Content

Inhibitory Effect of Mannitol Isolated from Mushroom *Mycoamaranthus cambodgensis* on LPS-Induced Inflammation of RAW264.7 Cells

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Abstract

Mycoamaranthus cambodgensis is an edible wild mushroom found in Thailand. The objective of this study was to isolate and elucidate the chemical structure of the bioactive compound from the mushroom, in addition, the anti-inflammatory effect of the compound on LPS-induced RAW264.7 cells was also studied by using RT-PCR technique. A white solid compound was isolated from methanolic extract of *M. cambodgensis* with a yield of 0.9% from crude extract. The results from NMR analysis showed that the compound was mannitol. The mannitol showed no cytotoxic effect on the test cells at concentration ranges of 1.25 to 20 µg/ml. The mannitol had no effect on inflammatory mediator gene expression of RAW264.7 cells, whereas the compound showed significant suppression on the expression of COX-2, IL-6, iNOS and TNF-α genes of LPS-induced RAW264.7 cells in a dose-dependent manner. In conclusion, the isolated mannitol from this mushroom may play an important role in health beneficial effecst of *M. cambodgensis* mushroom.

Keywords: *Mycoamaranthus cambodgensis*, Mannitol, Anti-Inflammatory Effect

Introduction

Inflammation is an immune mechanism in response to infections. Several pro- inflammatory biomarkers activated from pro-inflammatory cells play an important role in this inflammatory response including inducible nitric oxide synthase (iNOS), inducible type cyclooxygenase-2 (COX-2), interleukins (IL) -1β, IL-6, IL-8, prostaglandin E2 (PGE2) and tumor necrosis factor-α (TNF-α). The imbalance or excessive response of inflammation may lead to different kinds of damage, chronic inflammatory diseases, cancers, and autoimmune diseases. Therefore, decreasing the inflammatory response is one of the main treatment targets of those diseases.

Mycoamaranthus cambodgensis (Pat.) Trappe, S. Lumyong, P. Lumyong, Sanmee & Zhu L. Yang. is an edible mushroom found in Australia and southeastern Asia [1]. This brilliant yellow sequestrate fungus is in the family Hymenogasteraceae (Figure 1A) [2]. Its white gleba changes to dark brown gleba when it turns to maturity (Figure 1B) [1]. This mushroom was used as a traditional medicine for maintenance of a regular menstrual cycle and treatment of tumor. *M. cambodgensis* was reported to have high nutritional value and contain phenolics and flavonoids [3]. However, there was no scientific report of its bioactive compounds and pharmaceutical effects. Therefore, the objectives of this study were to elucidate the chemical structure of the isolated bioactive compound and study its anti-inflammatory activity by using RAW264.7 cells.

Methodology

Extract preparation

Fresh fruiting bodies of *M. cambodgensis* mushroom were collected from community forest in Wattana Nakorn district, Sa Kaeo province, Thailand. The mushroom was sliced and dried by using hot air oven at 50ºC. The dried mushroom was macerated by using 50% ethanol for 7 days at room temperature. The extract was filtered and concentrated by using rotary evaporator. Yield of crude extract from fruiting body dry weight was 15.1%.

Chemical isolation and structure elucidation

The crude extract (28.0 g) was dissolved in methanol and then partitioned by using n-hexane. The methanol fraction was concentrated by using rotary

evaporator. The crude methanol extract (9.73 g) was further purified by Quick Column Chromatography using CH_2Cl_2 as eluent and increasing polarity with methanol to give 10 fractions (S1-S10). Fraction S8 (612 mg) was recrystallized from methanol to give a white solid compound (252.3 mg) with the yield of 0.9% from the crude extract. The compound was structurally elucidated by using 400 MHz Bruker FTNMR Ultra ShieldTM spectrometers in DMSO-d6 solution.

Figure 1. The outside (A) and inside (B) appearances of *M. cambodgensis* fresh fruiting body

Study of cytotoxicity

The compound was dissolved in distilled water and filtered by using 0.4 μm syringe filter. RAW264.7 cells were cultured in 96-well plate (10,000 cells/well) by using RPMI culture medium supplemented with 10% FBS and 1% Penicillin-Streptomycin and incubated overnight at 37ºC in the humidified atmosphere with 5% CO_2. The cells were treated with various concentrations of compound and then incubated for 24 h. Cell viability was measured by using Alamar blue assay [4]. The cytotoxicity was expressed as % cell viability.

Determination of inflammatory-related gene expression

The RAW264.7 cells were cultured overnight in 12-well plates (1×10^5 cells/well) and treated with various concentrations of compound and Indomethacin as a positive control. After incubation at 37ºC in the humidified atmosphere with 5% CO_2 for 22 h, the *Escherichia coli* lipopolysaccharide (LPS) was added then further incubated for 2 h. Total RNA was extracted from the treated cells by using a GE Healthcare extraction kit. The first-strand cDNA was synthesized from total RNA (40 ng) with Omniscript reverse transcriptase kit. The primers were used for amplifying the respective fragments. Polymerase chain reaction (PCR) was performed by incubation of each cDNA sample with the primers, Taq polymerase, and deoxynucleotide mix. Amplification was completed for 30 cycles and the conditions for PCR amplification followed previous reports [5-6]. The PCR products were then analyzed on 1.5% agarose gel, visualized by NovelJoice staining and RT-PCR product densities measured by Gel Documentation and System Analysis machine. The inflammatory-related gene expressions were calculated for the relative mRNA expression level compared with β-actin and expressed as 50% inhibitory concentration (IC_{50}).

Statistical analysis

The results are reported as mean ± S.D. One-Way ANOVA and multiple comparison (LSD) were analyzed by using SPSS version 16.0.

Results

The white solid compound was structurally elucidated and the following data were described. The compound melting point was 166-168°C. The IR spectrum showed absorption bands of hydroxyl group (3385 cm^{-1}), and C-H stretching (2938 cm^{-1}). The ^{13}C NMR and DEPT spectral data exhibited 2 carbons of an oxymethylene (δ 64.3) and two oxymethines (δ 70.1 and 71.8) (Table 1). The ^1H NMR spectral data displayed the presence of a AB system at δ 3.37 (2H, m, H_A-1/H_A-6) and 3.61 (2H, m, H_B-1/ H_B-6), two oxymethine protons at δ 3.46 (2H, m, H-2/H-5) and 3.54 (2H, t, J = 7.2 Hz, H-3/H-4) and three hydroxyl groups at δ 4.14 (2H, d, J = 7.2 Hz, OH-3/OH-4), 4.33 (2H, t, J = 5.2 Hz, OH-1/OH-6) and 4.41 (2H, d, J = 5.2 Hz, OH-2/OH-5). The structure was confirmed by HMBC correlations. The oxymethine proton at δ 3.46 (H-2/H-5) showed correlation with carbons at δ 64.3 (C-1/C-6), 70.1 (C-3/C-4) and 71.8 (C-2/C-5) and an oxymetine proton at δ 3.54 (H-3/H-4) showed correlation with carbons at δ 64.3 (C-1/C-6) and 71.8 (C-2/C-5). The correlation of oxymethylene protons at δ 3.37 and 3.61 (2H-1/2H-6)

with carbons at δ 70.1 (C-3/C-4) and 71.8 (C-2/C-5) suggested the position of OH at C-1/C-6, C-2/C-5 and C-3/C-4. From the spectral data and comparison with those of mannitol [7-9], the compound was assigned as mannitol (Figure 2).

The mannitol was analyzed for its cytotoxicity against RAW264.7 cells prior to determination of anti-inflammatory activity. Without LPS addition, mannitol had no cytotoxicity to the tested cells and cell proliferation was slightly increased when treated with 1.25 to 20 µg/ml of mannitol. Mannitol slightly decreased cell viability in the LPS supplemented medium (Figure 3). Therefore, mannitol at concentration of 1.25 to 20 µg/ml was safe for RAW264.7 cells in this study.

Table 1. ^{1}H, ^{13}C, DEPT, COSY and HMBC NMR (400 and 100 MHz, DMSO-d6) spectroscopic data of mannitol in DMSO-d6 (δ in ppm, multiplicities, J in Hz)

positions	δ ^{1}H	δ ^{13}C	DEPT	COSY	HMBC
1	3.37 m, 3.61 m	64.3	CH_2	2, 1-OH	2,3
2	3.46 m	71.8	CH	1,3,2-OH	1,3
3	3.54 t (7.2)	70.1	CH	2, 3-OH	1,2
4	3.54 t (7.2)	70.1	CH	5, 4-OH	5,6
5	3.46 m	71.8	CH	4,6,5-OH	4,6
6	3.37 m, 3.61 m	64.3	CH_2	5,6-OH	4,5
1,6-OH	4.33 t (5.2)	-	-	1,6	1,2 (5,6)
2,5-OH	4.41 d (5.2)	-	-	2,5	1,2,3 (4,5,6)
3,4-OH	4.14 d (7.2)	-	-	3,4	2,3 (4,5)

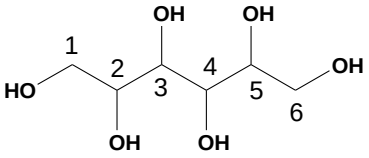

Figure 2. Chemical structure of mannitol isolated from *M. cambodgensis*.

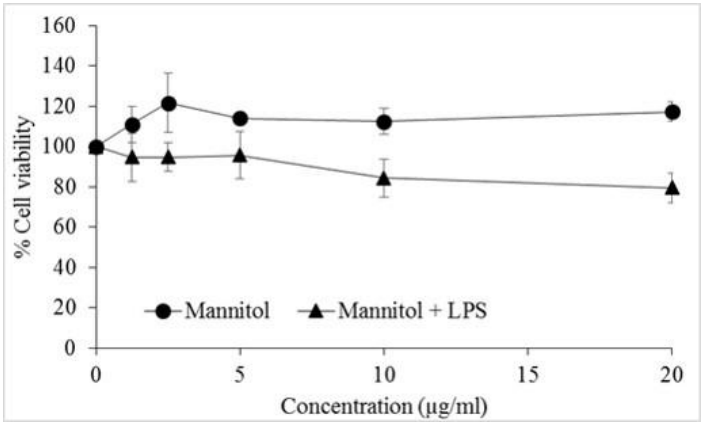

Figure 3. Cytotoxicity of mannitol against RAW264.7 with and without LPS induction

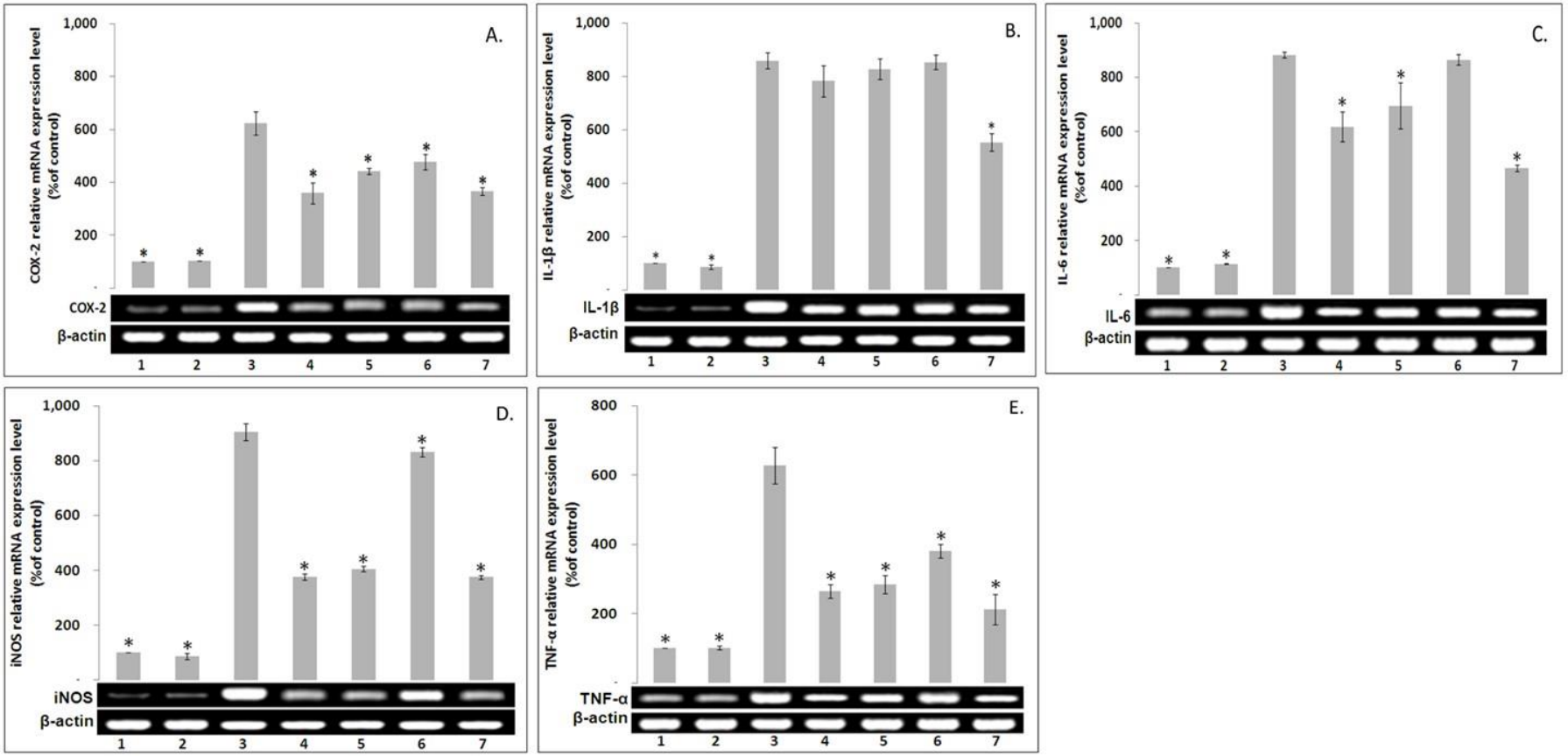

Figure 4. mRNA expression of pro-inflammatory mediators. A) COX-2, B) IL-1β, C) IL-6, D) iNOS and E) TNF-α. Lane 1: unstimulated cells, lane 2: 20 µg/ml mannitol stimulated cells, lane 3: LPS stimulated cells, lane 4: 20 µg/ml mannitol and LPS co-stimulated cells, lane 5: 10 µg/ml mannitol and LPS co-stimulated cells, lane 6: 5 µg/ml mannitol and LPS co-stimulated cells and lane 7: 50 µg/ml Indomethacin and LPS co-stimulated cells

In Figure 4, mannitol did not induce the expression of all studied pro-inflammatory mediator genes compared to the control, whereas LPS induced gene expression of all pro-inflammatory mediators. After co-treatment with mannitol and LPS, the expression of those genes, except IL-1β, was significantly suppressed in a dose-dependent manner. Interestingly, 10 to 20 µg/ml mannitol could decrease the expression of COX-2 (IC50 = 22.77±0.79 µg/ml), iNOS (IC_{50} = 14.95±0.61 µg/ml) and TNF-α (IC_{50} = 13.38±0.12 µg/ml) genes to levels similar to 50 µg/ml of Indomethacin. These results indicate that mannitol isolated from *M. cambodgensis* shows strong anti-inflammatory activity and may play an important role in the anti-inflammatory activity of the *M. cambodgensis* mushroom.

