hot plate inside an aluminum casing. The temperature is manually controlled and can range from 45 to 70 degrees Celsius. It is designed to be used with a herbal gel extracted from various herbs found in traditional compress balls, such as *Zingiber cassumunar*, lemongrass, and curcumin. This innovation has revolutionized the application of herbal hot compresses, making them more accessible and dependable in TTM clinics, spas, and wellness centers or for personal use at home. However, further improvements are required in the design and monitoring of temperature and charging for this innovation.

Keywords: Compress ball, Herbal gel, Electrical

IN-9

The development of an innovative sunscreen gel derived from the extract of *Acanthus ebracteatus* Vahl holds promise for preventing oxidation and establishing a sustainable community spa

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This research project centers around the creation of a groundbreaking sunscreen gel utilizing extracts derived from *Acanthus ebracteatus* Vahl. The study encompasses several key objectives. Firstly, the aim is to develop natural extracts obtained from *Acanthus ebracteatus* Vahl. Secondly, it intends to explore the extracts' antioxidant activity, anti-inflammatory effect, antimicrobial effect, and total phenolic content. Thirdly, the research investigates the stability of the product. Finally, the study strives to identify appropriate formulations for the sunscreen gel utilizing the extracts from *Acanthus ebracteatus* Vahl. The research commences with the selection of raw materials and the preparation of extracts to determine the total phenolic content. The antioxidant activity is assessed using the DPPH assay method, while the anti-inflammatory effect is evaluated through various tests such as inhibitory activity of nitric oxide production, inhibition of LPS-induced TNF-α secretion, and COX-2 anti-inflammatory activity. Once a suitable formulation is developed, tests are conducted to ensure the safety and physical properties of the sunscreen gel product. This includes a skin irritation test involving 10 volunteers to assess its effectiveness and safety. The results of the product stability study indicate that the sunscreen gel containing *Acanthus ebracteatus* Vahl extracts does not exhibit phase separation. Furthermore, the irritation test conducted on the volunteers after 12 hours of product use shows no allergic reactions, itching, or irritation. Additionally, toxicity tests confirm the absence of cytotoxicity. The anti-inflammatory effect test demonstrates potent anti-inflammatory properties when tested on RAW 264.7 macrophage cells. It is important to note that this study exclusively focuses on the development of an innovative sunscreen gel utilizing extracts from *Acanthus ebracteatus* Vahl and does not cover other aspects of skincare or cosmetic products.

Keywords: Sunscreen gel, Nature, Elasticity

Acknowledgement: This work was supported by Applied Thai Traditional Medicine Program, College of Allied Health Sciences, Suan Sunandha Rajabhat University, Samut Songkhram 75000, Thailand

IN-10

Hypoglycemic activity of the combined extract from Satagavata-Matumeho-Tubpikarn antidiabetic herbal formula

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The Thai Traditional Medicine has different theory in human physiology and different paradigm in diabetic treatment. In this study three herbal formula prescribed to diabetic patients; Satagavata, Matumeho and Tubpikarn were prepared and fed to the induced diabetic rats. These three formulas were separately extracted and combined at the ratio of 1:1:1. This combined extract was fed to the diabetic rats at the daily doses of 0.5 and 1.0 g/kg for 30 days. The antidiabetic drug (glibencamide) at a dose of 5 mg/kg was given as a positive control group. The results showed effectiveness of the herbal formula at the dose of 1 g/kg in rats equivalent to 10 g/day for 70 kg human.

Keywords: Thai Traditional Medicine, Antidiabetic, Herbal formula, Glibencamide

Poster Presentations

P-1 10.23177/NATPRO8.2023.06.22.013

A preliminary screening of the antioxidant activities and cytotoxicity of *Euphorbia heterophylla* L. extracts

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Euphorbia heterophylla L. was used by folk healers for constipation in cancer patients. This research aimed to study the antioxidant activity determined by DPPH assay and cytotoxic activities against gastric cancer cell lines (KATO III) and human colon adenocarcinoma cell lines (SW480) by using the Sulforhodamine B (SRB assay) method. The results showed that the underground part of its water extract (RW) exhibited the highest antioxidant activities with a percentage of inhibition of 49.11 ± 0.89 at a concentration of 100 μg/mL, which indicated little anti-oxidant activity. Meanwhile, its ethanolic extract of aerial part (AE) demonstrated the most effective cytotoxic activities against KATO III and SW480 cancer cells with IC_{50} values of 5.67 ± 0.62 μg/mL and 5.01 ± 0.31 μg/mL, respectively. In conclusion, *Euphorbia heterophylla* L. showed potentiality against gastrointestinal cancer cells but little antioxidant activity. However, the active cytotoxic compounds should be isolated and identified for future product development for cancer treatment.

Keywords: *Euphorbia heterophylla* L., Antioxidant, Cytotoxicity, KATO III, SW480

Introduction

Cancer is the leading cause of death in the world. Empirical evidence compiled by the National Center for Health Statistics reveals that the trend of the incidence and mortality rates is cancer. In the United States, an estimated 1,958,310 new cancer cases and 609,820 cancer-related fatalities are expected in 2023 [1]. In Thailand, the annual number of cancer related deaths is increasing every year. Among the various types of cancer prevalent in Thailand, liver and bile duct cancer, lung cancer, and colon and rectal cancer emergence are the most commonly diagnosed forms [2]. Various cancer treatments are available, including alternative medicine used in conjunction with modern medicine to boost patient immunity. Diet plays a crucial role in cancer prevention, with foods rich in antioxidants. Thai medicinal plants are a major source of those compounds such as phenolic, flavonoid, vitamin, and catechin which involve in preventing and reducing the risk of cancer [3].

In the Thai traditional medicine framework, cancer is the results of human body deterioration and an imbalance in the tri-elemental system consisting of Pitta, Wata, and Semha. This imbalance disrupts the body's equilibrium. Thai folk healers, with extensive cancer treatment knowledge, often rely on local herbal medicines. Among these remedies, one commonly used herb is from the Euphorbiaceae family, known for its laxative property and its ability to facilitate detoxification of the body. In the northern region of Thailand, folk healers in Umphang District, Tak Province often use *Euphorbia heterophylla* L. as a laxative, which can be beneficial for cancer patients who have constipation symptom in this regard. The plant has been investigated for its cytotoxicity against A549 (human lung cancer cells) and HepG2 (human liver cancer cells) [4]. Its extract demonstrated an impact on intestinal motility in mice similar to acetylcholine, histamine, and potassium chloride [5]. *Euphorbia heterophylla* L., a member of the Euphorbiaceae family, shows promise as a potential laxative and exhibits potential anti-cancer properties, particularly within the gastrointestinal system. However, there is no

report on the antioxidant and cytotoxic activities against human colon adenocarcinoma cell lines (SW480 ATCC CCL-228) and human gastric carcinoma cell lines (Kato III) of this plant. Therefore, the objective of this study was to investigate the antioxidant activity and cytotoxic effects of *Euphorbia heterophylla* L. on these cancer cell lines.

Materials and Methods

Plant materials

Plant parts were collected in The Umphang District, Tak Province. The collected plant materials were identified by authentication procedures, ensuring its accuracy and reliability. A voucher specimen were keep in Department of Thai Traditional and Alternative Medicine in Thailand annotated as TTM-C No.1000701-4.

Preparation of crude extracts

Maceration Mehod. The dried plant materials (300 g) were macerated with 95% ethanol or hexane for 3 days. It was then filtered and dried using an evaporator. Subsequently, the hexane residue, was extracted with 95% ethanol using the same procedure.

Decoction method. The dried plant materials (100 g) were boiled with water (decoction) for 15 minutes, then filtered and evaporated down to one-third of the volume, then freeze dried using a freeze dryer (Lyophilizer). All extracts of each plant part were calculated percentage of yield and kept in freezer (-20°C) until used.

DPPH Radical Scavenging Assay

The antioxidant activity was determined using DPPH assay, according to the modified method [6]. Briefly, testing procedure began with preparation of a stock solution of extracts and standard substances to a concentration equal to 1 mg/mL, then adjust the concentration to different concentrations. The final concentrations were as follow: 1, 10, 50, 100 µg/ mL which were tested for antioxidant activity in 96 well plates. A 100 µl of DPPH was added to 100 µl of test substance, held for 30 minutes, then absorbance was measured at 520 nm using a spectrophotometer. The scavenging activity of the samples were calculated as a percent inhibition in the formula below:

$$\text{Inhibition (\%)} = [(\text{ODcontrol} - \text{ODsample})/\text{ODcontrol}] \times 100$$

BHT (Butylated hydroxytoluene) was used as standard control. The obtained values were calculated for radical scavenging activity (EC_{50}). The EC_{50} value below 20 µg/mL indicates as good antioxidant activity.

Cell culture

Cancer cell lines used in this study were the human colon adenocarcinoma cell lines (SW480 ATCC CCL-228) and Human gastric carcinoma cell lines (Kato III) were purchased from RIKEN BRC, Japan. They were cultured in RPMI 1640 medium (BIOCHROMAG) supplemented with 10% heated fetal bovine serum, 1% of 2mM 1-glutamine, 50 IU/mL penicillin and 50 µg/mL streptomycin.

Sulforhodamine B (SRB) Assay

The cytotoxicity activity assay, SRB assay, was performed essentially according to the previous method [7]. This assay is used for cell density determination, which is performed to assess growth inhibition by a colorimetric assay by staining total cellular protein with the dye SRB. Cell cultures were fixed by 100 µl of ice-cold 40% Trichloroacetic acid (TCA) per well, incubated at 4 °C for 1 hour in the refrigerator and washed off non-viable cells with distilled water, add 50 µl of SRB solution (0.4% w/v in 1% acetic acid, Sigma) per well, held for 30 minutes and wash with 1% acetic acid. The plate was dried then 100 µl of 10 mM Tris, pH 10.5 was added. The absorbance (OD) of each well was measured at 492 nm. The IC50 values were calculated from the Prism program obtained by plotting the percentage of survival versus the concentrations, interpolated by cubic spine. According to National Cancer Institute guidelines extracts with IC50 values < 20 µg/mL were considered active. The IC50 values were calculated from the Prism program.

$$\text{Inhibition (\%)} = [(\text{ODcontrol} - \text{ODsample})/\text{ODcontrol}] \times 100$$

Statistical analysis

All experiments were carried out in triplicate and presented as mean ± SEM (standard error of the mean).

Results & Discussion

Plant materials

The percentage yield was calculated after the extraction process. The 95% ethanol extract of the hexane residue (AHE) of aerial part exhibited the highest yield of 11.43%. Similarly, the underground part was extracted using n-hexane (RH) and hexanes residue with 95% ethanol (RHE), yielded 13.43% and 13.32%, respectively (**Table 1**).

Table 1. Percentage yield of all *Euphorbia heterophylla* L. extracts.

Part use	Extract	Code	% yield
Aerial part	95% Ethanol	AE	8.193
	n-Hexane	AH	10.49
	Hexanes residue with 95% Ethanol	AHE	11.43
	Water	AW	10.16
Underground part	95% Ethanol	RE	10.99
	n-Hexane	RH	13.43
	Hexanes Residue with 95% Ethanol	RHE	13.32
	Water	RW	12.22

DPPH Radical Scavenging Assay

The results revealed that *Euphorbia heterophylla* L. exhibited little antioxidant activity, as assessed through the DPPH assay. The EC_{50} values for both aerial and underground parts in all extracts were found to more than 100 μg/mL. In comparison, the standard substance BHT demonstrated superior antioxidant activity, with an EC_{50} value of 15.49 ± 1.27 μg/mL. However, the underground part of the water extract (RW) demonstrated the highest percentage inhibition at a concentration of 100 μg/mL, with a value of 49.11 ± 0.89% (**Table 2**). These results suggested that this plant showed little antioxidant effect. This study agreed with previous research, which reported the maximum concentration of 500 μg/mL for the aqueous extract of Euphorbia heterophylla L. leaves which showed EC_{50} value as 141.11 ± 4.23 μg/mL [8].

Table 2. Antioxidant activity of Euphorbia heterophylla L. extracts using DPPH radical scavenging activity.

Botanical name	Part use	Extract	Code	% Inhibition Conc. 100 (μg/mL ± SEM)	EC_{50} (μg/mL)
Euphorbia heterophylla L.	Aerial part	95% Ethanol	AE	17.60 ± 0.11	> 100
		n-Hexane	AH	32.05 ± 0.73	> 100
		Hexanes Residue with 95% Ethanol	AHE	27.18 ± 0.21	> 100
		Water	AW	39.88 ± 2.26	> 100
	Underground part	95% Ethanol	RE	17.71 ± 0.01	> 100
		n-Hexane	RH	33.82 ± 2.90	> 100
		Hexanes Residue with 95% Ethanol	RHE	30.39 ± 1.56	> 100
		Water	RW	49.11 ± 0.89	> 100
			BHT	88.29 ± 1.70	15.49 ± 1.27

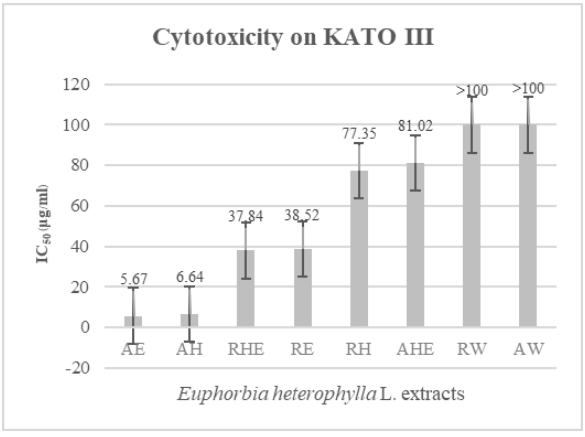
Table 3 Cytotoxicity of the *Euphorbia heterophylla* L. Extract against Gastric cancer cell-lines (Kato III), human colon adenocarcinoma cell lines (SW480) using SRB assay (n=3).

Botanical name	Part use	Extract	Code	IC_{50} (μg/mL ± SEM)	
				Kato III	SW480
Euphorbia heterophylla L.	Aerial part	95% Ethanol	AE	5.67 ± 0.62	5.01 ± 0.31
		n-Hexane	AH	6.64 ± 0.17	17.87 ± 1.28
		Hexanes Residue with 95% Ethanol	AHE	81.02 ± 1.02	6.17 ± 0.33
		Water	AW	>100	>100
	Underground part	95% Ethanol	RE	38.52 ± 0.79	12.56 ± 1.78
		n-Hexane	RH	77.35 ± 3.83	7.84 ± 0.23
		Hexanes Residue with 95% Ethanol	RHE	37.84 ± 0.75	7.76 ± 0.34
		Water	RW	>100	>100

Sulforhodamine B (SRB) Assay

According to the National Cancer Institute (NCI) of the US, plant extracts are classified as high cytotoxic activity if their IC_{50} is less than 20 μg/mL, moderate cytotoxic activity if the IC_{50} ranges between 21-200 μg/mL, weak cytotoxic activity if the IC_{50} ranges between 201-500 μg/mL, and no cytotoxic activity if the IC_{50} exceeds 500 μg/mL [9]. This study revealed the remarkable cytotoxic potential of *Euphorbia heterophylla* L. against gastric cancer and adenocarcinoma cells using diverse extraction methods. The aerial parts, including AE and AH, exhibited highly potent cytotoxic activity against gastric cancer cell lines (Kato III), with IC_{50} values of 5.67 ± 0.62 μg/mL and 6.64 ± 0.17 μg/mL, respectively. Conversely, the underground parts, RE and RHE, demonstrated moderate cytotoxic activity, with IC_{50} values of 38.52 ± 0.79 μg/mL and 37.84 ± 0.75 μg/mL, respectively.

Furthermore, the aerial parts in the AE, AHE, and AH extracts exhibited highly potent cytotoxic activity against human colon adenocarcinoma cell lines (SW480), with IC_{50} values of 5.01 ± 0.31 μg/mL, 6.17 ± 0.33 μg/mL, and 17.87 ± 1.28 μg/mL, respectively. Similarly, the underground parts, including RHE, RH, and RE, also displayed highly potent cytotoxic activity against these cell lines, with IC_{50} values of 7.76 ± 0.34 μg/mL, 7.84 ± 0.23 μg/mL, and 12.56 ± 1.78 μg/mL, respectively. Notably, these extracts exhibited high selectivity in their cytotoxic effects against all types of cancerous cell lines. However, all water extracts showed no effect on any of the cancer cell lines tested (IC_{50} > 100 μg/mL).



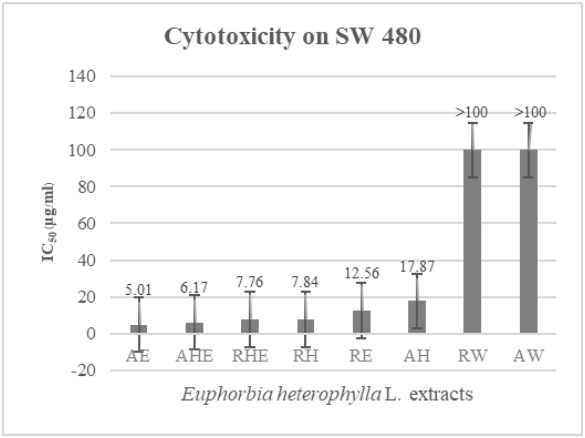


Figure 1 Cytotoxicity of the *Euphorbia heterophylla* L. extracts against Gastric cancer cell lines (Kato III), human colon adenocarcinoma cell lines (SW 480) using SRB assay (n=3).

Conclusion

Euphorbia heterophylla L. has been traditionally used by folk healers as a laxative for cancer patients. The present study provides substantial support for the potential utilization of *Euphorbia heterophylla* L. in the treatment of cancer, especially gastric cancer and adenocarcinoma cells. Despite the fact that all its extracts showed little antioxidant activity, but they showed high cytotoxic effects. This current study also showed that the extraction method by 95% ethanol demonstrated cytotoxic activity, suggesting its potential as a selective approach for extracting the active components. Nevertheless, the cytotoxic compound should be investigated for the development of its product for gastrointestinal cancer treatment.

Acknowledgement

This research was financially supported by the Thailand Science Research and Innovation Fundamental Fund through Grant No. TUFF 28/2565. The authors are grateful to the Center of Excellence in Applied Thai Traditional Medicine Research and the Faculty of Medicine, Thammasat University, for providing laboratory facilities.

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P-2 10.23177/NATPRO8.2023.06.22.015

In vitro anti-inflammatory testing on four Thai herbal formulations

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Inflammation is a response to tissue injuries, which is indispensable and important for human health, but excessive inflammation can potentially cause damage to the host organisms. Therefore, it is crucial to explore interventions that can effectively mitigate inflammation. In this study, the anti-inflammatory activity of Bhup-po, Mareng, Gastric, and Sahasa-rangsi, all of which are traditional herbal formulae, was investigated. The objective was to evaluate their potential in suppressing inflammation. The 95% ethanol extracts of Bhup-po, Mareng, Gastric, and Sahasa-rangsi were screened for their potential to reduce the production of nitric oxide (NO) in LPS induced inflammation. The Bhup-po, Mareng, Gastric, and Sahasa-rangsi extracts inhibited NO production in a concentration-dependent manner with IC50 values of 40.10±1.46, 20.77±3.41, 35.00±5.32 and 33.25±1.80 μg/mL, respectively. The results showed that these herbal extracts could inhibit the LPS-induced NO pathway suggests their effective properties in attenuating inflammation and further development of these innovative therapeutic agents is warranted.

Keywords: Anti-inflammatory activity, Nitric oxide, Bhup-po, Mareng, Gastric, Sahasa-rangsi

Introduction

Inflammation is a crucial biological response that helps protect the body from infections and injuries and maintains homeostasis. However, chronic inflammation can contribute to the development of various diseases, including rheumatoid arthritis, chronic hepatitis, pulmonary fibrosis, and cancer [1]. The initiation of inflammation involves innate immune cells, particularly macrophages, which release pro-inflammatory mediators such as nitric oxide (NO) and prostaglandin E2 (PGE2), as well as inflammatory cytokines like tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and interleukin-6 (IL-6). Macrophages and dendritic cells play a crucial role in detecting danger signals and triggering an inflammatory response. These cells release pro-inflammatory mediators to promote the migration of leukocytes, eliminate the cause of infection, and contribute to tissue repair [2, 3]. One of the important molecules released by activated macrophages and immune cells is nitric oxide (NO), which has dual functions in cells: homeostasis and cytotoxicity. Under normal physiological conditions, NO is produced in small amounts and serves regulatory functions such as vasodilation, platelet and neutrophil adhesion control, and involvement in neurotransmission. However, in abnormal situations, overproduction of NO can occur, leading to its role as a pro-inflammatory mediator and contributes to the pathogenesis of inflammatory disorders. The excessive production of NO can cause vasodilation and tissue damage during the inflammatory process [4, 5]. Therefore, the development of NO inhibitors represents a significant therapeutic advancement in the management of inflammatory diseases. NSAIDs are effective in reducing inflammation and also possess antipyretic (fever-reducing) and analgesic (pain-relieving) properties [6, 7]. However, their adverse effects, such as gastrointestinal, renal, and cardiovascular toxicity, have restricted their usage. To overcome these limitations, researchers are exploring natural compounds and herbs as alternative options.

Bhup-po is prescribed in clinics for the following symptoms; general wounds, abscess, sore throat, tonsilitis, chronic cough, chronic wounds, diabetic wounds, pressure sores, traumatic injury and long

COVID. The main herbs are Ya-Prag or Burmuda grass, *Cynodon dactylon* (L.) Pers (Poaceae) and Ya-teen-Nok or Fingergrass, *Digitaria ciliaris* (Retz.) Koel (Poaceae). Mareng formula is used to treat cysts, benign and malignant tumours of the internal organs (lung, liver, spleen, kidney, heart) and breast cancer. It is usually taken with Bhup-po. The main herbs are whole plants of Tong-pan-chang or White crane flower, *Rhinacanthus nasutus* (L.) Kurz. (Acanthaceae), Krob-jakra-wan or Indian Mallow, *Abutilon hirtum* and Krob-fun-si or Country mallow, *Abutilon indicum* (Malvaceae) and Pak-kra-sang or Pepperomia, *Peperomia pellucida* (L.) Humb., Bonpl & Kunth (Piperaceae). The gastric formula is used in the treatment of peptic and duodenal ulcers and GERD. The main herb is Kamin-oy, or White turmeric, *Curcuma zedoaria* (Christm.) Roscoe (Zingiberaceae). Sahasa-rangsi formula is used for the tendon and muscle inflammation in rheumatoid arthritis, gout, osteoarthritis, herniated disc, piriformis syndrome, and stiff neck and is usually taken with Bhup-po. The main herbs are Kamin-Kruea or Yellow Fruit Moonseed, *Arcangelisia flava* (L.) Merr. (Menispermaceae) and Plai, *Zingiber montanum* (Koenig) Link ex Dietr.

Therefore, the present study focuses, for the first time, on the evaluation of the anti-inflammatory potential of four herbal formulae: Bhup-po, Mareng, Gastric, and Sahasa-rangsi through the inhibition of NO production in LPS-activated RAW264.7 macrophages.

Materials and Methods

Preparation of plant extracts

The powdered of each recipe Bhup-po, Mareng, Gastric and Sahasa-rangsi was extracted for 5 days in 100 mL 95% ethanol. The separated extracts were then filtered through Whatman No. 1 filter paper and the ethanol filtrate evaporated to dryness using a rotary evaporator at room temperature (30 °C). The thick extracted mass was then dried at room temperature, and the dried extract stored in an air-tight container at 4 °C until further use. Percentage yields of the crude extracts were calculated asweight of crude mushroom extract (g)/weight of dry mushroom (g)x100%] [8].

Cell culture

RAW 264.7 macrophages were obtained from the American Type Culture Collection (ATCC). RAW 264.7 macrophages were cultured in 37°C, 5% CO_2 incubator with Dulbecco's modified Eagle's medium (DMEM; Sigma-Aldrich Co.) containing 10% fetal bovine serum (FBS; Gibco), and 1% Penicillin-Streptomycin solution (Sigma-Aldrich Co.), and were split twice a week.

Measurement of Nitric Oxide Production

Nitrate and nitrite concentrations were assayed by Griess reagent using a nitric oxide assay kit as described previously [9]. RAW 264.7 cells (4 × 105 cells/well) were plated in 96-well culture plates overnight. Under the serum-free condition, cells were pretreated with the Bhup-po, Mareng, Gastric and Sahasa-rangsi extract (12.50-200 μg/mL), for 1 h and subsequently treated with LPS (100 ng/mL) for 24 h. After 24 h, the supernatants (100 μL) were transferred into 96-well plates and mixed with 100 μL Griess reagent (20 μL of 1% sulfanilamide in 5% phosphoric acid and 20 μL of 0.1% naphthyl-ethylenediamine dihydrochloride). After 10 min, the absorbance was determined at 550 nm. The concentrations of nitrite were calculated from regression analysis using serial dilutions of sodium nitrite as a standard. Percentage inhibition was calculated based on the ability of extracts to inhibit nitric oxide formation by cells compared with the control (cells in media without extracts containing triggering agents and DMSO), which was considered as 0 % inhibition.

Results & Discussion

Crude yield of extracts

Table 1 presents the percentage yield of ethanolic extracts obtained from Bhup-po, Mareng, Gastric, and Sahasa-rangsi using ethanol as the solvent. The ethanolic extract yields for Bhup-po, Mareng, Gastric, and Sahasa-rangsi were determined to be 8.16%, 6.50%, 8.98%, and 8.14%, respectively.

Table 1. Percentage yield of ethanol extracts.

Extracts	% yield
Bhup-po	8.16
Mareng	6.50
Gastric	8.98
Sahasa-rangsi	8.14

Cytotoxicity assessment in RAW 264.7 cells

MTT assay was used to assess the cytotoxicity of each extract in RAW 264.7 macrophages (**Figure 1**). Sample concentrations with cell viability of ≥90% relative to the control group were considered to be safe, non-toxic concentrations. Cell viability measurement after treatment with 12.50, 25, 50, 100, and 200 μg/ml of each

extract showed cell viability of ≥90% up to the concentration of 200 μg/ml, revealing no cytotoxicity. On the basis of these results, the 12.50, 25, 50, 100, and 200 μg/ml samples were selected for further experiments.

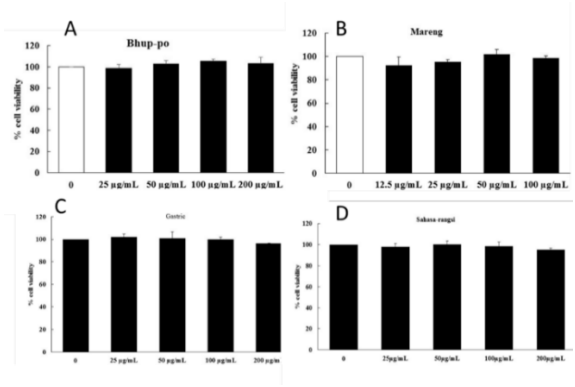

Figure 1 Cytotoxicity of Bhup-po, Mareng, Gastric, and Sahasa-rangsi extracts in RAW 264.7 cells. RAW 264.7 cells were incubated for 24 hr in the presence or absence of each extract at the indicated concentration. Cell viability was evaluated by the MTT assay. Data represent the mean ± SEM of triplicate determinations from three separate experiments (n=3). (A) Bhup-po (B) Mareng (C) Gastric (D)Sahasa-rangsi

Table2. NO inhibitory activity of extracts.

Extract	% **inhibition at various concentrations**					IC_{50} (μg/mL)
	12.50 μg/mL	**25 μg/mL**	**50 μg/mL**	**100 μg/mL**	**200 μg/mL**	
Bhup-po		28.32±2.67	63.05±2.56	91.53±6.90	101.82±0.89	40.10±1.46
Mareng	30.92±5.12	59.22±7.49	91.84±0.90	96.20±2.68	-	20.77±3.41
Gastric	-	34.05±6.24	71.03±0.62	92.26±3.47	99.47±2.63	35.00±5.32
Sahasa-rangsi		45.00±6.39	75.54±8.38	96.11±0.67	104.45±6.38	33.25±1.80
Dexamethasone 20 μM , % inhibition= 48.80±5.45						

Each value represents the mean ± S.E.M (n=3).

Inhibitory effects on NO production

Lipopolysaccharides are extracellular components of gram-negative bacteria and they act as powerful stimuli for various cells such as monocytes and macrophages. In particular, when macrophages are activated after stimulation by LPS, they produce and release inflammatory mediators such as NO via regulation of pro-inflammatory factors Therefore, in the present study, we examined the effects of Bhup-po, Mareng, Gastric, and Sahasa-rangsi extracts on the production of NO, which is one of the active oxygen species and is known to play a key role in inflammation induction. The study aimed to assess the inhibitory effects of Bhup-po, Mareng, Gastric, and Sahasa-rangsi extracts on nitric oxide (NO) production in LPS-stimulated RAW 264.7 cells. The amount of NO produced was quantified by measuring the NO2 content in the cell culture broth using the Griess reagent. Upon LPS stimulation, untreated cells exhibited an increase in NO production. However, treatment with Bhup-po, Mareng, Gastric, and Sahasa-rangsi extracts effectively inhibited this LPS-induced increase in NO production, and the inhibitory effects were observed to be concentration-dependent (**Figure 2**).

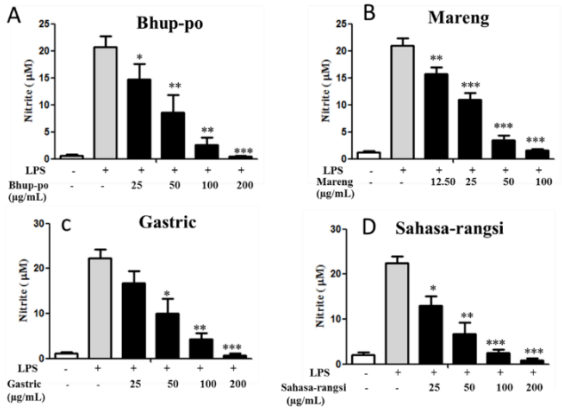

Figure 2. Effect of Bhup-po, Mareng, Gastric, and Sahasa-rangsi extracts on nitrite production in RAW 264.7 cells. After pretreatment with the indicated concentration of each extract for 1 hr, RAW 264.7 cells were treated with 100 ng/ml lipopolysaccharide (LPS) for 24 hr. Nitrite levels in the cell media were measured spectrophotometrically using Griess reagent. Data are provided as mean ± SEM (n=3). *p < 0.05 ***p < 0.01 ***p < 0.001 vs C LPS treatment. (A) Bhup-po (B) Mareng (C) Gastric (D) Sahasa-rangsi.

Four extracts were evaluated for their ability to inhibit NO production. Mareng extract demonstrated robust anti-inflammatory activity, with a significant inhibition of 96.20±2.68% at a concentration of 100 μg/mL, and its half-maximal inhibitory concentration (IC_{50}) was determined to be 20.77±3.41 μg/mL. Sahasa-rangsi extract also exhibited inhibition of NO production, with a notable 104.45±6.38% inhibition at a concentration of 200 μg/mL and an IC_{50} value of 33.25±1.80 μg/mL. Similarly, Bhup-po and Gastric extracts displayed strong inhibition of NO production, with percentages of inhibition of 101.82±0.89% and 99.47±2.63% respectively at a concentration of 200 μg/mL. The corresponding IC_{50} values for Bhup-po and Gastric extracts were 40.10±1.46 μg/mL and 35.00±5.32 μg/mL respectively (**Table 2**).

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P-3 10.23177/NATPRO8.2023.06.22.016

Analgesic effect of hot herbal compression (Massage ball) from *Kaempferia parvriflora* Wall., *Kaempferia galangal* L. and *Zingiber cassumnae* Roxb. upper trapezius of patients

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The objective of this study aimed to compare the effect of the hot herbal compression (HHC) from *Kaempferia parvriflora*, *Kaempferia galangal* and *Zingiber cassumnae* in the upper trapezius of patients. The sample comprised 48 volunteers aged 19-33 years. The subjects were randomly assigned to 3 groups (n=16) by different compresses. The former group received 30 minutes of HHC 3 times per week for 1 week, while also the participants' pressure pain threshold (PPT) and satisfaction prior to and after were determined. It was found that their PPT did not significantly increase and the 3 groups revealed no significant differences (p>0.05). Therefore, it can be concluded that the 3 group of HHC are all likely to be efficacious treatment options for the alleviation in patient of upper trapezius.

Keywords: Herb compression, *Zingiber cassumnae* Roxb., *Kaempferia parvriflora* Wall., *Kaempferia galangal* L., Upper trapezius

Introduction

At present, the concept and cultural dimensions of Thai people's health are more open. Therefore, many patients seek treatment solutions to fulfill their physical, mental and social needs. This is a traditional way of health care that people are familiar with and self-reliant. The World Health Organization has increasingly supported alternative and complementary therapies [1]. The holistic approach is consistent with the way of the community. It has become more popular, such as therapeutic massage, herbal compress, etc [2].

Herbal compress is a method of healing in Thai traditional medicine which can be used in conjunction with Thai massage. The benefits of herbal compresses have been discussed for a long time which are collected as follows are reduce aches and pains, sprains, swelling, inflammation and improves blood circulation of body. In addition, the aroma of herbs helps to relieve tension. Such qualities have been said since the time of our ancestors and there is a practice inherited from the present day [3].

