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 ºC 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**).

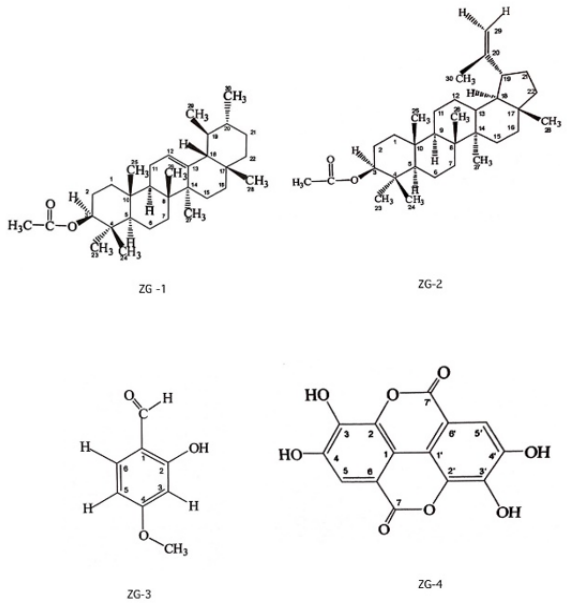

Figure 2. The structures of isolated compounds α-amyrin acetate (ZG-1), β-lupeol acetate (ZG-2), 2-hydroxy-4-methoxy-benzaldehyde (ZG-3), and ellagic acid (ZG-4).

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-CH_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-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*,-**H**CO), 11.40 (1H, *s*, O**H**)

^{13}C NMR: (δ: ppm, in MeOH d_4, 125 MHz): δ: 54.88 (CH_3, O-CH_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, CHO)

DEPT 135° : **CH_3** : 54.88 (O-CH3), **CH** :100.21 (C-3), 107.59 (C-5),134.71 ( ),194.28(CHO), **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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