Discussion and Conclusion

Mushrooms are a good source of nutritional and medicinal natural products. A number of bioactive substances have been discovered from mushrooms such as polysaccharides, phenolics, polyphenolics, flavonoids, terpenoids, ergosterols, and peptides. These substances were reported to have several pharmaceutical effects such as anti-tumor, anti-inflammation, anti-virus and immunomodulation [10-11]. Various compounds from mushroom were reported to show significant anti-inflammatory activity such as ganoderic acid from *Ganoderma lucidum* [12], fucomannogalactan from *Lentinus edodes* [13], ergosterol from *Inonotus obliquus* [14], and cordymin from *Cordyceps sinensis* [15]. In this study, *M. cambodgensis* isolated mannitol showed no cytotoxic effect against macrophages. This is the first report that mannitol can be found in *M. cambodgensis* fruiting bodies. Mannitol was found in several mushrooms such as *L. Edodes* [16], *Agaricus bisporus* [17], *Clitocybe nebularis* [18], *Pleurotus cornucopiae* [19] and *Agaricus silvaticus* [20]. Mannitol is important for NADPH regeneratiion in mushroom. Mannitol is found in the mycelium stage at low concentration and then dramatically increases during differentiation to sporophores [17]. Mannitol dehydrogenase catalyzes mannitol synthesis from fructose and NADPH is a cofactor in this metabolism [21]. Increasing mannitol synthesis is related to the initiation and development of mushroom fruiting bodies [22]. Therefore, mannitol metabolism plays an important role in the development of mushroom fruiting bodies. Moreover, the isolated mannitol in this study showed strong anti-inflammatory activity by suppression of pro-inflammatory mediator gene expression, especially COX-2, iNOS and TNF-α genes. There have been few reports on the anti-inflammatory activity of mannitol. Mannitol was reported to reduce inflammatory edema in a rat model of arthritis and also to reduce inflammation in adjuvant-induced arthritis in the rat [23-24].

In conclusion, *M. combodgensis* contains mannitol as a bioactive compound which shows a strong suppressive effect on inflammatory mediators *in vitro*. The anti-inflammatory activity of *M. combodgensis* crude extract and other compounds will be further studied. These results can support the use of *M. combodgensis* as a traditional medicine and as a good candidate for inflammatory treatment from natural products.

Acknowledgements

This work was financially supported by the Research Grant of Burapha University through National Research Council of Thailand (Grant no. 129/2560). Thank you to the Center for Research and Development of Herbal Health Products, Khon Kaen University, Khon Kaen, Thailand, for facility support.

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DPPH Scavenging Activities and Phytochemical Studies of Extracts and Embelin Isolated from *Ardisia elliptica* Fruits

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Abstract

Ardisia elliptica has been used for the treatment of various ailments for a long time. Its dried fruit is used for the treatment of diarrhea, fever, liver disease, leprosy and as a nourishing agent. *A. elliptica* fruits contain some phenolics and flavonoids. Embelin is a major compound in this plant. The aim of this study was to determine the antioxidant activities and phytochemistry study of extracts from *A. elliptica* fruits and the isolated embelin. The ethanolic and dichloromethane extracts from the fruits of *A. elliptica* were screened for 2,2-diphenyl-1-picryhydrazyl (DPPH) scavenging activities and total phenolic and total flavonoid contents were determined. The results demonstrated that the dichloromethane extract and filtrate fraction (the rest of dichloromethane extract after the separation of embelin) showed the highest DPPH scavenging activities with EC_{50} values of 63.33 ± 4.23 and 73.16 ± 2.98 µg/ml, respectively. The isolated embelin also promoted high antioxidant activity with EC_{50} value of 64.98 ± 6.44 µg/ml. Dichloromethane extract and the filtrate fraction had high total phenolic contents while the filtrate fraction had significantly higher total flavonoid content than other extracts. Thin layer chromatography (TLC) analysis showed that even though embelin was active as antioxidant compound, there are other active compounds in the filtrate fraction that have antioxidant potential for further pharmacological and phytochemical studies.

Keywords: *Ardisia elliptica*, Embelin, DPPH, Total Phenolic, Total Flavonoids

Introduction

Ardisia elliptica (Myrsinaceae) or Pilangkasa is a plant that has been used in Thai traditional medicine for a long time. The dried fruit has been used for the treatment of diarrhea, fever, liver disease, leprosy and as nourishing agent [1]. *A. elliptica* fruit was reported to contain phenolics and flavonoids such as malvadin-3-glucoside, delphinidin-3-glucoside, pentunidin-3-galactoside, bergenin, embelin, quercetin, isorhamnetin, bergenin and syringic acid and terpenoids such as α amyrin acetate and lanosterol [1, 2, 3]. Pharmacological activities of *A. elliptica* fruit that have been previously reported were antiproliferative activity against SKBR3 human breast adenocarcinoma cell lines determined by MTT assay of the fruit ethanolic extract [4], antimicrobial activities against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans* of the fruit ethanolic extract [5]. The fruit aqueous and methanolic extracts also showed antioxidant activity tested by DPPH scavenging, metal chelating, ferric reducing antioxidant power (FRAP), 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and oxygen radical absorbance capacity (ORAC) assays [2, 6]. Moreover, the polar fraction from the fruit ethanolic extract promoted anti-Salmonella activity [7]. Embelin was reported as one of the major compounds in *A. elliptica* fruit [8], however there could be other compounds that promote *in vitro* antioxidant activity in the crude extract and filtrate fraction of *A. elliptica* fruit. Therefore, the aim of this study were to screen for *in vitro* antioxidant activity of crude extracts from *A. elliptica* fruits using DPPH scavenging assay. Embelin was isolated using precipitation techniques, structurally elucidated by spectroscopic techniques and antioxidant activity was determined by DPPH scavenging assay. The crude extracts were also phytochemically studied using TLC and total phenolic and total flavonoid contents were determined by spectrophotometric techniques.

Methodology

1. Sample preparation

The *A. elliptica* ripe fruits were collected from Siri Ruckhachati Nature Park, Mahidol University Salaya Campus, Nakhonpathom province, Thailand in 2016. The plant was identified at the Bangkok Herbarium (BK), Department of Agriculture, Thailand. Plant sample was

dried in a hot air oven at 60 ˚C, powdered using an electric mill and passed through a sieve with mesh number 20.

2. Plant extract preparation

The fruit powder of *A. elliptica* was macerated with dichloromethane (1:4 w/v) for 8 hours and filtrated. The process was repeated 3 times and the obtain solution was combined and evaporated to dryness by rotary evaporator. Embelin was isolated from dichloromethane extract by crystallization. The rest of the extract after the separation of embelin was called the filtrate fraction. The isolated embelin was identified using thin layer chromatography (TLC) compared with standard embelin and structurally elucidated by spectroscopic techniques including nuclear magnetic resonance spectroscopy (NMR), infrared spectroscopy (IR), mass spectrometry (MS), ultraviolet spectroscopy (UV) and the analysis of melting point. The ethanolic extract was obtained from previous studied with the same extraction condition as dichloromethane extract.

3. Phytochemical studies

3.1 Thin layer chromatography (TLC)

A. elliptica fruit extract was phytochemically analyzed by thin layer chromatography (TLC) in the following conditions:

Stationary phase: silica gel 60 F254

Mobile phase: chloroform, ethyl acetate, formic acid (5:4:1)

Detector: UV detector at 254 nm and 366 nm, DPPH spraying reagent

3.2 Determination of total phenolic content

The total phenolic content of extracts was determined by the Folin–Ceiocalteu assay. The method was carried out according the method of Herald et al. (2012) with gallic acid as the standard solution [9]. A Folin-Ceiocalteu reagent was add to *A. elliptica* fruits extract or standard gallic acid solution then the solution was kept in the dark at room temperature for 90 min. After that, 20% w/v sodium carbonate solution was added to each reaction mixture. The absorbance of the reaction solution was determined at a wavelength of 765 nm using a micro-plate reader. The total phenolic compound was calculated from the standard curve of gallic acid and was expressed as gram gallic acid equivalent per 100 gram extract (g% GAE). Each experiment was carried out in triplicate. The average total phenolic content and standard deviation was then calculated.

3.3 Determination of total flavonoid content

The total flavonoids content was determined by the aluminium chloride colorimetric assay. The method was conducted using quercetin as the standard solution [10]. An aluminium chloride solution (2% w/v) was added to each sample or standard quercetin solution. The mixture was kept at room temperature for 30 min. The absorbance of the reaction solution was determined at a wavelength of 415 nm using a micro-plate reader. The total flavonoid content was calculated from the standard curve of quercetin and was expressed as gram quercetin equivalent per 100 gram extract (g% QE). Each experiment was carried out in triplicate. The average total flavonoid content and standard deviation was then calculated.

4. Determination of antioxidant activity

The antioxidant activity of extracts was investigated using 2,2-diphenyl-1-picryhydrazyl (DPPH) assay. The DPPH was dissolved in methanol to prepare the DPPH solution at a concentration of 207 µM. The DPPH solution (100 µL) was added to each sample solution with various concentrations ranging from 15–250 µg/mL in the same volume (100 µL).The mixture was mixed and kept in the dark for 15 min. The absorbance of each reaction solution was determined at the wavelength of 515 nm using a micro-plate reader. The percentage of inhibition for each reaction was calculated, and EC_{50} values (µg/mL) was calculated from the linear equation from the curve between the percentage of inhibition and the solution's concentration. Each experiment was conducted in triplicate. The EC_{50} value of each extract was expressed as the mean ± SD [11].

5. Data analysis

The scientific data was statistically analyzed by IBM SPSS Statistics 20 program (IBM, USA). *P-value* less than 0.05 was considered statistically significant.

Results

1. Phytochemical studies

1.1 Embelin structure elucidation

Embelin (Figure 1.) was obtained from dichloromethane extract as orange-yellow crystals after repeated crystallization by methanol (14.96 % w/w of the extract) with the melting point of 142 – 145 ºC. The UV-Vis spectrum showed maximum absorption at 290 nm. Its molecular formula was derived as $C_{17}H_{26}O_4$ from elemental analysis and m/z at 278.86 (M-CH_3) in the ESI-MS spectrum according to the mass spectra of

embelin standard (TRC, Canada). The IR spectrum (KBr disc) showed a band at 3299 cm^{-1} (-OH stretching), the vibration of methylene groups (CH_2) at 2919 and 2848 cm^{-1}, carbonyl stretching (C=O) at 1613 cm^{-1} and aromatic double bonds (C=C) at 1324 – 1180 cm^{-1}. The ^{1}H NMR (500 MHz, $CDCl_3$): δ 0.88 (3H, t, H-11′), 1.30-1.26 (16H, br s, H-3′-H-10′), 1.47 (2H, m, H-2′), 2.45 (2H, t, J = 7.68 Hz, H-1′), 6.00 (1H, s, H-6). The ^{13}C NMR (125 MHz$CDCl_3$): 116.9 (C-3), 102.2 (C-6), 31.9 (C9′), 29.6 – 27.9 (C-2′- C-8′), 22.7 (C-1′), 22.5 (C-10′), 14.1 (C-11′). According to the spectral data, the isolated compound is proposed to be embelin [8, 12, 13, and 14].

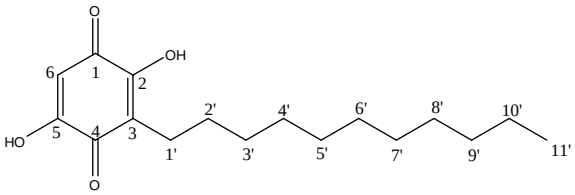

Figure 1. Structure of embelin

1.2 Thin layer chromatography (TLC)

All *A. elliptica* samples were phytochemically analyzed by TLC analysis detected under UV 254 and 366 nm and sprayed with DPPH spray reagent. They exhibited TLC fingerprints as shown in Figure 2. There was a clear chromatographic band that corresponded to embelin at an R_f value of 0.56 in dichloromethane extract (CD) while in ethanolic extract (CE) and filtrate fraction (FT), the bands in this position were reduced. After spraying with DPPH spray reagent, some chromatographic bands including the band that corresponded to embelin were positive to this spray reagent and appeared as yellow bands on purple background suggesting the antioxidant activity. There were more chromatographic bands in dichloromethane extract and filtrate fraction with higher R_f values than embelin that were also positive to DPPH spray reagent suggesting the presence of other antioxidant compounds.

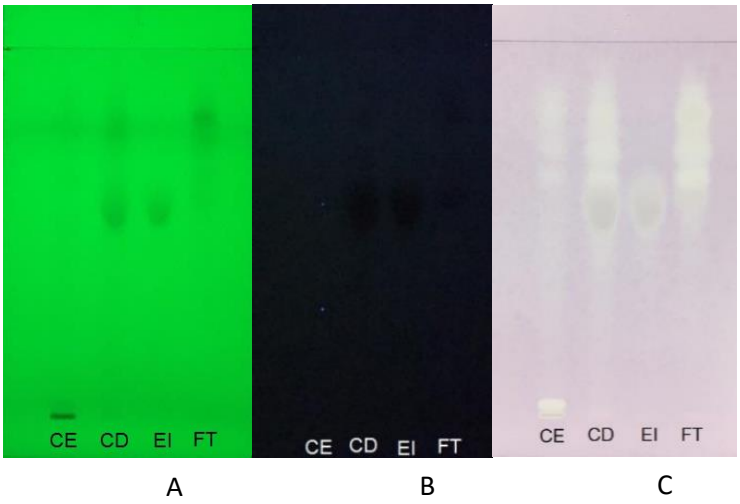

Figure 2. TLC fingerprints of ethanolic crude extract (CE), dichloromethane crude extract (CD), isolated embelin (EI) and filtrate (FT) A - 254 nm, B – UV 365 nm, C –after spraying with DPPH 30 minutes. Adsorbent: Silica gel GF254. Solvent system: chloroform-methanol-formic acid (5:4:1).

1.3 Determination of total phenolic and total flavonoid contents

As shown in Table 1, the *A. elliptica* extracts contained total phenolic contents in the range of 8.31 to 15.84 g gallic acid equivalent in each 100 g extract (g% GAE). The dichloromethane and filtrate extracts (CD, FT, respectively) showed significantly higher total phenolic contents. Total flavonoid contents in *A. elliptica* fruit extracts from ethanolic extract (CE) and filtrate (FT) were 0.12 and 0.27 g quercetin equivalent in 100 g extract (g% QE), respectively while total flavonoid content in CD could not be determined (Table 1).

2. Antioxidant activity

As shown in Table 1, the dichloromethane extract and filtrate fraction (CD and FT) and isolated embelin exhibited similar high antioxidant activities as determined by the DPPH scavenging assay, with EC_{50} values of 63.33, 73.16 and 64.98µg/ml, respectively (5-

6 times weaker than ascorbic acid). The ethanolic extract showed a lower antioxidant effect, with EC_{50} value of 100.37 µg/ml (8 times weaker than ascorbic acid).

Table 1. Antioxidant activity, total phenolic and total flavonoid contents of *A. elliptica* fruits extract.

Extract	DPPH assay EC_{50} (µg/ml)	Total phenolic content (g% GAE)	Total flavonoids content (g% QE)
CE	100.37 ± 3.93[b]	8.13 ± 0.8[b]	0.12 ± 0.01[b]
CD	63.33 ± 4.23[a]	15.69 ± 0.8[a]	cd
FT	73.16 ± 2.98[a]	15.84 ± 2.8[a]	0.27± 0.04[a]
EI	64.98 ± 6.44[a]	-	-
Ascorbic acid	12.63 ± 0.54	-	-

CE = ethanolic crude extract, CD = dichloromethane crude extract, EI = isolated embelin and FT = filtrate. Different letters in the same column indicate significant difference ($p < 0.05$). cd = cannot be determined.

Discussion and conclusion

DPPH is a stable free radical which has an unpaired valence electron at one atom of nitrogen in its structure. Scavenging effect to DPPH radical indicates the potency of the plant extracts to donate the proton to the radicals. Some phytochemicals in natural products, especially phenolics and flavonoids promote the hydrogen donating abilities and act as antioxidant compounds [15].

A. elliptica fruit extracts prepared by maceration with dichloromethane and its filtrate fraction exhibited higher antioxidant activity than ethanolic extract determined by DPPH scavenging assay. High total phenolic content in dichloromethane extract and filtrate fraction were found related to their high antioxidant effects.