How to use herb compression that is very comfortable for the patient to treat the pain is of paramount importance. The first step for herb compression use is by steaming. Drug or substancemust spread out into the location of the painful aches. *Zingiber cassumnae* is used in herb compression to reduces pain, swelling, and inflammation. The *Z. cassumnae* is the main herb mixed with other herbs. A disadvantage of *Z. cassumnae* is an unpleasant odor. As a consequence, it is necessary to compress the time available for patients who react adversely to the smell with severe dizziness. As a result, the treatment does not work optimally. *Z. cassumnae* also stains yellow and the clothes stain. As a consequence, *Z. cassumnae* is limited in its use. Alternative herbal remeides that share similar efficacy without the intense odor and that do not stain clothing may have greater utility. *Kaempferia galangal* and *Kaempferia parvriflora* are two alternatives that require further investigation for herb compression of upper trapezius pain.

Materials and Methods

This research was quasi-experimental research with three groups pre-post test design for a comparative study between the hot herbal compression (HHC) from *K. parvriflora*, *K. galangal* and *Z. cassumnae* in the upper trapezius. The sample group was the general population aged 18-35 years who were assessed for muscle spasm of shoulder, 16 cases per group, totaling 48 cases. The researcher and team jointly considered the criteria.

Inclusion criteria

1. Participants must have been diagnosed in operative medicine by Applied Thai traditional medicine as spasticity of the shoulder.
2. Participants were voluntarily admitted to receive treatment in the applied Thai traditional medicine unit, Mae Fah Luang University Hospital Mueang Chiang Rai District Chiang Rai.
3. Participants must have normal vital signs.
4. The subjects must not have heat/cold sensing impairment.
5. Participants must not have a history of allergies to the herbs used in the experiment.
6. A research subject shall not be subject to any restrictions on the tests conducted in the research.

Exclusion Criteria

1. During the experiment, there was an abnormality in the sense of heat and cold sensation, for example, numbness of the extremities. The toe type can't feel the heat and cold. It is often found in patients with diabetes. Abnormal cerebrovascular disease, etc.
2. Testing and experimenting if there is a congenital disease and using drugs that affect the heat and cold sensation system will be excluded from this study. If there is any doubt about the health of the study participant a consult with the doctor will be required as to whether to participate in this study or not.
3. There are other neurological complications. The musculoskeletal system hinders the experiment.
4. While testing, the subjects had allergies to the herbs used in the experiment.
5. Swelling that is not caused by inflammation because it will increase the swelling.

The pre-test pain was assessed using an Algorimeter (a measure of pain tolerance).

Volunteers were randomly drawn into 3 groups.

- The group that compresses with the hot herbal compression (HHC) from Kaempferia parvriflor ball.
- A group that is compressed with the hot herbal compression (HHC) from Kaempferia galangal ball.
- A group that is compressed with the hot herbal compression (HHC) from Zingiber cassumnae ball.

The subjects were assigned to receive herbal compress 3 days a week for 30 minutes per day. The assessment was performed before and after the experiment.

The pain level was assessed 2 days after the last herbal compress and 1 more time and this study was analyzed by means Standard deviation and ONE WAY ANOVA statistics were used.

Results & Discussion

The results determined that after receiving herbal compresses with *K. parvriflora* ball, the level of pain tolerance (Pain Threshold) decreased (before 4.76 and after 4.67 kg/m^2) with no statistically significant difference at the 0.05 level and was equal to 5.12 kg/m^2. There was no significant difference at the 0.05 level. After receiving a herbal compress with *K. galangal* ball, the level of pain tolerance (Pain Threshold) decreased (before 4.09 and after 3.82 kg/m^2) with no statistically significant difference at the 0.05 level and was equal to 4.64 kg/m^2. There was no significant difference at the 0.05 level. After receiving a herbal compress with *Z. cassumnae* ball, the level of pain tolerance (Pain Threshold) increased (before 4.03 and after 4.10 kg/m^2) with no statistically significant difference at the 0.05 level and was equal to 4.82 kg/m^2. There was no significant difference at the 0.05 level. There was no statistically significant difference in the level of pain tolerance of the participants in the 3 groups.

Results of satisfaction with herbal compress service by satisfaction with the smell of *K. parvriflor*, *K. galangal* and *Z. cassumnae* ball were in the very satisfied level (56.25%, 62.50% and 68.75% respectively). *K. parvriflora* ball treatment received the highest satisfaction level (50 percent and 50 percent, respectively).

Conclusion

The pain of the upper trapezius after being compressed with *K. parvriflora*, *K. galangal* and *Z. cassumnae* ball was assessed. The 3 groups showed that the pressure pain threshold in the group that received the compress with the *K. parvriflora* ball after the end of the experiment had an average increase of 0.36 kg/m^2. The group that received the *K. galangal* ball had an average increase of 0.55 kg/m^2. In addition, the group that received the *Z. cassumnae* ball had an average increase of 0.79 kg/m^2. Therefore, all 3 formulas of herbal compresses were able to reduce pain and stiffness in the shoulder musclesand to tolerate additional pain. The satisfaction of the 16 volunteers after receiving the compress service with the *K. parvriflora* ball were satisfied with the smell at a high level (56.25%) and also satisfied with the cleanliness of their clothes. At the area where the compress was applied the effect of treatment with Black Galingale Compress was at the highest level (50% and 50% respectively).

Therefore, it can be concluded that there is similar effectiveness of the use of *K. parvriflora*, *K. galangal* and

Z. cassumnae ball in reducing shoulder muscle spasms. The result is not different from the effectiveness of the use of *Z. cassumnae* ball which is a general formula that is widely used in Traditional Thai Medicine. this study also found that all 3 groups of compress balls had an effect on the shoulder muscles in increasing pain tolerance, especially the *Z. cassumnae* balls. However, there was no significant difference at the 0.05 level. So, *K. parvriflora*, and *K. galangal* ball can be used as an alternative to herbal compress treatment plans for people with allergies. Dizziness when inhaling the odour of *Z. cassumnae* can be clinically limiting. The clinical utility of alternative herbal compression balls should also promote the cultivation of *K. parvriflora* and *K. galangal* as cash crops to be used in the production of herbal dosage forms.

Acknowledgement

The researcher would like to express his utmost gratitude for the kindness of Dr. Samroeng Kanjanametakul, the chairman of the advisor who kindly gave advice. As well as correcting the shortcomings of the research and thanks to Ajarn Piyawan Pramualasap, Ajarn Wasana Netveera and Mr. Nopporn Suksawat for helping to compile and analyze the statistical data. I would like to thank Mae Fah Luang University for supporting the budget for the research project. and the place of collecting data for this study. As well as thanking my father and mother for their encouragement. Always helpful and generous and thanked the volunteers for their cooperation in carrying out the research project I would like to express my gratitude to all of you on this occasion.

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P-4 10.23177/NATPRO8.2023.06.22.010

Method comparison for mitragynine extraction from *Mitragyna speciosa* and evaluation of antioxidant activities of the extract

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Kratom (*Mitragyna speciosa*) is an ethnomedicinal plant prevalent in Southeast Asia. *M. speciosa* leaves were known to contain many bioactive substances, resulting in various pharmacological properties. Many studies reported different mitragynine extraction methods which were based on hazardous and non-environmentally friendly organic solvents. This research aims to optimize a method for mitragynine extraction using water and ethanol-based procedures at different extraction times and temperatures. Mitragynine concentration was analyzed using a high-performance liquid chromatography (HPLC) technique. The results showed that the most efficient extraction method was ethanol extraction for 1 day, proving the mitragynine content of 39.53 ± 0.34 mg mitragynine/ g extract. Moreover, the free radical scavenging properties of the extracts were determined by DPPH and ABTS assays. The leaf extracts showed the inhibitory activity against DPPH and ABTS radicals at the EC values of 186.59 ± 4.42 and 636.70 ± 9.52 μg/ mL, respectively. This work revealed that water and ethanol extraction methods could be considerably used for non-toxic mitragynine preparation, and which the extracts would be safe and suitable for the development of dietary supplements or food products in the future.

Keywords: *Mitragyna speciosa*, Kratom, Mitragynine, Extraction, Antioxidant

Introduction

Kratom (*Mitragyna speciosa*) is an indigenous herbal plant commonly found in Southeast Asia. Its leaves were traditionally known to relief several ailments, such as muscle pain, diarrhea, stomachache, fever, cough, diabetes, hypertension, and opium addiction. Also, the leaves were used for mood and stamina enhancement for outdoor workers.[1,2] It was reported that lower dose of consumption provides stimulant effects; however, higher dose leads to opioid-like and psychosis symptoms.[3,4] The major compounds that contribute to pharmacological effects of *M. speciosa* leaves are alkaloids, of which more than 50 compounds were previously characterized.[5,6]

The most abundant active compound is Mitragynine, which is an indole-alkaloid accounting for about two-thirds of the total alkaloid content.1 Mitragynine and related compounds have been reported to contribute to analgesic effects and opioid agonist activity.[7] Various extraction methods with different extraction yields have been reported. Most of the published reports employed organic solvent extraction, including hexane, chloroform, methanol, and ethanol, using Soxhlet, maceration, ultrasonication, and sequential extraction methods. The extraction yield of mitragynine ranged from 0.4 mg/g to 75 mg/g.[8,9] A limited number of studies reported the use of aqueous extraction, and the yield was shown to be lower than using solvent extraction. Parthasarathy et al. (2013) reported that methanolic extracts provided a 6-times higher yield of mitragynine than aqueous extract.[10]

Since mitragynine has a broad range of pharmaceutical properties, this compound is of interest for food-related product development. Therefore, it is important to avoid petrochemical solvents during the extraction process and production. This study focused on the use of water and ethanol, which is considered bio-solvent, for mitragynine extraction. The method for aqueous and ethanolic extraction was optimized to increase mitragynine yield. Furthermore, antioxidant activities of the extracts with a higher yield of mitragynine were evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assays. The information from this work would be beneficial for the future development of

functional food products with pharmaceutical properties.

Materials and Methods

Plant extract preparation

M. speciosa (red veined leaves) were harvested from the plantation in Pathum Thani province. The leaves were washed with distilled water and air-dried at room temperature. The petioles were removed from the leaves prior to grinding. The leave powder was kept at 4 (°C).

Extraction of mitragynine

The leaf powder was subjected to 13 different extraction methods as shown in Table 1. For Methods A, B, C, D, E, F, G, H, and I, 10 g of the leaves powder was immersed in 10 mL H2O and incubated in a water bath for 30, 45, and 60 min at 60, 80 and 95 C (Table 1). For Methods G, H, and I, the leaves powder (10 g) was immersed in 10 mL absolute ethanol for 60 min, 1 day, and 3 days at room temperature (**Table 1**). For Method M, the leaf powder was soaked in 40% ethanol and incubated for 1 day at room temperature prior to incubation at 100C for 30 min. After that, the extracts were filtrated using Whatman® No.1 filter paper. The filtrates were dried using a centrifugal evaporator (SC100, Savant, USA). Percent yield was determined using Equation 1. The extracts were further subjected to the determination of mitragynine concentration using HPLC.

% yield = (weight of the extract/ weight of leaf powder) / 100 Equation (1)

Determination of mitragynine concentration

The concentration of mitragynine from different extraction methods was evaluated by a reverse-phase HPLC following the method from Mudge and Brown (2017) with modifications.[11] Briefly, the extract powder was dissolved in acetonitrile, and 20 µL of the solution was subjected to an Agilent 1260 Infinity II HPLC system connected with a diode array detector (Agilent Technology, Wood Dale, IL, USA). A Phenomenex Luna C18 (150 mm x 4.6 mm, 5-µm) column was used for reverse-phase separation. The flow rate was set to 1 mL/min. Isocratic elution was performed using 50% solvent A (0.05% Formic acid in H2O, pH = 5.0) and 50% solvent B (acetonitrile) for 12 min. Mitragynine elution was monitored at 226 nm (bandwidth = 4 nm). The standard mitragynine (CAS Number 4098-40-2) was purchased from Cayman Chemical (Ann Arbor, MI, USA), and the standard concentration varied from 2.5 to 40 µg/mL.

Table 1. Extraction conditions of the Method A-M.

Extraction Method	Conditions		
	Solvent	Temperature (C)	Time
A	H_2O	60	30 min
B	H_2O	60	45 min
C	H_2O	60	60 min
D	H_2O	80	30 min
E	H_2O	80	45 min
F	H_2O	80	60 min
G	H_2O	95	30 min
H	H_2O	95	45 min
I	H_2O	95	60 min
J	Absolute ethanol	Room temperature	60 min
K	Absolute ethanol	Room temperature	1 day
L	Absolute ethanol	Room temperature	3 days
M	40% ethanol	Room temperature, 100	1 day, 30 min

DPPH radical scavenging assay

DPPH radical scavenging assay was modified from Molyneux (2006).[12] Kratom leaf extracts (50 µL) with concentrations of 50, 100, 150, 200, 250, 300, 350, and 400 µg/mL were mixed with 100 µL of 0.2 mM DPPH in methanol in 96 well-plate. The mixtures were incubated at room temperature for 30 min prior to absorbance measurement at 517 nm using a VersaMax microplate spectrophotometer. The ability of the leaf extracts to scavenge DPPH radical was determined by Equation (2). Ascorbic acid at the concentrations of 2, 4, 8, 12, 14, 16, and 20 µg/mL was used as the positive control.

% inhibition = $[(A_{control} - A_{blank}) - (A_{sample} - A_{blank\ sample})] / (A_{control} - A_{blank})$ Equation (2)

ABTS radical scavenging assay

The ABTS assay was modified from Shalaby and Shanab, 2013.[13] Prior to the assay, ABTS solution was prepared at the concentration of 7 mM in H_2O and oxidized by 2.45 mM $K_2S_2O_8$ for 16 h to form $ABTS^{\bullet+}$ solution. The leaf extracts at the concentrations of 100, 300, 500, 700, and 900 µg/mL were mixed with 1 ml $ABTS^{\bullet+}$ solution. The reaction was performed for 6 min at room temperature, and absorbance at 734 nm was

recorded. ABTS radical scavenging ability was determined by Equation (3). Ascorbic acid at concentrations of 120, 240, 360, 480, and 600 μg/mL was used as the positive control.

$$\% \text{ inhibition} = [(A_{control} - A_{blank}) - (A_{sample} - A_{blank\ sample})] / (A_{control} - A_{blank}) \quad \text{Equation (3)}$$

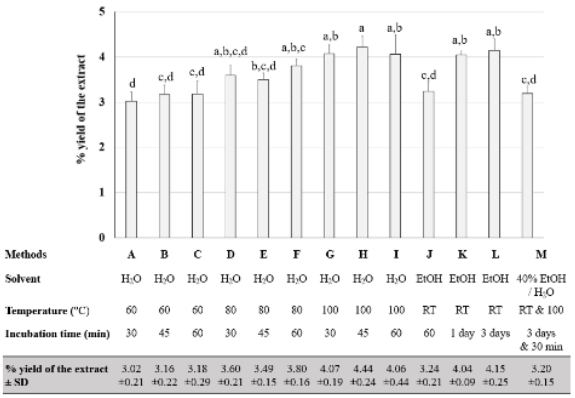
Statistical analysis

All experiments were performed in triplicate. The results were shown as mean ± standard deviation. Statistical analyses were performed by Minitab17 using one-way ANOVA with Tukey's Post-test ($p < 0.05$).

Results & Discussion

The efficiency of 13 different water- and ethanol-based extraction procedures were evaluated. The % yield of the extracts was in the range of 3.02 ± 0.21% and 4.44 ± 0.24% (**Figure 1**). Considering aqueous extraction, the higher temperature tends to provide a higher extraction yield (Method A-I). Using absolute ethanol at room temperature, maceration of *M. speciosa* for 1 day and 3 days (Method K and L) provided a higher % yield than incubation for 60 min (Method J). Also, using absolute ethanol (Method L) provided a higher extraction yield than 40% ethanol (Method M), using the same period of incubation. However, the overall trend showed that % yields from both aqueous and ethanolic extractions were not statistically different.

The concentration of mitragynine in the extracts was determined by reversed phase HPLC. An example of a chromatogram from mitragynine was shown in Figure 2(A), presenting the retention time at 3.282 min. The calibration curve of mitragynine at the concentration of 2.5 to 40 μg/mL was shown in Figure 2(B). The concentration of mitragynine extracted from 13 extraction methods was revealed in Figure 3. Absolute ethanol-based extraction methods (Method J, K, and L) were shown to be more effective than 40% ethanol and water-based procedures. In agreement with other published reports that the presence of a lower polarity solvent could enhance mitragynine extraction as compared to water due to the chemical structure and polarity of the compound.[10, 14] Method K, which employed maceration of the leaves in absolute ethanol at room temperature for 3 days, was shown to be the most effective method for mitragynine extraction. It provided 39.53 ± 0.34 mg mitragynine/ g extract, which this concentration was in between the values reported in the literature ranging from 0.4 mg/g to 75 mg/g.[8, 9] And it could be accounted for 1.59 ± 0.04 mg mitragynine/ g fresh leaf powder. The result from hot water extraction was also in accordance with the previous report. Mitragynine concentration from this work ranged from 0.50 ± 0.10 to 1.41±0.15 mg/ g (Figure 3); while Parthasarathy et al. (2013) revealed 0.80 ± 0.11 mg/g from aqueous extraction.[10]



Methods	A	B	C	D	E	F	G	H	I	J	K	L	M
Solvent	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	EtOH	EtOH	EtOH	40% EtOH / H_2O
Temperature (°C)	60	60	60	80	80	80	100	100	100	RT	RT	RT	RT & 100
Incubation time (min)	30	45	60	30	45	60	30	45	60	60	1 day	3 days	3 days & 30 min
% yield of the extract ± SD	3.02 ±0.21	3.16 ±0.22	3.18 ±0.29	3.60 ±0.21	3.49 ±0.15	3.80 ±0.16	4.07 ±0.19	4.44 ±0.24	4.06 ±0.44	3.24 ±0.21	4.04 ±0.09	4.15 ±0.25	3.20 ±0.15

Figure 1 % Yield of *M. speciosa* leaf extracts from 13 different extraction methods

The ethanolic extract from Method K was chosen for the determination of antioxidant activities using DPPH and ABTS radical scavenging assays. *M. speciosa* leaf extracts showed the ability to quench DPPH and ABTS radicals in a concentration-dependent manner (Figure 4(A) and Figure 4(B)). The half maximal effective concentration (EC50) values against DPPH and ABTS radicals were 186.59 ± 4.42 and 636.70 ± 9.52 μg/ mL, respectively. The difference in these EC50 values resulted from different mechanisms of action in these two assays. It was suggested that quenching the DPPH radical could be involved with hydrogen atom transfer, whereas ABTS radical scavenging could be via direct electron transfer.[15] The EC50 value against DPPH radical of ethanolic extract from our work was in between those from aqueous and methanolic extracts reported by Parthasarathy et al. (2009), which were shown to be 213.4 and 104.81 μg/mL, respectively.[16] The radical scavenging abilities of the leaf extracts from both DPPH and ABTS assays were lower than that of ascorbic acid, which was used as a positive control. The EC50 values of ascorbic acid against DPPH and ABTS radicals were revealed to be 19.99 ± 0.40 and 320.30 ± 2.51 μg/ mL, respectively.

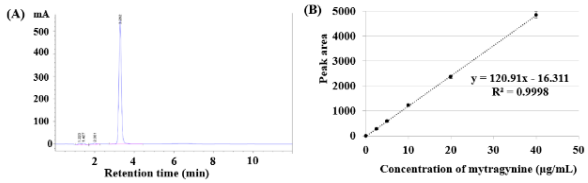
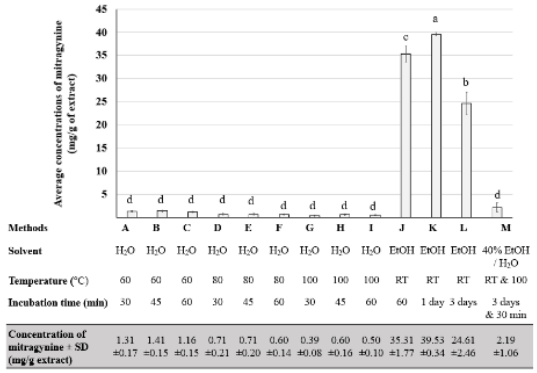


Figure 2. (A) Chromatogram showing mitragynine retention time at 3.282 min. (B) Standard curve of mitragynine from triplicate experiments.



Methods	A	B	C	D	E	F	G	H	I	J	K	L	M
Solvent	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	H_2O	EtOH	EtOH	EtOH	40% EtOH / H_2O
Temperature (°C)	60	60	60	80	80	80	100	100	100	RT	RT	RT	RT & 100
Incubation time (min)	30	45	60	30	45	60	30	45	60	60	1 day	3 days	3 days & 30 min
Concentration of mitragynine ± SD (mg/g extract)	1.31 ±0.17	1.41 ±0.15	1.16 ±0.15	0.71 ±0.21	0.71 ±0.20	0.60 ±0.14	0.39 ±0.08	0.60 ±0.16	0.50 ±0.10	35.31 ±1.77	39.53 ±0.34	24.61 ±2.46	2.19 ±1.06

Figure 3. Comparison of mitragynine concentrations (mean ± SD) obtained from different extraction methods. The bar charts with different letters (a, b, c, d) indicate significant differences between the samples (ANOVA and Tukey test, $p<0.05$).

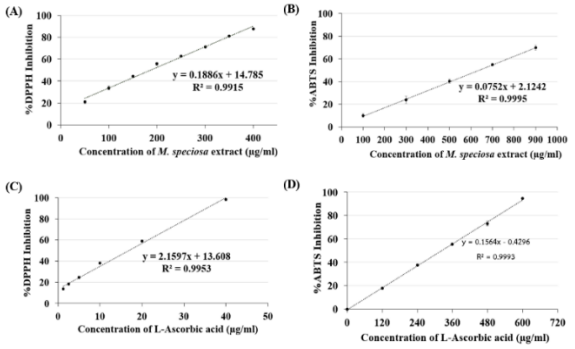


Figure 4. Antioxidant activities of *M. speciosa* extract at different concentrations were determined by (A) DPPH radical scavenging assay and (B) ABTS radical scavenging assay. And antioxidant activities of ascorbic acid solutions were evaluated by (C) DPPH radical scavenging assay and (D) ABTS radical scavenging assay.

Conclusion

This study revealed that mitragynine could be extracted from *M. speciosa* leaves using water and ethanol. The most effective condition was using maceration in absolute ethanol at room temperature for 1 day. And this ethanolic extract possessed antioxidant activities against DPPH and ABTS assays. Although the yield of mitragynine from aqueous and ethanolic extraction was shown to be lower than other organic solvent extractions, our method provided the advantage of using a green and non-toxic solvent which could be advantageous for food-based product development.

Acknowledgment

M. speciosa was supported by K.G.M. Thai Herb Co., Ltd. and Good Green Herb Co., Ltd. The authors would like to acknowledge the Science Innovation Facility, Faculty of Science, Burapha University, and the Center of Excellence for Innovation in Chemistry (PERCH-CIC), Ministry of Higher Education, Science, Research and Innovation for the instrument and financial support.

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NATPRO 8
The International Conference on Natural Products

P-5 10.23177/NATPRO8.2023.06.22.011

Validated HPLC method for mitragynine quantification in Kratom extract

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Kratom (*Mitragyna speciosa* Korth, Rubiaceae) was a household plant in Thailand for several decades. The fresh leaves were chewed by outdoor workers to give them stamina and withstanding the scorching sun. Unfortunately, the plant was under legal control by Kratom Bill 1943 and was later put in the Thai narcotic plant list in 1979. These regulations have prevented and delayed research and development of Kratom in Thailand as well as utilization of this plant. Despite strict rules for Thai consumption, North American consumers are turning to Kratom for self-management in pain and opioid addiction. Various Kratom products are available to North American people containing mitragynine ranging from 13.9 - 270 mg/g. This study provided a better validated HPLC method using C-18 column, with isocratic elution using methanol: water: acetic acid (95:4:1, v/v) at 35°C. Mitragynine was detected at 254 nm at 3.23 min reducing run time to 7 min. The linear relationship was found in the range of 6.25 – 200 µg/ml with R^2 of 1. The content of mitragynine in red vein Kratom leaf was 33.45 mg/g dry weight. Since Kratom was removed from Thai narcotic plant list in 2021, this validated method will be useful in monitoring Kratom products.

Keywords: Kratom, *Mitragyna speciosa*, Mitragynine, Validated HPLC system

Introduction

Kratom or *Mitragyna speciosa* Korth (**Figure 1**) is a member of Rubiaceae family. It was recently taken off of Thailand narcotic plant list in 2021. Before being legally noted as narcotic plant it was recognized as a household plant by Thai people and used for physical endurance and muscle pain relief [1] due to its muscle relaxant property [2]. The director of Thai Traditional Medicine Department, Ministry of Public Health, Thailand recently declared that Kratom had the following properties; pain relief, antidiarrheal, blood sugar control and CNS stimulation [3].

Figure 1. The leaves and plant of Kratom (*Mitragyna speciosa* Korth).

Mitragynine (Figure 2), an indole major alkaloid, was isolated and identified since 1921 [4], it has molecular structure $C_{23}H_{30}O_4N_2$ with molecular weight of 398.503 g/mole. It is soluble in alcohol, chloroform and acetic acid [5].

Figure 2. The structure of mitragynine.

The presence of other groups of compounds were also reported, these were flavonoids, triterpenes and phenolic acids [6]. Many pharmacological studies demonstrated that mitragynine had CNS stimulant effects [7], antinociceptive [8, 9], a similar effect on isolated ileum of guinea pig as morphine [9], anti-inflammatory [8], immunostimulant and wound healing [10]. The mechanism of anti-inflammatory action was reported to involve cyclooxygenase 2 and prostaglandin E2 [11]. The ethanolic extract exerted

analgesic effect in mice but the aqueous extract did not have the activity which was due to higher amount of mitragynine and flavonoids in the ethanolic extract, therefore the ethanolic extract has been recommended as a potentially effective analgesic [9]. Mitragynine could stimulate the nervous system similar to cocaine [12], administration of mitragynine acetate at a dose of 50 mg in 5 men gave the similar results but also caused nausea and vomiting [7, 13]. However, chewing 2-3 fresh Kratom leaves 3-10 times per day by 30 Thai male workers for 5 years could enhance their work efficiency with minor side effects such as dry mouth, constipation, loss of appetite and weight reduction [1]. From these studies the use of kratom extract was suggested to produce better efficacy than using pure mitragynine [6].

In the United States, many consumers are turning to kratom (*Mitragyna speciosa*) for self-management in pain and opioid addiction. Many forms of kratom products are readily available such as capsules, powders, and loose-leaf. Moreover, live kratom plants can be bought from several online sites. To understand how these products effect clinical outcomes, it is a prerequisite to establish quality control for the identification and quantitation of active alkaloid constituents within available products. An ultra-high performance liquid chromatography-high resolution mass spectrometry method was developed for the analysis of indole alkaloids content in these preparations available in the USA. These commercial products shared a qualitatively similar alkaloid profile, with 12 - 13 detected alkaloids and high levels of the indole alkaloid mitragynine (13.9 ± 1.1 - 270 ± 24 mg/g) [14].

The different strains of Kratom were noted from leaf vein colours by North American consumers, for more energetic individuals the white and green veins were recommended, while red vein Kratom offered a more relaxed mood and acts as a sedative when taken in large dose. The recommended starting dose is 2 g and with potential increase to 5-15 g. Doses and effects depend on each individual. The expected effects are pain relief, euphoria, relaxation, drowsiness and relief from opioid withdrawal symptoms [15].

An interesting survey conducted in 2017 found that the most beneficial effects were observed in doses of 1–3 and 3–5 g if taken 2–3 times per day; in contrast, most adverse effects required higher doses of >8 g and higher frequency of dosing between 4–5 times per day of daily use [16]. A more recent survey in 2022 reported a consumption of 6.85 g powder was effective while 8.68 g was "a bit too much". This was followed by 7.25 capsules ("too much") versus 5.88 capsules (effective); 3.93 spoonfuls ("too much") versus 2.87 (effective); and 3.44 cups of tea ("too much") versus 2.25 (effective) [17].

At present, Kratom are still being used in Thailand as single herb or in combination with other botanicals. Over 100 products are available and have been recorded [18]. The uses of Kratom by folk healers in 14 provinces of southern Thailand were as follows; diarrhea (67.4%), diabetes (63.3%), pain relief (32.7%) and cough suppressant (26.5%) [6]. Traditional uses of Kratom were continually used in Kiriwong Temple in Chumporn province, south of Thailand. Among 50 recipes there are 4 recipes that utilize a 40% alcoholic extract of Kratom leaves for topical muscle pain relief [19].

At present it is not clear how much mitragynine is present in these products. It is therefore the objective of this study to develop a validated HPLC method for mitragynine for quality control purpose of Kratom extract.

Materials and Methods

Plant Material

The fresh leaves of Kratom, red vein, were collected from Chumporn province. The herbarium specimen was identified as *Mitragyna speciosa* Korth by the Thai Traditional Medicine Herbarium, Bangkok and kept under ttm no.0005467.

Chemicals

The solvents and chemicals were all AR grade from Merck, Germany (methanol, glacial acetic acid, *n*-hexane, 28% ammonia, Silica gel G (9385) for column chromatography, TLC plate silica gel GF254 (105554). Chloroform and ethyl acetate were from Sigma Aldrich, USA, Deionized water was from RCI Labscan, Ireland. Mitragynine Reference Standard was from Chromadex, USA.

The HPLC system consisted of a Thermo, Ultimate 3000 series, Germany, with Photo diode array detector and automatic injector. The system was controlled and data analyses were performed with Thermo Scientific Chromeleon Chromatography Data System (CDS) software. The analysis was done on a Thermo, AcclaimTM 120, C-18 column (5 µm, 4.6 mm x150 mm). All solvents for the mobile phase and sample were filtered through a 0.45 µm nylon filter disc. HPLC analysis for mitragynine was performed by isocratic elution with a flow rate of 0.5 mL/min. The temperature was controlled at 35°C. The detector wavelength was

set at 254 nm. The mobile phase was Methanol: Water: Acetic acid (95:4:1, v/v). A volume of 10 µL of each prepared solution and samples were injected into the column. The chromatographic run time was 7 min.

Preparation of sample solution for method validation

Fresh Kratom leaves (10 kg) were washed and dried in a hot air oven at 45 °C yielded 2,602.86 g dried leaves. Then powdered and sieved through 60 mesh yielded 2,335.25 g Kratom powder. A 200 g of powdered Kratom was macerated in 2000 mL of 95% EtOH for 5 days with occasional stirring. After filtration, the ethanol extract was evaporated in a rotary evaporator at 50 °C, 60 rpm, followed by a freeze drying process. The freeze-dried powder was defatted with *n*-hexane, then the total alkaloid was extracted by acid-base shaking using 10% acetic acid and ammonia (28%). The alkaloid was extracted from the ammoniacal solution (pH 9-10) with chloroform in a separatory funnel. The chloroform layer was evaporated in a rotary evaporator at 50 °C to provide total alkaloid.

The separation of mitragynine

The separation was done by column chromatography (4 cm x 40 cm) using silica gel 9385 (50 g) as the adsorbent and hexane: ethyl acetate (60:40) as the developing solvent. The total alkaloid was dissolved in 5 mL chloroform and added to the column. The elution was made at the rate of 5 mL/min and collected 10 mL/tube yielded 60 fractions. The fractions 21-32 contained mitragynine as the major component by TLC (silica gel GF254, $CHCL_3$: MeOH 95:5). This fraction was further separated in the second column using silica gel 9385 (25 g) as the adsorbent and $CHCL_3$: MeOH (97:3) as the developing solvent. The fractions 21-32 from the first column was dissolved in chloroform and added to the second column. The elution was made at the rate of 5 mL/min and collected in 9 mL/tube yielded 25 fractions. The fractions 13-21 contained mitragynine as the major component by TLC (silica gel GF254, $CHCL_3$: MeOH 97:3). This fraction yielded picrate with saturated solution of picric acid and was used for HPLC in method validation.

Preparation of Standard Solution

Prepare mitragynine solution at 200 µg/mL in methanol by accurately weighed 1 mg of mitragynine dissolved in 5 mL methanol, then diluted to 100, 50, 25, 12.5, and 6.25 µg/mL. The solutions were filtered through 0.45 µm nylon filter disc before injection.

Method Validation (20)

Specificity: The standard solution of mitragynine at 50 µg/mL was injected to HPLC to observe the shape, separation and retention time of the peak.

Linearity: Each diluted solution of mitragynine at 6.25, 12.5, 25, 50, 100, 200 µg/mL were injected 3 times and the HPLC chromatograms were recorded. The standard curves were prepared by plotting the concentrations (*X*-axis) against peak areas (*Y*-axis). The coefficient of determination (r^2) must not be less than 0.9950.

Precision: Intra-day Precision. The solution of mitragynine at 3 concentrations; 12.5, 25 and 50 µg/mL were injected 7 times within the same day. The percentage of Relative Standard Deviation (% RSD) were calculated (Equation 1), the value should not be more than 2%.

Inter-day Precision. The solution of mitragynine at 3 concentrations; 12.5, 25 and 50 µg /mL were injected 7 times in 3 consecutive days. The percentage of Relative Standard Deviation (% RSD) were calculated (Equation 1), the value should not be more than 2%.

$$\%\,RSD = \frac{\text{Standard Deviation}}{\text{mean}} \times 100 \qquad \text{(equation 1)}$$

$$\text{Standard Deviation} = \frac{\sqrt{\sum(X_i - X)^2}}{n-1} \qquad \text{(equation 2)}$$

X_i = data

n = replicate

Accuracy: The accuracy of the method was done by addition of standard solution of mitragynine (50 µg/mL) to sample solutions in set 2.