The methanolic extract from *A. elliptica* fruits has been reported to promote antioxidant activity as determined by DPPH scavenging assay with EC_{50} value of 45.0 µg/ml [2]. The phytochemical study of this methanolic extract revealed the presence of volatile compounds by gas chromatography – mass spectrometry (GC-MS) technique such as 5-pentadecyl resorcinol derivatives, α-amyrin acetate and tocopherol. Moreover, some phenolic compounds such as aspidin, gingerol, kaempferol and stercuresin were also detected by liquid chromatography – mass spectrometry (LC-MS) technique [2]. These compounds in *A. elliptica* fruit including tocopherol, kaempferol and gingerol have been reported to exhibit antioxidant activities [16, 17]. The ethanolic extract of *A. elliptica* fruit has been shown to exhibit *in vitro* antioxidant activities determined by various methods such as DPPH assay, nitric oxide method and reducing power assay [18, 19]. A GC-MS analysis of ethanolic extract from *A. elliptica* fruit suggested that 2,4-bis(1,1-dimethylethyl)-phenol could be responsible for this antioxidant activity of *A. elliptica* [19]. Methanol, dichloromethane and hexane extracts from *A. elliptica* fruit were recently reported for their antioxidant activities tested by DPPH scavenging assay with EC_{50} values of 8.87, 14.24 and 50.01 µg/ml, respectively. Syringic acid, which exhibited strong DPPH scavenging activity, was also separated and identified from dichloromethane extract [20]. Embelin has been reported as one of the major compounds in *A. elliptica* fruit [8] and it also presented antioxidant activity when tested by DPPH scavenging assay with EC_{50} value of 75.06 µg/ml [21]. In this study, the filtrate fraction, after the separation of embelin, showed high antioxidant activity and had higher phenolic and flavonoid contents than ethanolic extract, while dichloromethane extract which also promoted high DPPH scavenging activity, only contained high total phenolic content but total flavonoid content could not be determined. TLC fingerprint detected by DPPH spray reagent confirmed that embelin was the active compound, however, it appeared that there were other active compounds in *A. elliptica* fruit dichloromethane extract and filtrate fraction that contribute to the antioxidant activity of *A. elliptica*.

Furthermore, there are some reports about other pharmacological activities that are related to antioxidant effects such as the antiproliferative activity against human breast adenocarcinoma (SKBR3) cells reported for ethanolic extract from *A. elliptica* fruit [4]. Three phenolic and flavonoid compounds including quercetin, syringic acid and isorhamnetin isolated from

ethanolic extract of *A. elliptica* fruits showed antibacterial activity against veterinary *Salmonella*, [7]. Ardisiphenols isolated from ethanolic extract of *Ardisia colorata* fruit exhibited antiproliferative effect against the murine breast cancer cells (FM3A) [22].

In the future, isolation of the active antioxidant compounds from the filtrate fraction should be conducted. The results from this study could be beneficial for further studies of related pharmacological activities such as antiproliferative and anti-inflammatory effects of the extracts and isolated compounds from this plant.

Acknowledgements

The authors would like to express sincere gratitude to Siri Ruckhachati Nature Park, Mahidol University to donate the plant sample used in this experiment.

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Antioxidant and Anticancer Activities of *Buchanania siamensis* Miq. Stem and Leaf Extracts

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Abstract

Buchanania siamensis (BS) is a local plant found in mixed deciduous forests of eastern and north-eastern Thailand. The plant has been used as traditional medicine for treatment of food poisoning, gingivitis and aphthous stomatitis. Its stems (S) and leaves (L) were extracted by using dichloromethane (1) and methanol (2) and the extracts were called BSS1, BSS2, BSL1 and BSL2, respectively. Thin layer chromatography fingerprint, antioxidant activity, total phenolic content and anticancer activity of the extracts were studied. The methanolic extracts contained higher total phenolic content than those of dichloromethane extracts but there was no significant difference between stems and leaves. BSS2 showed higher antioxidant activity (EC_{50} of 7.1±0.1 µg/ml) than BSL2, BSS1 and BSL1, respectively. BSL1 exhibited the highest cytotoxicity against HaCaT, HepG2, MCF-7 and MDA-MB-231 cells by IC_{50} of 119.41±4.80, 196.47±41.36, 264.76±8.50 and 289.81±36.57 µg/ml, respectively. However, the BSL1 showed the lowest selectivity index (SI) against those cancerous cell lines (SI of 0.61, 0.45 and 0.41, respectively). Interestingly, the extracts could significantly inhibit the proliferation of HepG2 human hepatoma cells better than MCF-7 and MDA-MB-231 human breast cancer cells. In addition, the SI between HepG2 and HaCaT cells of BSS2 was 2.25, which was higher than BSL2, BSS1 and BSL1 (SI of 1.58, 0.88 and 0.61, respectively) and correlated to its antioxidant activity. These results suggested that *B. siamensis* extracts have high antioxidant and anticancer activities, especially BSS2. Therefore, *B. siamensis* extracts are a good candidate for further studies in herbal medicine and natural product development.

Keywords: *Buchanania siamensis*, Antioxidant, Anticancer, Phenolic Content

Introduction

Buchanania siamensis Miq. (family: Anacardiaceae) has many local Thai names, such as Si thanon chai, Rang thai (Northeastern) and Phang phuai nok, Thanon chai (Central). It is a large perennial shrub or small tree that is widely distributed in tropical areas, and mixed deciduous forests in eastern and north-eastern Thailand. The young shoots and leaves are locally consumed as fresh vegetables and appetizers [1]. The plant has been used as a Thai traditional medicine and medicinal formulation according to local wisdom. Stems and roots have also been used to relieve food poisoning and fever [1-2]. The stem and bark have been used for the treatment of gingivitis, aphthous stomatitis and herpes simplex virus infection [1]. However, to our knowledge, there is no scientific report of its chemical and pharmacological activities. Therefore, *B. siamensis* stem and leaves were selected in order to study their chemical composition and the cytotoxic effect of extracts on cancerous and non-cancerous cell lines.

Methodology

Extract preparation

The stems and leaves of *B. siamensis* Miq. were collected from a community forest in Sa Kaeo province, eastern part of Thailand in April 2015. The plant was identified by Dr. Chakkrapong Rattamanee, Faculty of Agricultural Technology, Burapha University, Thailand. Its herbarium specimen is C. Rattamanee M273 and it is kept at the Faculty of Agricultural Technology, Burapha University and at the Bangkok Herbarium, Department of Agriculture, Bangkok. The air-dried stems (2.3 kg) of *B. siamensis* were extracted with CH_2Cl_2 and MeOH successively (each 2 × 10 L, for 5 days) at room temperature. The crude extracts were evaporated under reduced pressure to afford brownish CH_2Cl_2 (6.09 g) and MeOH (97.80 g) extracts, respectively. Ground-dried leaves (316.69 g) of *B. siamensis* were extracted with CH_2Cl_2 and MeOH (each 2 × 5 L, for 5 days) successively at room temperature and the solvent was evaporated under reduced pressure to afford CH_2Cl_2 (10.60 g) and MeOH (18.37 g) extracts, respectively. *B. siamensis* leaf extracts from CH_2Cl_2 and

MeOH fractions were called as BSL1 and BSL2, respectively. *B. siamensis* stem extracts from CH_2Cl_2 and MeOH fractions were called as BSS1 and BSS2, respectively.

Thin layer chromatography

The extracts were dissolved in methanol at 1 mg/ml and then spotted onto TLC Silica gel 60 F254 plates (Merck KGaA, Germany). One dimensional TLC analysis was performed with EtoAC:Hex:Acetic acid in volume ratio of 60:3.93:0.07, respectively, as a mobile phase. Lane 1 to 11 were epigallocatechin (EGC), vanillic acid, gallic acid, BSL1, BSL2, BSS1, BSS2, catechin, quercetin, caffeic acid and tannic acid, respectively. Spots were observed under Ultra-Violet light (UV light) at 254 nm and 360 nm. The plates were then sprayed with 30% sulfuric acid, anisaldehyde-sulfuric, DPPH solution and observed (Figure 1).

Study of Antioxidant

DPPH radical scavenging assay [3] was used for determination of antioxidant activity of the extracts. Briefly, 1 mM DPPH (2,2-diphenyl-picryl hydrazine) was freshly prepared by dissolving in methanol. The extracts were diluted to various concentrations by using methanol. The DPPH solution (200 µl) was added to diluted extracts (2800 µl) and then incubated at room temperature for 15 min. The absorbance of bleaching solution was measured at 515 nm. The percent of inhibition was calculated and expressed as 50% effective concentration (EC_{50}). Ascorbic acid and tocopherol were used as positive compounds for comparison.

Study of Total phenolic content

Folin-Ciocalteu method [4] was used for determination of total phenolic content. Briefly, tannic acid (0.5 ml), a standard phenolic compound, was diluted with methanol to various concentrations and Folin reagent (0.25 ml) was added with 20% sodium carbonate solution (0.25 ml). After incubation at room temperature for 40 min, the absorbance of the solution was measured at 725 nm. The standard curve was plotted. The extracts were diluted and then mixed with Folin reagent and 20% sodium carbonate solution as the description above. The sample absorbance was compared to the standard curve and expressed as tannic acid equivalent (mg TAE/g).

Anticancer determination

The extracts and the standard medicine, tamoxifen, were dissolved in dimethyl sulfoxide and filtered by using 0.4 µm syringe filter. The cell lines in this study were non-cancerous cells i.e. human keratinocytes or HaCaT and cancerous cells i.e. human hepatoma (HepG2) and human breast cancer cells i.e. MCF-7 and MDA-MB-231 cells. These cells were cultured in 96-well plate (10,000 cells/well) by using DMEM culture medium supplemented with 10% FBS and 1% Penicillin-Streptomycin and overnight incubated at 37ºC in a humidified atmosphere with 5% CO_2. The cells were added with various concentrations of either extracts or tamoxifen and then incubated for 24 h. Cell viability was measured by using Alamar blue assay [5]. The cytotoxicity was calculated to % cytotoxicity and then expressed as 50% inhibitory concentration (IC_{50}). The selectivity index (SI) was calculated by using the following equation.

SI = IC_{50} from non-cancerous cells / IC_{50} from cancerous cells

Statistical analysis

The results were reported in mean ± S.D. One-Way ANOVA and multiple comparison (LSD) were analyzed by using SPSS version 16.0.

Results

The phytochemical screening (Figure 1) of CH_2Cl_2 leaf extracts (BSL1) revealed the absence of vanillic acid and gallic acid while MeOH leaf extracts (BSL2) revealed the absence of caffeic acid and tannic acid. The CH_2Cl_2 stem extracts (BSS1) revealed the presence of vanillic acid and quercetin while MeOH stem extracts (BSS2) revealed the presence of EGC, vanillic acid, catechin, quercetin and caffeic acid. In addition, all extracts showed positive chemical reaction screening by using DPPH spray reagent (Figure 1E) suggesting that the extracts had antioxidative activity. In Table1, the methanolic extracts (BSL2 and BSS2) can be seen to contain higher total phenolic content than those of dichloromethane extracts (BSL1 and BSS1). However, there was no significant difference between stem and leaf extracts. For the DPPH assay, BSS2 showed stronger antioxidant activity (EC_{50} of 7.1±0.1 µg/ml) than BSL2, BSS1 and BSL1, respectively (Table 1).

The extracts were tested for their inhibitory effect on both cancerous and non-cancerous cell lines to determine the SI (Table 2). Although BSL1 exhibited the highest cytotoxicity against HaCaT, HepG2, MCF-7 and MDA-MB-231 cells with IC_{50} of 119.41±4.80, 196.47±41.36, 264.76±8.50 and 289.81±36.57 µg/ml,

respectively, BSL1 showed the lowest SI compared with the other extracts. BSL2 showed a lower cytotoxic effect than BSL1, especially in MCF-7 and MDA-MB-231 cells where the extract had no cytotoxic effect in the concentration range of 50 to 800 µg/ml. Similarly, BSS2 showed no cytotoxic effect against MCF-7 and low cytotoxicity against MDA-MB-231 cells. These results suggest that the dichloromethane extracts were more cytotoxic than the methanolic extracts. In addition, the extracts from leaves showed higher cytotoxic effect than stem extracts. Moreover, all extracts could significantly inhibit the proliferation of human hepatoma, HepG2 cells, better than human breast cancer cells, MCF-7 and MDA-MB-231 cells. The SI between HepG2 and HaCaT cells of BSS2 was 2.25, which was higher than BSL2, BSS1 and BSL1, respectively. However, there was no significant difference between IC_{50} of extracts against estrogen receptor positive MCF-7 breast cancer cells and estrogen receptor negative MDA-MB-231 breast cancer cells.

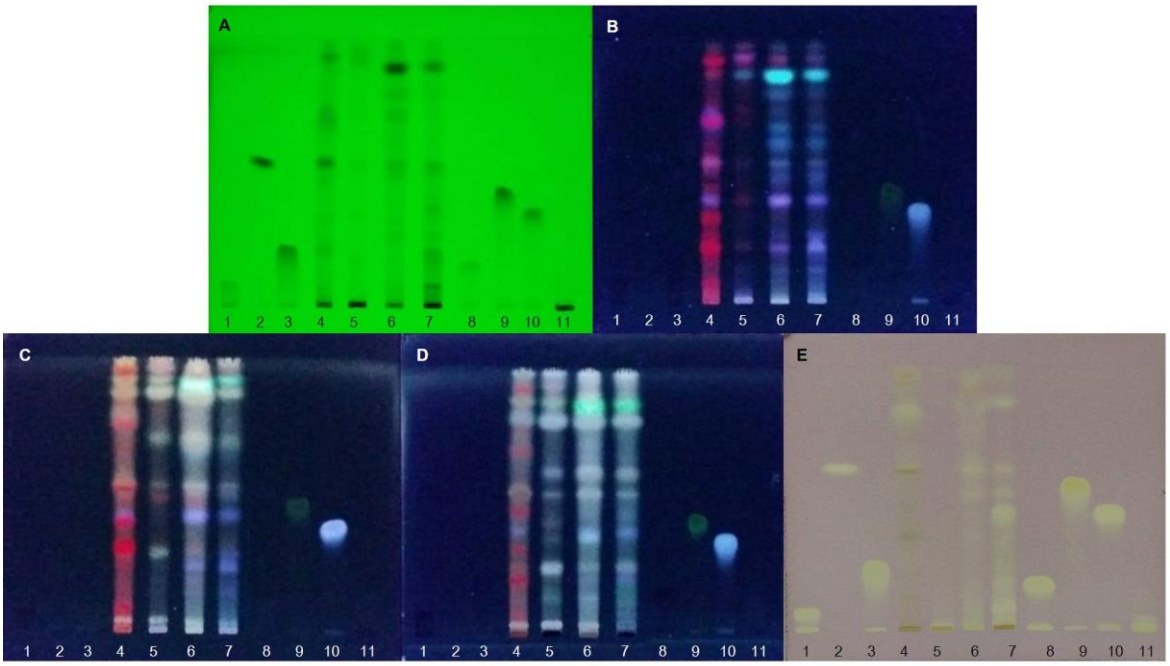

Figure 1. TLC fingerprint of *B. siamensis* extracts. A) 254 nm, B) 360 nm, C) Anisuldehyde-sulfuric under 360 nm, D) 30% sulfuric under 360 nm and E) DPPH spray reagent. Lane 1: EGC, 2: vanillic acid, 3: gallic acid, 4: BSL1, 5: BSL2, 6: BSS1, 7: BSS2, 8: catechin, 9: quecetin, 10: caffeic acid and 11: tannic acid.

Table 1 Antioxidant activity and total phenolic content of *B. siamensis* extracts

Extracts/Standard	Antioxidant	Total phenolic
BSL1	750.1±52.4^{a}	6.2±0.4^{A}
BSL2	10.0±0.2^{b}	206.7±12.8^{B}
BSS1	13.5±1.1^{c}	6.4±0.5^{A}
BSS2	7.1±0.1^{d}	192.3±8.7^{B}
Ascorbic acid	3.5±0.1^{e}	ND
Tocopherol	6.7±0.1^{f}	ND

Note: ND represents no determination.

Discussion and Conclusion

Phenolic compounds are widely dispersed in the plant kingdom with structures ranging from one aromatic ring with one or more hydroxyl groups attached to large and complex phenolic structures [6]. Phenolic substances are better extracted with high polarity solvents such as methanol, ethanol, acetone and ethyl acetate than low polarity solvents [7]. Therefore, the methanolic extracts of *B. siamensis* in this study contained higher amount of phenolics than dichloromethane extracts. Although the extracts were screened for their phenolic composition using HPLC, there was no compatible spectrum peak with the general standards of phenolic compounds (data not shown). However, the TLC mobile phase system and fingerprints from *B. siamensis* in this study can be used for quality control of the extracts.

Table 2 IC_{50} and SI values of *B. siamensis* extracts

Ext. /Cpd	Inhibitory effect						
	HaCaT	HepG2		MCF-7		MDA-MB-231	
	IC_{50} (µg/ml)	IC_{50} (µg/ml)	SI	IC_{50} (µg/ml)	SI	IC_{50} (µg/ml)	SI
BSL1	$119.41\pm4.80^{a,A}$	$196.47\pm41.36^{a,B}$	0.61	$264.76\pm8.50^{a,C}$	0.45	$289.81\pm36.57^{a,C}$	0.41
BSL2	$434.12\pm16.56^{b,A}$	$275.27\pm37.47^{b,c,B}$	1.58	$>800^{b,C}$	0.54	$>800^{b,C}$	0.54
BSS1	$256.34\pm6.63^{c,A}$	$291.25\pm13.75^{c,B}$	0.88	$312.97\pm32.74^{c,B,C}$	0.82	$341.43\pm5.50^{c,C}$	0.75
BSS2	$490.24\pm16.10^{d,A}$	$218.34\pm38.75^{a,b,B}$	2.25	$>800^{b,C}$	0.61	$778.48\pm15.09^{b,C}$	0.63
Tamoxifen	$5.62\pm0.05^{e,A}$	$11.03\pm0.47^{d,B}$	0.51	$4.18\pm0.14^{d,C}$	1.34	$8.42\pm0.05^{d,D}$	0.67

Note: a to e represented significant difference of IC_{50} between extracts in the same cell line (column).

A to D represented significant difference of IC_{50} between cell lines in the same extract (row).

B. siamensis extracts showed anticancer activity *in vitro*, especially BSS2 which showed the highest SI, which represents cytotoxicity against cancerous cells compared to non-cancerous cells. In addition, BSS2 showed higher antioxidant activity than the other extracts which correlated with the high amount of total phenolic content and TLC fingerprint. Plenty of phenolic compounds have been reported to have anticancer activity both *in vitro* and *in vivo* [6-7]. For example, gallic acid was reported to have a cytotoxic effect on several cancer cells and in mouse models via inhibition of cell proliferation, ribonucleotide reductase, cyclooxygenases and angiogenesis and also by apoptotic induction [8-10]. Several flavonoids, e.g. quercetin, kaempferol and catechin, are also well-known non-enzymatic antioxidants which show strong anticancer activity [6-7]. The antioxidants are reported to inhibit mechanisms of cancer progression including inhibition of phase I enzymes, induction of phase II enzymes, stimulation of DNA repair, induction of cell cycle arrest, induction of apoptosis, inhibition of cell proliferation and antiangiogenesis [7]. Therefore, natural phenolics are a great source of antioxidants and anticancer agents.

In conclusion, the extracts from *B. siamensis* stem and leaves are promising candidates for further studies and development of herbal health products, especially the methanolic extract from its stem.

Acknowledgements

This work was financially supported by the Research Grant of Burapha University through National Research Council of Thailand (Grant no. 118/2559). Thank you the Center for Research and Development of Herbal Health Products, Khon Kaen University, Khon Kaen, Thailand, for facility supports. Thank you Dr. Chakkrapong Rattamanee, Faculty of Agricultural Technology, Burapha University, Thailand, for identifying the plant and preparing the herbarium specimen.

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Antiproliferative Effect of *Eurycoma longifolia* Root Extract and Eurycomanone on Human Hepatocellular Carcinoma and Human Dermal Fibroblast Cell Lines

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Abstract

This study investigated the antiproliferative effect of *Eurycoma longifolia* root**.** The powdered root was macerated with 95%ethanol for 7 days. The ethanol extract had the drug-extract ratio (DER) of 51.27: 1. The standard compound used in the study was eurycomanone. The TLC and HPLC chromatogram showed the presence of the eurycomanone in the ethanol extract. The extract and eurycomanone strongly inhibited Hep3B (human hepatocellular carcinoma cell line induced by hepatitis virus type B) with the ED_{50} values of 27.78 $\pm$0.72 and 17.94 $\pm$0.18 µg/mL respectively. Doxorubicin, the positive control, had ED_{50} values of 24.36 $\pm$0.28 µg/ml. Eurycomanone also inhibited HDF (human dermal fibroblast), a normal cell line, with the ED_{50} of 45.98 $\pm$0.35 µg/mL. Eurycomanone was highly cytotoxic against liver cancer cell and eurycomanone was the antiproliferative compound in *Eurycoma longifolia* root. The cytotoxicity of eurycomanone was more potent than doxorubicin when tested with C3A cell line.

Keywords: *Eurycoma longifolia***,** Pla lai phueak**,** Antiproliferative Effect**,** MTT Assay

Introduction

Eurycoma longifolia or Plalai phueak is a tall, slender, shrubby tree, which grows in sandy soil. It belongs to the Simaroubaceae family. It has compound leaves on branches that can grow up to 1 m long. The leaves are pinnate in shape and green in color. The numerous leaflets are opposite or sub opposite, lanceolate to ovate-lanceolate, 5–20 cm by 1.5–6 cm, with smooth margins. Flowers are tiny, reddish, and unisexual and are densely arranged. The drupes are ovoid with a distinct ridge, 1–2 cm by 0.5–1.2 cm and they turn dark reddish brown when ripe [1–3]. Indigenous to South-East Asian countries like Malaysia, Indonesia, and Vietnam, some of the plant species are also found in regions of Cambodia, Myanmar, Laos and Thailand [5-8]. The root extract of *Eurycoma longifolia* was used as folk medicine for sexual dysfunction, aging, malaria, cancer, diabetes, anxiety, aches, constipation, exercise recovery, fever, increased energy, increased strength, leukemia, osteoporosis, stress, syphilis and glandular swelling. It was also used as an aphrodisiac, antibiotic, appetite stimulant and health supplement [9-15]. *Eurycoma longifolia* roots, stem, leaves and bark were reported for the isolation of sixty five phenolic compounds [9]. *Eurycoma longifolia* is a rich source of various classes of bioactive compounds, which include quassinoids, β-carboline alkaloids, canthin-6-one alkaloids, triterpene-type tirucallane, squalene derivatives and ligans [9, 15-18]. Among them, bitter tasting quassinoids account for a major portion of *Eurycoma longifolia* root. The quassinoids are a group of nortriterpenoids with C-19, C-20 and C-21 skeleton [13-14]. The presence of tirucallane and squalene-type triterpenes might be the quassinoids' biological precursors. Eurycomanone a quassinoid was found in the plant root [19] and it was reported to suppress the lung cancer cell tumor markers; prohibitin, annexin 1 and endoplasmic reticulum protein 28 [21-22]. It was also cytotoxic against human lung cancer (A-549), and human breast cancer (MCF-7) cell lines [23]. Recently it has been reported to be an antimalarial with activity against *P. falciparum* NF- ΚB inhibitor [20].

Eurycoma longifolia root has been used as one plant component of a Thai herbal medicine used by patients suffering from liver cancer for self-medicine. The present study was conducted to evaluate the antiproliferative activity of ethanol extract of the roots of *Eurycoma longifolia* and its major constituent, eurycomanone.

Material and method

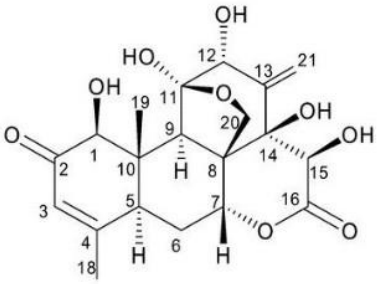
Plant Material

We collected *Eurycoma longifolia* (pla lai peauk) roots from Ubon Ratchathani, Thailand in 2012.

The plant was identified by comparing with the voucher specimen (BKF. 14760), which was kept at the office of the Forest Herbarium, Department of National Park, Wildlife and Plant Conservation, Thailand.

Chemical standard

Eurycomanone was purchased from Chengdu Biopurify Phytochemicals Ltd. as off-white powder with the melting point (m.p.) of 250-251 ^{O}C and hRf value of 28 on TLC chromatogram, using silica gel aluminum sheet as an adsorbent and MeOH: DCM, 30:70 as a mobile phase. The UV spectrum in MeOH had maximum absorption peak of 254 nm. The FTIR spectrum showed absorption peaks (KBr disc) at 3379.29 (OH-stretching); 2980.02 and 2879.72 (aliphatic C-H stretching); 1732.08, 1674.21 and 1618.28 (C=C stretching); and 1055.06 cm^{-1} (OH def). ESI-MS showed the molecular ion $[M+Na]^{+}$ of 431.1324. The corresponding compound had the molecular mass of 408 [$C_{20}H_{24}O_9$].^{1}H-NMR (CD_3OD, 500 MHZ), δ 4.30 (1H, s, H-1), 6.50-6.53 (1H, d, H-3), 3.02 (1H, d, H-5), 2.06-2.09 (1H, m, H-6a), 2.29-2.33 (1H, m, H-6b), 4.60-4.78 (1H, t, H-7), 3.06 (1H, s, H-9), 4.79 (1H, s, H-12), 3.95 (1H, s, H-15), 2.02 (3H, s, H-18), 1.19 (3H, s, H-19), 3.72-3.88 (2H, dd, H-20), 5.42 (1H, s, H-21a), 5.54 (1H, s, H21b). ^{13}C-NMR (CD_3OD, 125 MHz), δ 85.24 (C1), 199.70 (C-2), 126.86 (C-3), 166.09 (C4), 43.74 (C-5), 27.14 (C-6), 78.02 (C-7), 54.02 (C-8), 49.39 (C-9), 47.39 (C-10), 110.08 (C-11), 80.22 (C-12), 147.03 (C-13), 78.02 (C-14), 72.64 (C-15), 175.41 (C-16), 23.77 (C-18), 10.95 (C-19), 68.93(C-20), 122.41 (C-21) [24].



$C_{20}H_{24}O_9$ Mr 408

Figure 1. Structure of Eurycomanone

Preparation of ethanol extract

The roots were cleaned, cut into small pieces, dried in a hot-air oven (40^{O}C) for 24h, and crushed to powder by the disc mill. The powdered root (10 kg) were macerated with 95%ethanol (commercial grade) 10 L for 7 days. The extract was concentrated under reduced pressure at 45^{O}C. The ethanol extract had the drug-extract ratio (DER) of 51.27: 1.

Thin-layer chromatography

Five milligrams of *Eurycoma longifolia* ethanol extract was dissolved in 10 mL methanol and 20 µL was spotted onto a TLC plate. The TLC plate was developed with 95:5, 90:10 and 80:20 (dichloromethane: methanol) as a mobile phase. TLC was performed on aluminum sheets coated with Silica gel 60 $F_{254,}$ aluminum, pre-coated, layer thickness 0.25 cm (E. Merck, Germany). Detection was under ultra violet (UV) at 254 nm after spraying with anisaldehyde and heating on the hot plate (120^{O}C) for 1 minute. The hR_f values of ethanol extract were calculated.

Antiproliferativeactivity

Sample preparation

The stock solution of *Eurycoma longifolia* root extract (200 mg/mL) was diluted to the final concentrations of 100, 500, 1,000, 5000, 10,000 µg/mL.

Preparation of human hepatocellular carcinoma cell lines (Hep3B and C3A)

MTT assay for cytotoxicity against Hep3B and C3A was performed. The cell lines were cultured and grown as monolayer. They were maintained in MEM medium containing 10% FBS, 1% sodium pyruvate, 1% non-essential amino acid and 1% penicillin-streptomycin to prevent the microbial contamination. The cell lines of each experiment were incubated at 37^{o}C with 5% CO_2 [28].

Preparation of human dermal fibroblast (HDF)

Human dermal fibroblast (HDF) cells were maintained in MEM containing high glucose medium 10% FBS and 1% penicillin-streptomycin.

MTT assay for cytotoxicity

MTT assay measures the reduction of yellow 3-(4, 5-dimethythiazol- 2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) by mitochondrial succinate dehydrogenase of living cells. The salt enters the cells and passes into the mitochondria where it is reduced to an insoluble, colored (dark purple) formazan product. The optical density of the colored formazan is determined at the wavelength of 570 nm. Decreases in the color intensity indicate the measure of nonliving cells. Doxorubicin is used as a positive control. Calculation of ED_{50} (50% inhibition of cancer cell growth) is performed using the formula below:

Percentage of viability = OD sample/OD control x 100

Percentage of inhibition = 100 - % viability

Antiproliferative effect of Eurycoma longifolia root.

Human hepatocellular carcinoma cell lines Hep3B and C3A were prepared diluted with culture media to $1x10^5$ cells and human dermal fibroblast HDF $1x10^4$ cells were inoculated into 96-well plates. They were treated with five concentrations (1, 10, 50, 100 and 200 mg/mL) of *Eurycoma longifolia* root extract and doxorubicin (control). The cells were incubated under appropriate conditions for 48 hours. MTT (3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide) was dissolved at 5 mg/ml in PBS and used essentially as previously described. Briefly, 20 µL of MTT solution added to each well and incubated for 4 h. at 37^{o}C. After the incubation, MTT reagent solution was added in every plate. The medium in plate were removed gently and replaced with DMSO to dissolve the formazan crystals which were produced by the living cells. The optical density (OD) was measured by microplate reader at the wavelength of 570 nm. The result was generated from the dose-response established from the percentage of cells viability (Y-axis) and the concentrations of each sample (X-axis). Cytotoxicity was expressed the effective dose for 50% cells viability of *Eurycoma longifolia* root extracted (ED_{50}) [27].

Statistical analysis

The ED_{50} values were obtained by plotting the absorbance against the corresponding concentrations of the test compound, and were reported as the mean ± standard error (SE). Data were statistically analyzed using the Kruskal-Wallis One Way Analysis of Variance. Significance was accepted at the $P < 0.05$ level.

High Performance Liquid Chromatography

Sample preparation

Eurycoma longifolia dried root extract powder (100 mg) in a 50 mL screw-capped conical flask containing 10 mL of ethanol was placed in a water bath at 80^{O}C for 1 h before being centrifuged at 5,000 rpm for 20 min. The supernatant was evaporated until completely dry, reconstituted with 2 mL methanol and filtered through a 0.45 µm syringe filter before injection into the HPLC column.

Standard solution

Two milligrams of eurycomanone standard was dissolved in 10 mL of methanol to give a 200 µg/mL solution.

Optimal chromatographic condition

The optimal chromatographic condition was for a HPLC C18 column :BDS Hypersil® C_{18} column)250x4.6 mm i.d., 5µm(**.** Column temperature was maintained at 40°C, injection volume was 2 µl, and the gradient mobile phase was acetonitrile (A), 0.1 % formic acid in water (B) (Table 1). Flow rate was maintained at 0.7 ml/min and the UV detector operated at a wavelength 244 nm. RP-HPLC was performed using Shimadzu Class VP)consisting :CL-10A system, UV-VIS SPD-10A detector, LC-10 AD, SIL-10A injector).

Table 1. Mobile phase (gradient mode)

Runtime	A (%)	B (%)
0	10.0	90.0
7	12.5	87.5
9	12.5	87.5
11	15.0	85.0
13	15.0	85.0
18	90.0	10.0
22	10.0	90.0
35	10.0	90.0

A = acetonitrile and B = 0.1 % formic acid

Results and discussion

TLC chromatogram was used to separate and identify eurycomanone in *Eurycoma longifolia* root using solvent mixture of dichrolomethane: methanol. Figures 3A-3C show TLC separation of standard eurycomanone and ethanol extracts of *Eurycoma longifolia* root. Standard eurycomanone under ultra violet (UV) at 254 nm is shown as a dark spot as well as in the ethanol extracts of *Eurycoma longifolia* roots It was found that eurycomanone was detected in all concentrations of the ethanol extracts of *Eurycoma longifolia* root samples tested. The quantity spotted on the TLC plate was 20 µL.

HPLC analysis suggested that the *Eurycoma longifolia* ethanol extract spiked with eurycomanone showed higher intensity at the same retention time of eurycomanone standard compared to ethanol extract. This result suggested the presence of eurycomanone in the ethanol extract as shown in Figure 4.