Sample solution set 1: Prepare sample solution at 100 µg/ml in 10 ml volumetric flask, then pipette 2.5 1.5, 0.625 mL into 3 x 5 mL volumetric flasks to give 50, 30 and 12.5 µg/ml respectively.

Sample solution set 2: Prepare sample solutions in 3 x 5 mL volumetric flasks as in set 1, then add standard solution of mitragynine (50 µg/mL) to the volumetric flasks, make up volume to 5 mL.

$$\%\,\text{recovery} = \frac{(S2) - (S1)}{(S2)} \times 100 \qquad \text{(equation 3)}$$

where S1 = data from set 1

S2 = data from set 2

Limit of Detection (LOD) and Limit of quality (LOQ): **T**he value of LOD and LOQ were calculated from the graph (equation 4, equation 5).

$$LOD = 3.3\,\sigma/S \qquad \text{(equation 4)}$$

$$LOQ = 10\,\sigma/S \qquad \text{(equation 5)}$$

where σ = the standard deviation of y-intercepts.

S = the slope of the calibration curve

Preparation of freeze-dried 40% ethanol extract for analysis

Ten milligrams of freeze-dried powder of 40% ethanol extract was dissolved in 10 mL methanol to give 1000 µg/mL concentration. From this solution an aliquot of 0.2 mL was pipetted into a 5 mL volumetric flask and volume was adjusted with methanol to give 40 µg/mL analytical concentration. The solution was filtered through 0.45 µm disc prior to HPLC injection (n=3).

Results & Discussion

The developed HPLC method offered shorter time of analysis i.e. 7 min vs 10 min in prior study [21]. In this study mitragynine was detected at 254 nm and appeared at 3.23 min sooner than previous study at 5.97 min (**Figure 3**). This could be due to the use of C-18 column instead of C-8 column also acetic acid was used and the temperature of column was kept at 35°C.

Preparation of sample solution for method validation

From 200 g of powdered Kratom yielded 48.28 g freeze-dried powder. The powder (12 g) was defatted with n-hexane and left to dry, yielded 9.8622 g defatted powder. Acetic acid (10%) was added to defatted powder and filtered. The acidic filtrate was extracted with n-hexane and discarded. The aqueous acidic layer was then basified to pH 9-10 with ammonia (28%) and the total alkaloid was extracted with chloroform. After solvent evaporation the yield of total alkaloid was 1.29 g or 0.645% of Kratom dried powder.

Mitragynine was separated from the total alkaloid by two consecutive column chromatography procedures and kept in the form of picrate (0.0397 g). Mitragynine was extracted back from this picrate (10 mg) by acid-base shaking yielding 0.0064 g alkaloid which was dissolved in 5 mL methanol giving 1.28 mg /ml. This solution was used in method validation.

Method Validation Parameters

Specificity: The retention time of mitragynine in the standard solution and in the sample was 3.23 minutes (**Figure 3** and **Figure 4**).

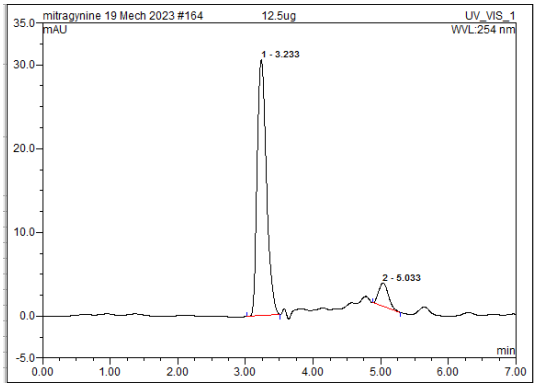

Figure 3 The HPLC chromatogram of standard mitragynine.

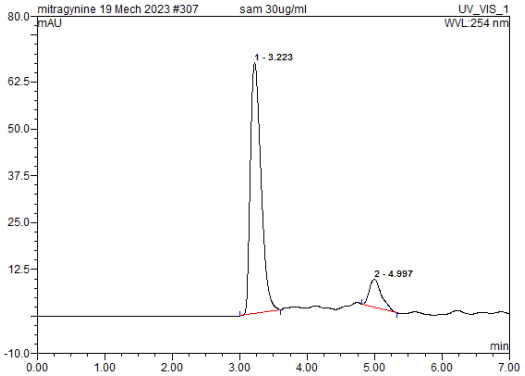

Figure 4. The HPLC chromatogram of mitragynine in sample solution.

Linearity

The linear relationship between peak area and concentration of standard mitragynine was found to be in the range 6.25 - 200 µg/ml. The correlation coefficient (r^2) from equation y = 0.6x-0.9901 was equal to 1 which passed the requirement of ICH guideline (> 0.995) (**Figure 5**).

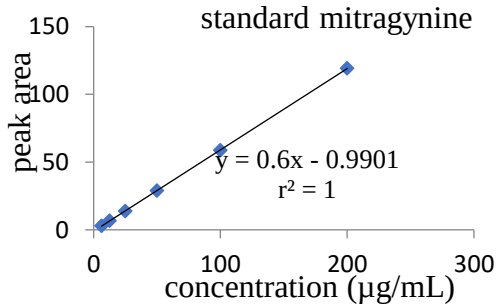

Figure 5. The linear regression line of standard mitragynine.

Precision

According to ICH guideline precision means that all measurements of an analyte should be very close together. There should be no more than a ±2% variation in the assay system. A useful criterion is the relative standard deviation (RSD) or coefficient of variation

(CV), which is an indication of the imprecision of the system (Equation 1). This study was performed with 3 concentrations of mitragynine standard at 50, 25 and 12.5 µg/ml within the same day (intra-day precision) showing %RSD to be 0.334, 0.178 and 0.355 respectively. The analysis of the 3 standard solutions in different days (inter-day precision) showed the %RSD to be 0.200 0.234 and 0.214 respectively. The results demonstrated that this method provides precision for the analysis within the same day or in different days.

Table 1. The analytical results from intra-day precision and inter-day precision study.

Mitragynine (µg/mL)	Intra-day Precision			Inter-day Precision		
	Mean	SD	%RSD	Mean	SD	%RSD
50	50.534	0.169	0.334	50.081	0.100	0.200
25	24.950	0.044	0.178	24.723	0.058	0.234
12.5	11.694	0.041	0.355	11.657	0.025	0.214

Accuracy

A method is said to be accurate if it gives the correct amount for the analyte. However, the results of several replicate tests may not give the same answer, so the mean or average value is taken as the estimate of the accurate answer. Accuracy is usually determined by measuring a known amount of standard material. For assay methods, spiked samples are prepared in triplicate at three levels, the per cent recovery should then be calculated. In the present study, the accuracy of the method was evaluated by adding 25 µg/ml of mitragynine standard to a known amount of sample, at 12.5, 30 and 50 µg/ml. The samples were analyzed, and mean recovery calculated. The data presented in Table 2 show that the recovery of mitragynine in spiked samples met the evaluation criterion for accuracy (80–120%).

Table 2. The percentage of mitragynine recovery in samples with and without mitragynine standard addition (25 µg/ml).

Sample concentration (µg/ml)	Sample with standard addition (µg/ml)	Sample without standard addition (µg/ml)	(%recovery)
50	64.777	41.651	92.507
	64.782	41.012	95.078
	64.880	41.041	95.356
30	46.853	22.064	99.157
	47.053	21.976	100.307
	47.664	22.004	102.640
12.5	37.158	13.316	95.370
	37.261	13.340	95.685
	37.284	13.338	95.785
Average			96.876

The limit of detection (LOD) and the limit of quantitation (LOQ)

The limit of detection (LOD) is defined as the lowest concentration of an analyte in a sample that can be detected. It is expressed as a concentration at a specified signal:noise ratio, 3:1. The limit of quantitation (LOQ) is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated operational conditions of the method. The ICH has recommended a signal:noise ratio 10:1. LOD and LOQ may also be calculated based on the standard deviation of the response (SD) and the slope of the calibration curve according to equation 4 and equation 5. The results showed that the values of LOD and LOQ for mitragynine determination were 0.266 and 0.807 µg/mL respectively (**Table 3**).

Table 3. The calculation data for LOD and LOQ of Mitragynine standard.

No. of experiment	Linear equation	slope	Y-intercept
1	y = 0.5993X - 0.9857	0.5993	0.9857
2	y = 0.5994x - 0.9441	0.5994	0.9441
3	y = 0.6013x - 1.0406	0.6013	1.0406
Mean		0.6000	0.990
SD			0.048
LOD		0.266 µg/mL	
LOQ		0.807 µg/mL	

Table 4. The content of mitragynine in freeze-dried extract of 40% ethanol based on linear regression and its content in dried Kratom leaves.

Injection	Area (Y)	µg/mL (X)	Mitragy-nine In freeze dried extract (%w/w)	Mitragy-nine In Kratom leaves (mg/g dry weight)
1	4.881	9.785	24.46333	33.573
2	4.877	9.778	24.44583	33.549
3	4.819	9.683	24.20625	33.220
mean	4.859	9.749	24.37181	33.447
SD				0.197
mean±SD				33.447±0.197

The content of mitragynine in freeze-dried 40% ethanol extract

The analytical sample was injected three times and the content of mitragynine was calculated based on the developed method. It was found that the freeze-dried 40% ethanol extract contained 24.37% mitragynine equivalent to 33.45 mg/g dry weight of Kratom leaves (**Table 4**). This finding is much higher than previously reported from HPLC chromatogram, 8.76 mg/g [8].

Conclusion

The fact that Thai workers chewed 2-3 fresh Kratom leaves 3-10 times per day for 5 years which could lead to the enhancement of their work efficiency with minor side effects such as dry mouth, constipation, loss of appetite and weight reduction [1] provided evidence of efficiency and safety. This also indicates that Kratom leaves are well tolerated. From our data a Kratom leaf yielded about 1 g of dry weight which would provide 33.45 mg of mitragynine, however, there are other groups of compounds which may contribute to the overall effects of Kratom leaf and its safety. Moreover, the red vein strain Kratom was use in this study which was considered to have a stronger effect than other strains. Whether other strains contain more or less mitragynine content should be investigated in future studies. In addition, the strength of ethanol used for extraction should be delineated. Overall, our findings support the traditional use of 40% ethanol in Kratom extraction.

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NATPRO 8
The International Conference on Natural Products

P-6 10.23177/NATPRO8.2023.06.22.008

Screening of phytochemical and biological activity in red cabbage extracts

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Red cabbage (*Brassica oleracea var. capitata* L.) has few reports concerning the effects of extraction conditions on the quality of the extracts. Therefore, the objective of this study was to evaluate the qualities including the yield, total phenolic, total flavonoid and total anthocyanin contents and in vitro biological activities including DPPH scavenging and antibacterial activities of the extracts prepared by different extraction methods and solvents. It was found that the dried red cabbage extract obtained from ultrasonic extraction with ethanol (RDUE) contained the highest total phenolic content (5.86 ± 0.06 g% gallic acid of dried extract) with the strongest DPPH scavenging activity (EC_{50} 137.91 ± 0.91 µg/mL). The dried red cabbage extract by maceration with ethanol (RDME) showed the highest yield (58.38 %w/w of dried plant), total flavonoid content (0.88 ± 0.02 g% quercetin of dried extract) and total anthocyanin contents (0.96 and 2.11 g% cyanidin-3-glucoside of dried extract using the pH-differential method by standard calculation and standard curve of cyanidin-3-glucoside, respectively). Thin layer chromatographic analysis of some red cabbage extracts showed the major chromatographic bands corresponded to some anthocyanins. RDME showed no inhibitory effect against five clinically pathogenic bacteria at a maximum concentration of 12.5 mg/disc.

Keywords: Red cabbage, Anthocyanin, Delphinidin-3-sambubioside, Cyanidin-3-sambubioside, Delphinidin-3-glucoside, Cyanidin-3-glucoside

Introduction

Brassica oleracea var. capitata L., commonly known as red cabbage, belongs to the Brassicaceae family. Red cabbage is an economic dietary vegetable in Asia, North America, and European countries (1). There were previous reports concerning about potential health benefits of red cabbage including its antioxidant, antiobesity, and antidiabetic properties, the beneficial effects against cardiac and hepatic oxidative stress, and hypocholesterolemic and hepatoprotective activities (2,3). Several phytochemicals have been reported from red cabbage such as chlorogenic acid, caffeic acid, ferulic acid, gallic acid, vanillic acid, syringic acid, catechin, epicatechin, naringin, rutin, myricetin, hesperidin (4). Moreover, anthocyanins are the active compound of red cabbage, it has been identified predominantly as derivatives of cyanidin-3-diglucoside-5-glucoside (5). However, many factors have been reported to affect the stability of anthocyanins such as pH, temperature, enzymes, light, and oxygen, especially, in the extraction process for the preparation of the red cabbage extract (2).

Antioxidants act as an inhibitor of the oxidation process that protects the human body against free radicals, especially reactive oxygen species (ROSs) (6). The excess amounts of ROSs could lead to the generation of oxidative stress which could be the cause of various pathologies and diseases such as cancer, rheumatoid arthritis, cardiovascular, and neurological diseases (7). Natural antioxidants from dietaries and plants have gained high attention nowadays. Green tea (*Camellia sinensis* (L.) Kuntze), turmeric (*Curcuma longa* L.), blackberry (*Rubus fruticosus* L.), ginkgo (*Ginkgo biloba* L.), and chamomile (*Matricaria chamomilla* L.) have been shown the strong antioxidant activities (8,9). The red cabbage was also previously reported to promote antioxidant activities (10,11). Furthermore, the

methanol extract of red cabbage has been reported to have antibacterial activities against Methicillin-resistant *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Bacillus subtilis*, and *Salmonella enterica* serovar Typhimurium with the minimum inhibitory concentration (MIC) in the range of 100 to 400 mg/ml (12).

Even though red cabbage has been popularly consumed as a vegetable all over the world, there are only a few reports concerning the effects of extraction conditions on the quality of the extracts. Therefore, this study was conducted to evaluate the yield, phytochemical contents including total phenolic, total flavonoid, and total anthocyanin contents, and also in vitro biological activities including DPPH scavenging and antibacterial activities of red cabbage extracts prepared from fresh and dried red cabbage leaves using various extraction methods including maceration, decoction, digestion and ultrasonic extraction and various extraction solvents including 80% ethanol, 80% ethanol with 1%HCl, water, and water with 1%HCl. The %yield, phytochemical contents including total phenolic, total flavonoid, and total anthocyanin contents determined by Folin-Ciocalteu, aluminium chloride, and pH-differential methods, respectively, and DPPH scavenging effects and antibacterial effect determined by disk diffusion of all obtained extracts were evaluated and compared. Analysis by thin-layer chromatography (TLC) was also conducted to study for phytochemical characteristics of all red cabbage extracts.

Materials and Methods

Plant material

The fresh cabbage leaves were purchased from a department store, in Bangkok, Thailand, and were identified according to the International Plant Names Index (IPNI). The IPNI Life Sciences Identifier (LSID) was urn:lsid:ipni.org:names:60452371-2. The plant samples were cleaned and dried in a hot air oven at 60 °C for 24 hours and ground into a powder being passed through a sieve with mesh number 20. All extracts were kept at -20 °C until use (13).

Preparation of plant extracts

Preparation of red cabbage extract by various extraction methods

Fresh and dried red cabbage leaves were extracted by various extraction methods including maceration, decoction, digestion, and ultrasonic extraction with a plant: solvent ratio of 1:10 w/v. Extraction processes were repeated 3 times, then the filtrates were combined. All extracts were dried using a rotary evaporator or a water bath. The percentage yields of all extracts (%yield) were calculated. All extracts were kept at -20 °C until use (14).

Preparation of red cabbage extract with various solvents

Red cabbage leaf extracts were prepared using the selected extraction method with various extraction solvents including 80% ethanol, 80% ethanol with 1%HCl, water, and water with 1%HCl. The percentage yields of all extracts (%yield) were calculated. The obtained extracts were kept at-20 °C until use.

Determination of the extraction yield

The yields of the extracts based on dry weight were calculated from the following equation:

$$\%\text{Yield} = \frac{\text{Weight of dry extract (g)} \times 100}{\text{Weight of the starting dry plant material (powder, g)}}$$

The crude extracts were weighed and stored at freezer (-20°C) until use.

Phytochemical analysis

Determination of total phenolic content (TPC)

The total phenolic content of all red cabbage extracts was determined using the modified Folin–Ciocalteu assay as mentioned by Sithisarn et al., 2015 (15). The total phenolic content was calculated from the standard curve of gallic acid and was expressed as g gallic acid equivalent in 100 g of extract (g% GAE).

Determination of total flavonoid content (TFC)

The total flavonoid content of all red cabbage extracts was determined by the aluminum chloride colorimetric assay (15). The total flavonoid content was calculated from the standard quercetin curve and expressed as g quercetin equivalent in 100 g of extract (g% QE).

Determination of total anthocyanin content (TAC)

Determination of total anthocyanin content using standard curve of cyanidin-3-glucoside

The total anthocyanin content of all red cabbage extracts was determined using the pH-differential method with some modifications (16). Each experiment was analyzed in triplicate. The total anthocyanin was calculated from the standard curve of cyanidin 3-glucoside and was expressed as g cyanidin 3-glucoside equivalent in 100 g of extract (g% C3G).

Where $A = (A_{520nm} - A_{700nm})_{pH\ 1.0} - (A_{520nm} - A_{700nm})_{pH\ 4.5}$

A = the absorbance

A520nm= the absorbance measured at 520 nm
A700nm = the absorbance measured at 700 nm

Determination of total anthocyanin content by standard calculation

The total anthocyanin content of all red cabbage extracts was determined using the pH-differential method according to the guidelines of AOAC (16). Each experiment was analyzed in triplicate. The total anthocyanin was calculated as cyanidin 3-glucoside (C3G) equivalent as follows:

Anthocyanin pigment (cyanidin-3-glucoside equivalents, mg/L) = (A x MW x DF x 1000) (εx1)
Where A = (A520nm – A700nm)pH 1.0 – (A520nm – A700nm)pH 4.5
A = the absorbance
A520nm= the absorbance measured at 520 nm
A700nm = the absorbance measured at 700 nm
MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside (C3G)
DF = dilution factor
l = pathlength in cm
ε= 26,900 molar extinction coefficient, in L x mol-1 x cm-1, for C3G
103 = factor for conversion from g to mg

Thin layer chromatographic (TLC) analysis

Thin layer chromatographic (TLC) analysis of some red cabbage extracts was evaluated using the analytical condition as following.

TLC system (17):

Stationary phase: silica gel 60 F254

Mobile phase: ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26 v/v/v/v)

Detector: UV cabinet under 254 and 366 nm

Spray reagent: natural product/polyethylene glycol reagent (NP/PEG) and 2,2-diphenyl-1-picrylhydrazyl (DPPH)

Determination of in vitro biological activities

Determination of in vitro DPPH scavenging activity

The DPPH (2,2-diphenyl-1-picrylhydrazyl) assay (18) was used for the antioxidant activity of all red cabbage extracts. The EC50 value was calculated from the linear equation obtained from the plot between sample concentration and percentage of inhibition. Each experiment was analyzed in triplicate.

% inhibition = A-B-C -D (A-B) x 100

When A = absorbance of control

B = absorbance of blank control

C = absorbance of sample

D = absorbance of blank sample

Determination of in vitro antibacterial activity

The disk diffusion method of the dried red cabbage extract by maceration with ethanol (RDME), according to Clinical and Laboratory Standards Institute (CLSI) guidelines (19), was used to determine the diameter of the inhibition zone of the extract. The assay was performed against five clinically pathogenic bacteria including Escherichia coli, Staphylococcus aureus, Staphylococcus intermedius, Proteus mirabilis, Pseudomonas aeruginosa. The bacteria strains were obtained from the Microbiological Laboratory, Veterinary Diagnostic Center, Faculty of Veterinary Medicine, Kasetsart University, Nakhon Pathom, Thailand and were isolated and characterized using differential bacterial culture and biochemical assays for clinical samples according to the standard method of Baron et al. (20). Standard amoxycillin (AML 10), gentamicin (CN10) and doxycycline (DO 30) were used as positive controls while DMSO was used as the negative control. All tests were conducted in triplicate and the average zone of inhibition was calculated with the standard deviation (21).

Statistical analysis

The results from all experiments were expressed as mean ± standard deviation of triplicates analysis. Significant differences between samples were analyzed by ANOVA using SPSS version 18.0 (StatSoft, USA). The Pearson Correlation test was used for correlation analysis.

Results & Discussion

Yield of the extract

As shown in **Table 1**, the dried red cabbage extracts promoted higher %yields than the fresh red cabbage extract. The dried red cabbage extract prepared by maceration with 80%EtOH (RDME) showed the highest yield (58.38 %w/w). The result demonstrated that maceration using 80% ethanol was the most effective extraction method to promote the highest yield extract.

Determination of total phenolic content (TPC)

The total phenolic contents of red cabbage extracts were shown in Table 1. All extracts contained total phenolic contents in the range of 0.20 to 5.86 g gallic acid equivalent in 100 g of extract (g% GAE). The dried red cabbage extract prepared by ultrasonic extraction with 80% ethanol (RDUE) contained the highest total phenolic content (5.86 ± 0.06 g% GAE). The result showed that ultrasonic extraction using 80% ethanol was the most effective extraction method to significantly promote the highest extract with total phenolic content ($P < 0.05$). Moreover, using solvents (80% ethanol or water) without acid (1% HCl) significantly promoted the extracts with higher total phenolic content than extracts obtained from using solvents of extraction with acid. However, the dried red cabbage extract ((RDUW) and the fresh red cabbage extract (RFUE) contained no different total phenolic contents ($P < 0.05$).

Determination of total flavonoid content (TFC)

The total flavonoid contents of red cabbage extracts were shown in Table 1. All extracts contained total flavonoid contents in the range of 0.15 to 0.88 g quercetin equivalent in 100 g of extract (g% QE). The dried red cabbage extract prepared by maceration with 80% ethanol (RDME) contained the highest total flavonoid content (0.88 ± 0.02 g% QE). The result showed that maceration using 80% ethanol was the most effective extraction method to significantly promote the extract with the highest total flavonoid content ($P < 0.05$). However, using solvents of extraction without acid or with acid did not promote any difference in total flavonoid content among the obtained extracts. The dried and fresh red cabbage raw materials also did not promote the extracts with any total flavonoid content difference ($P < 0.05$).

Determination of total anthocyanin content (TAC)

Using pH-differential with standard calculation method, total anthocyanin contents of red cabbage extracts are in the range of 0.17-0.96 g cyanidin 3-glucoside equivalent in 100 g of extract (g% C3G). RDME contained the highest total anthocyanin content (0.96 ± 0.00 g% C3G).

Using pH-differential with a standard curve of cyanidin 3-glucoside method, total anthocyanin contents of red cabbage extracts are in the range of g cyanidin 3-glucoside equivalent in 100 g of extract (g% C3G). RDME contained the highest total anthocyanin content (2.11 ± 0.00 g% C3G).

The total anthocyanin contents of red cabbage extracts obtained from 2 pH-differential methods are shown in Table 1. The results from the pH-differential with a standard curve of the cyanidin 3-glucoside method were significantly higher than the results obtained from the pH-differential with a standard calculation method ($P < 0.05$). Additionally, the dried red cabbage extract, the maceration with 80% ethanol contained the highest total anthocyanin contents from both methods. On the other hand, the fresh red cabbage extract prepared by decoction with water (RFDEW) contained the lowest total anthocyanin content followed by dried red cabbage extract prepared by the same method (RDDEW). The results suggested that the decoction which is the extraction using high temperature significantly decreased total anthocyanin content ($P < 0.05$) corresponded to the previous study which demonstrated that stewing at high temperature can cause a great decrease of anthocyanin contents in red cabbage (10). Another previous study reported that the degree of extraction of anthocyanin in red cabbage using acidified water-ethanol as extraction solvent increased in solvents with a low ratio of ethanol up to 50% (v/v) while the obtained anthocyanin content in the extracts decreased in solvents with the high ethanolic ratio (60-80%v/v) (22). The dried red cabbage extract prepared by ultrasonic extraction with 80% ethanol (RDUE) contained a moderate total flavonoid content (0.27 ± 0.01 g% QE) and the moderate total anthocyanin content using pH-differential with standard calculation method and pH-differential with a standard curve of cyanidin 3-glucoside method (0.26 ± 0.00 and 0.55 ± 0.00g% C3G, respectively). The results indicated that maceration with ultrasonic and shaking techniques effectively significantly promoted extracts with high total anthocyanin contents ($P < 0.05$). The effective effects could come from the induction of physical ruptures of the membrane of anthocyanoplasts in plant cells, leading to the simple release of anthocyanins (23).

Thin Layer Chromatography (TLC) Analysis

TLC fingerprints of red cabbage extracts were investigated with white light, UV 254 nm, and UV 366 nm with NP/PEG and DPPH spray reagents as detectors. As shown in Figure 1, all red cabbage extracts showed specific chromatographic fingerprints with some chromatographic bands corresponding to standard anthocyanins. After being detected under white light, there were violet and purple bands corresponding to delphinidin-3-sambubioside and cyanidin-3-sambubioside at Rf values of 0.15 and 0.20,

respectively in all red cabbage extracts except RFDEW (track 14) (Figure 1; A). After being detected under UV 254 nm, there were dark quenching bands on a green background which suggested the presence of substances with chromophores in the red cabbage extracts while detection under UV 366 nm showed the blue, yellow, and green fluorescence chromatographic bands suggested the presence of compounds with some fluorescence functional groups such as conjugated double bonds (Figure 1; B and C). After spraying with NP/PEG and detecting under UV 366 nm, there were bright blue and yellow fluorescence chromatographic bands suggesting the presence of phenolics and flavonoids in red cabbage extracts (Figure 1; D). Moreover, after spraying with DPPH spray reagent and detected under white light, some chromatographic bands were positive to this spray reagent and appeared as yellow bands on purple background suggesting the compounds with DPPH scavenging activities.

Table 1. Total phenolic, total flavonoid, total anthocyanin contents and in vitro antioxidant activity of fresh and dried red cabbage extracts from various conditions.

Sample	Yield (%w/w)	TPC (g% GAE)	TFC (g% QE)	TAC* (g% C3G)	TAC** (g% C3G)	DPPH Assay EC_{50} (µg/ml)
RFME	7.45	0.89 ± 0.02^{b}	$0.17 \pm 0.01^{b,c}$	0.17 ± 0.00^{d}	0.36 ± 0.00^{d}	535.03 ± 25.90^{h}
RFDIW	0.664	1.59 ± 0.18^{d}	0.19 ± 0.01^{c}	0.25 ± 0.00^{f}	0.54 ± 0.00^{f}	224.91 ± 5.81^{b}
RFDEW	4.636	1.34 ± 0.01^{c}	0.23 ± 0.01^{d}	0.05 ± 0.00^{c}	0.08 ± 0.00^{c}	730.49 ± 14.37^{i}
RFUE	5.05	3.28 ± 0.02^{i}	$0.15 \pm 0.01^{a,b}$	0.89 ± 0.00^{m}	1.94 ± 0.00^{m}	235.07 ± 5.71^{b}
RDME	**58.38**	4.67 ± 0.03^{k}	**0.88 ± 0.02^{f}**	**0.96 ± 0.00^{n}**	**2.11 ± 0.00^{n}**	239.09 ± 14.17^{b}
RDMEH	55.884	0.20 ± 0.01^{a}	$0.16 \pm 0.00^{a,b,c}$	0.04 ± 0.00^{b}	0.07 ± 0.00^{b}	1294.60 ± 20.26^{j}
RDMW	41.69	1.90 ± 0.02^{e}	0.27 ± 0.01^{e}	0.25 ± 0.00^{e}	0.53 ± 0.00^{e}	$280.30 \pm 3.83^{c,d}$
RDMWH	21.742	0.74 ± 0.04^{b}	0.13 ± 0.01^{a}	0.35 ± 0.00^{i}	0.75 ± 0.00^{i}	$304.72 \pm 0.54^{d,e}$
RDDIW	20.878	3.07 ± 0.07^{h}	$0.25 \pm 0.01^{d,e}$	0.27 ± 0.00^{h}	0.58 ± 0.00^{h}	473.30 ± 9.38^{g}
RDDEW	14.008	2.13 ± 0.03^{f}	$0.25 \pm 0.01^{d,e}$	0.03 ± 0.00^{a}	0.05 ± 0.00^{a}	364.94 ± 10.78^{f}
RDUE	30.592	**5.86 ± 0.06^{l}**	0.27 ± 0.01^{e}	0.26 ± 0.00^{g}	0.55 ± 0.00^{g}	**137.91 ± 0.91^{a}**
RDUEH	28.446	3.67 ± 0.07^{j}	$0.16 \pm 0.00^{a,b,c}$	0.41 ± 0.00^{j}	0.88 ± 0.00^{j}	229.85 ± 1.38^{b}
RDUW	20.516	$3.17 \pm 0.03^{h,i}$	$0.26 \pm 0.00^{d,e}$	0.69 ± 0.00^{k}	1.51 ± 0.00^{k}	271.05 ± 1.71^{c}
RDUWH	21.742	2.57 ± 0.03^{g}	$0.15 \pm 0.01^{a,b}$	0.69 ± 0.00^{l}	1.51 ± 0.00^{l}	324.17 ± 3.77^{e}
Ascorbic acid	-	-	-	-	-	7.89 ± 0.01
D3S	-	-	-	-	-	15.19 ± 0.18
C3S	-	-	-	-	-	17.59 ± 0.04
D3G	-	-	-	-	-	22.19 ± 0.14
C3G	-	-	-	-	-	25.13 ± 0.20

TPC = total phenolic content, TFC = total flavonoid content, TAC* = total anthocyanin content by standard calculation, TAC** = total anthocyanin content using standard curve of cyanidin 3-glucoside, DPPH = 2,2-diphenyl-1-picrylhydrazyl, RFME = fresh red cabbage extract by maceration with 80%EtOH, RFDIW = fresh red cabbage extract by digestion with water, RFDEW = fresh red cabbage extract by decoction with water, RFUE = fresh red cabbage extract by ultrasonic with 80%EtOH, RDME = dried red cabbage extract by maceration with 80%EtOH, RDMEH = dried red cabbage extract by maceration with 80%EtOH and 1%HCl, RDMW = dried red cabbage extract by maceration with water, RDMWH = dried red cabbage extract by maceration with water and 1%HCl, RDDIW = dried red cabbage extract by digestion with water, RDDEW = dried red cabbage extract by decoction with water, RDUE = dried red cabbage extract by ultrasonic with 80%EtOH, RDUEH = dried red cabbage extract by ultrasonic with 80%EtOH and 1%HCl, RDUW = dried red cabbage extract by ultrasonic with water, RDUWH = dried red cabbage extract by ultrasonic with water and 1%HCl, D3S = delphinidin-3-sambubioside, C3S = cyanidin-3-sambubioside, D3G = delphinidin-3-glucoside, C3G = cyanidin-3-glucoside, - = The experiment was not performed. Different letters in the same column are significant differences ($P < 0.05$)

The appearances of 4 standard references (delphinidin-3-sambubioside, cyanidin-3-sambubioside, delphinidin-3-glucoside, and cyanidin-3-glucoside) were positive to all detectors and were found in RDUE, RDUEH, and RFUE with Rf values of 0.15, 0.20, 0.29, and 0.35, respectively (**Figure 1; E**).

Determination of DPPH scavenging activity

All red cabbage extracts exhibited low to moderate DPPH scavenging effects as shown in **Table 1**. The EC_{50} values of all extracts ranged from 137.91 to 1294.60 µg/ml. RDUE which contained the highest total phenolic content was found to promote the highest DPPH scavenging activity while RDME which contained the highest total phenolic and total flavonoid contents promoted moderate DPPH scavenging activity (EC_{50} 239.09 ± 14.17 µg/mL). Total phenolic content and total anthocyanin contents of red cabbage extracts obtained from 2 pH-differential methods showed moderate correlation with DPPH scavenging activity (r= 0.6283, 0.5085, and 0.5080) whereas total flavonoid content showed low correlation with DPPH scavenging activity (r= 0.2061).

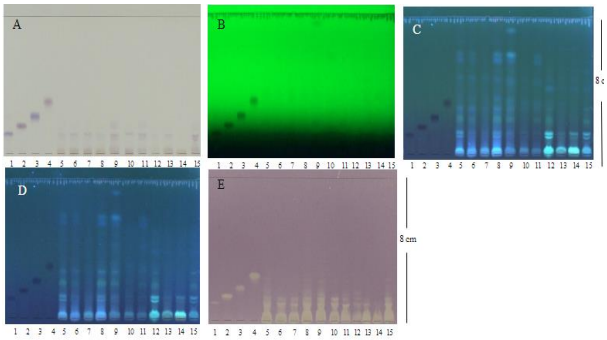

Figure 1. TLC chromatogram of red cabbage extracts; 1 = delphinidin-3-sambubioside, 2 = cyanidin-3-sambubioside, 3 = delphinidin-3-glucoside, 4 = cyanidin-3-glucoside, 5 = RDME (dried red cabbage extract by maceration with 80%EtOH), 6 = RDDIW (dried red cabbage extract by digestion with water), 7 = RDDEW (dried red cabbage extract by decoction with water), 8 = RDUE (dried red cabbage extract by ultrasonic with 80%EtOH), 9 = RDUEH (dried red cabbage extract by ultrasonic with 80%EtOH and 1%HCl), 10 = RDUW (dried red cabbage extract by ultrasonic with water), 11 = RDUWH (dried red cabbage extract by ultrasonic with water and 1%HCl), 12 = RFME (fresh red cabbage extract by maceration with 80%EtOH), 13 = RFDIW (fresh red cabbage extract by digestion with water), 14 = RFDEW (fresh red cabbage extract by decoction with water), 15 = RFUE (fresh red cabbage extract by ultrasonic with 80%EtOH). Adsorbent: Silica gel GF254. Solvent system = ethyl acetate: glacial acetic acid: formic acid: water (100:11:11:26). A = detection under white light, B = detection under UV 254 nm, C = detection under UV 366 nm, D = detection with NP/PEG spray reagent under UV 366 nm, E = detection with DPPH spray reagent under white light

According to TLC analysis, four standard anthocyanins including delphinidin-3-sambubioside, cyanidin-3-sambubioside, delphinidin-3-glucoside, and cyanidin-3-glucoside should be presented in red cabbage extracts. There was no previous report on DPPH scavenging assay for determination of antioxidant activity of these 4 compounds, therefore, in this study, they were tested for DPPH scavenging effect. It was found that delphinidin-3-sambubioside, cyanidin-3-sambubioside, delphinidin-3-glucoside, and cyanidin-3-glucoside exhibited strong DPPH scavenging effects with EC_{50} values of 15.19 ± 0.18, 17.59 ± 0.04, 22.19 ± 0.14, and 25.13 ± 0.20 µg/ml, respectively. Furthermore, there was a study showed a high correlation (r = 0.926) between the concentration of the major red cabbage anthocyanins and the antioxidant activity of red cabbage extracts determined by DPPH assay (10). Therefore, these compounds including delphinidin-3-sambubioside, cyanidin-3-sambubioside, delphinidin-3-glucoside, and cyanidin-3-glucoside could be used as active markers for quality control of raw materials and extracts from red cabbage for their antioxidant properties.

Determination of in vitro antibacterial activity by disk diffusion method

The dried red cabbage extract by maceration with ethanol (RDME) was investigated for inhibitory effects against five clinically pathogenic bacteria including Escherichia coli, Staphylococcus aureus, Staphylococcus intermedius, Proteus mirabilis, Pseudomonas aeruginosa using disk diffusion method. As shown in **Table 2**, the extract showed no inhibition zone against all the tested bacteria at a maximum concentration of 12.5 mg/disc. The absence of the

inhibitory effect of the extract in this tested condition could be related to the sensitivity of the bacteria to the extract. However, find the alternative results, a concentration adjustment of the tested extract and determination of antibacterial activity using other testing methods such as broth microdilution should be conducted. From previous studies, it was found that the methanol extract of red cabbage promoted inhibitory effects against Methicillin-resistant Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Staphylococcus aureus, Bacillus subtilis, and Salmonella enterica serovar Typhimurium with the inhibition zones for the gram-positive bacteria ranged from 7 to 17 mm whereas the inhibition zones for gram-negative bacteria were in the range of 12 to 20 mm (6).

Table 2. Antibacterial activity of the dried red cabbage extract by maceration extraction with 80%EtOH (RDME) against some cli nical isolated bacteria by the disk diffusion method.

Sample	Concentration (mg/disc)	Zone of inhibition (mm)				
		E. coli	*S. aureus*	*S. intermedius*	*P. mirabilis*	*P. aeruginosa*
RDME	12.5	0	0	0	0	0
	6.25	0	0	0	0	0
	3.125	0	0	0	0	0
	1.5625	0	0	0	0	0
	0.78125	0	0	0	0	0
AML 10	0.01	0	42 ± 0.00	8 ± 0.00	28 ± 0.00	0
CN 10	0.01	0	25.5 ± 0.45	15 ± 0.00	26.5 ± 0.00	22 ± 0.00
DO 30	0.01	26 ± 0.00	18 ± 0.00	13.5 ± 0.00	7.3 ± 0.00	0

E. coli = *Escherichia coli*, *S. aureus* = *Staphylococcus aureus*, *S. intermedius* = *Staphylococcus Intermedius*, *P. mirabilis* = *Proteus mirabilis*, *P. aeruginosa* = *Pseudomonas aeruginosa*,

AML 10 = Amoxycillin 10 μg, CN 10 = Gentamicin 10 μg, DO 30 = Doxycycline 30 μg, 0 = no inhibition zone

In this study, red cabbage extracts were prepared with various extraction methods and solvents. Ultrasonic extraction with 80% ethanol promoted the extract with the highest total phenolic content and the strongest DPPH scavenging activity while maceration with 80% ethanol promoted the extract with the highest yield, total flavonoid, and total anthocyanin contents and also high total phenolic content and moderate DPPH scavenging activity. From TLC analysis, four anthocyanins including delphinidin-3-sambubioside, cyanidin-3-sambubioside, delphinidin-3-glucoside, and cyanidin-3-glucoside could be used as chemical markers in red cabbage bands. Furthermore, moderate correlations between total phenolic content and total anthocyanin contents of red cabbage extracts and DPPH scavenging activity were found while total flavonoid content showed a low correlation to DPPH scavenging activity. The dried red cabbage extract by maceration with ethanol (RDME) showed no inhibitory effect against five clinically pathogenic bacteria at a maximum concentration of 12.5 mg/disc, the results could affect by the sensitivity of the tested bacteria to the extract and also the tested concentration of the extract which should be adjusted and repeated the experiment in the future.

Conclusion

In this study, the red cabbage extracts prepared from fresh and dried red cabbage leaves using various extraction methods and solvents were evaluated for the yield, phytochemical contents consisting of total phenolic, total flavonoid, and total anthocyanin contents, and in vitro biological activities including DPPH scavenging and antibacterial activities. The dried red cabbage extract obtained from ultrasonic extraction with ethanol (RDUE) contained the highest total phenolic content with the strongest DPPH scavenging while the dried red cabbage extract by maceration with ethanol (RDME) promoted the highest yield, total flavonoid, and total anthocyanin contents. According to TLC analysis, all red cabbage extracts showed specific fingerprints with chromatographic

bands that could be corresponding to phenolics and flavonoids. Four anthocyanins including delphinidin-3-sambubioside, cyanidin-3-sambubioside, delphinidin-3-glucoside, and cyanidin-3-glucoside were found in most red cabbage extracts and promoted strong DPPH scavenging activities which could be used as active markers. Moreover, the study demonstrated a moderate correlation between total phenolic content and total anthocyanin contents of red cabbage extracts with DPPH scavenging activity whereas total flavonoid content was low. The dried red cabbage extract by maceration with ethanol (RDME) showed no inhibitory effect against five clinically pathogenic bacteria at a maximum concentration of 12.5 mg/disc.

Acknowledgement

This presentation is supported by the Scholarship to Promote Master and Doctoral Students' Competency for Academic Presentations from the Faculty of Graduate Studies, Mahidol University.

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P-7 10.23177/NATPRO8.2023.06.22.009

Active compounds of *Zygostelma benthamii* and their validated methods of analysis

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The roots of *Zygostelma benthamii* have Thai traditional uses as carminative, heart tonic, antispasmodic, and to treat vertigo. Prior to our study there was no phytochemical study on this plant in Thailand, however *Hemisdesmus indicus* or Indian Sarsaparilla with similar phenotypes and various traditional uses includingin food and cosmetics has high commercial values and has received more significant biomedical interests. Many active compounds with biological activities have been reported to be contained within the roots. Recently, the roots of *Z. benthamii* have become popular in Thailand based on traditional uses only. This study reports the presence of α-amyrin acetate, β-lupeol acetate, 2-hydroxy-4-methoxybenzaldehyde, and ellagic acid which were also present in *H. indicus*. Validated methods of analysis by TLC-densitometry for α-amyrin acetate and β-lupeol acetate as well as HPLC for 2-hydroxy-4-methoxybenzaldehyde for quality control and future utilization are provided. The parameters studied included specificity, linearity, precision, accuracy, limit of detection and limit of quantification. Our findings suggest the potentiality of *Z. benthamii* as an economic plant and further investigation on these plant chemicals and biological activities as well as its conservation are required.

Keywords: *Zygostelma benthamii*, 2-Hydroxy-4-methoxybenzaldehyde, α-Amyrin acetate, β-Lupeol acetate, Ellagic acid

Introduction

The plant *Zygostelma benthamii* Baill (**Figure 1**) previously a member of Asclepiadaceae but now belongs to Apocynaceae known in Thai as "Op Choei Thao", the root has been used in folk medicine as a carminative, a heart tonic, an antispasmodic, and to treat vertigo. The stems are used to treat headache and lumbo-sacral pain. It is a slender, laticiferous, twining, sometimes prostrate or semi-erect shrub. Roots are woody and aromatic. The stem is numerous, slender, terete, thickened at the nodes. Often with milky latex, leaves are sparse, opposite and highly variable in shape and length; linear-lanceolate to elliptic-oblong, 2.5-10 cm long and 0.7-3.8 cm broad. Dorsal parts are often variegated in white with silvery white and pubescent beneath. Flowers are small, opposite, crowded in subsessile axillary cymes and greenish outside and purplish inside. Fruit is 10-15 cm long cylindrical, glabrous follicle with several black flattened seeds with long white silky hairs [1,2].

Op Choei Thao has not been extensively investigated, however, there is an Indian plant with very similar botanical characteristics, *Hemisdesmus indicus* (L.) R.Br. was formerly a member of Asclepiadaceae but now belongs to Apocynaceae known as Indian Sarsaparilla, Ananthamoola or Sugandha in Sanskrit. The roots of the plant are woody with a strong fragrance and have a sweet taste, with cooling effect. An unusual phenolic compound, 2-hydroxy-4-methoxybenzaldehyde is responsible for the fragrance in the root [3]. According to Ayurveda, *H. indicus* root is considered a tonic, demulcent, diaphoretic, diuretic and blood purifier. It is employed in nutritional disorders, syphilis, rheumatism, respiratory disorders, gravel and other urinary diseases and skin affections. It is said to be useful in afflictions of the kidneys, scrofula, thrush, venereal disease, nephritic complaints, and for sore mouths of children and is a component of several medicinal preparations. The roots are also used as an adjunctive therapy in treatment of snakebite and scorpion sting [3-7].

Other uses include preparation of curd milk with the roots in the daily diet to obtain relief from piles. Washing the wounds with a decoction of *H. indicus*'s leaves and root hasten recovery of wounds. An eye wash with mixture of the decoction of *H. indicus*'s root

and some honey reduce eye inflammation due to its astringent action [8-10]. The national medicinal plant board (NMPB) India has identified H. indicus as a highly traded medicinal plant because of its multipurpose nature [4].

The chemical constituents of the root are an essential oil composed of 80% 2-hydroxy-4-methoxybenzaldehyde and also contain 2-hydroxy-4-methoxy acetophenone, 2-hydroxy-4-methoxybenzoic acid, aromadendrene, terpinen-4-ol, salicylaldehyde, limonene, α-terpinyl acetate, amyl cinnamate, benzophenone, benzyl benzoate, borneol and others [4]. The roots also contain lupeol, α-amyrin, β-amyrin, its acetate and sitosterol among others [11].

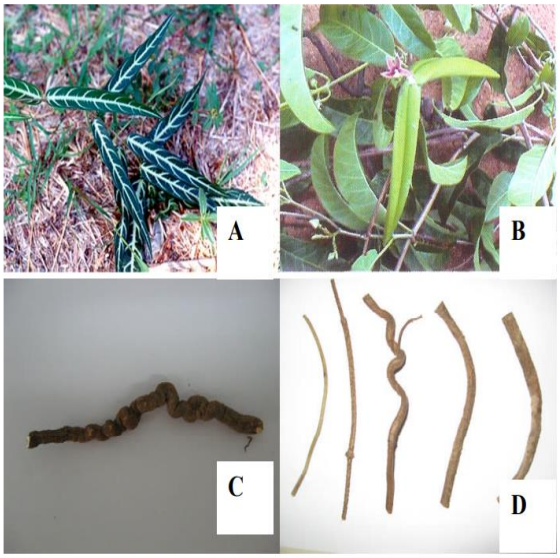

Figure 1. *Zygostelma benthamii* Baill A. Creeping plant on the ground showing white variegation, B. Climbing mature plant with fruits, C. The fresh root, D. The dried stems (Photographs taken by Pongpan, N.)

Biological tests suggested many pharmacological activities such as antiinflammatory in the treatment of Viper venom and *Propionibacterium acne* have been reported to be treated by root extract possibly by reducing reactive oxygen species and inflammatory cytokines IL8 and TNF-α [12]. Topical application of root powder paste reduced swellings, inflammation and rheumatism [13]. The ethanolic extract of roots showed significant anti-nociceptive effects in mice by reducing pain through acetic acid (writhing test), formalin (Paw licking test) and hot plate test in mice. The extract showed dose-dependent anti-nociceptive effect in all models for anti-nociception and it could block both neurogenic and inflammatory pain [14].

The aqueous extract of the roots exhibited bacteriostatic activity in mice infected with *Mycobacterium leprae*. The presence of *p*-methoxysalicylic aldehyde in the extract was considered to be responsible for the activity [15]. The methanolic root extract proved to possess anti-diarrheal activity in *in vivo* and *in vitro* studies [16]. The chloroform and methanol extracts of *H. indicus* root have demonstrated potent anti-enterobacterial activity, the presence of minerals in the methanol extract might supplement the antidiarrheal activity of this plant. Therefore, it can be used as a complementary medicine for antibiotics or as a supplement to antibiotics to treat diarrhea and other foodborne diseases caused by multidrug resistant strains and is an effective remedy for Salmonellosis and also for other forms of gastroenteritis [17]. Moreover, the root aqueous ethanolic extract showed anti-ulcer activities and significantly reduced the formation of gastric and duodenal lesions in Wistar strain albino rats induced by various ulcerogenic procedures and cyto-destructive agents. It has muco-protective activity by selectively increasing prostaglandins [18], therefore it provides another alternative for ulcer treatment. It aims to enhance defensive factors so that the normal balance between offensive and defensive factors can be achieved [19].

The screening of antioxidant activity of *H. indicus* has revealed its capacity to scavenge the superoxide and hydroxyl radicals at low concentrations. The methanolic extract of *H. indicus* roots was found to inhibit lipid peroxidation with an IC_{50} of 217 μg/ml and inhibits superoxide radical and hydroxyl radicals with an IC_{50} of 73.5 and 6.3 μg/ml respectively. The plant also showed potent neutralizing effect against the venom of *Crotalus adamenteus* (rattlesnake) and produced significant protection against venom induced changes in serum superoxide dismutase (SOD) and lipid peroxide levels [20]. The pure compound lupeol acetate from methanolic root extract of *H. indicus* could neutralize venom induced action of *Daboia russellii* (Russell's viper) and *Naja kaouthia* (Indian spitting cobra) on experimental animals. The neutralization activity against these venoms was due to lupeol acetate [19]. Inhibition of viper venom-induced hemorrhagic and coagulation in albino mice was due to 2-hydroxy-4-methoxy benzoic acid [21].

In vivo studies confirmed beneficial anti-oxidant status in response to *H. indicus* treatment. Root extract in

ethanol challenged rats was found to increase the enzymatic and non-enzymatic anti-oxidant status like superoxide dismutase, glutathione peroxidase, catalase, ascorbic acid levels in serum and hepatic tissue. It was found that this plant extract could protect against free radical-mediated oxidative stress in plasma, erythrocytes and liver of animals [22]. The elevated serum levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase and lactate dehydrogenase activity were also decreased [23]. The methanolic root extract of *H. indicus* at the dose of 1500 mg/kg showed a more effective reduction of diarrhea than the drug Lomotil® (diphenoxylate / atropine), a well-known antidiarrheal drug [12]. The diabetic effects (glycosuria, hyperglycemia, polyphagia, polydipsia) induced by administration of alloxan (150 mg/kg) in Wister albino rat can be reversed by an aqueous root extract of *H. indicus* via antihyperglycemic activity by increasing the secretion of insulin or enhanced transport of blood glucose to peripheral tissue. An increase in lipid levels in the serum by alloxan administration can also be reduced by antihyperlipidemic action of root extract, which serves as a defense mechanism against atherosclerosis development [24]. The aqueous (200 mg/kg) and ethanolic (400 mg/kg) *H. indicus* root extracts were administered orally to the dehydrated rats and both extracts showed a significant increase in urine output in higher doses, the diuresis induced by aqueous extract (400 mg/kg) in 5 hours showed similar results for the drugs furosemide and hydrochlorothiazide and an increase in urinary Na^+ and K^+ levels [25].

Because of these properties and utilization in traditional medicine, food and cosmetics about 1,614 tons per year of roots are harvested in India mostly from the wild which also includes the collection of roots from the immature plants. *H. indicus* is a slow-growing plant and the yield of root biomass is low, in-vitro micropropagation to induce organogenesis and somatic embryogenesis from callus induction are being recommended [4]. At present, the price of 227 g (1/2 lb) is $21.24 plus shipping price [26] or 25 kg pack of root for $8.1/kg or $202.5 per pack [27].

Previous studies showed that root extract of *Z. benthamii* exerted a skin lightening property [28] and 2-hydroxy-4-methoxybenzaldehyde isolated from some African plants contained potent tyrosinase activity [29]. Due to the similarities between *H. indicus* and *Z. benthamii* in their botanical characteristics and traditional uses this led us to investigate phytochemicals in *Z. benthamii* roots including validated analytical methods for a quality control purpose.

Materials and Methods

Plant materials

Zygostelma benthamii Baill roots were collected from Phetchaburi province, Thailand in May 2004, A herbarium specimen was labeled as NP 2004-01 and deposited at the Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University. Authentication was achieved through comparison with herbarium specimens in the Forest Herbarium National Park, Wildlife and Plant Conservation Department Ministry of Natural Resources and Environment, Thailand.

Extraction and isolation

The fresh roots of *Zygostelma benthamii* root (1 kg) were reduced to fine particles in a blender and macerated with MeOH (3x1000 mL) for one week at room temperature. The extracts were combined and filtered. The filtrate was concentrated under reduced pressure yielding white precipitate (20 g) which was filtered off. The filtrate was evaporated to dryness giving a brownish residue (50.0 g) with characteristic odor. The crude MeOH extract (50.0 g) was suspended in 300 mL water and partitioned with n-hexane, EtOAc, and n-BuOH respectively. The organic layers were evaporated under reduced pressure to provide *n*-hexane solubles (2.0 g), EtOAc solubles (3.3 g), and n-BuOH solubles (6.0 g). The aqueous layer was evaporated to dryness on a water bath to give a water-soluble fraction (30 g).

Isolation of compounds from the white precipitate: The white precipitate (3.0 g) was dissolved in 5 ml of hexane and then triturated with Silica gel 60 (3.0 g) and fractionated on a column chromatography (Silica gel 60, 70-230 mesh, Merck, column size id 2 cm × 60 cm), using a stepwise gradient of n-hexane:chloroform with increasing polarity (10:0, 9.5:0.5, 9:1,8:2). Collecting 25 ml per tube to give 40 fractions, examined by TLC and similar fractions were combined. The combined fraction 6-15 (1.2 g) were further sub-fractionated on a second column (Silica gel 60, 230-400 mesh, column size id 2 cm × 60 cm) using a stepwise gradient of *n*-hexane:chloroform (9:1-4:1) as the eluents. The eluate was collected and monitored by TLC using n-hexane: chloroform (5:2) and the like fractions were combined to give 25 fractions. The subfraction 4-6 from the column produceda white precipitate which was

recrystallized in a mixture of chloroform and methanol and washed with methanol to give a colorless crystal (ZG-1). Another combined fraction 9-11 from the column also produced another white precipitate which was recrystallized in n-hexane and washed with methanol to give colorless needles (ZG-2).

Isolation of compound from ethyl acetate fraction: The ethyl acetate fraction (3.0 g) was fractionated on a silica gel column (silica gel no.09385, 70-230 mesh, column size id 3 cm × 30 cm) and eluted with chloroform:ethyl acetate mixture (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, 0:10). Fifty fractions (25 mL each) were collected and examined by TLC (Silica gel GF_{254}, *n*-hexane:dichloromethane 1:1). The fractions showing the same pattern were combined. The combined fraction 14-20 were rechromatographed on Sephadex LH-20 using MeOH as the eluent. The combined fraction 3-6 gave out a pale-yellow residue with characteristic odor. This residue was recrystallized in petroleum ether at 4ºC to give aromatic white needle like crystals (ZG-3).

Isolation of compound from the water-soluble fraction: The water-soluble fraction (10.00 g) from the methanol extract was dissolved in 200 ml of methanol, filtered and concentrated under reduced pressure and applied to a Sep-Pak C_{18} cartridge and eluted with water, ethyl acetate, and methanol respectively (90 mL each). The ethyl acetate fraction was dried under reduced pressure, dissolved in methanol and fractionated on Sephadex LH-20 column (column size id 3.8 cm × 40 cm) using methanol as the eluent. Ten fractions (100 ml each) were collected and monitored by a TLC method and screening for antioxidant activity by the TLC-DPPH method. Based on the antioxidant activity of the fractions and TLC profile, the active fractions were combined and further sub-fractionated. The combined fraction 10-15 was chromatographed on a silica gel column (230-400 mesh, column size i.d. 2 cm × 60 cm) and eluted with a stepwise gradient of chloroform, ethyl acetate and methanol. The eluate was collected in fractions of 25 ml and monitored by TLC (silica gelGF254 toluene:ethyl acetate:methanol:formic acid (3:3:0.8:0.2 v/v)) the similar fractions were combined to give 35 fractions. The combined fraction 11- 15 was further sub-fractionated on a Sephadex LH-20 column (column size, id 3.8 cm × 40 cm) and eluted with methanol yielding 25 fractions of 20 ml. Thecombined fractions 7-10 provided a yellowish white precipitate. The residue was recrystallized in methanol and washed with chloroform to yield yellowish white prisms (ZG-4).

The isolated compounds were chemically characterized by a variety of spectroscopy including UV, IR, MS, 1D NMR (such as ^{1}H-NMR, ^{13}C-NMR, DEPT-90, 135) and 2D NMR (such as ^{1}H-^{1}H COSY, NOESY, HMQC and HMBC). The known compounds were identified by comparison to their spectroscopic data with the values in the literature previously reported.

The UV spectra (in methanol or chloroform) were obtained on a Milon Roy Spectronic 3000 Array Spectrophotometer and the UV λ_{max} values were determined from the HPLC chromatograms. All data were recorded at the Faculty of Pharmacy, Mahidol University, Bangkok, Thailand). The IR spectra (KBr disc) were recorded on a Perkin Elmer Spectrometer at the Faculty of Sciences, Mahidol University, Bangkok, Thailand. Electron Impact and high-resolution electron impact mass spectra (El-MS and HREI-MS) were recorded on a Mass Finigan mat GCQ-Mass spectrometer at the Faculty of Sciences, Mahidol University, Bangkok, Thailand. The ^{1}H NMR (500 MHz) and ^{13}C NMR (125.00 MHz) spectra were obtained with a Bruker Advance DPX-300 FT-NMR spectrometer at the Faculty of Science, Mahidol University, and National Science and Technology Development Agency (NSTDA), Thailand Science Park, Bangkok, Thailand. Deuterated solvents for NMR spectra were $CDCl_3$ (Deuterated chloroform), CD_3OD (Deuterated methanol), and C_5D_5N (Deuterated pyridine). The chemical shifts were reported in ppm scale using the chemical shift of trimethyl silane (TMS) signal as reference. Melting points were obtained on an Electrothermal 9100 apparatus at the Department of Pharmacognosy, Faculty of Pharmacy, Mahidol University, Bangkok, Thailand.

Quantitative analysis by TLC-densitometry method

Instruments: Sample syringe 100 µL (Hamilton, Switzerland), TLC Twin Trough Chamber 20x10 cm (Camag, Switzerland), Sample Application: Linomat V (Camag, Switzerland), Chromatogram Evaluation: TLC Scanner (Camag, Switzerland), Computer Integrator: winCATS 1.2.6 software, UV Lamp Carbinet (Camag, Switzerland)

TLC system: Silica gel 60 GF254, pre-coated on TLC aluminum sheets 20 x 20 cm, layer thickness 0.25 cm, Merck, Germany, Solvent: Hexane: Chloroform (5:2).

Preparation of standard solution of α-amyrin acetate and β-lupeol acetate: Each stock solution of authenticated α-amyrin acetate (100µg/mL) and β-lupeol acetate (100 µg/mL) was prepared by dissolving 10 mg of each standard in methanol and diluted to 10 ml with methanol. The aliquots (0.1 to 1.0 mL) of the stock solution were transferred to 10 mL standard volumetric flasks and adjusted volume with methanol to obtain working standard solutions containing 1.0 to 10.0 µg/mL.

Preparation of sample Solution: The freshly ground powder of *Z. benthamii* Baill root (10.00 g) was macerated with 100 mL of methanol for 2 days. After filtration, the filtrate was dried under reduced pressure. The extract was dissolved in methanol and adjusted to 10 mL with methanol in a volumetric flask to a concentration of 1.0 mg/mL. Samples were filtered through a 0.45 µm filter before analysis.

Calibration Curve for α-amyrin acetate and β-lupeol acetate: Ten microlitres of the standard solutions (1, 2, 4, 6 and 8 µg/spot) were applied at a band width of 8 mm in triplicate on a TLC plate (Silica gel 60 GF254). The plate was developed in a twin trough chamber that has previously been equilibrated with solvent system for 30 min. The solvent system used was hexane–chloroform (5:2 v/v) and the solvent front was allowed to travel to a distance of 8 cm. After development, the plate was dried and sprayed with anisaldehyde-sulfuric acid reagent followed by heating at 110 ºC for 5 min. The plate was scanned at 520 nm with a TLC scanner 3, and the peak areas were recorded. The calibration curve of each standard was obtained by plotting the peak areas versus concentrations.

Quantification of α-amyrin acetate and β-lupeol acetate in the root of *Z. benthamii*: Standard solutions of α-amyrin acetate and β-lupeol acetate and extracts of the root of *Z. benthamii* were applied to a precoated silica gel 60 GF254 TLC plate 20x10 cm, with Linomat V Automatic sample spotter. The plate was developed with hexane: chloroform (5:2 v/v) solvent system, then dried and sprayed with anisaldehyde sulfuric acid reagent followed by heating at 110 °C for 5 min. The plate was scanned at 520 nm with TLC Scanner 3. The amount of α-amyrin acetate and β-lupeol acetate present in the extracts were determined from areas of the peaks in the sample solutions of *Z. benthamii* using the calibration curves of α-amyrin acetate and β-lupeol acetate.

Method validation: The method was validated according to the ICH guidelines on the validation of analytical procedures (30).

Linearity: The α-amyrin acetate and β-lupeol acetate solutions were prepared at five concentration levels (1.0, 2.0, 4.0, 6.0, and 8.0 µg/spot) and were analyzed in triplicate.

Precision: The precision of the method was affirmed by analyzing 6 µg/spot) of standard solutions of α-amyrin acetate and β-lupeol acetate individually (n = 5) and was expressed as % RSD. Variability of the method was studied by analysis aliquots of standard solutions of α-amyrin acetate (8.0 µg/spot) and β-lupeol acetate (3.40 µg/spot) on the same day (intra-day precision) and on different days (inter-day precision) and the results were expressed as % RSD.

Accuracy: The accuracy of the method was tested by performing recovery studies at two levels for α-amyrin acetate and β-lupeol acetate. To 0.20 g of fresh powdered root of *Z. benthamii*, known amounts of α-amyrin acetate and β-lupeol acetate (4.0 and 6.0 µg) were added. The percentage recovery as well as average percent recovery for α-amyrin acetate and β-lupeol acetate were calculated.

Limit of detection (LOD) and limit of quantification (LOQ): Methanol was applied six times to represent the noise signal. The LOD and LOQ were obtained by applying the standard solution at varying concentrations to give signal-to-noise ratio (S/N) of 3 and 10, respectively.

Quantitative analysis by HPLC method (2-hydroxy-4-methoxybenzaldehyde)

Instrumentation: Column: Mightysil RP-18 GP column (150 x 3.0 mm i.d.; 5µm) with guard C18 column, Flow rate: 0.8 mL/min, Mobile phase: linear gradient elution Solvent A: 0.1%TFA in water Solvent B: Acetonitrile, Sample injection: Rheodyne TM 7725i injection were carried out using a 5 µL loop, Pump: Shimadzu LC-10AD (Shimadzu, Kyoto, Japan), Detector: SPl-M10A UV photodiode array detector, λ= 280 nm (Shimadzu, Kyoto, Japan), Data processing: LC-MS solution software (Shimadzu, Kyoto, Japan), Statistical Analysis: software and graph Microsoft Office Excel 2003.

Preparation of standard solution of 2-hydroxy-4-methoxybenzaldehyde: A 10 mg of accurately weighed 2-hydroxy-4-methoxybenzaldehyde (Fluka, Switzerland) was dissolved in methanol and made up volume to 10 mL with methanol in a volumetric flask. Working standard solutions in the range of 5–500

μg/mL were prepared by dilution with methanol. All stock solutions were stored at 4 ºC. Five different calibration levels (5–500 μg /mL) were used for the calibration plot, the analysis was done in triplicate. The calibration curve was drawn by plotting the peak area against the concentration of the compound.

Preparation of sample solution: The fresh root of *Z. benthamii* (20.0 g) was coarsely ground in a blender and macerated with 200 ml of methanol for 6 hours and left standing overnight at room temperature. After filtration, the filtrate was dried under reduced pressure. The extract (0.01 g) was redissolved with 10 ml methanol in a volumetric flask to give a concentration of 1.0 mg/ml. The solution was filtered through a 0.45 μm nylon filter before injection.

HPLC conditions: The mobile phase consisted of: (A) 0.1% (v/v) TFA in water and (B) acetonitrile. A gradient elution program of 7–10% B at 0–13 min, 10–18% B at 13–30 min, 18–28% B at 30–45 min, isocratic 28% B at 45–55 min. Re-equilibration duration was 15 min between individual runs. Detection wavelength was set at 280 nm. The flow rate was 0.8 ml/min and aliquots of 5 μL were injected. The column temperature was maintained at 28 ºC. The peak identification of the analytes was based on the comparison of retention time with those of 2-hydroxy-4- methoxy benzaldehyde standards in the same condition. Peak purity was assessed off-line. UV-spectral scans (190–400 nm) of HPLC eluents were carried out in a Diode-Array detector and data were analyzed with LC-MS solution software (Shimadzu, Kyoto, Japan).

Method validation

Specificity: The selectivity of the method was evaluated by processing blank samples with and without the addition of analyte to test for interferences.

Linearity: The linearity of the method was evaluated by analyzing a series dilution of standard 2-hydroxy-4-methoxy benzaldehyde stock solution. Each standard solution (25-200 μg /mL) was injected into HPLC system in triplicate. The standard calibration curves were obtained by plotting the peak area versus concentration. The slope and intercept values were calculated using the least-square linear regression method. Linearity was expressed as a coefficient of determination (r^2).

Precision: The precision of the method was determined in terms of intra-day and inter-day precision. Intra-day tests were performed by five consecutive injections of two different concentrations (100 and 1000 μg/ml) within the same day. Inter-day precision tests were performed with five injections of a solution (100 μg/ml) for three consecutive days. The precision was expressed as percent relative standard deviation (%RSD).

$$\% \text{ RSD} = 100 \times (SD) / \bar{x}$$

SD = the standard deviation from the mean value

$\bar{x}$ = the mean value

The value (%RSD) should be less than 2.0%

Accuracy: The accuracy was determined by the recovery of the added 2-hydroxy-4-methoxy benzaldehyde standard to the analytical sample. An accurate weight of extract (100 mg) was transferred to a 10 ml volumetric flask and methanol was added to volume (final concentration 10 mg/ml). Aliquots of 200 μL of this solution were separately transferred into 10 ml volumetric flasks containing 500 and 1,000 μL of 2-hydroxy-4-methoxybenzaldehyde standard solution (1,000 μg/ml) and methanol was added to give final concentrations of 50 and 100 μg/ml. The accuracy was expressed as the percentage of the analyte concentration measured in each sample relative to the known amount of analyte spiked to the sample. The percentage of recovery was calculated by the following equation:

$$\% \text{ Recovery} = \frac{[X_{found}] - [X_{added}]}{X_{intial}} \times 100$$

X_{found} = The concentration of 2-hydroxy-4-methoxy benzaldehyde found in the spiked sample

X_{added} = The concentration of 2-hydroxy-4-methoxy benzaldehyde added

X_{intial} = The concentration of 2-hydroxy-4-methoxy benzaldehyde found in the sample

The value (%Recovery) should be within 80-120 % at each level.

Limit of detection (LOD) and limit of quantitation (LOQ): The LOD and LOQ was calculated with 2-hydroxy-4-methoxy benzaldehyde standard solution on the basis of signal-to-noise ratio (S/N) of 3 and 10, respectively.

Anti-tyrosinase activity

Determination of anti-tyrosinase activity: The method followed a general procedure of anti-tyrosinase testing previously published (29) with some adjustment. The solution of 20 mM phosphate buffer (pH 6.8) was used in the preparation of 0.85 mM of L-DOPA. Mushroom tyrosinase enzyme (0.80 mg, 3320 U/mg) was dissolved in 5 ml of the phosphate buffer. The sample solution was prepared in methanol to provide a final concentration 1 mg/ml in the well (total volume = 200 μL).