Other chemical constituents in the ethanol extract of *Eurycoma longifolia* root included fluorescing substances, Figure 5. We isolated stigmasterol from *Eurycoma longifolia* root. The stigmasterol we isolated

was a white powder with a melting point (m.p.) of 154-156 ^{O}C and hR_f value of 45 on TLC chromatogram, using silica gel aluminum sheet as an adsorbent and MeOH: DCM, 10:90 as a mobile phase. The UV spectrum in MeOH had maximum absorption peak of 206 nm. The FTIR spectrum showed adsorption peak (KBr disc) at 3353 cm^{-1} (OH-stretching); 2935 cm^{-1} and 2867 - cm^{-1} (aliphatic C-H stretching); 1458 cm^{-1} (C=C absorption peak); other absorption peaks included 1383 cm^{-1} (CH_2 def); 1054 cm^{-1} (OH def); 971 cm^{-1} (cycloalkane) and 799 cm^{-1}. ESI-MS showed the molecular ion $[M+Na]^+$ of 435.3614. The corresponding compound had the molecular mass 412 [$C_{29}H_{42}O$]. ^1H NMR ($CDCl_3$, 500 MHz): δ 0.68, 0.79, 0.82, 0.86, 0.92, 1.02 (each 3H, s, Me × 6), 3.53 (1H, m, H-3), 5.36 (1H, t, H-6), 5.15 (1H, s, H-22), 5.01 (1H, s, H-23). ^{13}C NMR ($CDCl_3$, 125 MHz): δ 140.76 (C- 5), 138.30 (C-22), 121.9 (C-6), 71.79 (C-3), 56.87 (C-14), 56.07 (C-17), 51.23 (C-24), 50.17 (C-9), 31.90 (C-25), 42.30 (C-13), 40.46 (C-20), 39.78 (C-12), 42.30 (C-4), 37.26 (C-1), 36.51 (C-10), 31.90 (C-8), 31.66 (C-7), 28.90 (C-16), 31.87 (C- 2), 28.39 (C-28), 24.36 (C-15), 21.21 (C-21), 21.07 (C-11), 19.38 (C-27), 21.07 (C-26), 12.23 (C-19), 12.04 (C-29), 11.97 (C-18) [25].

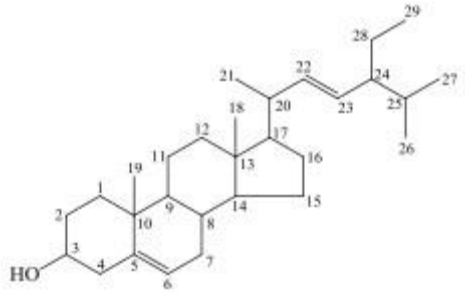


Figure 2. Stigmasterol from *Eurycoma longifolia* root

Eurycoma longifolia ethanolic root extract, doxorubicin and eurycomanone were investigated for cytotoxicity by MTT assay. The results (Table 2) showed the antiproliferative effect of *Eurycoma longifolia* root, doxorubicin and eurycomanone.

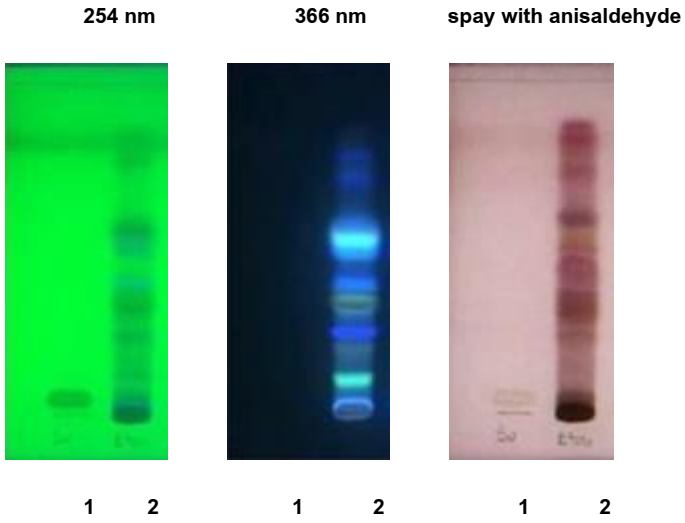


Figure 3A. TLC chromatograms of the ethanol extract and eurycomanone *(1: Eurycomanone standard and 2: Ethanol extract of Eurycoma longifolia root); Adsorbent: silica gel 60 F254 aluminum sheet; Mobile phase: dichloromethane: methanol = 95:5; the hR_f value of eurycomanone = 10*

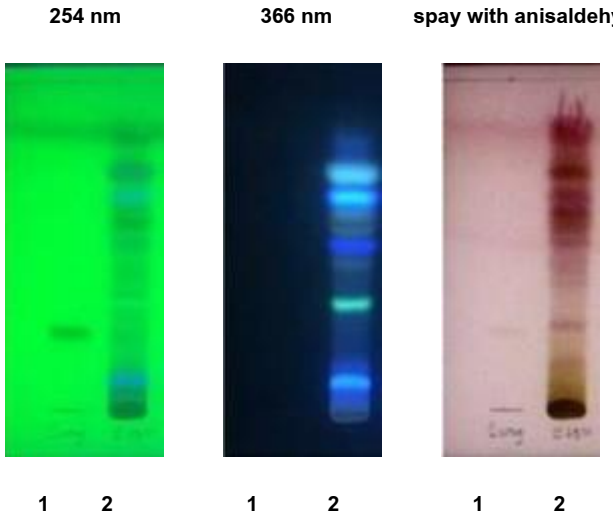


Figure 3B. TLC chromatograms of the ethanol extract and eurycomanone ***(1: Eurycomanone standard and 2: Ethanol extract of Eurycoma longifolia root); Adsorbent: silica gel 60 F254 aluminum sheet; Mobile phase: dichloromethane: methanol = 90:10; the hR$_f$ value of eurycomanone = 26***

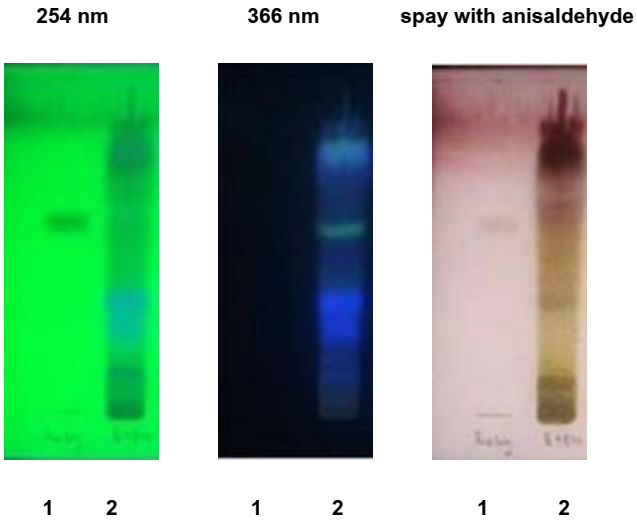


Figure 3C. TLC chromatograms of the ethanol extract and eurycomanone ***(1: Eurycomanone standard and 2: Ethanol extract of Eurycoma longifolia root); Adsorbent: silica gel 60 F254 aluminum sheet; Mobile phase: dichloromethane: methanol = 80:20; the hR$_f$ value of eurycomanone = 64***

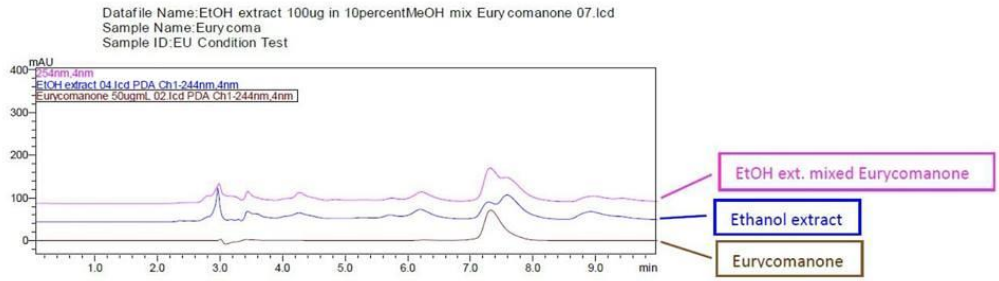


Figure 4. HPLC chromatograms of ethanol extract and eurycomanone

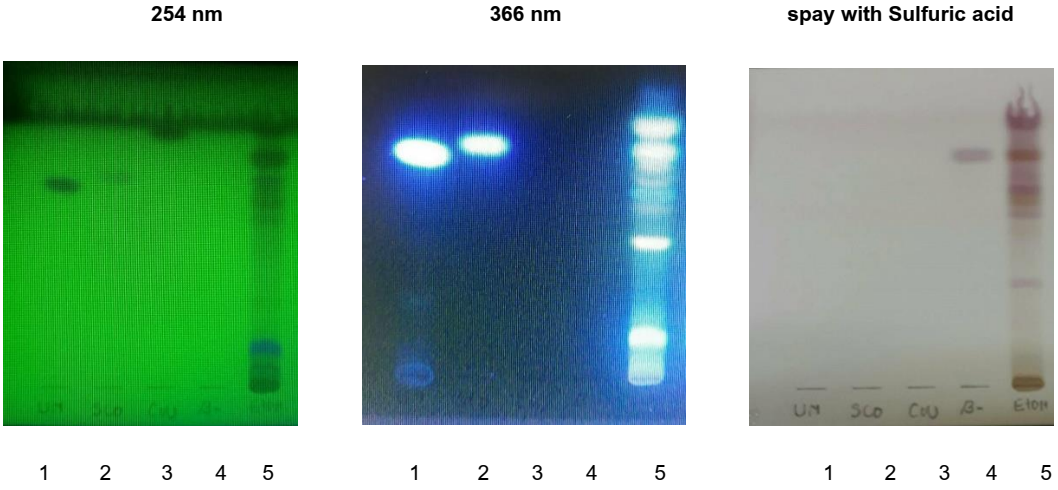


Figure 5. TLC chromatograms of the stigmasterrol and fluorescing compounds of *Eurycoma longifolia* roots *(1: Umbelliferon, 2: Scopolatin, 3: Coumarin, 4: Stigmasterrol and 5: Ethanol extract of Eurycoma longifolia root); Adsorbent: silica gel 60 F254 aluminum sheet; Mobile phase: dichloromethane: methanol = 90:10*

Table 2. Antiproliferative effect of *Eurycoma longifolia* root

Cell lines	**ED_{50} (μg/ml)**		
	Doxorubicin (positive control)	**EtOH extract of *Eurycoma longifolia* root**	**Eurycomanone**
Hep3B	41.44 ±0.12	27.78 ±0.72	17.94 ±0.18
C3A	27.73 ±0.14	310.23 ±0.33	45.98 ±0.35
HDF (normal cells)	24.36 ±0.28	-	1342.29 ±0.33

Note: The values are shown as mean ± SE, n = 3

Previously, the antiproliferative activity of eurycomanone from *Eurycoma longifolia* has been investigated in cancerous cell lines (Caov-3, HeLa, Hep G2, HM3KO and MCF-7) and it has been found to be relatively nontoxic on noncancerous cell lines (MDBK, Vero). Eurycomanone proved to be cytotoxic towards HeLa cells by triggering apoptotic cell death [26]. Our study showed that *Eurycoma longifolia* root ethanol extract had cytotoxic activity against human hepatocellular carcinoma cell lines (Hep3B and C3A). Differences in the activity might come from the differences of assay method for cytotoxicity [27], type of cancer cell line and the duration of incubation time.

Conclusion

Eurycoma longifolia contained eurycomanone. TLC and HPLC chromatogram revealed the presence of eurycomanone in ethanol extract of *Eurycoma longifolia* root. We discovered that eurycomanone was highly cytotoxic against liver cancer cell and eurycomanone

was the antiproliferative compound in *Eurycoma longifolia* root. The cytotoxicity of eurycomanone was more potent than doxorubicin when tested with C3A cell line. Further studies for detailed chemical characterization and more extensive biological evaluation with various cancer call line are needed. Such information are necessary for the research and development of anticancer drug (s) from this plant in the future.

Acknowledgment

This study was supported by Faculty of pharmacy and Faculty of Medicine Siriraj Hospital, Mahidol University for financial support.

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Antifungal Activity of Alginate Microparticles of Blended Essential Oils

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Abstract

Humans have used natural substances for several benefits because they are hypoallergenic, exhibit low environment pollution and are safer than synthetic substances. Essential oils have been used popularly in medication. The antifungal activity of a blended essential oil (BEO) composed of lavender, clary sage and ylang ylang oils in the ratio of 4:4:2, has been demonstrated against *Candida albicans*. However, this activity can be lost by volatilization and essential oils are easily degraded by heat or UV light. The objective of this study was to encapsulate BEOs in microparticles to preserve their optimal antifungal activity and protect them from the external environment. Alginate microparticles were prepared by using the ionic gelation method and encapsulated by calcium chloride cross-linking agent and sodium alginate polymer. The effects of varying the sodium alginate (5 and 7 %w/v) and calcium chloride concentrations (3, 5, 7 and 10 %w/v) were evaluated for %encapsulated efficiency (%EE) and %loading capacity (%LC). The antifungal activity of encapsulated and non-encapsulated BEOs against *C. albicans* was evaluated. The formulation prepared by 7 %w/v sodium alginate and 10 %w/v calcium chloride provided the highest %EE and %LC, which were 58.36±3.24 % and 12.45±0.59 %, respectively. The antifungal activity of non-encapsulated BEOs rapidly decreased, as detected by decreasing inhibition zone, within storage period of 2 days. The antifungal activity of encapsulated BEOs could be maintained for up to 8 days of storage at room temperature. In conclusion, encapsulation may effectively reduce the volatility rate of BEOs, thus prolong the antifungal activity.

Keywords: Blended Essential Oils, Antifungal Activity, Microparticle

Introduction

Natural compounds offer advantages over synthetic substances in terms of safety. Natural substances that are popular in medicinal field are essential oils, aromatic oily liquid extracted from plants. Essential oils do not only serve as food and cosmetic additives but also exhibit antimicrobial and antioxidant properties [1]. However, essential oils can be easily volatilized at room temperature and are easily degraded when they are exposed to light, especially UV radiation and heat. Protection is needed to increase essential oils duration of action and provide controlled release. Encapsulation technology may be applied for protecting the volatility of essential oils [2]. Several studies have demonstrated the application of microparticles of essential oils in medicinal field, for example insect control [3], antibacterial [4] and antifungal [5]. The popular method of encapsulation technology is ionic gelation because it is simple, low cost and suitable for substances which have hydrophobic properties. The principle of this method is the use of polar polymers connected with oppositely charged cross-linking agents. The microparticles are formed to encapsulate the active ingredients inside. One of the interesting properties of essential oils in medical field is antifungal activity. The study showed that blended essential oils (BEOs) from lavender, clary sage, and ylang ylang at a ratio of 4:4:2 (v/v) showed antifungal activity against *Candida albicans* [6]. The objective of this study was to encapsulate BEOs using ionic gelation technique and evaluate their antifungal activity against *C.albicans*.

Materials and Methods

Materials

Cary sage oil was purchased from Make Scents Limited) Bangkok, Thailand). Lavender oil and ylang ylang oil were purchased from Thai-China Flavours and Fragrances (Nonthaburi, Thailand). Linaool, *p*-Cymene, and Clotrimazole were purchased from Sigma-Aldrich (Germany). *Candida albican* (ATCC 10231) was obtained from Department of medical science (Nonthaburi, Thailand). Calcium chloride was purchased

from Ajax Finechem (New Zealand). Sodium alginate was purchased from T. C. Sathaporn group (Bangkok, Thailand).