The absorbance of the reaction mixture was measured in a set of four wells (A, B, C and D). In each well, the substance was added in the order of mixing (final volume = 200 µL) as follows:

A (control)	20 µL of mushroom tyrosinase (480 unit/ml) 140 µL of 20 mM phosphate buffer (pH 6.8) 20 µL of methanol
B (blank of A)	160 µL of 20 mM phosphate buffer (pH 6.8) 20 µL of methanol
C (sample)	20 µL of mushroom tyrosinase (480 unit/ml) 140 µL of 20 mM phosphaste buffer (pH 6.8) 20 µL of test sample solution
D (control)	140 µL of 20 mM phosphaste buffer (pH 6.8) 20 µL of test sample solution

After each well was mixed and preincubated at 25 °C for 10 minutes, a 20 µL of 0.85 mM L-DOPA was added, and the mixture was further incubated at 25 °C for another 20 min. The absorbance of each well was measured at 492 nm in a microplate reader, in triplicate. Kojic acid was used as reference for the anti-tyrosinase activity. In addition to the methanol crude extract of *Z. benthamii* root, other extracts and isolated compounds were also tested for the tyrosinase inhibitory activity.

Calculation of the percent inhibition of tyrosinase enzyme as follow:

$$\% \text{ Tyrosinase inhibition} = \frac{(A-B)-(C-D)}{(A-B)} \times 100$$

A graph between the percentage of tyrosinase inhibition versus concentration of the inhibitor was plotted. The IC_{50} of the test sample was then obtained from the graph. All the experiments were carried out in triplicate (n=3) and the data expressed as mean ± SD.

Results & Discussion

The methanolic extract of *Z. benthamii* roots was concentrated under reduced pressure which gave out a white precipitate (20 g, 2.015%) and the filtrate was evaporated to dryness to provide a brownish residue with characteristic odor (50 g, 5.0%). The crude methanol extract was suspended in water and partitioned between n-hexane, EtOAc, and n-BuOH yielding 2g (0.2%), 3.3 g (0.33%), and 6 g (0.6%) respectively. The aqueous part was evaporated to dryness on a water bath yielded 30 g (3.0%).

Two compounds were isolated from the white precipitate as colorless crystal (ZG-1) and colorless needles (ZG-2). Examination by TLC (Silica gel GF254, Hexane; Chloroform 5:2) and sprayed with anisaldehyde-sulfuric acid reagent followed by heating at 110°C for 5 min, ZG-I showed deep violet and ZG-2 was pinkish-violet bands, the Rf values were 0.46 and 0.44 respectively. The structure elucidations were determined by general procedures of various spectroscopy and comparison with the literature values. The compound ZG-I was identified as α-amyrin acetate [31,32], and the compound ZG-2 was identified as β-lupeol acetate [33,34] (**Figure 2**).

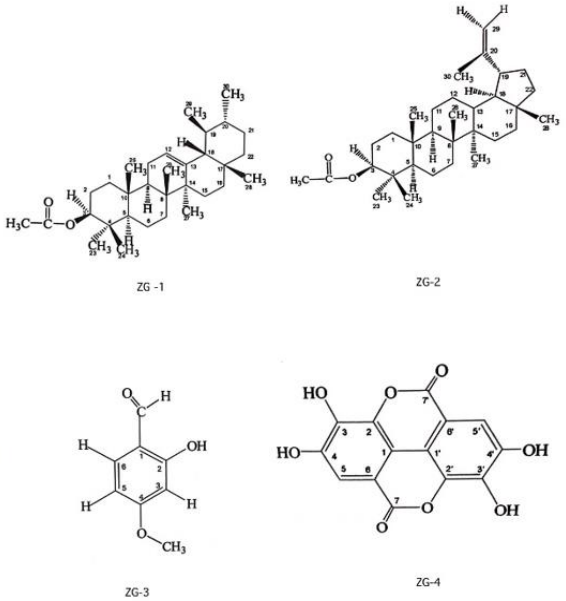

Figure 2. The structures of isolated compounds α-amyrin acetate (ZG-1), β-lupeol acetate (ZG-2), 2-hydroxy-4-methoxy-benzaldehyde (ZG-3), and ellagic acid (ZG-4).

Another compound was isolated from the EtOAc soluble fraction as pale yellow crystals with characteristic odor (ZG-3). Examination by TLC (Silica gel GF254, toluene:ethyl acetate:methanol 8:2:0.5) and sprayed with anisaldehyde-sulfuric acid reagent followed by heating at 110°C for 5 min, revealed ZG-3 as brown band, the Rf values was 0.57 as that of reference standard (Fluka Chemicals, Switzerland). The structure elucidations were determined by general procedures of various spectroscopy and comparison with the literature values [35]. The compound was identified as 2-hydroxy-4-methoxy-benzaldehyde (**Figure 2**).

The fourth compound was isolated from the water-soluble fraction as yellowish-white solid (ZG-4) (**Figure 2**). Examination by TLC (Silica gel GF254, toluene:ethylacetate:methanol:formic acid 3:3:0.8:0.2 v/v) and detected after spraying with methanolic ferric chloride reagent, showed ZG-4 as a dark blue band, the Rf values was 0.30 as that of reference standard ellagic acid (Fluka Chemicals, Switzerland).

ZG-1: Melting point 241-243 °C, UV λ_{max} ($CHCl_3$): 208 nm, IR ν_{max} (KBr disc):3397, 2932, 1730, 1380, 1370, 1250,

1030, 1000, 985, 960 cm^{-1}, EI-MS: m/z (% relative intensity): 468.32 ([M^+,14.5], 408.33 ([M-HOAc] $^+$, 32.31), 218.22 (RDA, 100), 203.25([218-CH_3] $^+$, 48), 189.20 ([249-HOAc] $^+$, 72.54)

^{1}H NMR: (δ: ppm, in $CDCl_3$, 500 MHz): δ 0.806 (3H, *s*, H-28), 0.814 (3H, *s*, H-29), 0.87 (3H, *d*, H-5, 15',16'), 0.88 (3H, *s*, H-23), 0.89 (3H, *s*, H-24), 0.94 (3H, *d*, H-30), 0.98 (H, *d*, H-20), 0.99 (3H, *s*, H-25), 1.02 (3H, *s*, H-26), 1.07 (3H, *s*, H-27), 1.65 (2H, *s*, H-2), 1.66 (2H, *s*,H1), 2.07 (3H, *s*, O-CH_3), 4.52 (1H, *dd*, *J*=1.9, 6.1 Hz, H-3α), 5.14 (1H, *t*, *J*=3.5 Hz, H-12)

^{13}C NMR: (δ: ppm, in $CDCl_3$, 125.65 MHz): δ 38.52 (CH_2, C-1), 23.57 (CH_2, C-2), 81.00 (CH, C-3), 37.76 (C, C-4), 55.31 (CH, C-5), 18.29 (CH_2, C-6), 32.92 (CH_2, C-7), 40.08 (C, C-8), 47.69 (CH, C-9), 36.85 (C, C-10), 23.42 (CH_2, C-11), 124.37 (CH, C-12), 139.68 (C, C-13), 42.12(C, C14), 28.14 (CH_2, C-15), 26.65 (CH_2, C-16), 33.80 (C, C-17), 59.11 (CH, C-18), 39.70 (CH, C-19), 39.66 (CH, C-20), 31.30 (CH_2, C-21), 41.58 (CH_2, C-22), 28.12 (CH_3, C-23), 16.92 (CH_3, C-24), 15.79 (CH_3, C-25), 16.79 (CH_3, C-26), 23.28 (CH_3, C-27), 28.80 (CH_3, C-28), 17.56 (CH_3, C-29), 21.45 (CH_3, C-30), 171.06 (C, CO-CH_3), 21.37 (CH_3, CO-$\underline{C}H_3$)

ZG-2: Melting point 216-217 °C, UV λ_{max} (MeOH): 210 nm, IR ν_{max}(KBr disc): 3072, 2917, 2851, 1730, 1641,1471,1382, 977, 912, 718 cm^{-1}, EI-MS: m/z (% relative intensity): 468([M^+]26), 408 ([M-HOAc]$^+$, 45), 365 ([M-HOAciPr]$^+$, 67), 218 ([$C_{16}H_{16}$] $^+$) ,49), 189([$C_{14}H_{23}$O-H_2O] $^+$,100)

^{1}H NMR:(δ: ppm, in $CDCl_3$, 500 MHz): δ 0.78 (3H, *s*, H-28), 0.83 (3H, *s*, H-24), 0.84 (3H, *s*, H-23), 0.85 (3H *d*, H-25), 0.94 (3H, *s*, H-27), 1.03 (3H, *s*, H-26), 1.68 (3H, *s*, H-30), 2.04 (3H, *s*, O-CH_3), 2.37(1H, *dt*, *J*=5.5,11.0 Hz, H-19), 4.47 (1H, *dd*, J 10.6Hz, H-3α), 4.57 (IH, *dd*, *J* =1.4Hz, H-29 α), 4.69 (1H, *dd*, *J* = 2.4 Hz, H-29β)

^{13}C NMR: (δ: ppm, in $CDCl_3$, 125 MHz): δ: 38.39 (C-1), 23.71 (C-2), 80.98 (C-3), 37.79 (C-4), 55.39 (C-5),18.20 (C-6), 34.22 (C-7), 40.85 (C-8), 50.35 (C-9), 37.08 (C-10), 20.94 (C-11), 25.11(C-12), 38.05 (C-13), 42.83 (C-14), 27.43 (C-15), 35.57 (C-16), 42.99 (C-17),48.29 (C-18), 48.00 (C-19), 150.94 (C-20), 29.84 (C-21), 39.99 (C-22) 27.94 (C-23),16.48 (C-24), 16.17(C-25), 15.97 (C-26), 14.50 (C-27), 17.99 (C-28), 109.34 (C-29),19.28 (C-30), 21.30 (CO-CH_3), 170.99 (CO-$\underline{C}H_3$)

ZG-3: Melting point 39-41 °C, UV λ_{max} (MeOH): 256, 276 nm, IR ν_{max} (KBr disc): 3345, 1730, 1207, 2851,1760, 1500, 1238, 977, 912,718 cm^{-1}, EI-MS: m/z (% relative intensity): 152 ([$M]^+$, 52), 151([M-H]$^+$, 100), 140 (22), 125 (35), 97 (20)

^{1}H NMR (δ: ppm, in MeOH d_4, 500 MHz): δ 3.86 (3H, *s*, O-$\underline{C}H_3$), 6.45 (H, *d*, *J* = 2.31 Hz, H-3), 6.59 (1H, *dd*, *J*= 2.32, 8.66 Hz H-5), 7.34 (1H, *d*, *J*= 8.67 Hz, H-6), 9.79 (1H, *s*,-$\underline{H}$CO), 11.40 (1H, *s*, O$\underline{H}$)

^{13}C NMR: (δ: ppm, in MeOH d_4, 125 MHz): δ: 54.88 (CH_3, O-$\underline{C}H_3$), 100.21 (CH, C-3), 107.59 (CH, C-5), 115.49 (C, C-1), 134.71 (CH, C-6), 164.10 (C, C-2), 167.05 (C, C-4), 194.28 (C, $\underline{C}$HO)

DEPT 135°: **CH_3** : 54.88 (O-$\underline{C}$H3), **CH** :100.21 (C-3), 107.59 (C-5),134.71 ( ),194.28($\underline{C}$HO), **C** :115.49 (C-1), 164.10(C-2), 167.05(C-4)

ZG-4: Compound ZG-4 was obtained as yellow colorless amorphous powder soluble in methanol or water and insoluble in n-hexane, chloroform or ethyl acetate. Melting point: > 360 °C, UV: λ_{max} (in MeOH): 214, 273 nm, IR: ν_{max} (KBr): 3426, 3762 (-OH), 1700 (CO) cm^{-1}, API-ES-MS: m/z (% relative intensity): 303 [M+H]$^+$

^{1}H NMR (δ: ppm, pyridine-d_6, 500 MHz): δ: 7.61 (2H, *s*, *Ar*-H)

^{13}C NMR: (δ: ppm, in Pyridine-d_6, 125.65 MHz): δ: 108.61 (C-1, 1'), 111.71 (C-5,5'), 113.44 (C-6,6'), 135.10 (C-2,2'), 137.10 (C-3,3'), 141.83 (C-4,4'), 170.10 (C-7,7')

Validated Methods for Quantitative analysis

The linearity between peak area ratio versus concentrations was obtained in the range of 1.00-8.00 µg/spot for both α-amyrin acetate and β-lupeol acetate with good correlation coefficients. These values were within the acceptance criteria (r^2should be 0.0995 or greater).

The precision of the method was expressed as the percentage of the relative standard deviation (%RSD). The low value indicating that the method has good precision. Both Intra-day precision and Inter-day precision for α-amyrin acetate and β-lupeol acetate showed %RSD less than 2.0.

The accuracy of the method was determined from recovery studies at two levels of addition (4.0 and 6.0 µg.) of α-amyrin acetate and β-lupeol acetate. The average recovery percentage complied with the USP requirement (80-120%).

The smallest visible quantity determined by at least three different analysis was regarded as LOD. The LOQ was the smallest concentration of analyte which could give a response that can be quantified. The LOD values for α-amyrin acetate and β-lupeol acetate were found to be 0.10 and 0.020 µg, respectively. Their LOQ values were 0.050 and 0.60 µg, respectively.

Determination of α-amyrin acetate and β-lupeol acetate in root of *Z. benthamii*: The quantitative analysis of α-amyrin acetate and β-lupeol acetate showed the

contents in the extract to be 0.49% and 0.38% g fresh weight, respectively.

Table 1. Parameters of method validation for α-amyrin acetate and β-lupeol acetate quantification by TLC densitometry.

Parameter	α-amyrin acetate	β-lupeol acetate
Linearity range (μg/spot)	1.0-8.0	1.0-8.0
Linearity (r^2, Correlation coefficient)	0.996	0.997
Precision (%RSD)		
Repeatability of measurement (n=5)	0.60	0.96
- Intra-day precision	0.62	1.83
- Inter-day precision		
Accuracy (% recovery)		
- Level 1 (4 μg/spot)	98.45	98.24
- Level 2 (6 μg/spot)	97.06	97.85
Limit of detection (μg/spot)	0.01	0.02
Limit of quantification (μg/spot)	0.05	0.06

Quantitative analysis by High performance liquid chromatography (HPLC)

The specificity of the method was determined by injecting the standard solution of 2-hydroxy-4-methoxybenzaldehyde and sample solution of *Z. benthamii* root extract on to the chromatographic system previously described. The retention time of 2-hydroxy-4-methoxybenzaldehyde in standard solution and sample was the same.

Seven concentrations of 2-hydroxy-4-methoxybenzaldehyde solutions were prepared by serial dilutions (6.25-500 μg/mL). Each standard solution was subjected to HPLC analysis in triplicate. The correlation coefficient (r^2) lied within the acceptance criteria.

Both Intra-day precision and Inter-day precision showed %RSD less than 2.0, which complied with the USP requirement.

Two concentrations i.e. 50 and 100 μg/ml of 2-hydroxy-4-methoxybenzaldehyde were added to the *Z. benthamii* root extract. The mean percentage recoveries were 107.77% and 103.94% respectively. The results complied with the USP requirement (80-120%).

The determination was done at concentration range of 0.1-1.0 μg/ml. Limit of detection (LOD) was calculated on the basis of signal-to-noise ratio (S/N) equal to 3 which was 0.10 μg/ml. Limit of quantification (LOQ) was calculated on the basis of signal-to-noise ratio (S/N) equal to 10 which was 0.45 μg/ml.

Table 2. Parameters of method validation for 2-hydroxy-4-methoxybenzaldehyde quantification by HPLC.

Parameter	2-hydroxy-4-methoxybenzaldehyde
Linearity range (μg/mL)	5-500
Linearity (r^2, Correlation coefficient)	0.997
Precision (%RSD)	
Repeatability of measurement (n=5)	1.845
- Intra-day precision	1.230
- Inter-day precision	
Accuracy (% recovery)	105.85
Limit of detection (μg/ mL)	0.10
Limit of quantification (μg/ mL)	0.45

Determination of 2-hy droxy-4-methoxybenzaldehyde content in Z. benthamii root extract by the validated HPLC method

The methanolic extract of *Z. benthamii* root was prepared by maceration at room temperature. The quantitative analysis was performed three times (n=3) using the validated HPLC method. The content of 2-hydroxy-4-methoxybenzaldehyde was found to be 8.74% g dry weight. The content in the fresh root sample was 0.51% g fresh weight.

Tyrosinase Inhibitory activity

There was a linear correlation between the concentration and the inhibitory activity of all test sample solution in the concentration below 2,000 ppm, increasing to 4,000 ppm gave little change in the activity. This demonstrated that 2,000 ppm was the maximal concentration that gave linear correlation.

The reference compound kojic acid showed two steps of linear correlation, it could inhibit tyrosinase activity with the IC_{50} of 0.16 mM but the 100% inhibition was found to be 0.70 mM.

The methanolic extract of *Z. benthamii* root showed IC_{50} value of 3.87 μg/ml. The white precipitate which separated out from the crude methanolic extract contained little activity i.e. less than 30% inhibition. Further partition of the crude methanolic extract yielded more active subfractions. The n-hexane soluble fraction and the water-soluble fraction contained comparable anti-tyrosinase activity with slightly higher activity in the n-hexane fraction (IC_{50} = 1.55 vs 1.68 μg/ml). The ethyl acetate and the n-butanol fractions exhibited less activity in decreasing order (IC_{50} = 2.02 and 3.26 μg/ml).

Among the isolated compounds ellagic acid showed highest anti-tyrosinase activity (IC_{50} = 0.33 mM),

followed by α-amyrin acetate (IC_{50} = 0.39 mM), and 2-hydroxy-4-methoxybenzaldehyde (IC_{50} = 2.06 mM). Ellagic acid was, therefore responsible for anti-tyrosinase activity of the water-soluble fraction. The anti-tyrosinase activity in the ethyl acetate fraction was due to 2-hydroxy-4-methoxybenzaldehyde and the activity in hexane fraction was due to α-amyrin acetate and 2-hydroxy-4-methoxybenzaldehyde (**Table 3**).

Table 3. Anti-tyrosinase activity of various fractions and isolated compounds from *Z. benthamii* root extract.

Sample	IC_{50} (μg/ml)*
Methanol extract	3.87 ± 0.12
White precipitate	n.a.
n-Hexane fraction	1.55 ± 0.15
Ethyl acetate fraction	2.02 ± 0.05
n-Butanol fraction	3.26 ± 0.08
Water-soluble fraction	1.68 ± 0.12
α-amyrin acetate (ZG-1)	0.39 ± 0.05 (0.83 mM)
β-lupeol acetate (ZG-2)	n.a.
2-hydroxy-4-methoxybenzaldehyde (ZG-3)	0.46 ± 0.15 (2.06 mM)
Ellagic acid (ZG-4)	0.18 ± 0.05 (0.33 mM)
Kojic acid	0.14 ± 0.05 (0.16 mM)

*mean ± SD (n=3), n.a. not applicable

Conclusion

Zygostelma benthamii Baill is known in Thai as "Op Choei Thao". The botanical characteristics of this plant resembles *Hemidesmus indicus* (Linn.) R.Br., an Indian medicinal plant, which belongs to the same family, Apocynaceae. The most obvious characteristics of both plants are the dark green leaves variegated with white midribs and large obvious veins. Their traditional indications shared some similarities. The roots of *Z. benthamii* have been used traditionally as carminative, heart tonic, antispasmodic, and antivertigo. These uses suggested anti- inflammatory, antioxidant, and antimicrobial properties. Previous study also found that the root extract also possessed anti- tyrosinase activity [28].

The roots of *H. indicus* have many indications such as tonic, demulcent, diaphoretic, diuretic and blood purifier. It is employed in nutritional disorders, syphilis, rheumatism, gravel and other urinary diseases and skin affections. The roots are also used as an additive in the main treatment of snakebite and scorpion sting, etc. It is often called 'Sugandha' because of the fragrance of its roots. This Indian plant has been studied extensively in vitro and in vivo and revealed the following activities; anti-nociceptive effects, anti-inflammatory, antibacterial, antifungal, anti-diarrheal, anti- ulcer, antihyperlipidemic, otoprotective, antithrombotic, antioxidant, hepato- protective and anti- snake venom. These findings supported the traditional uses.

Apart from botanical characteristics and indications, both plants also share similar chemical constituents. The dried root of *H. indicus* contain β- sitoserol, β-lupeol, α-amyrin, β-lupeol acetate, α-amyrin acetate, hexatriacontane, coumarins, essential oil, tannic acid, 2- hydroxy- 4- methoxybenzoic acid. 2- hydroxy- 4-methoxybenzaldehyde, p- anisaldehyde, vanillin and two sterols; hemidosterol and hemidesmol. From this study 4 compounds were isolated and identified from the crude methanolic extract of *Z benthamii* root. These were α-amyrin acetate, β-lupeol acetate, 2-hydroxy-4-methoxybenzaldehyde and ellagic acid. Validated analytical methods were developed for the quantification of α-amyrin acetate and β-lupeol acetate by TLC-densitometry with good correlation coefficient (0.9966 and 0.9977 respectively). The validated HPLC method was developed for 2- hydroxy- 4- methoxy benzaldehyde with correlation coefficient of 0.9997.

The analysis of *Z benthamii* root extract by these validated methods showed that the two triterpenes isolated were the major components in the root. The fact that α-amyrin acetate and β-lupeol acetate are well known triterpenes with anti-inflammatory activity [36], the presence of these two constituents as major chemical components in this plant supports its traditional indications for antispasmodic, and inflammatory treatment.

The presence of 2-hydroxy-4-methoxybenzaldehyde, the root specific fragrance resembles vanillin, which exhibited anti-tyrosinase activity (IC_{50} value of 0.46 μg/ml or 2.06 mM) suggested the potential use of *Z benhamii* root extract as another ingredient for whitening cosmetics. In addition to this, the isolated ellagic acid also showed good anti-tyrosinase activity which further supports the whitening application in cosmetics.

The compound 2- hydroxy- 4- methoxybenzaldehyde was determined in earlier studies to scavenge superoxide activity and have inhibitory activity on microsomal lipid peroxide (IC_{50} 0.57 and 2.46 μM/ml respectively). In the same study, it was reported that this compound showed a broad spectrum antifungal activity which was beneficial to the food industry [37]. These findings support the external uses of *Z. benhamii* root extract.

The polar fractions of the methanolic extract and

ellagic acid contained antioxidant activity which supported the traditional uses. Moreover, β-lupeol acetate had been reported by earlier studies that it could inhibit lipid peroxidation which is the cause of cell damage and was scientifically proven to be the active ingredient for anti-snake venom activity of *H. indicus* roots [19]. These findings demonstrated that *Z. benthamii* root extract contained antioxidant compounds in both polar and non-polar fractions.

Ellagic acid, the fourth compound isolated from water soluble fraction of methanolic extract, is a naturally occurring phenolic compound found in many plant sources. Recently, many biological activities of ellagic acid were reported, for examples, anti-tyrosinase, antioxidant and anticancer. The pomegranate extract which contained 90% ellagic acid exhibited anti-tyrosinase activity with IC_{50} value of 182 μg/ml while that of arbutin was 162 μg/ml [38]. This study revealed the IC_{50} value of the isolated ellagic acid from *Z. benthami* were 0.18 μg/ml or 0.33 mM equivalent to half the activity of kojic acid (0.16 mM). In another study, ellagic acid was found to have antioxidant properties with an IC_{50} value of 0.33 μg/ml (1.1μM) which was comparable to ascorbic acid (IC_{50} value 0.35 μg/ml or 1.9 μM) [39]. In the same study ellagic acid was reported to have antimicrobial activity when tested against *Escherichia coli*, *Pseudomonas aeruginosa, Candida albicans*, *Cryptococcus neoforman*, methicillin-resistant *Staphyloccus aureus*, *Aspergillus fumigatus* and *Mycobacterium intracellulare* [39, 40]. Ellagic acid also exhibited the ability to initiate cell cycle arrest, apoptosis and antitumorigenic activity in animal models for breast, leukemia, human bladder, colon and prostate cancer cells [41, 42]. The presence of ellagic aid in *Z. benthamii* root supports the traditional indications in GI complaints and whitening application in cosmetics.

The above findings suggest the potentiality of *Z. benthami* root as an economic plant material providing that further investigation on its phytochemicals and biological studies are performed. The developed validated quantification of three active compounds and the presence of ellagic acid in *Z. benthami* root could be used in grading the raw material, its commercial value and for quality control. Furthermore, learning from the Indian research, experience, and investigations in *H. indicus* over exploitation and plant conservation should be planned along with the promotion of *Z. benthami* root utilization.

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P-8 10.23177/NATPRO8.2023.06.22.012

Antiviral activity on selective bitter plants against Influenza A (H1N1) virus

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Influenza remains the most common annual recurrent global respiratory infection that uncontrollably spreads. Thai traditional practitioners use herbal medicine with bitter taste to reduce fever. Thus, three plants [*Dracaena loureiri* (DL), *Myristica fragrans* (MF) and *Mammea siamensis* (MS)] which have bitter taste and are used as antipyretic drugs in Thai traditional medicine were investigate for antiviral activity against influenza A (H1N1) virus. The cytotoxic activities of DL, MF and MS extracts were assessed in Madin-Darby Canine Kidney cells using the MTT assay. Antiviral activity against 100 $TCID_{50}$ of influenza A (H1N1) virus was performed using a cytopathic effect reduction assay and results confirmed with a hemagglutination assay. The test was divided into 3 sub-tests; pre-treatment, co-treatment and post-treatment. The study found that in the co-treatment assay the aqueous extract of MF demonstrated the most potent inhibitory effect on influenza A virus with an IC_{50} value of 2.42±1.08 μg/mL and a selectivity index (SI) of 13.37. In addition, the aqueous extract of MS revealed lower anti-influenza A activity than MF with an IC_{50} value of 22.15±19.44 μg/mL providing a SI of >9.03. While there was no plant examined which inhibited influenza A virus in both the pre-treatment and post-treatment assay. This study supports the use of these bitter-tasting plants to treat early viral infections in order to reduce the severity of infection.

Keywords: Anti-influenza, Anti-virus, *Myristica fragrans*, *Mammea siamensis*, Thai Traditional Medicine

Introduction

Influenza, which is caused by the influenza virus, remains the most common annual recurrent global respiratory infection that uncontrollably spreads [1]. The Influenza viruses is consequently responsible for a considerable amount of morbidity and mortality in both children and adults worldwide [2]. The World Health Organization (WHO) has reported that seasonal influenza is estimated to cause 290,000-650,000 fatalities each year owing to respiratory illnesses alone with the second highest mortality rates estimated in southeast Asia (3.5-9.2 per 100,000 individuals) [3]. Common signs and symptoms of influenza virus infection include fever, cough, sore throat, runny or stuffy nose, muscle or body aches, headaches, fatigue and some patients experience vomiting and diarrhea. Most people who contract the influenza virus recuperate in a few days to less than two weeks, while others develop complications including pneumonia, which can be life-threatening and lead to death [4]. At present, vaccination and two major classes of antiviral drugs, oseltamivir and amantadine, are used as prevention and treatment for influenza virus infection [5]. However, antiviral drugs have several side effects, such as, nausea, vomiting, diarrhea, abdominal pain, rash and dizziness [6]. Furthermore, the mutations of surface antigens hemagglutinin and neuraminidase, and drug resistances constantly emerge [7]. Therefore, the development of new effective drugs for treatment of influenza infection requires further research. Natural products and herbal medicines are considered as potential sources of antiviral drug candidates and

may also be safer alternatives.

The symptoms of influenza are similar to Khai-Wad-Yai from Tak-Ka-Si-La scripture [8]. In Thai traditional theory, disease is caused by an imbalance of four elements (fire, wind, water and earth), this scripture describes different fevers particularly involving the fire element. When the fire element is increased (symptom: fever), it stimulates an increase of the wind element or blood circulation (symptoms: fatigue, muscle pain, headaches). The increased fire and wind elements cause abnormality inof the water element (symptoms: runny or stuffy nose, diarrhea). To balance the fire element, the therapy focuses on heat reduction. Thus, the herbal medicine with bitter and cool taste are suggested to be used to reduce the fire element.

Dracaena loureiri Gagnep (Chan-dang), *Myristica fragrans* Houtt. (Chan-thet) and *Mammea siamensis* Kosterm. (Sa-ra-phi) all have bitter taste and have been used in Thai folk medicine to treat fever [9]. Three plants were selected to evaluate their antiviral activity. A literature review indicates that, *D. loureiri* has been shown to possess potent anti-inflammatory [10-12], anti-pyretic and antinociception [13] and antiviral activities [14,15]. *M. fragrans* has been shown to have an anti-inflammatory effect [16,17]. *M. siamensis* exhibits anti-inflammatory [18] and antiviral activities [19]. However, these three plants have never been scientifically studied for antiviral activity against influenza A virus. Therefore, the objective of this study is to investigate the antiviral activity against influenza A (H1N1) virus of *D. loureirin, M. fragrans* and *M. siamensis*.

Materials and Methods

Plant materials

All plant materials were identified by comparison with authentic herbarium specimens at the herbarium of Thai Medicinal Plants at Faculty of Pharmaceutical Science, Prince of Songkla University, Songkhla Province, Thailand.

Preparation of crude extracts

Plants were rinsed with water, chopped into little pieces, and dried in a hot air oven at 45-50 degrees Celsius. Then they were crushed into a coarse powder. Finally, each plant was macerated in 95% ethanol and decocted in distilled water.

Maceration: Each powdered plant materials were macerated with 95% EtOH for 72 h then filtered through a Whatman filter paper No.1 and re-macerated for two times. The combined extract was dried by using a rotary evaporator.

Decoction: Each powdered plant materials were boiled in distilled water for 15 minutes and filtered through a Whatman filter paper No.1. This boiling was repeated twice with the residue and dried by lyophilizer.

Cytotoxicity assay

Animal cell line: Madin-Darby Canine Kidney (MDCK) cells (obtained from the American Type Culture Collection; CCL-34) were maintained in Eagle's Minimum Essential Medium (EMEM) containing penicillin-streptomycin, amphotericin B and supplemented with 10% heat-inactivated fetal bovine serum (FBS). The cells were maintained at 37°C in a 5% CO_2 incubator and sub passage every three days.

Preparation of sample solution: The aqueous extracts were dissolved in sterile distilled water (DI water) and adjusted to a concentration of 10 mg/mL. Subsequently, a 0.22 μm sterile filter was used to filter the stock solution of the aqueous extract. The ethanolic extracts were dissolved in sterile dimethyl sulfoxide (DMSO) and adjusted to a concentration of 10 mg/mL. Until used, each stock solution was kept at -20°C.

Cytotoxicity activity by MTT assay

The cytotoxicity of plant extracts on MDCK cells were determined using the MTT assay. A confluent monolayer of MDCK cells were seeded in a 96-well flat bottom plate at with density of 2.5 x 10^6 cell/plate and incubated for 24 h at 37°C in a 5% CO_2 incubator. The supernatant was removed from each well and washed twice with 200 μl/well of 1X EMEM. Plant extracts were prepared at various concentrations by serial 2-fold dilutions with 1X EMEM. A total volume of 200 μl/well of each dilution was added into the MCDK cell and incubated at 37°C with 5% CO_2 for 3 days. After incubation, the supernatant (100 μL/well) was removed from each well. Then 10 μL of MTT solution (5 mg/mL) was added into each well and incubated at 37°C with 5% CO_2 for 2 h. Finally, the supernatant was removed and dissolved in formazan product with 100 μL/well of isopropanol containing 0.04 M HCl. The formazan solution was measured by the spectrophotometer at wavelength of 595 nm. The density of formazan was proportion to the number of viable cells. The calculation below was used to calculate the percentage of cell viability. The concentrations of extract that show survival rate ≥70% were selected for antiviral testing.

The 50% cytotoxic concentration (CC_{50}) was defined as the extract concentration that reduced the cell viability by 50% when compared to untreated controls.

% Cell viability = (OD sample ×100)/ OD cell control

Antiviral activity assay

Cells and virus: Madin-Darby Canine Kidney (MDCK) cells (obtained from the American Type Culture Collection; CCL-34) were maintained in Eagle's Minimum Essential Medium (EMEM) containing penicillin-streptomycin, amphotericin B and supplemented with 10% heat-inactivated fetal bovine serum (FBS). The cells will be maintained at 37°C in a 5% CO_2 incubator and sub passage every three days. The influenza virus (A/Puerto Rico/8/1934 (H1N1) (ATCC VR-95) was propagated in MDCK cell monolayer maintained in the virus growth medium (VGM). The VGM composed of 1X EMEM with 2 µg/mL of trypsin-TPCK (trypsintosyl phenylalanyl chloromethyl ketone) without FBS supplement. The titer of virus was estimated and stored at -80 °C until used.

Cytopathic effect (CPE) reduction assay

Cytopathic effects (CPE) are indicated by the changes in host cell morphology which are caused by the target infecting virus. The common visual observations of the host cells are swelling or shrinkage, rounding, lysis, plaques, clumping, syncytia, and inclusions. The CPE reduction assay is a widely-employed assay format to screen for antiviral agents. This test was performed to see how effective extracts are at inhibiting virus-induced CPE in mammalian cell culture. The test was divided into three time points to determine the stage at which extracts inhibit virus infection. A viral control was used in the absence of extracts, while a drug control was used in the absence of a virus, and cell control was used in the absence of a virus and extracts. All experiments were performed in triplicate. The 50% inhibitory concentration (IC_{50}) was defined as the concentration of the extract that inhibited 50% of viral replication when compared to the virus control.