Encapsulation of Blended Essential Oils by ionic gelation

Blended essential oils (BEOs) were composed of lavender, clary sage and ylang ylang oils in the ratio of 4:4:2 v/v. Microparticles containing BEOs were prepared using ionic gelation method described by Gab et.al [7]. Sodium alginate was dissolved in distilled water to produce sodium alginate solution with concentration of 5 and 7 % (w/v). Calcium chloride was dissolved in distilled water to produce calcium chloride solution with concentration of 3, 5, 7 and 10% (w/v). The alginate solution was then mixed with 5 g of BEOs and 9 mL of 1.25% polyvinyl alcohol (PVA). The mixed solution was gradually dropped into 200 mL of calcium chloride solution (3, 5, 7 and 10% w/v) with stirring speed at 300 rpm to obtain microparticle of BEOs. The obtained microparticles were left in calcium chloride solutions for 30 minutes. The microparticles were collected from calcium chloride solution by using sieve, rinsed with distilled water 3 times and left to dry on tissue paper to absorb the excess water for 3 hours. Blank microparticles were prepared using the same method described above without adding BEOs. The quantity of BEOs in the microparticles was evaluated using gas chromatography as described by Patheetin et.al [8]. Loading Capacity (%LC) and encapsulation efficiency (%EE) were calculated from the following equations.

$$Loading\ Capacity\ (\%) = \frac{Quantity\ of\ loaded\ BEO}{Quantity\ of\ microparticles}\ x\ 100$$

$$Encapsulation\ Efficiency\ (\%) = \frac{Quantity\ of\ loaded\ BEO}{Quantity\ of\ initial\ BEO}\ x\ 100$$

BEOs (50 μL) and BEOs microparticles containing 50 μL of BEOs were kept at room temperature, and protected from light at various time points (0, 1, 2, 7, 8, 14 days). The BEOs and BEOs microparticles were then placed on the inoculated agars for antifungal study as described below. The average of three inhibition zone diameters for each sample was used to determine the “inhibition zone”.

Gas Chromatography

GC-FID was performed using a Perkin-Elmer AutoSystem gas chromatograph fitted with a fused-silca PB-WAX capillary column (60 m, 0.25 mm, 0.32 μm film thickness). Column temperature was 80^{O}C, 2 minutes and then was programmed from 80-230^{O}C at 6^{O}C/min. Injector and detector temperatures were 190^{O}C and 260^{O}C, respectively. The carrier gas was helium at 1.0 mL/min. The samples (1.0 μL) were injected using a split ratio of 1:10. Quantitative analysis of the BEOs component was calculated by internal standard method. Linalool was used as a marker of the BEOs. *p*-Cymene was used as an internal standard.

Antifungal activity

The anti-fungal activity of BEOs and BEOs microparticles was conducted using agar disc diffusion methods described by Tadtong et.al [6]. The anti-fungal activity was evaluated by measuring diameter of inhibition zone against *C. albicans*. The BEOs (50 μL) were applied to a paper disc (9 mm in diameter) and placed on the inoculated agar. Microparticles from the most promising formula containing 50 μL of BEOs were placed on the well on the inoculated agar. The disc were kept at 37^{O}C for 24 hours before measuring inhibition zone. Clotrimazole 15 mg/mL was used as positive control. Microparticle without BEOs was used as negative control.

Statistical Analysis

The statistical analysis of the data was done by using Kruskal Wallis test for %LC and %EE and student t-test for the inhibition zone. The inhibition zone of BEOs and BEOs microparticles from antifungal study were compared. The differences between the samples were considered significant if *p-value* < 0.05.

Results and Discussion

1. Factors affecting the encapsulation of BEOs in microparticles.

In this study the effects of the concentration of polymer, sodium alginate, and cross-linking agent, calcium chloride, on loading capacity (%LC) and encapsulation efficiency (%EE) of BEOs in microparticles were studied. Various concentrations of sodium alginate (5, 7 % w/v) and calcium chloride (3, 5, 7 and 10 %w/v) were used to prepare microparticles by ionic gelation.

Even though the statistical analysis showed that there was no statistical difference in %LC (p-value 0.064) and %EE (p-value 0.066) in formula A1-A8, we can see the trend. The results showed in **Fig 1** and **Fig 2** indicate that microparticles are formed at the appropriate concentration of sodium alginate and calcium chloride. The increase of sodium alginate and calcium chloride concentration leads to higher %EE and %LC. Microparticles prepared from 7% (w/v) of sodium alginate and 10% (w/v) of calcium chloride showed the highest loading capacity (% 0.59±12.45) and encapsulation efficiency (% 3.24±58.36). Alginate microparticles were prepared by crosslinking the alginate phase with calcium ions. This cross link leads to the forming of a dense network structure of cohesive pores that can trap BEOs inside. More connections of cross link structures results in a stronger wall of microparticles. However, too dense network structures might lead to less room for the entrapped essential oil, which results in low encapsulation efficiency and low loading capacity. These results are in agreement with those reported by Soliman et.al. These authors represented that higher proportions of alginate chain led to increased %LC and %EE of clove oil and thyme oil in microparticles. However, by increasing alginate concentration to 2% and calcium chloride concentration to 0.5%, the encapsulation efficiency and loading capacity were decreased [5].

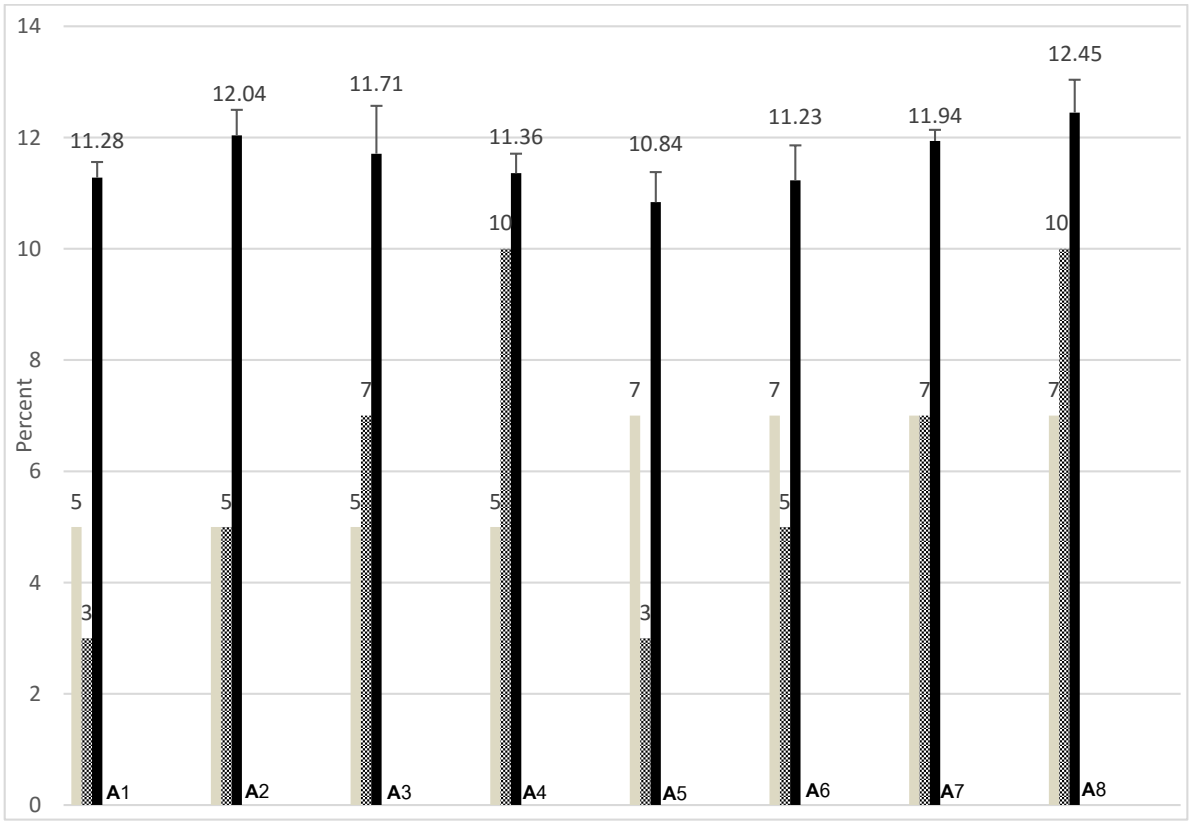

Figure 1 Effect of the concentration of sodium alginate and the concentration of calcium chloride on loading capacity (%LC) of BEOs in microparticles. Sodium Alginate (%w/v), Calcium Chloride (%w/v), Loading Capacity (%), **A1-8** formula A1-8

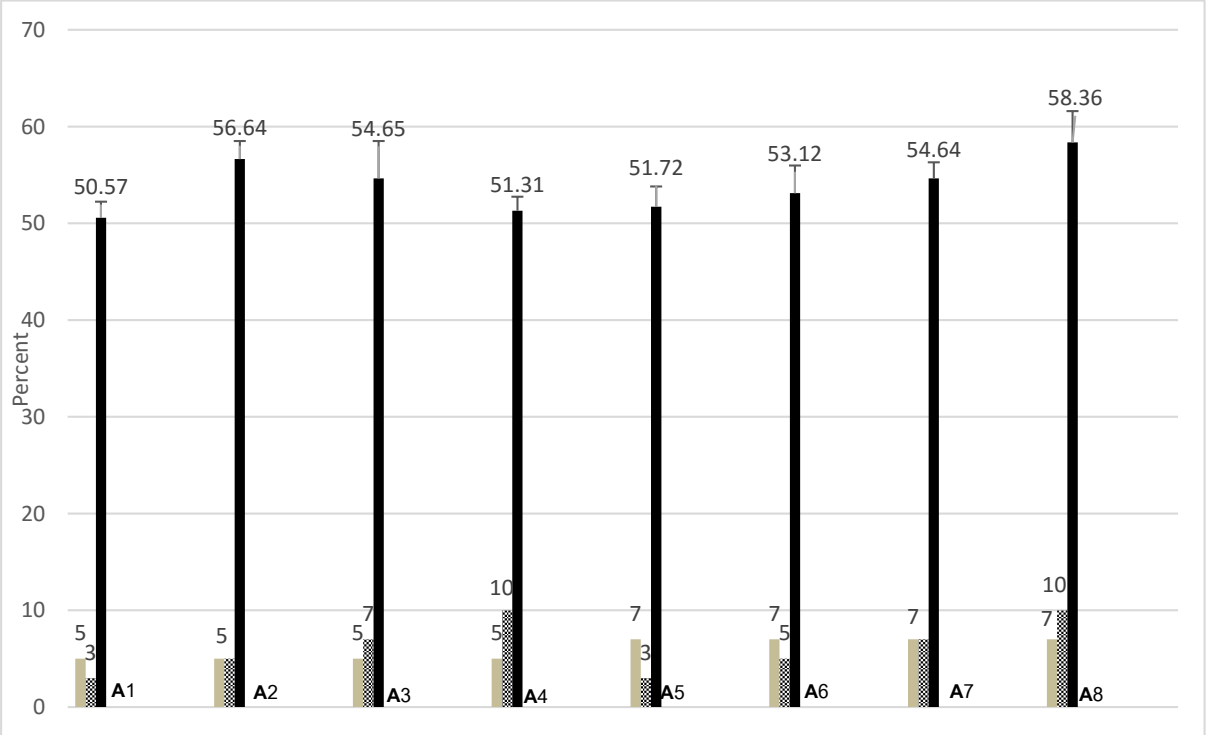

Figure 2 Effect of the concentration of sodium alginate and the concentration of calcium chloride on Encapsulation Efficiency (%EE) of BEOs in microparticles. Sodium Alginate (%w/v), Calcium Chloride (%w/v), Encapsulation Efficiency (%), **A1-8** formula A1-8

2. Antifungal activity of encapsulated BEOs and non-encapsulated BEOs against *C. albican*

Microparticles containing BEOs in this study were prepared from 7% sodium alginate and 10% calcium chloride since it gave the highest %EE and %LC. Encapsulated BEOs and non-encapsulated BEOs were evaluated for antifungal activity by using agar diffusion method. BEOs and BEOs microparticles (with equivalent amounts of encapsulated BEOs) were kept at room temperature for various time points (0, 1, 2, 7, 8, 14 days). The preparations were placed on the inoculated agar by the time they reached each time point and incubated for 24-hours before measuring inhibition zone. **Fig 3** shows antifungal activity, described by inhibition zone, of the encapsulated BEOs and non-encapsulated BEOs. The results indicate that the antifungal activity of BEOs was undetectable within 2 days. On the other hand, the antifungal activity of the encapsulated BEOs lasted for 8 days. The inhibition zone of non-encapsulated BEOs was significantly lower than those of encapsulated BEOs (p-*value* < 0.05) on storage day of 2, 7, and 8. These results are in agreement with Solliman et.al [5]. These authors reported that the antifungal activity of encapsulated clove and thyme oils against *Aspergillus niger* decreased by 50% after 8 days of storage whereas the antifungal activity of non-encapsulated clove and thyme oils decreased by 90% after 2 days of storage.

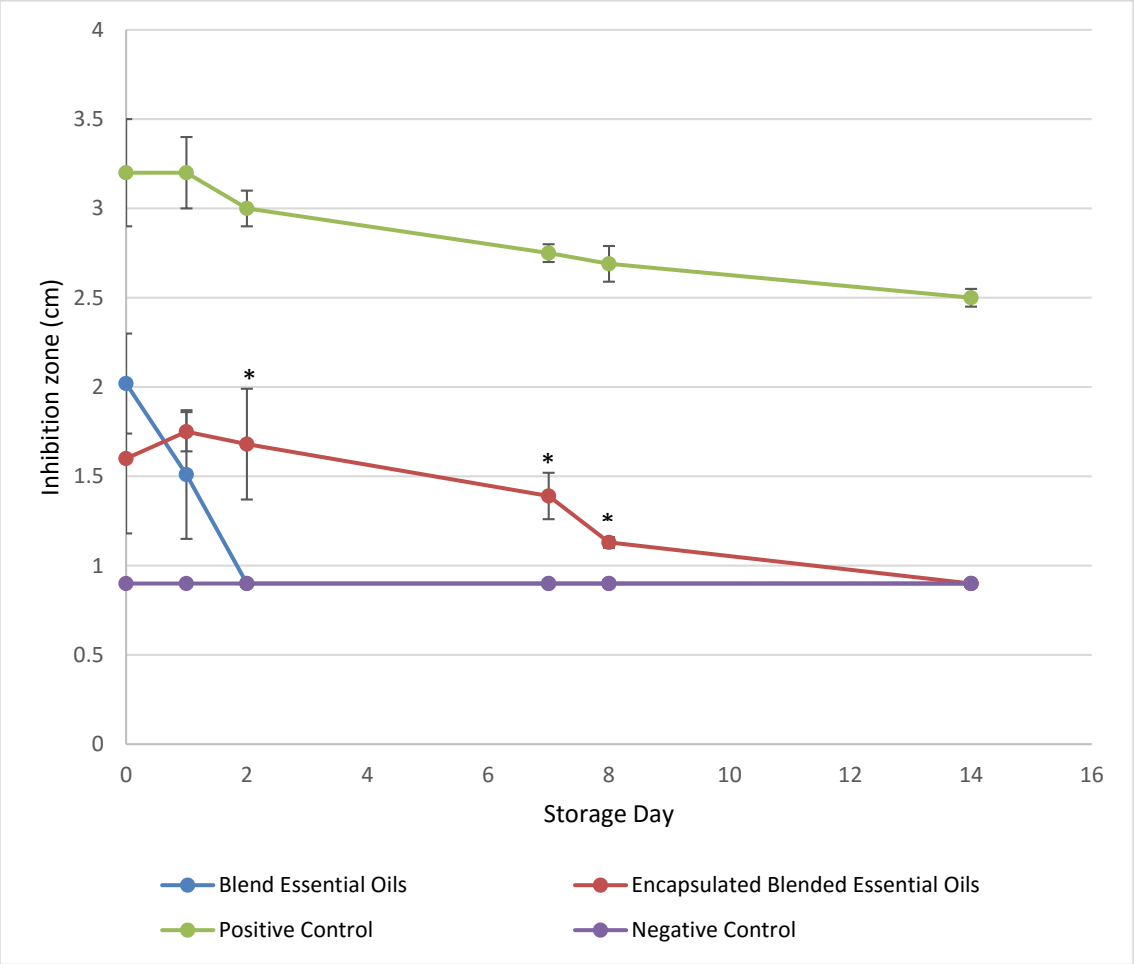

Figure 3 Antifungal activity (inhibition zone) of Blended Essential Oils (BEOs) and Encapsulated Blended Essential Oils against *C. albican*. Positive control is 15 mg/mL Clotrimazole. Negative control is microparticle without BEOs. * Statistically significant between BEOs and encapsulate BEOs (*p-value* < 0.05, t-test)

Conclusions

Blended essential oils (BEOs), composed of lavender, clary sage and ylang ylang oils in the ratio of 4:4:2 v/v were encapsulated in microparticles by using ionic gelation technique. Microparticles prepared from 7% (w/v) of sodium alginate and 10% (w/v) of calcium chloride gave the highest encapsulation efficiency and loading capacity. The encapsulated BEOs retained antifungal activity for 8 days of storage at room temperature whereas antifungal activity of non-encapsulated BEOs was undetectable after 2 days of storage. The result obtained from this study suggest that encapsulation by ionic gelation is suitable to protect the volatility of BEOs and increase the antifungal property of BEOs.