Pre-treatment assay

The pre-treatment assay was used to determine whether the extracts could inactivate or prevent influenza virus from entering MDCK cell. Firstly, MDCK cells were seeded in a 96-well plate with density at 2.5 x 10^6 cell/plate and incubated for 24 h at 37°C in 5% CO_2. Secondly, a MDCK cell monolayer was washed twice with 1X EMEM (200 µL/well) before 1X EMEM (50 µL/well) was added.hen a MDCK cell monolayer was treated with two-fold serial dilutions of extracts (50 µL/well) and incubated for 1 hour at 37°C in a 5% CO_2 incubator. Thereafter, the supernatant was removed, infected by 100 $TCID_{50}$ virus (100 µL/well) for 1 hour at 37°C 5% CO_2 and washed twice to remove the inoculum. Finally, virus growth media (VGM) in a volume of 200 µl/well was added into each well and further incubated at 37°C in 5% CO_2 for 3 days.

Co-treatment assay

The co-treatment assay was used to investigate the effect of the extracts on virus inactivation before infection. Firstly, MDCK cells were seeded in a 96-well plate with density of 2.5 x 10^6 cell/plate and incubated for 24 h at 37°C in 5% CO_2. Secondly, the 200 $TCID_{50}$ of virus (60 µL/well) was co-incubated with two-fold serial dilutions of extracts (60 µL/well) in a 96-well plate for 1 hour at 37°C 5% CO_2. MDCK cell monolayers in 96-well plate were washed twice with 1X EMEM. Then, the mixture of extracts and virus (100 µL/well) was transferred to the MDCK cells monolayers for infection and incubated for 1 h at 37°C in 5% CO_2 and washed twice to remove the inoculum. Finally, VGM (200 µl/well) was added into each well and further incubated at 37°C in 5% CO_2 for 3 days.

Post-treatment assay

The post-treatment assay was used to determine whether extracts could inhibit the replication of influenza virus in MDCK cell. Firstly, MDCK cells were seeded in a 96-well plate with density at 2.5 x 10^6 cell/plate and incubated for 24 h at 37°C in 5% CO_2. Secondly, a MDCK cell monolayer was washed twice with 1X EMEM (200 µL/well) before being infected by 100 $TCID_{50}$ virus (100 µL/well) incubated at 37°C in 5% CO_2 for 1 h to allow binding and attachment of virus to the host cells. Subsequently, the unbound virus was removed and the cells were washed twice. Two-fold serial dilutions of extracts of extracts (100 µL/well) were performed and added to the infected cells in quadruplicate wells and incubated at 37°C 5% CO_2 for 3 days. The appearance of CPE was assessed by observing cell morphological under a microscope.

Hemagglutination assay

Hemagglutination assay is used to detect progeny virus in culture medium, since influenza viruses contains surface hemagglutinin glycoproteins which bind and agglutinate red blood cells. This assay was used to confirm the results of CPE observation in each well. Fifty microliters of culture supernatant was

transferred from the tested plate (pre-, co-, and post-treatment) to the corresponding wells of V-bottom 96-well plate. The 0.5% goose red blood cells (RBCs) were added to each well in a volume of 50 µl/well and incubated for 30 minutes at room temperature. When the RBCs control wells had settled completely, the hemagglutination reaction in each well was recorded. The presence of virus particles in the culture supernatant is shown by a positive hemagglutination reaction which indicates that the tested extracts could not inhibit viral replication.

The inhibition percentage (%inhibition) at each concentration of extracts was calculated from the number of wells showing CPE-inhibition (negative for hemagglutination) among quadruplicates. The CC_{50} and IC_{50} were obtained from nonlinear regression analysis of concentration-effect curves by the GraphPad Prism 5 Demo program and represent the means ± standard error of the mean of three independent experiments. The selectivity index (SI) was calculated from the ratio CC_{50}/IC_{50}.

Statistical analysis

All data are presented as means ± standard error of the mean (Mean ± SEM) from at least three separate experiments. The GraphPad Prism 5 Demo program will be used to calculate CC_{50} and IC_{50} values.

Results & Discussion

Plant materials

The voucher specimen number of each plant are shown in **Table 1**.

Table 1. Description and voucher specimen number of *D. loureiri*, *M. fragrans* and *M. siamensis*.

Plant species	Family	Voucher specimen number	Part used	Collected from
Dracaena loureiri Gagnep.	DRACAENACEAE	SKP 065 04 12 01	Heartwood	Phitsanulok
Myristica fragrans Houtt.	MYRISTICACEAE	SKP 121 13 06 01	Heartwood	Indonesia
Mammea siamensis Kosterm.	CALOPHYLLACEAE	SKP 083 13 19 01	Flower	Chantaburi

Cytotoxicity (MTT assay)

The cytotoxicity of the aqueous and the ethanolic extracts of *D. loureiri*, *M. siamensis and M. fragrans* were evaluated on MDCK cells using MTT assay to determine percentage of toxicity. All extracts were diluted in 1X EMEM at various concentrations of 200-0.04 µg/mL for cytotoxic testing on MDCK cells using the MTT assay. The results of cytotoxic activities of all extracts were shown in **Table 2** and **Figure 1**. The results showed that DLA had no toxic on MDCK cell at all concentrations. DLE and MFA were non-toxic on MDCK cell at concentrations of 12.5, 6.25 and 3.13 µg/mL. The percentages of cell viability of DLE were 101.99±13.78, 103.52±10.47 and 118.66±13.20%, respectively and MFA were 77.01±8.12, 94.97±7.43 and 90.81±3.75%, respectively. MFE was non-toxic on MDCK cell at 25, 12.5, 6.25 and 3.13 µg/mL. The results of the cytotoxicity of *M. siamensis* were highly variable.

Table 2. The percentage of MDCK cell viability of aqueous and ethanolic extracts of *D. loureiri, M. fragrans* and *M. siamensis* (mean±SEM), (n = 3).

Sample	Extract	Code	Cell viability (%) ± SEM / Concentration (µg/mL)						
			200	100	50	25	12.5	6.25	3.13
D. loureiri	aqueous	DLA	96.12±10.32	83.97±3.96	80.25±6.71	82.84±6.88	83.10±6.14	105.40±2.21	107.22±7.04
	95% EtOH	DLE	25.92±1.84	24.81±1.65	25.43±1.49	35.98±2.62	101.99±13.78	103.52±10.47	118.66±13.20
M. fragrans	aqueous	MFA	20.07±1.33	22.48±1.31	27.95±5.06	57.71±11.46	77.01±8.12	94.97±7.43	90.81±3.75
	95% EtOH	MFE	33.38±0.63	60.57±18.94	67.31±9.28	122.44±2.06	133.54±11.93	156.92±4.86	138.62±2.19
M. siamensis	aqueous	MSA	59.72±16.75	71.08±9.10	82.47±5.51	83.76±5.80	80.76±3.73	93.14±1.07	88.00±5.68

Sample	Extract	Code	Cell viability (%) ± SEM / Concentration (µg/mL)						
			3.13	1.56	0.78	0.39	0.19	0.09	0.04
M. siamensis	95% EtOH	MSE	15.93±1.62	16.13±0.89	21.30±0.97	25.71±2.08	31.95±3.23	96.22±9.64	101.56±4.72

MSA was shown to be non-toxic on MDCK cells at concentrations of 100 50, 25, 12.5, 6.25, and 3.13 µg/mL, the percentages of cell viability were 71.08±9.10, 82.47±5.51, 83.76±5.80, 80.76±3.73, 93.14±1.07 and

88.00±5.68%, respectively. While MSE was non-toxic on MDCK cells at 0.09 and 0.04 μg/mL. All extracts that were non-toxic to MDCK cells (cell viability more than 70%) would be selected to evaluate antiviral activities.

Antiviral activities

The antiviral activities of *D. loureiri*, *M. fragrans*, and *M. siamensis* aqueous and ethanolic extracts were examined using three methods: pre-treatment, co-treatment and post-treatment based on CPE inhibition assay in quadruplicates against 100 TCID50/0.1 mL of influenza virus (A/PR/8/34 H1N1 (ATCC VR-95)). Three separate tests evaluated the antiviral effects of each extract. Oseltamivir as the first line antiviral medication for treating influenza virus infection was used as a positive control drug.

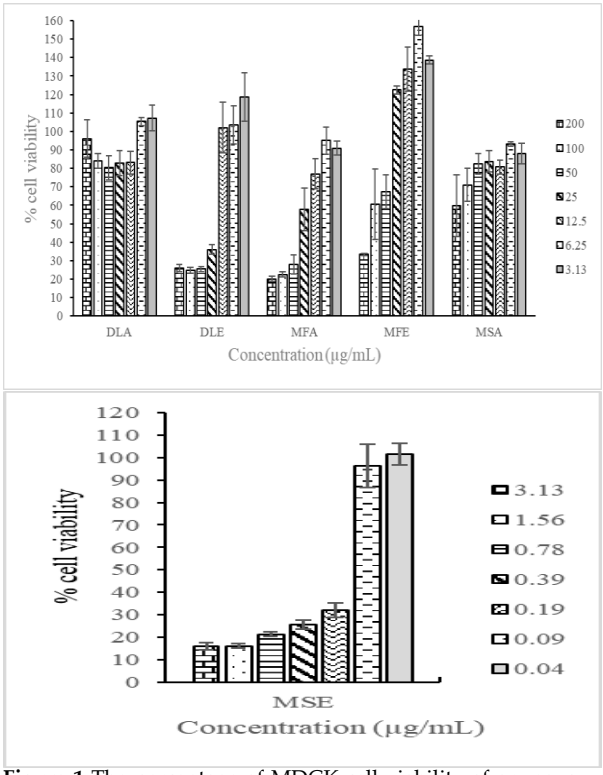

Figure 1 The percentage of MDCK cell viability of aqueous and ethanolic extracts of *D. loureiri*, *M. fragrans* and *M. Siamensis*.

Pre-treatment

The results of anti-influenza A (H1N1) virus pre-treatment are shown in Table 3. Pre-treatment with all extracts did not show inhibitory activity against influenza virus A (H1N1) virus. These results demonstrated that the aqueous and ethanolic extracts of *D. loureiri*, *M. fragrans* and *M. siamensis* could not inhibit or prevent virus entering the host cell.

Co-treatment

The result of anti-influenza A H1N1 virus of co-treatment was shown in Table 4 and Table 6. Co-treatment with DLA and DLE did not inhibit influenza A (H1N1) virus (100 $TCID_{50}$/0.1 mL). MFA was the most active for inhibition of influenza A (H1N1) virus with an IC_{50} value of 2.42±1.08 μg/mL giving a selectivity index (SI) of 13.37. In addition, MSA showed lower anti-influenza A (H1N1) activity with IC_{50} value of 22.15±19.44 μg/mL giving a SI of >9.03. These results demonstrated that MFA and MSA exhibited viral inactivation prior to infection and displayed safe antiviral activity. While MFE and MSE could not inhibit influenza A (H1N1) virus.

In this study, the selective index or SI of MFA and MSA were more than 10 thus indicative of safety. Selectivity index reflects both antiviral activity and eventual toxicity of the test compounds. The high SI value indicates the low toxicity of the test compound and high activity against the virus. Relatively low of SI (< 1) means the sample could be toxic and likely cannot be developed into a drug. If the calculated SI value is between 1 and 10, re-evaluating using another biosystem is advocated for confirmation. A SI value more than 10 was assumed to indicate that this potential treatmentshould undergo further investigation [20].

Post-treatment

The outcomes of post-treatment anti-influenza A (H1N1) virus testing displayed in Table 5. The results demonstrated that none of the extracts showed any inhibition of the influenza A (H1N1) virus. Therefore, the aqueous and ethanolic extracts of *D. loureiri*, *M. fragrans* and *M. siamensis* could not inhibit the replication of influenza A (H1N1) virus in MDCK cells

Table 3. The percentage of inhibition of influenza virus of aqueous and ethanolic extracts of *D. loureiri*, *M. fragrans* and *M. siamensis* in **pre-treatment** method (mean ± SEM), (n = 3).

Sample	% inhibition /Concentrations (μg/mL)								IC_{50} (μg/mL)
	200	100	50	25	12.5	6.25	3.13	1.56	
DLA	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	>200
DLE	-	-	-	-	0±0.00	0±0.00	0±0.00	0±0.00	>12.5
MFA	-	-	-	-	0±0.00	0±0.00	0±0.00	0±0.00	>12.5
MFE	-	-	-	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	>25
MSA	-	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	>100
Osel	-	-	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	>50

Table 4. The percentage of inhibition of influenza virus of aqueous and ethanolic extracts of *D. loureiri*, *M. fragrans* and *M. siamensis* in **co-treatment** method (mean ± SEM), (n = 3).

Sample	% inhibition /Concentrations (μg/mL)								IC_{50} (μg/mL)
	200	100	50	25	12.5	6.25	3.13	1.56	
DLA	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	>200
DLE	-	-	-	-	0±0.00	0±0.00	0±0.00	0±0.00	>12.5
MFA	-	-	-	-	100±0.00	100±0.00	66.67±33.33	0±0.00	2.42±1.08
MFE	-	-	-	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	>25
MSA	-	100±0.00	66.67±22.05	58.33±22.05	0±0.00	0±0.00	0±0.00	0±0.00	22.15±19.44
Osel			0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	>50

Table 5. The percentage of inhibition of influenza virus of aqueous and ethanolic extracts of *D. loureiri*, *M. fragrans* and *M. siamensis* in **post-treatment** method (mean ± SEM), (n = 3).

Sample	% inhibition /Concentrations (μg/mL)								IC_{50} (μg/mL)
	200	100	50	25	12.5	6.25	3.13	1.56	
DLA	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	>200
DLE	-	-	-	-	0±0.00	0±0.00	0±0.00	0±0.00	>12.5
MFA	-	-	-	-	0±0.00	0±0.00	0±0.00	0±0.00	>12.5
MFE	-	-	-	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	>25
MSA	-	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	0±0.00	>100
Osel	-	-	100±0.00	100±0.00	100±0.00	100±0.00	66.67±8.33	0±0.00	2.66±0.20

Table 6. Cytotoxicity concentration (CC_{50}), inhibition concentration (IC_{50}) and selective index (SI) of *M. fragrans*, *M. siamensis* and oseltamivir

.Sample	CC_{50} (μg/mL)	IC_{50} (μg/mL)	SI
MFA	32.361	2.42	13.37
MSA	>200	22.15	>9.03
oseltamivir	>200	2.66	>75.19

Conclusion

According to Thai traditional medicine principles, bitter tasting plants can reduce heat, and balance the fire element in the body. Thai traditional practitioners often use bitter herbs to treat fever, cooling or reducing body temperature, which may be similar or correlate to the action of antipyretic, antiviral, antiinflamatory and antimicrobial drugs in modern medicine.

In the pre-treatment and post-treatment assay, the aqueous and ethanolic extracts of all samples had no antiviral effect against influenza virus (A/PR/8/34 H1N1 (ATCC VR-95)). From these results, it can be concluded that all extracts cannot prevent and treat flu but they might be decreasing signs and symptoms of flu. In the co-treatment assay, the aqueous extract of *M. fragrans* demonstrated potent viral inactivation and *M. siamensis* showed moderate viral inactivation prior to entering the normal cells. Both MFA and MSA showed high selective index values, which suggest safe antiviral activity.

This study supported the use of bitter-tasting plants to treat early stages of viral infections to reduce the severity of infection. Further research on the action mechanism of *M. fragrans* and *M. siamensis* would be required. In addition, the isolation of active compounds for anti-influenza viral infection should be undertaken.

Acknowledgement

This research was financially supported by the Thailand Science Research and Innovation Fundamental Fund through Grant No. TUFF 28/2565 and Bualuang Professorship of Thammasat University. The authors are grateful to the Center

of Excellence in Applied Thai Traditional Medicine Research and the Faculty of Medicine, Thammasat University.

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P-9 10.23177/NATPRO8.2023.06.22.014

Anti-oxidant, anti-inflammatory activity and total phenolic content of *Cleome viscosa* L.

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Phak sian phi (*Cleome viscosa* L.) as a member of Cleomaceae, is a commonly used herb in Thai traditional remedies for longevity. It has long been used by folk doctors for making the body healthy. However, there is no research on the antioxidant and anti-inflammatory activities of its extracts. Thus, this study aimed to investigate the antioxidant activity by DPPH and ABTS assay, anti-inflammatory activity by production inhibition of nitric oxide (NO) and tumor necrosis factor (TNF-α), and also to determine the total phenolic contents of the various extracts from *C. viscosa* extracts by Folin-Ciocalteu assay. Its dried herb was extracted by maceration in 95% ethanol, it was also boiled in water or decoction. The results revealed that the 95% ethanol extract exhibited an excellent anti-inflammatory effect by inhibitory effect on nitric oxide (NO) release and tumor necrosis factor (TNF-α) with IC_{50} = 27.04±3.96 and 22.19±6.80 µg/mL respectively. The phenolic content of the 95% ethanol and water extracts were 32.08±2.96 and 26.23±0.39 mg GAE/g, respectively. However, its extracts should be further studied to isolate active compound for anti-inflammatory and antioxidant activities. It should be developed as anti-inflammatory drugs in the future.

Keywords: Anti-oxidant, Anti-inflammatory, Nitric oxide (NO), Tumor necrosis factor (TNF-α), Total Phenolic, Thai traditional medicine

Introduction

Cleome viscosa L. is a commonly used herb in Thai traditional medicine for longevity. It is a medicinal plant believed to have anti-aging properties. In Thai traditional medicine, it is used for longevity, strengthens the body, and prevents illnesses. (W. Kanthasuk, 2005). Therefore, *C. viscosa* might have anti-aging benefits, which could be due to its anti-oxidant and anti-inflammation activities. There has been no report on C. viscosa on its anti-oxidant and anti-inflammatory activities. This study was aimed to investigate anti-oxidant, anti-inflammatory activities and total phenolics content of extracts of *C. viscosa*.

Materials and Methods

Plant Materials

C. viscosa was collected from Pathum Thani Province, Thailand. Plant materials were defined through the identification of voucher specimens at the Thai Traditional Medicine Herbarium, Thai Traditional Medicine Research Institute, Department of Thai Traditional and Alternative Medicine, Nonthaburi, Thailand. Where a voucher specimen of this collection was deposited TTM-c No.1000670.

Preparation of crude extracts

Plant materials were cleaned, sliced into small pieces, oven-dried at 50°C, and ground into a crude powder. After that, C. viscosa was extracted using two methods, maceration with 95% ethanol and decoction in water.

Ethanolic extraction

Dried plant material (200 grams) was macerated with 95% ethanol (1,500 mL) at room temperature for three days, then the extracts were filtered through Whatman No.1 filter paper. The maceration of the residue was repeated twice with the same volume of solvent. The combined 95% ethanol extracts were evaporated by using a rotary evaporator. Then, the extracts were kept in a freezer at -20 °C.

Decoction method
The water extract was obtained by boiling dried plant material (500 grams) in 1 L distilled water for 15 minutes then filtered, the residue was repeated twice using the same volume of water all extracts being filtered through a Whatman paper No.1 filter paper and freeze dried by a lyophilizer. The crude extracts were kept in freezer (-20 °C).

An inhibitory effect on NO production
The RAW 264.7 cell line was cultured in RPMI 1640 medium supplemented with 10% heated fetal bovine serum (FBS), 50 IU/ml penicillin, and 50 µg/ml streptomycin. Sample solutions were prepared with various concentrations ranging from 1-100 µg/ml and added to the wells of sample and blank samples. The wells of control and blank control were added with RPMI medium. To determine NO production, the accumulation of nitrite in the culture supernatant was measured using the Griess reagent and the color was detected at a wavelength of 570 nm (Tewtrakul and Itharat, 2007.)

Determination of lipopolysaccharide (LPS)-induced tumor necrosis factor-α (TNF-α) release from RAW 264.7 cells
The RAW 264.7 cells were seeded in 96-well plates at a density of 1 x 105 cells/well and allowed to adhere for 24 hours at 37°C in 5% CO2. After that, the medium was replaced with RPMI medium 1640 (100 µl/well) containing 10 ng/ml of LPS, along with test samples at various concentrations (100 µl/well). The cells were then incubated at 37°C in 5% CO_2 for 24 hours. Subsequently, the inhibitory effect of the extract on TNF-α release from RAW 264.7 cells was determined using Quantikine mouse TNF-α ELISA kit according to the manufacturer's protocol. The supernatant was transferred into 96-well ELISA plate, and the TNF-α concentration was measured. The absorbance was measured at 450 nm using a microplate reader. DMSO was used for dissolving the test sample, and the solution was added to RPMI medium 1640. (Obermeier F)

DPPH radical scavenging assay
The 10 mg sample was taken from the ethanol extract and water extract. To test for DPPH scavenging assay, Butylated hydroxytoluene (BHT) was used as a standard antioxidant. The sample solution was then diluted to four concentrations. Next, 100 µL of the sample solution was added to each well of a 96-well microplate. Subsequently, 100 µL of DPPH solution was added to each well. The control solution were absolute ethanol and distilled water, each mixed with 100 µL DPPH. The absorbance was measured at 520 nm using a microplate reader. All concentrations were determined in triplicate. (Koleva et al., 2001).

ABTS radical scavenging activity
The ABTS cation radical decolorization method was used to determine the ABTS radical-scavenging activity of the extracts. To prepare radical ABTS, 5 mL of ABTS (7 mM) was mixed with 5 mL of potassium persulfate (2.45 mM) and kept in the dark for 12-16 hours to form ABTS·+radicals. The solution was then diluted with ethanol to obtain an absorbance of 0.700±0.020 at 734 nm. The extracts were homogenized with 1 mL of ABTS solution at different concentrations, and their absorbances were recorded at 734 nm. The ABTS radical-scavenging activity of the extract was determined which based on Gupta et al. with slight modifications. The ability of the extract to scavenge ABTS radicals was expressed as IC50 (mg/mL) (Gupta et al., 2016).

Determination of total phenolic content
The Folin-Ciocalteu method was described by Gomes et al, 2016. with slight modifications, was used to determine the total phenolic content in the extracts. The 500 µL of the sample was mixed with Folin-Ciocalteu's phenol reagent and incubated at 37°C for 5 minutes. Then 3 mL of 2% saturated Na2CO3 was added, and the mixture was left in the dark at room temperature for 60 minutes. Absorbance was measured at 760 nm using a UV-Vis spectrophotometer (Perkin-Elmer Lambda, US) against a reagent blank. Gallic acid served as the reference standard, and the total phenolic content was expressed as mg of gallic acid equivalents per gram of each extract on a dry weight basis (mg GAE/g extract) (Gomes et al., 2016).

Results & Discussion
The results showed that the anti-inflammatory properties of its ethanolic extract by using the NO production inhibition method exhibited an IC_{50} value of 27.04±3.96 µg/mL, as well as TNF-α with an IC_{50} value of 22.19±6.80 µg/mL, indicating that ethanol extract of C. viscosa has anti-inflammatory effect. This result was not corresponding with the previous report (Anuthakoengkun and Itharat, 2014) because this study showed high anti-inflammatory activity but the previous report showed less anti-inflammatory effect

on NO production inhibition. This may be due to fresh plants being used in this study which may contribute to the good anti-inflammatory effect. Both water extract and ethanolic extract showed less anti-oxidant activity by the tested methods. Other antioxidant testing methods should be performed in further studies. In addition, ethanolic and aqueous extracts have total phenolic content as 32.08±2.96 and 26.23±0.39 mg Gallic eq./g, respectively which could not be related with the activity. This suggested that other compounds may contribute to the anti-inflammatory effect which requires further investigation.

Conclusion

In conclusion, the 95% ethanolic extract of C. viscosa possessed potent anti-inflammatory activity as determined by production inhibition on NO and TNF-α. These findings supported Thai traditional use of C. viscosa for maintain a healthy body by reducing inflammation. Thus, its ethanolic extract should be further studied in the animal model and isolated active compound for anti-inflammatory activity.

Table1. Percent inhibition and percent survival of C. viscosa extracts using NO production inhibition assay (n=3)

Scientific name	Extract	% inhibition at conc. µg/mL (% survival)				IC_{50} (µg/mL ±SEM)
		100	50	10	1	
C. viscosa	95% Ethanol	92.84±2.01 (87.70±3.00)	73.99±4.61 (91.18±11.33)	19.77±3.06 (95.99±5.00)	-3.19±3.49 (92.89±1.78)	27.04±3.96
	Water					>100
Prednisolone						0.15±0.03

Table 2. Percent inhibition of *C. viscosa* extracts using tumor necrosis factor (TNF-α) assay (n=3)

Scientific name	Extract	% inhibition at conc. µg/mL				IC_{50} (µg/mL ±SEM)
		100	50	10	1	
C. viscosa	95% Ethanol	63.35±0.78	53.86±2.84	24.12±10.56	-14.50±14.29	22.19±6.80
	Water					>100

Table 3. Anti-inflammatory, Anti-oxidant activity and Total Phenolic content of C. *viscosa* extracts

Scientific name	Extract	Anti-inflammatory activity		Anti-oxidant activity		Total Phenolic content
		NO production inhibition (IC_{50} mean±SEM; µg/mL)	TNF-α assay (IC_{50} mean±SEM; µg/mL)	DPPH assay EC_{50} (mean±SEM; µg/mL)	ABTS assay EC_{50} (mean±SEM; µg/mL)	mean±SEM; mg Gallic eq./g
C. viscosa	95% Ethanol	27.04±3.96	22.19±6.80	>100	>100	32.08±2.96
	Water	>100	>100	>100	94.03±1.57	26.23±0.39
Prednisolone		0.15±0.03				
BHT				17.20±0.39		
Trolox					5.02±0.32	

Acknowledgement

This research was financially supported by the Thailand Science Research and Innovation Fundamental Fund through Grant No. TUFF 28/2565. The authors are grateful to the Center of Excellence in Applied Thai Traditional Medicine Research and the Faculty of Medicine, Thammasat University, for providing laboratory facilities.

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P-10 10.23177/NATPRO8.2023.06.22.022

Preliminary study on the gut metabolism of cannabidiol

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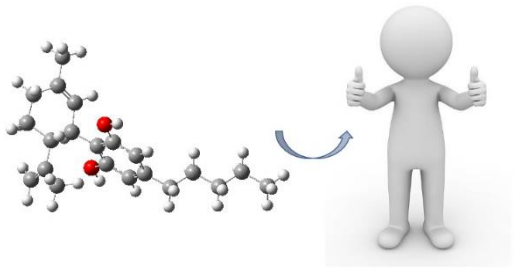
To provide basic information related to the in-body metabolism, gut metabolism of cannabidiol was studied with human microflora under anaerobic conditions. For the analysis of the metabolites, TLC and HPLC were adopted, and the gut bacteria exhibiting cannabidiol biotransformation activity were screened from fecal samples and isolated anaerobically by the serial dilution technique on GAM plates.



Keywords: Biotransformation, Cannabidiol, Cannabinoids, Gut bacteria, Gut metabolism, Terpenoids

Acknowledgement This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2021R1A2C2007712).

P-11 10.23177/NATPRO8.2023.06.22.019

Characterization of *O*-demethylase for the aryl ether cleavage

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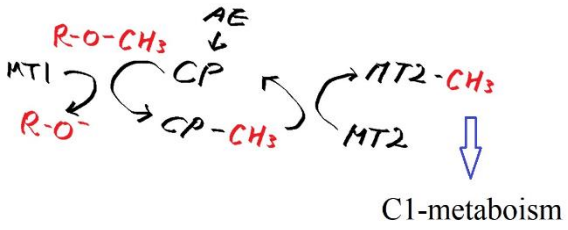
Human gut bacterium Blautia sp. MRG-PMF1 harbors O-demethylase exhibiting broad substrate spectrum for the various aryl allyl ethers and aryl methyl ethers to initiate one-carbon metabolism. The genes for this putative corrinoid-dependent enzyme, methyltransferase I, corrinoid protein, methyltransferase II, activating enzyme, were cloned and over-expressed to investigate the biochemical reaction mechanism. Here, preliminary characterizations of the proteins were reported.



Keywords: C-O bond cleavage, Corrinoid protein, Gut metabolism, Methyltransferases

Acknowledgement This work was supported under the framework of international cooperation program managed by National Research Foundation of Korea (NRF-2022K2A9A1A01097855).

P-12 10.23177/NATPRO8.2023.06.22.021

Reactivity of DgpBC, C-deglycosidase from *Dorea* sp. MRG-IFC3

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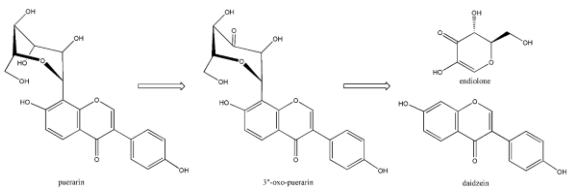
To study the biochemical reaction mechanism of C-deglycosidase which cleaves the glycosidic C-C bond, the genes of dgpBC were cloned from human gut bacterium *Dorea* sp. MRG-IFC3. DgpBC was overexpressed from pETDuet-1 plasmid in *E. coli* BL21(DE3) and the expressed recombinant protein was purified. Various glycosides, as well as synthetic compounds, were reacted with DgpBC. The reactivity of DgpBC was related to the biochemical reaction mechanism.



Keywords: C-glycoside, C-C bond cleavage, Gut metabolism, Mechanism, Flavonoids

Acknowledgement: This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of

Education (NRF-2021R1A2C2007712).

P-13

Microscopical characteristics, total phenolic content and antioxidant activities of root and stem from *Sida acuta* Burm. F. extracts

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Sida acuta (SA) belongs to the family Malvaceae. It is a medicinal plant that has long been used in Thai traditional and alternative medicine. It is used in the composition of various medicinal formulations such as anti-edema, analgesic and nourishing elements. The objectives for this study were to characterize microscopically, analyze the total phenolic content by Folin-Ciocalteu method and investigate antioxidant activities by DPPH and FRAP methods of roots and stems from SA. SA were extracted using three methods as follows; maceration with 95% ethanol, maceration with 50% ethanol and decoction. The microscopical characteristics of SA powder have distinguishing features such as a large number of fibers and vascular bundles both in stems and roots. The most vascular bundle types are bordered pitted and reticulate vessels. The result of total phenolic content found that the roots and stems extracts from SA are in the range of 16.97±0.42 to 86.19±1.00 mg GAE/g extract. The 95% ethanolic extract from roots of (SARE95) showed the highest total phenolic content of 86.19±1.00 mg GAE/g extract. The DPPH method showed that the roots had better antioxidant activity than the stems compared to extraction with the same solvent. It was determined that 50% ethanolic extract of the root (SARE50) had the best antioxidant activity, followed by 95% ethanolic extract of the root (SARE95) with EC50 values of 48.05±1.23 and 55.38±0.56 μg/mL, respectively. The 95% ethanolic extract of roots (SARE95) contained the highest FRAP and TEAC (137.07±2.73 mg Fe(II)/g extract and 57.77±1.31 mg Trolox/g extract). The roots and stems extracts demonstrated good antioxidant activities. Therefore, future investigations of the active substances and other biological activities are warranted.

Keywords: Antioxidant activity, Microscopical Characterization, *Sida acuta*, Total phenolic content

P-14

Embryotoxicity test of the anti-anemia drug from Amaranthaceae family

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A plant extract from Amaranthaceae family (APE) is available as a natural product that can combat anemia in the Philippines. This study aims to evaluate the effects of APE as a supplement for pregnant mothers by testing the embryotoxic and possible teratogenic effects on pregnant mouse. Pregnant dams were given low and high dosages of APE, as well as 10,000 IU/kg of isotretinoin (PC), soybean oil (VC), and distilled water (NC). Reproductive, histological parameters, and gene expression pattern changes were observed on embryos of pregnant dams. Dams and embryos exposed to isotretinoin have decreased reproductive performance and induced teratogenicity. Low dose of APE had no effect on dam reproductive performance or embryo development (E11.5). However, high dose of APE caused slight reduction in reproductive performance and embryo growth, however this was not statistically significant when compared to negative control group. During immunohistochemical analysis, expression of BCL-2 was significantly reduced in neuroepithelium, neural crest cell derivatives, and liver in isotretinoin group. For APE treated groups, a significant increase of BCL-2 antiapoptotic protein was observed in liver of embryos (E11.5). The findings in this study suggests the use of the APE as a supplement at low dose can be beneficial during pregnancy.

Keywords: Amaranthaceae, BCL-2, immunohistochemistry, isotretinoin, Teratogenicity

P-15

Anticancer and antioxidant activity of *Alstonia scholaris* (Linn.) R. Br. (Gentianales: Apocynaceae) ethanolic leaf extract on human liver cancer cell line (HepG2)

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Alstonia scholaris (Linn.) R. Br., commonly known as blackboard tree, is used in traditional medicine and reported to possess several biological activities. The present study aimed to investigate the anticancer and antioxidant potential of A. scholaris ethanolic leaf extract on HepG2 cells. Percent inhibition and IC_{50} were calculated through MTT assay. The anticancer activity was evaluated through investigating the apoptotic activity by DAPI/PI staining, determining the inhibition of cell migration using wound healing assay and exploring the effect the extract on the expression of biomarker genes. Results showed that *A. scholaris* ethanolic leaf extract was significantly cytotoxic to liver cancer cells and selective to normal cells. Furthermore, percentage of apoptotic cells and inhibition of cell migration was greater in cells treated with the extract than with positive control, 5-fluorouracil. These findings were concordant with the gene expression analysis. The extract caused upregulation of tumor

suppressor gene p53, pro-apoptotic gene Bax and apoptosis executioner gene Caspase-7. The evaluation of antioxidant activity using DCFDA staining assay is currently in progress. The findings suggest that *A. scholaris* ethanolic leaf extract could potentially be a source of anticancer agents.