Acknowledgement

The authors would like to thank Faculty of Pharmacy, Srinakharinwirot University for financial support.

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Comparison of Total Phenolic Content and Antioxidant Activity between the Ethanolic Leaf Extracts from Two Local Sapodilla Varieties

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Abstract

Manilkara zapota L. is also known as Sapodilla. In Koh Yor, Songkhla province, Thailand, two local Sapodilla varieties, i.e. Khai-Han and Mha-Kok varieties, are extensively grown. The aim of this study was to determine total phenolic content and antioxidant activity of the ethanolic leaf extracts from these two varieties. Initially, the dried old leaves were extracted by 80% ethanol, and they were subsequently lyophilized until solid extracts were obtained. The extracts were investigated for total phenolic content using the Folin-Ciocalteau assay and evaluated for free radical scavenging activity using the DPPH assay. The total phenolic content of the ethanolic leaf extracts from the Mha-Kok varieties (38.50 ± 1.16 mg GAE/g extract) was significantly higher than that from the Khai-Han varieties (26.12 ± 1.29 mg GAE/g extract; $p<0.05$). Increases in DPPH radical scavenging activity of both varieties were dose-dependent (1-100 µg/ml). IC_{50} values, determined from the DPPH assay, for the Khai-Han and Mha-Kok varieties were 20.63 ± 0.91 and 21.93 ± 1.66 µg/ml, respectively. Even though there was no significant difference between those IC_{50} values ($p>0.05$), the Mha-Kok varieties exhibited a noticeably higher total phenolic content than that observed in the Khai-Han varieties. This result, therefore, suggested that the Mha-Kok varieties can be a better source of phenolic substances than the Khai-Han varieties.

Keywords: *Manilkara zapota*, DPPH Assay, Total Phenolic Content, Ethanolic Leaf Extract

Introduction

Free radicals naturally occur by intracellular metabolism or by external factors such as UV radiation and pollution. If these free radicals are derived from oxygen, they will be termed as reactive oxygen species (ROS). The examples for this are superoxide ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$). Moreover, if these free radicals originated from nitrogen, they will be known as reactive nitrogen species (RNS), such as nitric oxide ($^{\cdot}NO$) [1]. These free radicals play a role in oxidative stress which occurs from the imbalance between the production and destruction of ROS and can lead to many diseases such as arteriosclerosis, Alzheimers, Parkinsons, cancer, aging processes as well as neurodegenerative diseases [2]. High levels of ROS, generated by the intracellular metabolism, can cause damage to intracellular molecules such as DNA, protein and phospholipids. DNA damage caused by ROS is known as oxidative DNA damage [3]. Among the ROSs, hydroxyl radicals can cause the most damage to DNA by either interacting with hydrogen bonds on DNA molecules or splitting hydrogen atoms from methyl group of thymine bases [4-5]. Most hydroxyl radical-induced DNA damage appears at the C8 position on guanine bases, which is known as 7,8-dihydro-8-oxoguanine (8-hydroxylguanine or 8-oxoG) [4]. This 8-oxoG will mismatch with adenine bases, resulting in G-to-T transversion mutations [6]. A number of studies have shown that antioxidants could reduce oxidative DNA damage. Li *et al*. (2013a) studied the protective effect (on DNA damage) and antioxidant activity of root extracts from *Radix Glycyrrhizae* or liquorice [7]. They found that an increase in DNA damage protective effect and antioxidant activity was dependent on the concentration of ethanolic liquorice root extracts. They then summarized that the protective effect on DNA damage of liquorice root extracts was related to their antioxidant ability, and this effect might be due to the phenolic and flavonoid compounds found in the extracts [7]. Moreover, Li *et al*. (2013b) reported that the protective effect on DNA damage of antioxidants found in *Citri reticulatae* Pericarpium extracts is dose-dependent. The mechanisms of this effect might be from two ways. First, the antioxidants interact with hydroxyl radicals before they can bind to DNA, and eventually reduce the DNA damage. Second, DNA is repaired by antioxidants after the DNA has interacted with hydroxyl radicals. Moreover, they also concluded that the antioxidant ability of *C. reticulatae* Pericarpium extracts might be from flavonoid molecules, especially hesperidin and narirutin [8].

Manilkara zapota L. belongs to the Sapotaceae family. There are number of common names for *M. zapota* L., including Sofeda or Sobeda in Bengali, Sapota or Chikku in Hindi, Simaiyiluppai in Tamil, Sapotasima in Telugu, Sapotille or Sapodilla in French, and American bully in English [9]. In Thailand, *M. zapota* L. is locally called “La Mood” or “Sa Wa”. In Songkhla Province, Thailand, *M. zapota* L. fruits are very well-known, especially in the area called Koh Yor. Two *M. zapota* L. varieties are extensively grown in Koh Yor i.e. Khai-Han and Mha-Kok varieties. It has been well-known for a long time that the *M. zapota* L. fruits from Koh Yor are considerably sweeter than those from other areas in Thailand. However, there is no previous evidence to support this observation. Various biological activities of *M. zapota* L. have been reported, for example, antioxidant activity [10-14], antiinflamatory activity [9], antipyretic activity [9], anticollagenase activity [14], and antielastase activity [14]. Even though many studies have reported the antioxidant activity as well as total phenolic contents in *M. zapota* L. extracts [10, 13-14], no reports have compared such activity and their phenolic compounds between different local varieties of *M. zapota* L. Also, the leaf extracts from *M. zapota* L. have been reported to have higher content of phenolic compounds as well as greater DPPH radical scavenging activity, when compared to other parts, such as seeds, fruit pulps and fruit peels, of *M. zapota* L. (Table 1 and Table 2). Therefore, this study aimed to determine the total phenolic content as well as antioxidant activity of leaf extracts from two *M. zapota* L. varieties collected from Koh Yor, Songkhla Province, Thailand.

Methodology

1. Plant collection

Fresh and mature leaves of two *M. zapota* L. varieties, i.e. Khai-Han varieties (Figure 1A) and Mha-Kok varieties (Figure 1B) were collected in the month of May 2017 from Koh Yor in Songkhla province, Thailand. The leaves were washed thoroughly with distilled water, dried at 45°C for 24 h [13], ground into powder and stored in desiccator (DRY-70, Weifo, Taiwan) until used.

Figure 1. Khai-Han and Mha-Kok varieties of *M. zapota* L. Fresh and mature leaves of Khai-Han (A) and Mha-Kok (B) varieties of *M. zapota* L. were collected from Koh Yor in Songkhla province, Thailand. The leaves were washed thoroughly with distilled water, dried at 45°C for 24 h, ground into powder, and stored in desiccator until used.

2. Plant extraction

20 g of leaf powder was soaked in 80% ethanol (200 ml; RCL Labcan) under orbital shaking at 150 rpm, room temperature, for 24 h. The solvent was filtered using Whatman® No. 1 filter paper. This extraction was repeated three times. The collected filtrate was combined and concentrated to about 40 ml using rotary evaporator (Hei-VAP, Heidolph, Germany) at 40 rpm, 37°C. This concentrated filtrate was lyophilized (FTS Systems FlexiDry, USA.) until the solid extract was obtained. This extraction gave the yield for Khai-Han and Mha-Kok varieties of 26.51 and 30.06%, respectively. Then, the solid ethanolic extract was stored in desiccator until further used.

3. Estimation of total phenolic content

Total phenolic content of the extracts was determined according to Ainsworth and Gillespie [15]. Briefly, diluted sample (100 µl) was mixed with Folin-Ciocalteau reagent (10%v/v, 200 µl; Loba Chemie). Sodium carbonate solution (700 mM, 800 µl; Rankem) was added, and the mixture was incubated in the dark at room temperature for 2 h. This mixture was centrifuged at 15,000 rpm for 10 min. Absorbance of supernatant was detected at 765 nm using Spectrophotometer. Total phenolic content was calculated from a standard curve prepared with various concentrations of gallic acid (2.5 – 30 µg/ml; Sigma), with $y = 0.07x - 0.0688$ ($R^2 = 0.9336$). The results were expressed as milligrams of gallic acid equivalent (GAE) per gram of the extract. Three replicates were performed, and mean ± S.D. was presented.

4. DPPH radical scavenging assay

The free radical scavenging activity of the extracts was measured using 2,2-Diphenyl-1-picrylhydrazyl (Sigma) as described in previous publication [16]. The dried ethanolic extract of Sapodilla was dissolved with 80% ethanol to prepare a stock solution of 1 mg/ml. Various concentrations of Sapodilla (1, 5, 10, 20, 40, 50, 75 and 100 µg/ml, 470 µl) were incubated in the dark with DPPH solution (0.1 mM in methanol, 940 µl) at room temperature for 30 min. This mixture was centrifuged at 15,000 rpm for 10 min. Absorbance of supernatant was detected at 517 nm using Spectrophotometer (Evolution 201, Thermo Fisher Scientific, china). The DPPH radical scavenging activity (%) of Sapodilla was calculated as follows:

$$\text{DPPH radical scavenging activity (\%)} = \frac{(\text{Absorbance of the control} - \text{Absorbance of the sample})}{\text{Absorbance of the control}} \times 100$$

Three replicates were performed, and mean ± S.D. was presented. Ascorbic acid (1, 2, 5, 10, 20 µg/ml) was employed as a positive control. IC_{50} values were calculated from the graph plotted between DPPH radical scavenging activity and concentrations of the extracts. The IC_{50} value is defined as the concentration of the extract which is able to reduce the DPPH radical concentration by 50% [12].

5. Statistical analysis

All data were analyzed by independent t-test using SPSS statistics version 22, and a *p* value of less than 0.05 was considered as a significant difference.

Results

1. Total phenolic content

Total phenolic contents determined in the extracts from Khai-Han and Mha-Kok varieties are shown in Figure 2. Significantly higher phenolic contents were detected in Mha-kok varieties than those observed in Khai-Han varieties ($p<0.05$). The total contents of phenolic compounds found in Khai-Han and Mha-Kok varieties were 26.12 ± 1.29 and 38.51 ± 1.16 mg GAE/g extract, respectively (Table 1). This also showed that Mha-kok varieties had about 12 mg GAE/g extract higher than that observed in Khai-Han varieties.

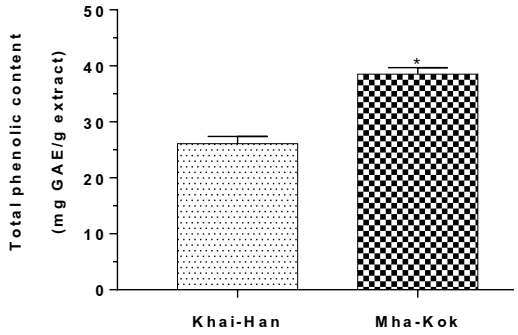

Figure 2. Total phenolic contents in leaf extracts of local Sapodilla varieties. Local Sapodilla varieties, i.e. Khai-Han and Mha-Kok varieties, were detected for total phenolic contents using the Folin-Ciocalteau assay. The diluted extract was incubated with Folin-Ciocalteau reagent and sodium carbonate solution in the dark at room temperature for 2 h. The results were expressed as milligrams of gallic acid equivalent (GAE) per gram of the extract (n = 3). Asterisks indicate a significant difference at $p<0.05$, when compared to Khai-Han varieties.

Table 1. Comparison of Total phenolic content of the extracts from various parts of *M. zapota* L. as being measured using Folin-Ciocalteu reagent (mean ± S.D.)

Extracts					References
Sources	**Varieties**	**Parts**	**Solvents**	**Total phenolic content**	
Songkhla, Thailand	Khai-Han	Leaf	80% ethanol	26.12 ± 1.29 mg GAE/g	-[a]
	Mha-Kok			38.50 ± 1.16 mg GAE/g	
Am-jodhpur, Jamnagar, Gujarat, India	-	Leaf	Toluene	6.68 ± 0.57 mg GAE/g	[10]
			Ethyl acetate	82.21 ± 3.60 mg GAE/g	
			Acetone	110.50 ± 0.66 mg GAE/g	
			Water	102.22 ± 3.13 mg GAE/g	
Pallamudir, Coimbatore, Tamil Nadu, India	-	Seed	Ethanol	4.00 ± 0.01 mg GAE/g	[11]
			Acetone	2.26 ± 0.01 mg GAE/g	
			Ethyl acetate	2.54 ± 0.02 mg GAE/g	
			Water	1.21 ± 0.01 mg GAE/g	
Rajshahi, Bangladesh	-	Leaf	Ethanol	89.67 ± 3.074 mg GAE/g	[12]
Pathum Thani, Thailand	-	Fruit pulp	95% ethanol	38.56 ± 1.98 mg GAE/g	[14]
Coimbatore, Tamil Nadu, India	-	Fruit pulp	Absolut methanol	14.97 ± 2.72 mg catechol/g	[17]
			Absolute ethanol	11.43 ± 1.09 mg catechol/g	
		Fruit peel	Absolut methanol	20.85 ± 0.44 mg catechol/g	
			Absolute ethanol	21.17 ± 0.45 mg catechol/g	

[a] is the results obtained from this present study.

2. DPPH radical scavenging activity

The DPPH radical scavenging activity of ethanolic leaf extracts of Khai-Han and Mha-Kok varieties is presented in Figure 3. An increase in the DPPH radical scavenging activity of these extracts was in a dose-dependent manner (1-100 µg/ml). IC_{50} values for the leaf extracts from Khai-Han and Mha-Kok varieties were 20.63 ± 0.91 and 21.93 ± 1.66 µg/ml, respectively, and the IC_{50} value for ascorbic acid was 8.57 ± 0.16 µg/ml (Figure 4; Table 2). No significant difference between those IC_{50} values was observed (p>0.05).

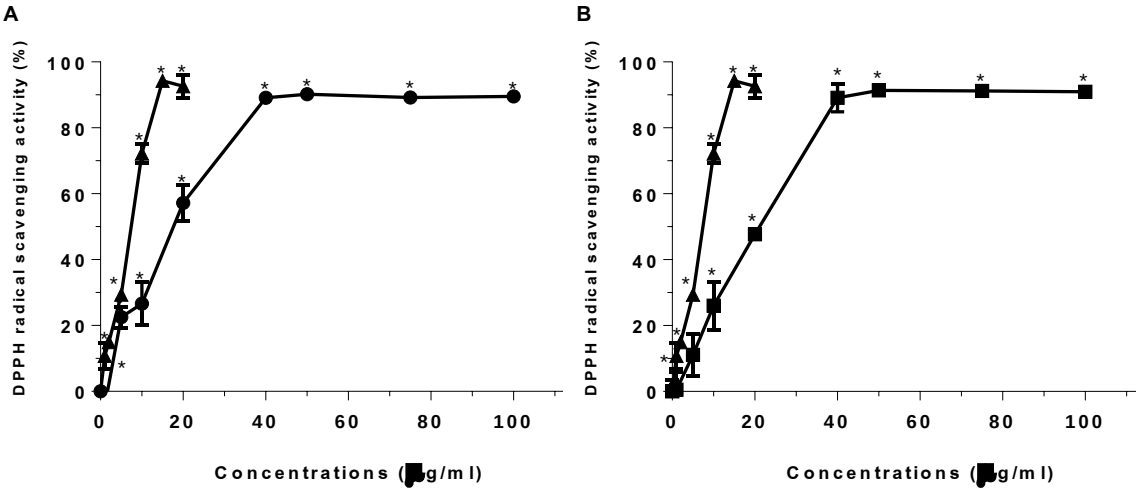

Figure 3. DPPH radical scavenging activity of two *M. zapota* L. varieties. The antioxidant activity of ethanolic leaf extracts of two *M. zapota* L. varieties, i.e. Khai-Han (A; ●) and Mha-Kok (B; ■) varieties, was determined using the DPPH radical scavenging assay. The extracts with various concentrations from 1 to 100 µg/ml were incubated with DPPH radical solution in the dark at room temperature for 2 h, and their absorbance were detected at 517 nm. Ascorbic acid was used as a positive control (▲). Data were presented as mean ± S.D. (n = 3). Asterisks indicate a significant difference at p<0.05, when compared with vehicles.