Keywords: *Alstonia scholaris*, Liver cancer, Anticancer, Antio xidant, HepG2

P-16

Pro-myogenic actions of crude leaf extract from Clusiaceae toward C2C12 muscle cell line

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Crude leaf extracts (CLE) from Clusiaceae were tested on C2C12 cells to determine its effect on myoblast count and myotube formation in a dose dependent manner. For myoblast count, C2C12 myoblast were observed after 24 and 48 hrs. of CLE treatment. Based on the results, CLE did not confer cytotoxic effects and precocious myotube formation was observed after 48 hours of treatment. Ki67, marker of proliferating cells. expression was not statistically significant among treatment groups. For induction of differentiation assay, myotube area, stained with May-Grünwald Giemsa, was examined at 1-, 3-, and 7-day post treatment (dpt), and images were obtained and analyzed using ImageJ. For induction assay at 3 dpt, higher myotube area was observed as the concentration of the extracts decrease. Concentrations of 0.781, 0.391, and 0.195 μg/mL showed significant differences compared to growth medium control in 3 dpt ($p<0.05$) and 7 dpt ($p>0.05$) and no significant differences were observed. There was also no observed significant difference in the expression of the muscle marker, MyoD. The increased size of the myotube may be explained based on glucose consumption, this was examined by acquiring glucometer readings from cell culture media. The glucometer reading shows a statistically significant lower glucometer reading in the CLE-treated group. These indicate that extract may contain compounds that promotes myoblast growth and increased myotube size.

Keywords: C2C12, cell culture, pro-myogenic, plant extract

P-17

The effects of *Tabernaemontana pandacaqui* Poir. leaf extracts on the nonspecific immune response of cyclophosphamide-treated Balb/C mice

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Tabernaemontana pandacaui Poir. is a plant species native to the Philippines that is traditionally used to treat various diseases such as microbial infections, inflammation, and fever. This study evaluated the immunomodulatory activity of *T. pandacaqui* leaf extracts in immunosuppressed Balb/C mice using different immune response assays in vivo. The experimental setup includes 3 groups of mice – the negative control group with sterile phosphate-buffer saline (50 mL/kgBW), another group with cyclophosphamide (30mg/kgBW), and the last group with plant extract (5mg/kgBW) combined with cyclophosphamide. The plant extract induced the lowest superoxide anion production which suggests that *T. pandacaqui* further reduced the superoxide anion produced by the immunosuppressed Balb/C mice and possibly has a high antioxidant capacity. It also had the lowest % cell proliferation of splenic lymphocytes which suggests that *T. pandacaqui* has antiproliferative effects. On the other hand, the plant extract induced higher plasma lysozyme activity compared to cyclophosphamide indicating greater pathogen killing capacity of macrophages from mice treated with *T. pandacaqui*. The leaf extract of *T. pandacaqui* was observed to exhibit antioxidative and antiproliferative activities with increased lysozyme activity. This is a preliminary study of the immunomodulatory potential of *T. pandacaqui* and therefore, it is recommended that further studies be undertaken to fully elucidate its bioactivity by employing other immune response and complementary assays.

Keywords: *Tabernaemontana pandacaqui*, Pandakaki, Immunomodulation, Cyclophosphamide

P-18

Formulation development of vegetable oil-based nanoemulsions loaded with a Thai traditional remedy used for osteoarthritis

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Benjakul (BJK) combines five herbs widely used in Thai traditional medicine. It has been reported to treat osteoarthritis patients. This study was to investigate for development of BJK-loaded formulas to be an anti-inflammatory product. The novel topical BJK-loaded

nanoparticles containing natural vegetable oil as an internal oil phase was developed. Vegetable oils are preferred for direct caring of the skin due to their biocompatible and present advantages on the skin. The preparation method was done using D-phase emulsification (DPE), a low-energy process. The formulas used four elements: oil, water, polyethylene surfactant, and polyol. Various oils were studied to find an appropriate oil with an excellent solubilizing capacity for Benjakul extract. The solubility of BJK extract was measured by HPLC using piperine as a chemical marker. The influence of the nanoemulsions compound was carried out using the Box-Behnken experimental design. Fifteen formulations, including three central points, were prepared. BJK extract would be added to each formula at a concentration of 0.5% w/w. Even though the previous study reported that stable nanoemulsions were carried up to 40.0% w/w of vegetable oil, applying the DPE process. From this study, nanoemulsions were formed with a particle size of 200 – 300 nm.

Keywords: Benjakul, Nanoemulsions, D-phase emulsification, Vegetable oil-based nanoemulsion

Acknowledgement: This work was supported by Thailand Research Fund through the Royal Jubilee PhD Program (Grant No .PHD/0071/2557), the National Research University Project of Thailand, Office of Higher Education Commission, Thammasat University, Centre of Excellence in Applied Thai Traditional Medicine Research, Bualuang ASEAN Chair Professorship, Faculty of Medicine, Thammasat University, Thailand and Drug Development and Innovation Centre, University of Alberta, Canada.

P-19

The estrogenic activity and chemical contents of *Asparagus racemosus* extract for women's health

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Asparagus racemosus Willd (Shatavari) has long been used to treat women's diseases following Ayurvedic principles in India and Thai traditional medicine because there are many hormone-like substances. Thus, this research aimed to investigate the estrogenic and anti-estrogenic activities of *A. racemosus* ethanolic extract and examine its chemical constituents, such as total isoflavone and saponin contents. Total isoflavone and saponin contents were determined by colorimetric method, using genistein and sapogenin as standard substances, respectively. The extract was studied on the activity to stimulate the growth of two types of breast cancer cells (T-47D and MCF-7), which respond to estrogen by using 17β-estradiol as a positive control. The results found that the extract contained total isoflavone and saponin contents of 7.34 ± 0.82 mg GE/g dry extract and 291.67 ± 7.24 mg DE/g dry extract, respectively. This extract showed anti-estrogenic activity at high concentrations by 99% suppressive activity against T-47D and MCF-7 of 57.30 and 32.29 μg/mL, respectively. However, it showed stimulatory activity of E2-enhance breast cancer cell proliferation at low concentrations, therefore called "Phytoestrogen". These results can support using *A. racemosus* ethanolic extract for women's health for estradiol-like hormone and anti-estrogen in breast cancer treatment by adjusting suitable doses.

Keywords: *Asparagus racemosus*, Estrogenic activity, Anti-estrogenic activity, Phytoestrogen

Acknowledgement: This research was financially supported by the Thailand Science Research and Innovation Fundamental Fund through Grant No. TUFF 28/2565. The authors are grateful to the Center of Excellence in Applied Thai Traditional Medicine Research and the Faculty of Medicine, Thammasat

P-20

Mass production of naringenin by elicitor treatment in the prothallium culture of *Crypsinus hastata* using a bioreactor

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Since *Crypsinus hastatus* is effective for diuresis and detoxification, the whole plant except for the root has been used as a medicine since ancient times. Recently, bioreactors have been used as systems for mass production of high value-added plant cells. In this experiment, we tried to investigate the content of Naringenin, an effective substance, in the prothallium of *Crypsinus hastatus* using a bioreactor. As an experimental material, an MS medium containing 3% sucrose was used as a prothallium of *Crypsinus hastatus*. In order to investigate the growth of the prothallium of *Crypsinus hastatus* in solid culture, liquid suspension culture, and bioreactor culture, as a result of culturing for 5 weeks with different culture methods, the biomass was the highest with 206g/L Fresh weight in the bioreactor. In order to investigate the difference in the content of effective substances according to the elicitor treatment, MJ (Methyl Jasmonate) and SA (Salicylic acid) were treated at 100μM and 200μM, respectively, and as a result of one-week culture, the Naringenin content was the highest at 4.6g/L in the treated 200μM MJ. As a result of this study, mass production of prothallium of *Crypsinus hastatus* using a bioreactor suggests the possibility of being a useful resource for food and drug development.

Keywords: *Crypsinus hastatus*, bioreactor, naringenin, prothallium, methyl jasmonate

P-21

Total isoflavone, estrogenic effect and anti-androgenic effect of coconut and palm haustorium

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Young coconut juice is a good source of phytoestrogen which helps to reduce menopausal symptoms and some pathologies associated with Alzheimer's disease, moreover it can also promote wound healing. Palm was used in Thai traditional remedy for reduce hair loss and gray hair. However, the relative knowledge about estrogen has never been studied about comparative reproductive hormone effect in coconut (*Cocos nucifera* L.) and palm (*Borassus flabellifer* L.) haustorium. Therefore, this study aims to investigate the contents of total isoflavone and in vitro activities in terms of estrogenic and anti-androgenic effect. Coconut haustorium (CH) and palm haustorium (PH) were extracted by maceration with 95% ethanol, decoction, and squeezing(juicing) methods. PH and CH decoction extracts showed the highest total isoflavone contents at 2.02±0.97 and 0.99±0.27 mg Genistein equivalent/g extract respectively. The concentration at 33.41 μg/mL of CH and 7.35 μg/mL of PH decoction extracts enhance the E2 effect equivalent level of 100 pM (EqE100) and 1 pM (EqE1) respectively when positive control genistein occurred EqE100 and EqE1 as 0.86 and 0.003 μg/mL. Determination of anti-androgenic effect showed that 100 μg/mL of CH juicing extract and 10 μg/mL of PH ethanolic extract inhibited LNCaP cell proliferation on 124.73±2.36% and 143.38±23.88%, while positive control finasteride 10 μg/mL was 141.58±7.68%. These results suggest that coconut haustorium and palm haustorium show both estrogenic and anti-androgenic effect. It may be used and support for hormone supplement in the Thai traditional knowledge.

Keywords: Coconut, Palm, Isoflavone, Estrogen, Androgen, Haustorium

Acknowledgement: The author would like to express sincere thanks the Center of Excellence in Applied Thai Traditional Medicine Research, Faculty of Medicine Thammasat University for counseling and supporting laboratory facilities.

P-22

Pharmacognostic studies of Ko-klan selected from pharmacies shop in Thailand

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Ko-Klan is commonly used for treatment pain relief. There are three species of Ko-klan: such as *Mallotus repandus* (Willd) Mull. Arg. (MR); *Anamirta cocculus* (L.) Wight & Arn. (AC); and *Croton caudatus* Geiseler (CC). The objective of this study was to investigate Ko-klan in pharmacy shops in Thailand which types were sold in Pharmacy shop and also comparative determined anti-inflammatory effect on both types of Ko-klan. Pharmacognostic studies included the macroscopic, microscopic, and chemical thin-layer chromatography were investigated. The similarities and differences in *C. caudatus* and *M. repandus* regarding ethnopharmacology, pharmacognosy, and pharmaceutical science. Differences existed in the Macroscopic method: leaves, stems, flowers, fruits, and seeds of *C. caudatus* (CC) were found to have central holes and be odorless, whereas *M. repandus* (MR) had no central holes and was fragrant and thorny. Identification of Ko-klan which were collected from 20 pharmacies, M. repandus was found higher type than *C. caudatus* (50.00% and 10.00%, respectively), Eight sample showed mix herbs. These results imply that Ko-Klan herbs in pharmacies in Thailand should be studied for bioactivity related inflammation. The results may need to be more tested on anti-inflammatory activity for treating neuromuscular disease.

Keywords: *Mallotus repandus* (Willd) Mull. Arg., *Croton caudatus* Geiseler, Anti-inflammatory, Ko-Klan

P-23

Extract from shoots of a native Cucurbitaceae plant display seasonal variation in phytoestrogenic activities and phenolic content

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The Cucurbitaceae family include several species which serve as important agricultural crops. Cucurbits are rich in phytochemicals, such as phenols, which have estrogenic and anti-estrogenic properties that may serve as potential pharmacologic agents. Hence, this study sought to determine whether extracts obtained from shoots of a native cucurbit cultivated in the Philippines display estrogenic response using an estrogen- inducible luciferase reporter gene reporter system. We found that a certain fraction (F5.6) isolated through solid-phase extraction inhibited the transactivation of estrogen-induced luciferase reporter gene without affecting cell viability in T47D breast cells, independently of season and geographical location. Inhibition was also observed in the E2-induced expression of SOD2, an antioxidant enzyme

upregulated in advanced breast cancer cells. The F5.6 fraction also exhibited high phenol concentrations, but concentrations varied depending on season, with a higher concentration found during northeast monsoon season. The fraction with the highest phenol content induced the highest mRNA expression of ESR1 and the estrogen-responsive gene GREB1A. Overall, this study demonstrated that the native cucurbit plant contains bioactive phenols that vary depending on the season, providing insights into a possible cultivation approach for generating bioactive phenols.

Keywords: Cucurbits, Estrogen response, Monsoon, Plant extract, Phenolic content

Acknowledgement: This work was funded by the University of the Philippines Diliman, Office of the Vice President for Academic Affairs, Emerging, Interdisciplinary Research Program (OVPAA-EIDR, EIDR-C08-006, MCV).

P-24

The antioxidant activity, inhibition of nitric xxide production, and stability of Thai yellow curry paste extract

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Thai progenitors imparted the recipe for curry paste through their culinary knowledge. In the southern region of Thailand, Yellow Curry Paste is a popular use in all curry. Its ingredients are chilli, turmeric, and garlic. This research aims to determine the antioxidant and the anti-inflammatory activities. The 95% ethanolic Thai Yellow Curry Paste extract (YCE) was also evaluated for stability. The extraction methods were maceration in 95% ethanol (YCE) and decoction in water (YCW). DPPH assay was used to examine antioxidant activity. Anti-inflammatory activity was evaluated by measuring the inhibitory effect on nitric oxide production from the RAW 264.7 macrophage cell line. Accelerated conditions were also used to test the stability of the best extract which showed high activity. YCE showed high antioxidant activity (EC_{50} =19.54 ± 2.23 µg/ml), but it was less effective than BHT as a positive antioxidant compound (EC_{50} =13.60 1.73 g/ml). However, YCE is also an effective inhibitor of nitric oxide production, its activity was lower than that of the positive control, prednisolone (IC_{50} = 15.95 ± 0.85 and 0.56 ± 0.2 µg/ml). The YCW was inactive in every assay. The stability of YCE was then evaluated. Its antioxidant stability was stable. Therefore, YCE is suitable for future development into pharmaceutical or health food products due to its high antioxidant activities, anti-inflammaory effect on inhibitory nitric oxide production, and it is antioxidative stable when is keep in room temperature for two-year.

Keywords: Yellow Curry Paste, Stability, Antioxidant, Nitric Oxide Production

Acknowledgement: This study was funded by the Department of Applied Thai Traditional Medicine and Center of Excellence in Applied Thai Traditional Medicine Research (CEATMR), the Thailand Science Research and Innovation Fundamental Fund through Grant No. TUFF 28/2565.

P-25

Safety and preliminary study on allergic rhinitis treatment with Prabchompoothaweep Remedy at U-Thong hospital: Historical analysis of data from 2017 to 2022

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Prabchompoothaweep (PCW) remedy is Thai folk medicine which was listed in the National Herbal Drug List of Thailand 2011. This remedy is used to relieve common cold and allergic rhinitis (AR). It is composed of twenty-three herbs that mostly ingredients are arial part of sea holly (*Acanthus ebracteatus* Vahl.), arial part of motherwort (*Leonurus sibiricus* L.) and seed of black pepper (*Piper nigrum* L.). Several biological activities of PCW remedy related allergy have ever been reported including anti-allergic, anti-inflammatory and antioxidant activities. In addition, PCW remedy was prescribed for patients with AR symptoms at U-Thong hospital (Suphanburi province), Kapchoeng hospital (Surin province) and Wang-namyen (Sa-kaeo province) but the literature report of PCW remedy is scarce. Thus, this retrospective observational study was aimed to assess the safety and preliminary therapeutic effect of PCW in patients with AR at U-Thong hospital. The data was reviewed from medical records of AR patients diagnosed by modern physician and Thai Traditional Doctors. Patients who received 2 capsules of 400 mg PCW before meals 3 times per day continuously and were treatment at least 2 times from year 2017 - 2022 at U-Thong hospital, Suphan buri province. The 431 AR patients, 111 received at least 2 treatments with mean age 51-70 years. 71.17% were females and 89.19% received only PCW, the other 10.81% received PCW and Loratadine. Patients treated with PCW reported improvement 96.97% which was itchy eyes/conjunctivitis 100%,

stuffy nose 97.78%, runny nose 97.47% and sneeze 96.97%. The other group received PCW with Loratadine 1 tablet of 10 mg before breakfast, after treatment all symptoms get better 100%. There were no adverse events occurred on both groups. In conclusion, there was no significant difference in symptom improvement between patients receiving PCW alone and patients receiving combination of PCW and Loratadine. (Z = 0.575, p= 0.05)

Keywords: Prabchompoothaweep, Clinical Trial, Allergic rhinitis

Acknowledgement: This research was financially supported by the Thailand Science Research and Innovation Fundamental Fund through Grant No. TUFF 28/2565. The authors are grateful to the Center of Excellence in Applied Thai Traditional Medicine Research and the Faculty of Medicine, Thammasat University, for providing preliminary data of PCW.

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P-26

Effect of elicitation period on secondary metabolite contents of in vitro *Boesenbergia rotunda* Shoots

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Secondary metabolite enhancement from plant tissue culture through elicitation has been widely used for medicinal plants. Elicitation period is one of the key factors in production of secondary metabolites in many plant species. Therefore, the objective of this study was to determine elicitation period of 200 μM MeJA on pinostrobin, total phenolic and total flavonoid contents, and radical scavenging activity of in vitro *B. rotunda* shoots. The in vitro shoots of *B. rotunda* were cultured on MS medium supplemented with 8.87 μM BA and 2.69 μM NAA in combination with 0 and 200 μM MeJA for 3, 4 and 5 weeks. The results indicated that the 4 and 5 week-elicited shoots contained higher contents of pinostrobin (18.34±0.18 and 18.46±3.30 mg/g dry extract, respectively), total phenolics (87.23±3.39 and 84.44±2.44 mg GAE/g dry extract, respectively) and flavonoids (195.42±1.82 and 194.44±4.83 mg QE/g dry extract, respectively) than the 3 week-elicited shoots and the control at the same elicitation period. These two elicitation period treatments also produced the greatest enhancement in DPPH radical scavenging (136.51±1.91 and 132.22±1.84 μg/ml, respectively). This study indicated that elicitation with 200 μM MeJA for 4 weeks was suitable for secondary metabolite enhancement in *B. rotunda* shoots.

Keywords: Antioxidant, Elicitation period, MeJA, Phenolic, Pinostrobin

Acknowledgement: This study was supported by the Thammasat University Research Fund.

P-27

Anti-oxidant and anti-inflammatory activities of various extracts of *Etlingera Elatior* inflorescences

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The therapeutic potential of the Inflorescence of *Etlingera elatior* (E. elatior), an edible plant belonging to the Zingiberaceae family, has been used in traditional medicine for the management of non-communicable diseases (NCDs). In this study, we aimed to evaluate the DPPH scavenging activity and inhibitory effect on nitric oxide production of various extracts obtained from *E. elatior* inflorescences. The bracts of *E. elatior* inflorescences were dried and extracted through maceration (using ethyl acetate, 95% ethanol, 70% ethanol, and 50% ethanol) and decoction methods. All extracts were evaluated for their ability to scavenge DPPH radicals and inhibit nitric oxide production in LPS-induced RAW264.7 cells. The 50% ethanolic extract exhibited the highest DPPH scavenging activity, with an EC_{50} value of 16.73±1.58 μg/mL, followed by the 70% ethanolic extract (EC_{50}=23.78±3.46 μg/mL). Conversely, the ethyl acetate and 95% ethanolic extracts displayed no scavenging effect. Regarding the inhibition of nitric oxide production, the 70% ethanolic extract demonstrated the most significant effect, with an IC_{50} value of 16.36±4.19 μg/mL, comparable to the 95% ethanolic extract (IC_{50}=16.78±7.21 μg/mL). The other extracts exhibited moderate inhibitory effects with IC_{50} values exceeding 50 μg/mL while

the aqueous extract showed no activity. Notably, the 70% ethanolic extract demonstrated both DPPH scavenging and nitric oxide inhibitory activities, marking the first report on its effectiveness in both antioxidant and anti-inflammatory properties. Nevertheless, additional investigations encompassing other NCD-related activities and in vivo studies are imperative to facilitate further development utilizing *E. elatior* extracts for NCD management.

Keywords: *Etlingera elatior*, Anti-inflammation, DPPH scavenging, Nitric oxide

Acknowledgement: This work was supported Regional Medical Sciences Center 12 Songkhla and funded by Department of Medical Sciences, Ministry of Public Health, Thailand.

P-28

Multistage and multiple molecular dissociation tandem mass spectrometry based searchable natural product spectrum library

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Multiple tandem mass (MSn) spectral library is the fastest way to accurately annotate mass spectra from screening metabolites in fields such as natural product analysis, drug screening, peptidomics, lipidomics, and metabolomics. The confidence in search result of chemical structures is impacted by instrumental settings and requirements. In this study, we standardize the mass spectral DB, which enables cross-search between instrument and laboratories. In order to acquire the mass spectrum, 3 types of Orbitrap MS instrument (XL, Velos, Fusion) were tested in two laboratories. Furthermore, in order to improve the accuracy of the search results, it is possible to cross-verity the spectral results of the CID (collision induced dissociation) and HCD (higher energy collisional dissociation) type tandem mass spectrum. We developed the searchable tandem mass spectral library to improve convenient natural product identification using UPLC-MSn. The library is working in the NIST MS search platform and contains all compound information, such as including tandem mass spectrum, retention time, and chemical properties. Recently, the searchable 2,119 tandem mass spectra DB has been expended using 264 natural product standards with CID and HCD fragmentation method.

Keywords: Mass spectrometry, Spectral library, Tandem mass spectrum search

P-29

Combined nanoemulsion formulation of tea tree and cypress oil for enhanced physical stability, antioxidant and antimicrobial properties

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Tea tree oil and cypress oil are both renowned for their potential therapeutic properties, such as antimicrobial and anti-inflammatory effects. By combining these oils in a nanoemulsion, it is anticipated that their synergistic effects can be harnessed to create a novel formulation with enhanced efficacy. This study presents the development of a combined nanoemulsion formulation using tea tree oil and cypress oil (1:1 ratio) with increased antibacterial characteristics, high physical stability, and tiny droplet size. The stability of various NEs formulations was then assessed at three different temperatures: 25°C, 45 °C, and 60 °C. The range of combined NE preparations had a mean droplet size of 80.1 to 96.2 nm and a zeta potential of (-)21.3 to (-)22.0. The antibacterial properties of the NEs against Escherichia coli, Staphylococcus aureus, and Bacillus subtilis were examined and showed a greater than 75% reduction in bacterial growth inhibition, followed by confirmation of cell viability via AO/EB dual staining. The preparation also showed significant antioxidant activities. The combined formulation was then mixed with various ingredients to develop two cosmetic products i.e., nano mist and nano lotion and then these products were also assessed for their biological activities. Our research indicates that NEs might be used as nanomedicines to treat pathogenic bacterial infections without causing any visible negative effects.

Keywords: Nanoemulsion, Formulation, Tea tree oil, Cypress oil, Nanomedicines

P-30

Effects of Hua-Khao-Yen extract on vascular endothelial growth factor hepatocellular carcinoma-bearing rats

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Hepatocellular carcinoma (HCC) is the most common type of liver cancer. However, the treatment for HCC is still limited and expensive. There are many biomarkers for HCC, including vascular endothelial growth factor (VEGF). VEGF is a protein that promotes growth of blood vessels allowing

HCC growth and VEGF is also associated with HCC metastasis. *Dioscorea membranacea* (DM) is a traditional Thai drug that is used as a complimentary treatment for patient with HCC. Sorafenib is a standard drug prescribed for HCC patients. This research aims to study the effect of *D. membranacea* in VEGF regulation compared to Sorafenib. Six groups of Wistar rat were used consist of the following: Control group (CPG), control group with DM (CDM), HCC control group (HPG), HCC treated with 4 mg/kg of DM (HDM4), HCC treated with 40 mg/kg of DM and HCC treated with 30 mg/kg of Sorafenib (HSF). HCC was induced by intraperitoneal injections of diethyl nitrosamine (DEN) and thioacetamide (TAA). All groups were euthanized, and livers were collected to analyze with real-time PCR and immunohistochemistry. HDM4 and HDM40 group showed similar expression to HSF group both Vegfa gene in real-time PCR and VEGF protein in Immunohistochemistry ($p<0.01$). Different dosage of HDM4 and HDM40 did not show a significant difference in Vegfa expression ($p<0.01$). HDM4, HDM40 and HSF have showed a significant reduction in expression of Vegfa, and level of VEGF compared to HPG ($p<0.01$). Thus, 4 mg/kg was the most efficient dose to regulate VEGF in rats. Immunohistochemistry results also showed similar dispersal of VEGF in sample tissues. Furthermore, CPG and CDM did not show differences in expression of Vegfa. Therefore, *D. membranacea* did not affect in normal VEGF dosage ($p<0.001$).

Keywords: Hepatocellular carcinoma, Vascular endothelial growth factor, Sorafenib and *Dioscorea membranacea*

P-31

Physicochemical stability of an herbal remedy formulated from sappanwood heartwood and Indian gooseberry fruit extracts

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Physicochemical stability of bioactive compounds in herbal products is crucial for their quality and effectiveness. Thus, this research investigated the physicochemical stability, degradation kinetics, and antioxidant activity of an herbal remedy (HBR) formulated from ethanolic extracts of Sappanwood heartwood and Indian gooseberry fruit under experimental conditions. Spectrophotometry analysis revealed that increasing pH values resulted in a more intense color due to the deprotonation of brazilein, the compound responsible for the pink color in Sappanwood heartwood extract. However, higher pH values negatively affected the stability of the HBR, as indicated by a decrease in the A540-to-A445 ratio. The addition of sodium chloride (0.9-2.7%), as a representative of ionic strength, prolonged the deprotonation process, enhancing the stability of the HBR. As a result, phosphate buffer saline at pH 5.8 and its hypertonic solution were identified as potential stabilizers for the HBR. Temperature also played a pivotal role in stability. Heating the solution to 80 °C for 0.5 h caused color fading, indicating heat-induced degradation of brazilein. This degradation was confirmed by a decrease in the A445-to-A405 ratio. Additionally, storing the non-heated HBR at 6 °C for 156 h resulted in a hyperchromic shift of A445, suggesting oxidation of brazilin to brazilein and affecting stability. For the antioxidant activity of the HBR, it correlated with the availability of deprotonatable compounds. These findings contribute to the understanding of HBR stability and have potential implications for pharmaceutical and nutraceutical product developments.

Keywords: Physicochemical stability, Herbal remedy, Sappanwood heartwood, Indian gooseberry fruit

P-32

Development of extraction process for relieving hyperglycemia from Thai herbs

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Currently, non-communicable diseases, particularly diabetes and hyperglycemia, have a significant impact on the Thai public health system. Hyperglycemia has been caused by abnormal insulin secretion, which has led to the development of diabetes. The complications caused by diabetes can result in increased mortality rates and disability among the population. Therefore, this research will focus on five herbal extracts, i.e., Indian gooseberry fruit (IG), Chebulic Myrobalans fruit (CM), Beleric Myrobalan fruit (BM), Sappanwood heartwood (SH) and Licorice fruit (LI), were extracted by macerating in 95% ethanol (95EtOH), 70% ethanol (70EtOH), 50% ethanol (50EtOH), methanol (MeOH) and ethyl acetate (EtOAc). The extraction yield of herbal extracts extracted with 50EtOH was found to be higher than that of other solvents. SH extract and IG extract had the highest levels of antioxidant activity, total phenolic content (TPC),

total flavonoid content (TFC), and alpha-glucosidase inhibitory activity. As the product, the evaluation of medicinal plants will be prepared.

Keywords: Antioxidant activity, Alpha-glucosidase Inhibitory activity, Herbal extract, Hyperglycemia

P-33

Cardioprotective activity of *Hibiscus rosa-sinensis* L. (Malvales: Malvaceae) extract and fractions on doxorubicin-induced zebrafish embryo

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Cardiovascular diseases are the leading cause of death not only globally, but also in the Philippines. Existing available synthetic cardioprotective drugs have exhibited adverse side effects and are costly. Thus, there is a need for safer and cheaper drug alternatives sourced from the bioactive compounds of natural products. Hibiscus rosa-sinensis (HRS) has been reported to have various medicinal properties. This study investigated the cardioprotective activity of crude HRS methanolic extract and fractions on doxorubicin-induced zebrafish embryos by assessing changes in structure and rhythm during cardiac development. Qualitative phytochemical screening was also conducted to determine which compounds were responsible for their cardioprotective activity. It was found that HRS methanolic extract and ethyl acetate and aqueous fractions have cardioprotective potential elucidated in their activity to attenuate doxorubicin-induced cardiac failure. Cardioprotective activity is attributed to the presence of phenolics and saponins in the HRS extract and fractions. The highest cardioprotective effect of HRS was achieved by the ethyl acetate fraction at 25 μg/mL with statistically significant capacity expressed by the normal cardiac morphology and heart rate values. However, all fractions exhibit cardiotoxicity at concentrations higher than 200 μg/mL. The findings suggest that HRS ethyl acetate fraction could be a lead for therapeutic agents for CVDs in drug development.

Keywords: Cardioprotective assessment in vivo, *Hibiscus rosa-sinensis*, Zebrafish embryo, Doxorubicin cardiotoxicity

P-34

Validation of An HPLC method for determination of mitragynine in *Mitragyna speciosa* Korth extracts

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Mitragyna speciosa Korth. or Kratom, a traditional Thai herbal medicine, has gained popularity in industrial production since its legalization in Thailand. Consequently, the need for quality control methods for product development purposes has become crucial. This study aimed to develop and validate an HPLC method for the determination of mitragynine in Kratom extract as a quality control measure. A C18 column (4.6 mm id. x 150 mm length, particle size 5 micron) was utilized as stationary phase. The gradient elution mobile phase comprising acetonitrile and formate buffer was employed with a flow rate of 1.0 ml/min. The diode array detector was set at 245 nm for the detection of mitragynine. The method validation was conducted according to ICH guideline with the validation parameters including specificity, linearity, range, accuracy and precision, limit of detection (LOD) and limit of quantitation (LOQ). The developed method exhibited specific identification of mitragynine peak in sample solutions, confirmed by its retention time and absorption spectrum corresponding to the standard solution. The linearity of the method was demonstrated as the coefficient of determination(r2) exceeding 0.999 within the concentration range of 8.23 - 205.82 μg/mL. The LOD and LOQ of the detection method were 0.863 μg/mL and 0.215 μg/mL, respectively. Accuracy, expressed as %recovery, fell within the range of 97.22 - 104.13%. The precision of the method was illustrated by the relative standard deviation (RSD, %), with within-run and between-run precision below 2.75%. Additionally, intermediate precision, assessed by analyzing with two HPLC instruments and columns, was found to be less than 2.38%. Mitragynine remained stable in the autosampler for up to three days. The content of mitragynine in 95%, 70% and 50%ethanolic macerated extracts and a decoction extract were 54.00±1.17, 32.14±0.64, 25.05±0.05 and 18.08±0.30 μg/mL, respectively. The developed HPLC method exhibited good specificity, linearity, accuracy, and precision, which can be applied as a routine quality control method of Kratom extract.

Keywords: *Mitragyna speciosa*, Kratom extract, HPLC, Method validation

Acknowledgement: This work was supported by The Regional Medical Science Center 12 Songkhla and funded by Department of Medical Sciences, Ministry of Public Health, Thailand.

P-35

In vitro cytotoxic activity against human cancer cell lines of different used parts of *Cannabis sativa* L. extracts

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Cannabis sativa L. is an annual and dioecious plants in the family Cannabaceae and domestic from Asia, and Europe.[1] Cannabis has been reported to be active against some kinds of cancer cells.[2] However, the cytotoxic activity of the Thai cannabis, *Cannabis sativa* L. have not been revealed. This study aimed to investigate the cytotoxic activity against human cancer cell lines of *Cannabis sativa* L. extracts from different used parts. *Cannabis sativa* L. leaves, stems and roots were extracted by maceration with 95% ethanol and decoction. The cytotoxic activity of all extracts was investigated via the Sulforhodamine B (SRB) assay against four different types of human cancerous cell lines such as lungs, breast, intrahepatic bile duct, and colon cancer cell lines (COR-L23, T-47D, KKU-M156, and SW480). The ethanolic extracts of leaves showed the highest cytotoxicity against breast, Intrahepatic bile duct, lungs, and colon cancer cell lines with IC50 values 2.46 ± 0.44, 12.04 ± 1.05, 14.24 ± 0.63, 28.15 ± 6.32µg/ml, respectively. Therefore, all used part ethanolic extracts showed potent in vitro cytotoxic activity against all human cancer cells. It should be further investigated for the active compounds against the human lungs, breast, intrahepatic bile duct, and colon cancer cells.

Keywords: *Cannabis sativa* L., Cannabis, Cytotoxic activity, Sulforhodamine B assay, Human cancer cells

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P-36

Evaluation of the cytotoxicity and anti-inflammatory properties of *Mentha cordifolia* crude extract against H9 and Jurkat cancer line

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The discovery and isolation of bioactive compounds from medicinal plants opened a new avenue in pharmacology, revolutionizing the production of medicine for cancer diseases. Most of these bioactive compounds are known to affect the hallmarks of cancer such as unrestrained proliferation, and inflammation. Hence, this study aims to determine the cytotoxic, and anti-inflammatory property of the crude extract of *Mentha cordifolia*, one of the Philippines' Sampung Halamang Gamot against the acute T lymphocytic leukemia Jurkat cells and H9 cell line. MTS assay result shows *M. cordifolia* cytotoxic activity to H9 cells at 400 ug/ml, while jurkat and PBMC shows no decrease in cell viability. In the coming weeks we will perform Annexin-PI in the H9 cells for early cell death confirmation and also perform qPCR analysis to determine cytokine expression and potential anti-inflammatory activity of the M.c.treated Jurkat cells. Results of this study may provide insights for selective inhibitory activity of M.c extract against two acute T lymphocytic leukemia cell lines.

Keywords: T lymphocytic leukemia, *Mentha cordifolia*, Cancer, Medicinal plant

P-37

In silico screening and molecular docking of isolated compounds from medicinal plant *Mentha cordifolia* shows potential HIV-1 protease inhibitor and Tat1-cyclin T1 competitor

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The silent epidemic HIV-1 infection has reached a total of 38 million infections worldwide. While treatment with antiretroviral drugs have extended the survival of the people living with HIV, recent data has shown that drug resistance has emerged, especially in the Philippines. The crude extract of *Mentha cordifolia*, one of the Philippines' Sampung Halamang Gamot, is able to reduce viral p24 production in vitro in HIV-1 latently infected cells (OM10.1). Because the active compound has not yet been identified, this research aimed to predict in silico the potential candidates. Out of the 89 collated compounds, catechin showed promising ADMET parameters and bioactivity. Molecular docking of catechin resulted in a binding affinity of -12.23 kcal/mol and an inhibition constant value (ki) of 1.09 nM. Furthermore, we collated analogs of catechin using the Zinc15 database and performed autodock vina to identify possible competitors of Tat1 binding against Cyclin T1. The catechin analog icosyl palmitate showed the lowest binding affinity of -8.9kcal/mol to cyclin T1. Lastly, in the coming weeks we will conduct molecular dynamics to further confirm poses found by molecular docking.

Keywords: HIV, Antiretroviral, *Mentha cordifolia*, Molecular docking, Medicinal plant

P-38

Anticancer activity of *Calophyllum inophyllum* L. (Malpighiales: Calophyllaceae) on human colorectal cancer cell line (HCT116)

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Plant natural products have long been considered as an alternative to current systemic treatments and have previously made impact as it is found to be more effective yet less cytotoxic. *Calophyllum inophyllum* – a common ethno-medicinal native tree in Cebu, Philippines, was found to have high percentage of inhibition hence was subjected to solvent-solvent partitioning to determine the most cytotoxic fraction and was further evaluated using anticancer assays and gene expression analysis against five marker genes – Caspase -3 and -7, Bax, Bcl-2, and TP53 using qRT-PCR. *C. inophyllum* hexane and chloroform fractions presented highest percentage inhibition with an IC_{50} value of 16.00 ± 3.48µg/mL and 14.70 ± 2.13µg/mL, respectively. *C. inophyllum* hexane fraction presented an SI of 2.518 while *C. inophyllum* chloroform parition an SI value of 3.867. In both *C. inophyllum* fractions, morphological examination using DAPI/PI staining presented condensed brightly colored nuclei suggestive of apoptosis, while cell migration in a dose- and time- dependent manner was suggestive of its antimetastatic properties as seen in Scratch (Wound Healing) assay observed in time intervals of 0,4,8, and 24 hours. Qualitative results of JC-1 staining also indicated changes in mitochondrial membrane potential of HCT116 cells. The upregulation TP53 and Bax and downregulation of Bcl-2 in the gene expression analysis further indicated the anticancer activity of the *C. inophyllum* fractions. These findings suggest that *C. inophyllum* hexane and chloroform fractions from its leaf extract has anticancer activity against HCT116 cell line, affecting several cancer hallmarks. Further investigation could promote the use of *C. inophyllum* as a potential alternative to current treatments.

Keywords: HCT116, Colorectal cancer, Plant natural products, *Calophyllum inophyllum*, MTT assay, Scratch (Wound Healing) Assay, Gene Expression Analysis, JC-1 staining, DCFDA assay

Acknowledgement: This work was supported and funded by the Department of Science and Technology–Accelerated Science and Technology Human Resource Development Program (DOST-ASTHRDP), Philippines. Laboratory materials and equipments used are courtesy of the University of San Carlos – Tuklas Lunas Laboratory.

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Subchronic oral toxicity study of Pluk Fai that herbal formula extract in rats

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Pluk Fai That is an herbal formula in Maha-Chotirat scripture and has been included in the National List of Essential Medicines since 2012. It consists of 11 medicinal plants used in Thai traditional medicine to promote milk production and increase blood circulation in postpartum mother. However, research on Pluk Fai That remedy extract (PFT) is limited. This study was thus conducted to evaluate the subchronic toxicity of PFT in rats. Each of 24 adult male or female Wistar rats was treated orally with PFT at a dosage of 250 and 500 mg/kg BW, or no treatment, for 90 consecutive days (n=8 per group). Body weight and abnormalities in rats such as seizures, anorexia, vomiting, staggering, urination and death were observed throughout the study. At the end of the period, all rats were blood collected from the tail vein to assess functional parameters of the liver (albumin, globulin, ALT, AST) and kidney (BUN, creatinine). All doses of PFT did not affect body weight gain or cause any abnormalities in rats. There were no treatment-related changes in liver and kidney parameters of male and female rats. This study showed that PFT was relatively non-toxic to male and female rats upon subchronic exposure.

Keywords: Pluk Fai That remedy, Subchronic toxicity, Maha-Chotirat scripture, Body weight, Functional parameters

Acknowledgement: This study was supported by Department of Thai Traditional and Alternative Medicine, Ministry of Public Health, Thailand and the Center for Research and Development of Herbal Health Products, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand.

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P-40

Subchronic oral toxicity study of Ya Khao remedy extract in rats

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Ya Khao remedy is an herbal medicine formula inscribed on a stone slab of Wat Pho. Such a remedy containing fifteen medicinal plants in equal proportions has long been used to treat various fevers in Thai traditional medicine. The purpose of this study was to evaluate the subchronic toxicity of Ya Khao remedy extract (YKE) in adult male Sprague-Dawley rats. Sixteen rats were orally treated with 500 mg/kg BW of YKE (n=8) or no treatment (n=8) for 12 consecutive weeks. The body weights were recorded daily. At the end of the experiment, the organ weights, biochemical and hematological parameters, and histopathology of organs were examined. The YKE did not affect body weight gain as compared to the control group. No significant differences were found in the relative organ weights, biochemical parameters (HbA1c, albumin, globulin, ALT, AST, BUN, cholesterol, triglyceride, HDL and LDL), and hematological parameters (Hct, WBC count, neutrophil, lymphocyte, monocyte and eosinophil) between the groups. Histopathological examination of the liver and kidney tissues in both groups showed normal structure without morphological changes. The results indicated that oral administration of YKE at a dose of 500 mg/kg BW for 12 weeks did not cause toxicological damage in rats.

Keywords: Ya Khao remedy, Subchronic oral toxicity, Body weights, Organ weights, Histopathology

Acknowledgement: This study was supported by Department of Thai Traditional and Alternative Medicine, Ministry of Public Health, Thailand and the Center for Research and Development of Herbal Health Products, Faculty of Pharmaceutical Sciences, Khon Kaen University, Khon Kaen, Thailand.

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P-41

Comparative evaluation of mass peptides in serum from patients with allergic rhinitis before and after treatment with Benjakul extract by MALDI-TOF mass spectrometry

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Benjakul remedy (BJK) is a traditional Thai medicine that helps to balance body elements and has long been used to relieve allergic rhinitis (AR). Previous clinical trials have shown that an ethanolic extract of BJK can alleviate AR symptoms. However, the effect of BJK extract on altering peptide patterns in serum has never been studied. Therefore, the purpose of this study was to analyze the mass signals of small peptides in serum from AR patients before and after treatment with BJK using MALDI-TOF MS. The study included healthy subjects (n=12), AR patients who received a complete treatment course (n=20), and those who received loratadine (10mg/day) as a standard drug (n=20). After treatment with BJK extract, the intensities of mass signals at 1225.66, 1364.14, and 1758.60 Da were significantly increased compared to the AR group, which is likely identical to healthy. Meanwhile, mass signals at 1545.30 and 2115.69 Da were also increased in treatment groups, but not significantly comparative with loratadine. Only after treatment with BJK extract exhibited mass signals at 1465.67 Da significantly decrease compared with the AR group before treatment. This study suggests that BJK extract has the potential to help improve the characteristics of some mass peptides in AR patients' serum. However, MALDI-TOF MS has limitations for identifying candidate mass peptides.

Keywords: Serum, Small peptide, Mass signal, Allergic rhinitis, MALDI-TOF MS

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P-42

Effect of germination on chemical composition and in vitro anti-inflammatory activities of Thai paddy rice RD47 extracts

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Germinated paddy rice has long been used by Thai folk doctors to treat fever and inflammation. However, there has been no scientific report. The aim of this study was to investigate the effect of germination on the chemical composition and anti-inflammatory activities of Thai paddy rice RD47 extracts which showed high production. The preparation of germinated rice was that the paddy rice was soaked for 12 hours, drained, and germinated at room temperature for 2 days. Paddy rice and germinated paddy rice were extracted by maceration with 95% and 50% ethanol, by decoction with distilled water, and by acid hydrolysis method. The extracts were determined for chemical composition using High-Performance Liquid Chromatography (HPLC) and anti-inflammatory activities on lipopolysaccharide-induced nitric oxide and tumor necrosis factor-alpha (TNF-α) in RAW 264.7 murine macrophage cells. The results showed that the germination process significantly ($p < 0.05$) increased α-tocopherol, p-coumaric acid, ferulic acid, γ-oryzanol, and GABA and increased NO and TNF-α inhibition levels more than ungerminated paddy rice. The results suggested that germinated paddy rice RD47 can be considered as an alternative source of γ-oryzanol, vitamins, and phenolic acids with high anti-inflammatory activity related to fever mediators i.e., NO and TNF-α.

Keywords: Germinated paddy rice, Fever, Anti-inflammatory activity, Nitric oxide, Tumor necrosis factor-alpha, γ-Oryzanol

P-43

Anti-inflammatory and antioxidant activities of *Cleome gynandra* and fermented *Cleome gynandra*

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Cleome gynandra L. has been used for traditional medicine such as treatment of rheumatoid arthritis in Taiwan and healing a wound in India. In addition, it is popular for food processing by fermentation, which is called "Som Pak Sian" in accordance with folk wisdom in Thailand for eliminating poisons from cyanogenic glycoside. The objective of this study is to evaluate anti-inflammatory effects by NO inhibition assay in RAW 264.5 cell line, antioxidant effects by DPPH assay, the amount of total phenolic content by Folin-Ciocalteu assay and the amount of total flavonoid content by aluminium trichloride method in 95% ethanol extracts of *C. gynandra* and fermented *C. gynandra*. The results indicated that inhibitory effect on NO production, antioxidant activity and total phenolic content of *C. gynandra* was slightly higher than fermented *C. gynandra*, while total flavonoid content of fermented *C. gynandra* was slightly higher than *C. gynandra*. In conclusion, fermentation process slightly decreased the anti-inflammatory and antioxidant activities of *C. gynandra*, but made it is edible.

Keywords: *Cleome gynandra*, Fermented *Cleome gynandra*, Anti-inflammatory activity, Antioxidant activity

P-44

Antimicrobial activity of *Smilax china* L. root extract against *Cutibacterium acnes*: Exploring potential natural products

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The root of *Smilax china* L. has been traditionally used in Asian traditional medicine for its various therapeutic properties. In this research, the root extract of *Smilax china* L. was obtained through solvent extraction and subjected to antimicrobial susceptibility testing against two *Cutibacterium acnes* strains (KCTC3314 and KCTC3320). To unravel the underlying mechanism of action, further investigations were conducted to identify and characterize the active compounds present in the *Smilax china* L. root extract. These analyses utilized modern analytical techniques such as high-performance liquid chromatography (HPLC) and liquid chromatography-hybrid quadrupole time-of-flight mass spectrometry (LC/QTOF/MS). The identification of specific bioactive constituents may shed light on the molecular targets involved in the antimicrobial activity against *Cutibacterium acnes*.

Keywords: Natural Products, *Cutibacterium acnes*, *Smilax china* L., Cosmetic applications, Antimicrobial effect

Acknowledgement: This study was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (No. 2021R1A6A1A03046418).

P-45

A novel of ethnopharmacological analysis and anti-inflammatory activity of cough medicine form Thai traditional wisdom

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Prasamawang remedy (PSM) is an antitussive drug for adults and children in Thai traditional medicine used and is described in Thailand's National List of Essential Medicine (NLEM). Additionally, Thai traditional wisdom was described the most common of ingredient of cough remedy that used as a *Phyllanthus emblica* (PE). Also, we were prepared a novel Thai traditional remedy for antitussive drug that mixed PSM with PE in ratio 1:1 (PSME). In addition, relationship between the flavor of this remedy, ethnopharmacological used and its pharmacological properties was reviewed. The aims of this study were to investigate the efficacy related anti-inflammatory on nitric oxide production (NO). PSME remedy was extracted by decoction with distill water (PSME1), by maceration with 95%ethanol (PSME2) and by powdered (PSME3). The inhibition activity on nitric oxide production was determined by Griess's reagent assay. PSME1 and PSME2 exhibited strong anti-inflammatory activity via nitric oxide production with the IC_{50} values of 4.73±1.15 and 6.21±2.31 μg/mL, respectively. In addition, PSME3 exhibited moderate anti-inflammatory activity via nitric oxide production with the IC_{50} values of 16.37±0.65 μg/mL. A standard drug Ibuprofen exhibited of NO production with the IC_{50} values of 12.84±4.01 μg/mL. All extracts of PSME exhibited significant different between PSME1, PSME2 and Ibuprofen ($^*p < 0.05$). Interestingly, Thai traditional wisdom for treating cough is to reduce the sputum and increasing blood circulation. In its wisdom, sour and spicy flavor is still considered an important characteristic of a medicine for cough symptoms. The results suggest that the PSME remedy should be further studied using in vivo models and clinical trials to support its use as an antitussive drug in Thai traditional medicine.

Keywords: Cough medicine, antitussive, Thai traditional medicine

Acknowledgement: We are grateful to the Center of Excellence in Applied Thai Traditional Medicine Research (CEATMR), Department of Applied Thai Traditional Medicine, Thammasat University and Faculty of Public Health and Faculty of Pharmaceutical science at Naresuan University, for providing necessary facilities and financial support to carry out this research.

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Anti-allergic rhinitis effect of an adaptogenic Thai traditional remedy on an ovalbumin-induced mouse model

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Benjakul (BJK), a Thai traditional medicine remedy, has been extensively used by Thai folk doctors for balancing health or as an adaptogenic drug in Thai traditional medicine. It is also used for controlling abnormal elements in the body and curing flatulence. Its beneficial effect in treating allergies has not been studied in an animal model. In this study, we investigated the anti-allergic effect of BJK ethanolic extract on ovalbumin (OVA)-sensitized allergic rhinitis in mice. Animals were administered BJK (37.5, 75, and 150 mg/kg) once a day, 3 hours before nasal challenge, for a week. Then, the anti-allergic activity of BJK was examined with the following tests: OVA-specific IgE, IgG1, and IgG2a levels in serum, histopathological determinations, and cytokine expression levels. The results found that BJK (37.5 mg/kg) decreased the levels of OVA-specific IgE and IgG1 in serum and reduced the number of grades of inflammatory cells and mast cells in allergic mice. Additionally, BJK (37.5 mg/kg) showed down-regulation of IL-5 and IL-13 and up-regulated of IL-10 mRNA expression. However, the expression of IFN-, IL-12p35, and IL-12p40 were down-regulated, and the TGF- mRNA level was unchanged in nasal tissues. These results suggest that the BJK remedy can be used as a candidate for treating allergy-related diseases such as allergic rhinitis.

Keywords: Allergic rhinitis, Benjakul, Cytokines, Ovalbumin

Acknowledgement: The authors would like to thank the

Center of Excellence in Applied Thai Traditional Medicine Research and Faculty of Medicine, Thammasat University for support and providing laboratory facilities.

P-47

Characterization of novel RNA silencing suppressor of bean yellow mosaic virus

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In this study, we aimed to isolate and characterize the RNA silencing suppressor found in the Bean Yellow Mosaic Virus (BYMV). The effectiveness of a gene expression system in plant biotechnology depends on our ability to control the transcriptional unit of the gene circuit. Unfortunately, gene silencing caused by transgenes poses a significant obstacle to achieving successful gene expression, which limits the applications for biotechnological use. To address this issue, we employed a novel gene silencing suppressor in a transient expression vector to alleviate transgene silencing. To establish a silencing suppressor system for plant expression, we isolated P1/helper component-proteinase (HcPRO) proteins encoded by the BYMV from virus-infected gladiolus plants. We then observed the effects of BYMV_P1/HcPRO on GFP expression through transient expression in Nicotiana benthamiana. Co-expressing BYMV_P1/HcPRO, as a potential suppressor of post-transcriptional gene silencing (PTGS), led to over a six-fold increase in the production of GFP fused with EGF. To gain insight into the mechanism underlying the enhanced production of GFP, we analyzed the expression profiles of main components involved in PTGS, including Dicer-like (DCL), Argonaute (AGO), and RNA-dependent RNA polymerase (RDR) proteins. The results revealed that the presence of P1/HcPRO consistently suppressed the expression of PTGS-related genes, leading to elevated levels of both transcription and translation of GFP. Furthermore, BYMV_P1/HcPRO has the potential to be utilized for the efficient production of various recombinant proteins in plants.

Keywords: RNA silencing suppressor, BYMV, P1/HcPRO, PTGS

Acknowledgement: This work was supported by Advanced Technology Center Plus (ATC+, 20017936, Development of growth factors and antibody drugs using plant cell-based platform technology and global sales expansion of fragrance materials.) funded By the Ministry of Trade, Industry and Energy (MOTIE, Korea).

P-48

Driving innovation in plant cell biofoundry: Exploring promising technologies for industrial applications

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Plant Cell Biofoundry is a technology for designing plant cell lines to efficiently produce desired substances using plant cells. It involves the use of genetic recombination or gene editing techniques to design plant cells and build them so that vector constructs, which enable efficient gene expression into proteins, can enter and function within the plant cells. Testing is conducted to ensure that the desired protein, a genetic product, is well expressed in the plant cell bioreactor. Gathering relevant data, employing machine learning and AI technologies for learning purposes, is also part of the process. In simple terms, plant cell line engineering refers to the design and mass production of plant cell lines through a cycle of Design-Build-Test-Learning, aiming to efficiently obtain the desired target substances. Unlike animals, plants are free from animal virus infection, safe, low in byproducts, and have mechanisms that contribute to carbon neutrality by removing carbon. Furthermore, the cost of cultivating plant cells is low, and they have good profitability for substance production. However, there is a lack of research in this field, and efficient techniques for large-scale cultivation or increasing substance yields during cultivation of plant cells are currently limited. The Plant Cell Research Institute of BIO-FD&C Co., Ltd. Has laid the foundation of Plant Cell Biofoundry technology through years of research in plant cell cultivation and genetic recombination. They are now designing a system that efficiently produces the desired substances in plant cells, from cell line design to manufacturing, by adding Manufacturing to the existing Design-Build-Test-Learning concept of Plant Cell Biofoundry. By introducing radiofrequency into the Plant Cell Bioreactor during plant cell cultivation, the activation of the phenylpropanoid pathway in plant cells is enhanced, increasing secondary metabolites in plants. They are developing promoters, a key element in genetic recombination technology, that can increase protein expression in plant cells, identifying genes related to that process. This allows for the introduction of foreign genes into plant cells, enabling efficient protein production and the construction of a Plant Cell Biofoundry. In the future, the industry is increasingly emphasizing the importance of environment, social, and governance aspects, as well as policies that promote carbon neutrality and sustainable preservation of the environment. In this context, the Plant Cell Biofoundry will bring about a significant paradigm shift in the industries involved in manufacturing and producing the desired substances, both economically and environmentally.

Keywords: Plant cell, Biofoundry, Radiofrequency, Design-Build-Test-Learning

Acknowledgement: This work was supported by the research

and development project "Construction, Design, and Commercialization of Plant Cell Culture/Production Biofoundry System based on Synthetic Biology.") funded by the Incheon Techno Park of Incheon Metropolitan City.

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Pre-formulation study of the Pra-Sa-Ka-Phrao remedy ethanolic extract

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Keywords: Pre-formulation study, Pra-Sa-Ka-Phrao remedy, Ethanolic extract

Pra-Sa-Ka-Phrao is a traditional remedy in Thailand's National List of Essential Medicines. It has long been used to treat flatulence and colic pain for centuries. It has a spicy taste and consists of 8 medicinal plants, 50% of the ingredients of the remedy as *Ocimum sanctum* L. This study aims to investigate the force degradation (stress test) or pre-formulation study of the 95% ethanolic extract of the remedy (PSKPE). The 5 conditions of temperature-forced degradation, moisture hydrolysis, acid hydrolysis (3N HCl), alkaline hydrolysis (3N NaOH), and oxidation (30% H_2O_2) of PSKPE were tested by inhibition of LPS-induced nitric oxide production (NO) in murine macrophage cell lines (RAW 264.7) and was determined NO by Griess reagent and compared to the control. The results showed that PSKPE still inhibit NO production in the stress test condition. It was stable in 5 stress conditions because IC_{50} on inhibitory effect on NO production did not change significantly difference (p-value > 0.05) when compared with the control, which was not treated in a stress test. The IC_{50} of temperature, moisture, oxidation, acid, and alkaline conditions compared with control were IC_{50} = 32.04±0.58, 32.37±1.31, 34.73±2.29, 35.23±1.29, 33.58±0.39, and 29.05±1.13 μg/mL, respectively). Thus, this remedy was stable in every stress condition. Furthermore, the base knowledge for the pre-formulation study could be supported for formulations of PSKPE remedies that all conditions cannot change the anti-inflammatory effect of PSKPE. It is easy to design a form of preparation and ingredient in developing formulas.

Acknowledgement: This research was supported by the Thailand Science Research and Innovation Fundamental Fund through Grant No. TUFF 28/2565, the Department of Applied Thai Traditional Medicine and Center of Excellence in Applied Thai Traditional Medicine Research (CEATMR), and the research group in Thai herbs and traditional remedy for cancer patients from Faculty of Medicine, Thammasat University.

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A preliminary study in rats with a learned helplessness model of depression from Thai traditional medicine called Sa-Tri-Lhung-Klod remedy

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Postpartum depression is caused by physical and emotional changes in postpartum mothers. It is a common psychiatric disorder and a public health concern. In Thai traditional medicine, the Sa-Tri-Lhung-Klod remedy (ST) is prepared by decoction as aqueous extract and used for postpartum care on prevention postpartum depression. Thus, the effect on the aqueous extract of Sa-Tri-Lhung-Klod (STW) was investigated on depressive-like behaviors in rats, the learned helplessness test. Rats were given 60 inescapable electric foot shocks (0.8 mA) on day 1 and exhibited a deficit in escape performance in three subsequent shuttle-box sessions (on days 3-5). Male Sprague-Dawley rats (weight 250 ± 10 g) were randomly divided into five groups of three rats each. 3% (w/v) acacia solution, fluoxetine (35 mg/kg), and STW (100, 200, and 400 mg/kg) were administered once daily via the oral route for 4 days. Rats were treated with fluoxetine (35 mg/kg) and STW (100, 200, and 400 mg/kg) showed the reduced number of escape failures which caused by exposure to the uncontrollable aversive situation on 3rd day of avoidance task (3.33 ± 0.88, 4.25 ± 3.33, 1.00 ± 0.71, and 1.67 ± 0.88 escape failures, respectively, while the control group showed escape failure values as 5.67 ± 5.67. Therefore, STW exhibited the possibility in order to prevent the occurrence of depressive-like behaviors in rats. To confirm this effect, larger numbers of animals should be necessarily studied in the experimental step.

Keywords: Sa-Tri-Lhung-Klod remedy, Anti-depressive activity, Learned helplessness test

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Phytochemical compositions of Thai traditional medicine called Trikanrapit remedy

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The Trikanrapit a Thai traditional herbal remedy used to cure the impairment of the body, nourish the blood, nourish sexual desire and carminative drug. This study aimed to investigate the chemical constituents of the water extracts were also analyzed by Gas Chromatography-Mass Spectrometry (GC-MS). The result found that the water extracted from Trikanrapit remedy showed contained about 21 different compounds. Hexadecanoic acid, methyl ester had the most remarkable peak area percent of the twenty-one compounds. The data given in this study reveals the phytochemical compositions of the herb recipe. However, the bioactivity of Trikanrapit Remedy should be further studied.

Keywords: Thai Traditional Medicine, Trikanrapit remedy, gas chromatography-mass spectrometry (GC-MS)

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Effect of salicylic acid foliar application times on bioactive compounds and antioxidant activity in holy basil leaves

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Foliage of holy basil (*Ocimum sanctum* L.) is a rich source of bioactive compounds and antioxidant activity. The objective of this study was to investigate the effect of salicylic acid (SA) foliar application times on bioactive compounds and antioxidant activity in holy basil leaves. The leaves were sampled at 0, 1, 2, 3, 4 and 5-day after 1 mM SA foliar application. The contents of eugenol, total phenolic and total flavonoid as well as antioxidant activity were determined. SA treatment at 3-day before harvest exhibited the highest contents of eugenol (7802.83 ± 51.67 μg/g dry extract), total phenolic (423.37 ± 1.73 mg GAE/g dry extract) and total flavonoid (216.17 ± 6.79 mg QE/g dry extract), where 2.5, 1.6 and 1.5-fold increase was obtained, respectively. The strongest antioxidant activity with EC_{50} of 7.61 ± 0.62 g/mL was also achieved under SA treatment at 3-day before harvest. These suggest that SA foliar application at 3-day before harvest enhances the accumulation of beneficial bioactive compounds and antioxidant activity in holy basil leaves.

Keywords: Antioxidant activity, Eugenol, Holy basil, Total flavonoid, Total phenolic

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Antibiofilm activities of lupinifolin in combinations with antibacterial drugs against methicillin-resistant *Staphylococcus aureus*

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The biofilm formation activity of methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the crucial virulence factors that essentially contributes to therapeutic challenges. Inhibition of biofilm formation provides an alternative strategy to combat this pathogenic bacterium. Lupinifolin, a prenylated flavanone isolated from Derris reticulata stem, has been reported to possess antibacterial activity against various gram-positive bacteria, including MRSA. This study aimed to investigate the effects of lupinifolin in combination with antibacterial drugs acting as protein synthesis inhibitors on the biofilm formation activity of MRSA. The biofilm formation activity was evaluated by the crystal violet biofilm formation assay. Lupinifolin produced a significant inhibition against MRSA biofilm formation, with the lowest IC_{50} of 6.73±3.47 g/mL found at 10-hour incubation. The combination of lupinifolin and the antibacterial drug (tetracycline, streptomycin, or clindamycin), at their sub-MICs, also exhibited significant inhibition against MRSA biofilm formation. The maximal antibiofilm activity was found with a combination of lupinifolin (8 g/mL) and tetracycline (16 g/mL) at 10-hour incubation, with a % inhibition of biofilm formation of 102.97±1.78% ($p<0.01$, $n=6$). This combination also produced significant antibiofilm activities at the subsequent incubation periods of 18, 24, and 36 hours, with % inhibitions of 99.43±2.00, 65.39±9.33 and 92.06±4.47, respectively. These findings suggest a potential role for lupinifolin as an antibacterial enhancer for the treatment of biofilm-associated MRSA infections. Nonetheless, more research is required to investigate the mechanisms of the antibiofilm activities of lupinifolin, either alone or in combination with antibacterial drugs.

Keywords: lupinifolin, *Derris reticulata* Craib., Biofilm, Methicillin-resistant *Staphylococcus aureus*, Antibacterial enhancer

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Acute toxicity and amount of tumor promoter, phorbol-12-myristate-13-acetate in Croton tiglium L. seed before and after treatment by Thai traditional medicine process

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Aim of study: The objective of the current study is to study the amounts of phorbol-12-myristate-13-acetate (PMA) and acute toxicity of *Croton tiglium* seeds before (CB) and after (CA) treated by Thai traditional process (Kharith). Materials and methods: The amount of PMA in CB and CA was analyzed by HPLC and acute toxicity was tested on Wistar rats compared to the control group Results: The amounts of PMA in croton seed extract decreased from 1.59 to 1.26 mg/g when compared before and after treatment, respectively. The acute toxicity test was started at the dose of 300 to 2,000 mg/kg. The result showed no sign of toxicity and no rat died in all doses of both groups. Conclusion: Croton tiglium seeds before and after being treated with the Thai traditional medicine process showed no acute toxicity at a 2000 mg/kg BW dose. However, 2 hematological parameters, mean corpuscular volume and mean corpuscular hemoglobin showed a significant difference when compared to the control group, it needs to be confirmed in the chronic toxicity test.

Keywords: *Croton tiglium*, phorbol-12-myristate-13-acetate (PMA), Thai traditional medicineprocess or Kharith

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Effects of solvents and extraction methods on anti-inflammatory and antioxidant activities of *Etlingera pavieana* rhizomes

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Etlingera pavieana, belonging to Zingiberaceae family, has been reported numerous biological activities. This study examined the impact of different solvents and extraction techniques on the anti-inflammatory and antioxidant effects of *E. pavieana* rhizomes. The rhizomes were extracted with two solvent systems, methanol and ethanol at concentrations of 95%, 70%, and 40% (v/v) by maceration and reflux methods. Anti-inflammatory activity was measured through the inhibition of nitric oxide (NO) production in LPS-stimulated RAW 264.7 macrophages. Antioxidant activity was assessed using DPPH scavenging activity assay, and the total phenolic content was measured with the Folin-Ciocalteu method. The 95% ethanol extracts from maceration and reflux methods showed a higher NO inhibitory effect and antioxidant activity than that of 70% and 40% extracts. Anti-inflammatory activities of the extracts using refluxing were higher than the maceration method. Additionally, the bioactivities of E. pavieana rhizome extracts using refluxing were higher than the maceration method. The obtained data indicated that 95% ethanol extract using the reflux method exhibited the greatest anti-inflammatory and antioxidant activities. These results provide the basic information for the preparation of extracts from E. pavieana rhizomes which may be developed into dietary supplements or functional foods to prevent inflammation-related diseases.

Keywords: Anti-inflammatory activity, Antioxidant activity, *Etlingera pavieana*, Reflux, Maceration

Acknowledgement: This work was financially supported by the Research and Development Fund Burapha University (Grant no. 10/2565) and the Center of Excellence for Innovation in Chemistry (PERCH-CIC), Commission on Higher Education, Ministry of Higher Education, Science, Research and Innovation, Thailand.

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Development of a tool for school nurses to manage students with food allergies

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School nurses face challenges in identifying and managing food allergies in the school setting. Given their crucial role in helping children avoid allergens and providing appropriate care during allergic reactions, improving their competency is necessary. Therefore, the purpose of this study was to develop a tool to guide school nurses in managing students with food allergies. This tool was developed using the following methods: 1) DACUM analysis method, involving seven focus group interviews with experts in the school health field; 2) examination of processes based on manuals distributed by Ministries of Education in South Korea, the United States, and Canada to ensure the accuracy and lack of bias in the identified items; and 3) validity verification from 166 school nurses currently managing the health of students with food allergies. Content validity confirmed that 14 items did not meet the standard index (CVI=.80) and were thus deleted. Construct validity was verified through factor analysis, resulting in a cumulative percentage of 68.6. Finally, a tool

consisting of 82 items were developed to assist school nurses in managing the health of students with food allergies. The tool developed in this study will provide guidance to school nurses in identifying and managing students with food allergies.

Keywords: Anaphylaxis; Food Allergy; Schools, Nursing; Tool

Acknowledgement: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT: Ministry of Science and ICT) (No. 2018R1C1B5086483). This was supported by Korea National University of Transportation in 2023.

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Development and validation of a tool to assess emergency care competency for school nurses managing food allergy incidents in elementary school settings

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Food allergies are a common pediatric emergency and pose significant concerns for elementary schools. School nurses need to be prepared to manage life-threatening allergic events and ensure the safety of these students. The purpose of this study was to develop a tool for assessing the emergency care competency of school nurses in effectively managing food allergy incidents. A panel discussion involving school health experts was conducted to ascertain the essential competencies required for school nurses to deliver emergency care. Content validity and construct validity were evaluated to establish the tool's validation. Items that demonstrated a content validity index of ≥80% were selected. Exploratory factor analysis was conducted with a sample of 166 school nurses who were actively responsible for the well-being of students with food allergies. As a result, the emergency care competency tool was ultimately comprised of 16 items. The content validity of the results was CVI=0.94. Through factor analysis, two sub-factors were extracted with the Eigen-value of 1.14 and a cumulative percentage of 81.9. The developed tool will provide valuable resources for school nursing practice, particularly in the care of students experiencing severe allergic reactions.

Keywords: Anaphylaxis; Food Allergy; Schools, Nursing; Emergency Treatment

Acknowledgement: This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea government (MSIT: Ministry of Science and ICT) (No. 2018R1C1B5086483). This was supported by Korea National University of Transportation in 2023.

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NATPRO8

The 8th International Conference on Natural Produts

NATPRO Conference Series

ISSN 2635 - 6643

Publication Date: July 15 2023

Publisher: The Asian Society of Natural Products

Rm 4353, Bd 809, Chung-Ang University,
Seodongdaero 4726, Angseong-si,
Gyeonggi-do, Korea. Zip code 17546

Printed by Phetkasem Printing Group Co., Ltd.

18/49 Tambon Lam Phaya, Mueang
Nakhon Pathom District, Nakhon
Pathom 73000, Thailand

NATPRO 8

The International Conference on Natural Products

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The Asian Society of Natural Products
Rm 4353, Bd 809, Chung-Ang University
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