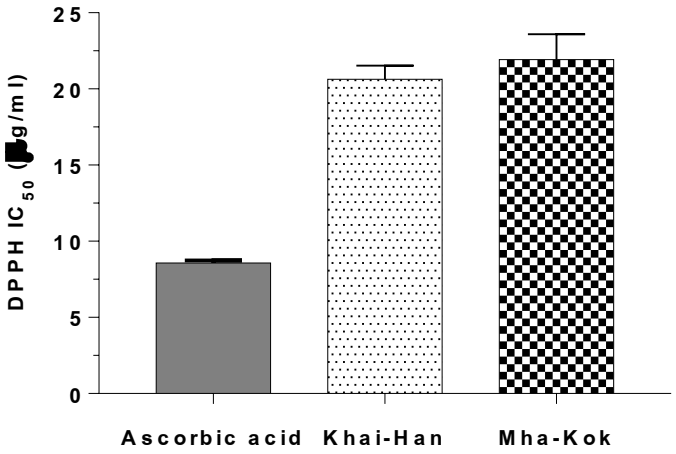

Figure 4. IC_{50} values determined using DPPH radical scavenging assay. IC_{50} values were calculated from the graph plotted between DPPH radical scavenging activity and concentrations of the extracts (Figure 2). No significant difference between IC_{50} values (mean ± S.D.) of ethanolic leaf extract from two *M. zapota* varieties was observed (p>0.05).

Table 2. Comparison of antioxidant activity of the extracts from various parts of *M. zapota* L. as being measured by DPPH radical scavenging assay (mean ± S.D.)

Extracts					Controls		References
Sources	**Varieties**	**Parts**	**Solvents**	**IC_{50} (µg/ml)**	**Chemicals**	**IC_{50} (µg/ml)**	
Songkhla, Thailand	Khai-Han	Leaf	80% ethanol	20.63 ± 0.91	Ascorbic acid	8.57 ± 0.16	-[a]
	Mha-Kok			21.93 ± 1.66			
Am-jodhpur, Jamnagar, Gujarat, India	-	Leaf	Toluene	93	Ascorbic acid	11.4	[10]
			Acetone	20			
			Water	160			
Pallamudir, Coimbatore, Tamil Nadu, India	-	Seed	Ethanol	290	-	-	[11]
			Acetone	400			
			Ethyl acetate	490			
			Water	500			
Rajshahi, Bangladesh	-	Leaf	Ethanol	68.27	Ascorbic acid	16.17	[12]
Palau Gadong, Malacca, Malaysia	-	Fruit pulp	20% ethanol	~800	-	-	[13]
			40% ethanol	446.45 ± 30.78			
			60% ethanol	~600			
			80% ethanol	~1,000			
			100% ethanol	~900			
			Water	~1,200			
		Fruit peel	20% ethanol	~400			
			40% ethanol	~200			
			60% ethanol	~200			
			80% ethanol	146.85 ± 1.01			
			100% ethanol	~150			
			Water	~800			
Pathum Thani, Thailand	-	Fruit pulp	95% ethanol	37.63 ± 1.18	Ascorbic acid	1.38 ± 0.05	[14]
Coimbatore, Tamil Nadu, India	-	Fruit pulp	Absolut methanol	950	-	-	[17]
			Absolute ethanol	500			
		Fruit peel	Absolut methanol	450			
			Absolute ethanol	130			

[a] is the results obtained from this present study.

Discussion and conclusion

Phenolic compounds have been reported to have an effect on biological activities, such as an antibacterial activity [18] and antioxidant activity [10, 12, 14]. In this present study, therefore, the total phenolic content and antioxidant activity of two different varieties of *M. zapota* L., Khai-Han and Mha-Kok varieties, were determined.

Total phenolic content of the extracts from the Khai-Han varieties were significantly lower than that observed in the Mha-Kok varieties. This revealed the fact that the total phenolic contents detected in *M. zapota* L. leaves depended on their variety. Different amounts of total phenolic compounds have been detected between different cherry varieties (four sweet and four sour cherry varieties) [19]. These cherries were extracted by absolute methanol and then were determined for the total phenolic contents by the Folin-Ciocalteau assay. For the sweet cherry varieties, total phenolic contents were between 92.1 to 146.8 mg GAE/100 g extracts with an average of 109.8 mg, whereas the sour cherry varieties exhibited the total contents of phenolic compounds of 146.1 to 312.4 mg GAE/100 g extracts with an average of 228.9 mg. Higher levels of phenolics were found with sour cherry varieties than that observed with sweet cherry varieties [19]. Among the different parts of *M. zapota* L, the leaf extracts have been shown to have the highest contents of phenolic compounds (Table 1). Chanda and Nagani (2010) reported that the total phenolics extracted by acetone, aqueous and ethyl acetate from *M. zapota* L. leaves were 110.50 ± 0.66, 102.22 ± 3.13 and 82.21 ± 3.60 mg GAE/g extract, respectively [10], while Shanmugapriya et al. (2011) presented that the *M. zapota* L. seeds extracted by acetone, aqueous and ethyl acetate exhibited the total phenolic contents of 2.26 ± 0.01, 1.21 ± 0.01 and 2.54 ± 0.02 mg GAE/g extract, respectively [11]. Also, ethanolic leaf extracts of *M. zapota* L. (89.67 ± 3.074 mg GAE/g extract) [12] had the higher phenolic contents than those observed in ethanolic seed extract (4.00 ± 0.01 mg GAE/g extract) [11]. Although previous researches have revealed the contents of phenolic compounds found in different parts of *M. zapota* L. (Table 1), to the best of our knowledge, the total phenolics observed in different varieties of *M. zapota* L. have not yet reported.

In this present study, no difference in the antioxidant activity between Khai-Han and Mha-Kok varieties was observed. IC_{50} values of the extracts from the Khai-Han and Mha-Kok varieties were 20.63 ± 0.91 and 21.93 ± 1.66 µg/ml, respectively, when determined using the DPPH assay. The DPPH radical scavenging IC_{50} values, reported in this present study, were much lower than those reported previously (Table 2), indicating the more potent antioxidant activity of *M. zapota* L. leaf extracts observe in this current study. Better DPPH radical scavenging activities were observed with leaf extracts rather than those observed with the extracts from other parts of *M. zapota* L. (Table 2). Acetone *M. zapota* L. leaf extracts showed considerably lower DPPH IC_{50} values (20 µg/ml) [10], when compared to the *M. zapota* L. extracts from seeds (IC_{50} value of 400 µg/ml) [11]. Also, aqueous *M. zapota* L. leaf extracts exhibited less IC_{50} values (160 µg/ml) [10] than those observed in seed extracts (IC_{50} value of 500 µg/ml) [11]. Moreover, the lowest IC_{50} values (68.27 µg/ml) was detected in ethanolic extracts of *M. zapota* L. leaves [12], while higher IC_{50} values were observed in fruit peel (130 µg/ml) [17], seed (290 µg/ml) [11] and fruit pulp (500 µg/ml) [17], respectively. It is well known that the antioxidant activity of phenolic compounds might be from their ROS scavenging ability [10]. Moreover, Ghasemzadeh and Jaafar (2013) reported that the extracts of *Pandanus amaryllifolius* leaves collected from different locations had different DPPH radical scavenging activities [20]. The leaves collected from the North (Bachok) appeared to have the highest value (64.27%) of DPPH activity, followed by those collected from the Central (Klang; 52.16%) and the south (Pontian; 50.10%), respectively. These were related to the content of phenolics found in the North (Bachok; 6.72 ± 0.355 mg/g extract), followed by the Central (Klang; 5.07 ± 0.406 mg/g extract) and the south (Pontian; 4.88 ± 0.477 mg/g extract), respectively. DPPH activity was also linked with the species of phenolic acid detected in the *P. amaryllifolius* leaf extracts. The leaves collected from the North (Bachok) consisted of gallic acid, cinnamic acid and ferulic acid, whereas those collected from the Central (Klang) were gallic acid and cinnamic acid, and only gallic acid was found in the leaves collected from the south (Pontian) [20]. Even though a correlation between the phenolic content and the antioxidant activity of the leaf extracts of Khai-Han and Mha-Kok varieties was not observed, we believe that further experiments should be

conducted to identify the specific phenolics in these leaf extracts.

In summary, the results obtained in this study showed an effect of plant variety on the total phenolic content of *M. zapota* leaf extracts, in which the Mha-Kok varieties exhibited significantly higher total phenolic contents than those observed in the Khai-Han varieties. This suggested that Mha-Kok varieties would be a better source of phenolic compounds than the Khai-Han varieties.

Acknowledgements

The authors would like to thank Faculty of Science and Technology, Songkhla Rajabhat University for providing the financial supports.

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Comparison of Total Phenolic Content, Antioxidant and Antibacterial Activity in Young and Old Leaf Extracts of *Azadirachta excelsa* (Jack) Jacobs.

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Abstract

Azadirachta excelsa (Jack) Jacobs, or its local name "Sa-Dao Tiam", is widely grown in the southern part of Thailand. Compared to *A. indica* (or Indian neem), the biological activities of *A. excelsa* have not been evaluated. This study, therefore, aimed to investigate antioxidant and antibacterial activity in young and old leaf extracts of *A. excelsa*. Dried leaves were extracted by 80% ethanol, and they were subsequently lyophilized until semi-solid extracts were obtained. The extracts were investigated for total phenolic content using the Folin-Ciocalteau assay. The antioxidant activity of the extracts (5-150 µg/ml) was measured using the DPPH radical scavenging assay, and the antibacterial activity of the extracts (5-100 mg/ml) was assessed using agar well diffusion method. Significantly higher phenolic content was detected in old leaf extracts (16.63 ± 3.30 mg GAE/g extract) than that detected in young leaf extracts (8.72 ± 1.72 mg GAE/g extract; $p<0.05$). Both young and old leaf extracts showed a dose-dependent DPPH radical scavenging activity. However, no significant difference between IC_{50} values, determined using the DPPH assay, for young (75.00 ± 4.82 µg/ml) and old (74.59 ± 2.05 µg/ml) leaf extracts of *A. excelsa* was observed ($p>0.05$). The antibacterial activity of both extracts exhibited a dose-dependent increase. Old leaf extracts exhibited significantly greater inhibition zones against *E. coli* than those observed in young leaf extracts at all tested concentrations ($p<0.05$). These results suggest that the higher antibacterial activity of old leaf extracts compared to young leaf extracts against *E. coli* might result from the higher total phenolic content found in old leaves of *A. excelsa*.

Keywords: *Azadirachta excelsa*, Total Phenolic Content, DPPH Assay, *Escherichia coli*

Introduction

Herbal medicines are of increasing interest nowadays. Even though synthetic medicines are regularly used as a potent drugs, patients somewhat prefer a natural way to cure diseases. Phenolic compounds have been suggested to be responsible for various biological activities, such as antiinflamatory, antioxidant and antibacterial activity [1-3]. Elansary *et al*. (2017) reported that the methanolic extracts of *Eucalyptus camaldulensis var obtusa* leaves exhibited the highest phenolic content, antioxidant and antimicrobial activities, when compared to extracts from *E. camaldulensis* L. and *Eucalyptus Gomphocephala* [4]. The antimicrobial effects of extracts from *Thymus caramanicus* and *Zataria multiflora* against *Staphylococcus aureus*, *Shigella dysenteriae*, *Salmonella typhimurium*, *Escherichia coli*, *Staphylococcus epidermis*, *Bacillus subtitis*, Methicillin-resistant *S. aureus* (MRSA) and *Pseudomonas aeruginosa* were dependent upon the amount of detected phenolic compounds[2]. Moreover, higher amounts of phenolic compounds were observed in methanol and ethyl acetate extracts from leaves of *Albizia odoratissima* than in chloroform and hexane extracts [5]. The higher phenolic contents of methanol and ethyl acetate extracts were correlated with better growth inhibition of *Klebsiella pneumoniae*, *E. coli*, *P. aeruginosa*, *Proteus vulgaris* and *S. aureus* as well as greater antioxidant activity [5]. Moreover, greater antioxidant and antibacterial activity has been reported to be due to the higher amount of phenolic compounds present in leaf extracts of *Sambucus australis* [3]. It has been suggested that phenolic compounds could inhibit bacterial growth and metabolism [1, 6].

Neem belongs to the Meliaceae family. There are three species, (i) *Azadirachta indica* A. Juss (ii) *Azadirachta siamensis* Valeton and (iii) *Azadirachta excelsa* (Jack) Jacobs [7]. *A. indica* or Indian neem is generally found in India, Africa and America [8], and *A. siamensis* or Thai neem grows in all parts of Thailand [7]. Moreover, *A. excelsa* or marrango, which is locally called as "Sa-Dao Tiam or Sa-Dao Chang", is widely found in the southern part of Thailand [7]. Among these three species, *A. indica* has been widely studied, and a number of biological activities have been reported such

as anti-inflamatory activity, anti-pyretic activity, anti-histamine activity, anti-fungal activity, and anti-bacterial activity [9]. Ethanolic leaf extract of *A. indica* has been reported as a potent antibacterial against various tested organisms, including both Gram positive and Gram negative bacterial species [10]. The link between antioxidative and antibacterial activity of bark extract from *A. indica* has been reported [11]. Greater total phenolic contents were detected in ethanol (1.462 ± 0.017 mg GAE/g extract) and methanol (2.680 ± 0.08 mg GAE/g extract) bark extract of *A. indica* than in aqueous (0.762 ± 0.29 mg GAE/g extract) and acetone (0.592 ± 0.06 mg GAE/g extract) bark extracts. Also, ethanol (IC_{50} of 0.202 ± 0.04) and methanol (IC_{50} of 0.590 ± 0.0081) extracts showed better antioxidant activity than aqueous (IC_{50} of 0.829 ± 0.021) and acetone (1.494 ± 0.005) extracts, as determined by the DPPH assay. These findings were also correlated to antibacterial activity against *S. paratyphi* A. The inhibition zones for ethanol extract (20 ± 0.6 mm) were greater than those for methanol (12 ± 0.4 m), aqueous (10 ± 0.2 mm), and acetone (10 ± 0.5 mm) extracts. Thus, this study suggested that extracts of *A. indica* bark could be used as an antioxidant as well as an antibacterial drug [11].

In this study, *A. excelsa* was employed to investigate its total phenolic content, antioxidant activity and antibacterial activity against *E. coli*. This is because *A. excelsa* is widely grown and easily found in Songkhla Province, Thailand. While the biological activity of *A. excelsa* has not been extensively studied, some studies have revealed some biological activities of *A. Excelsa* [12-13]. Nur *et al*. (2016) has reported that the ethanolic extract of *A. excelsa* leaves had a total phenolic content of 202 ± 0.42 mg GAE/g extract and total flavonoid content of 198 ± 0.67 mg rutin/g extract. The IC_{50} value of the *A. excelsa* leaf extract was 308 µg/ml, as determined by the DPPH radical scavenging assay. Moreover, the leaf extract of *A. excelsa* could reduce systolic blood pressure in rats by 19%, compared to 28%by captopril drug. This decreased pressure might be due to the high amount of phenols and flavonoids, as well as the antioxidant activity observed in the *A. excelsa* leaf extracts [13]. Even though total phenolic content and antioxidant activity of *A. excelsa* leaves has been reported by this previous study, the effect of leaf age has not been investigated. Therefore, this study aimed to determine the total phenolic content, antioxidant activity and antibacterial activity of ethanolic extracts from young and old leaves of *A. excelsa*.

Methodology

6. Plant collection

Fresh young) Figure 1A (and old) Figure 1B (leaves of *A. excelsa* were collected in the month of May 2017 from Sa-Dao district in Songkhla province, Thailand . The leaves were washed thoroughly with distilled water, dried at 45°C for 24 h [14], ground into powder and stored in desiccator) DRY-70, Weifo, Taiwan (until used .

Figure 1 . Young and old leaves of *A . excelsa* . Fresh young(A) and old (B) leaves of *A .excelsa* were collected from Sa-Dao district in Songkhla province, Thailand .The leaves were washed thoroughly with distilled water, dried at 45°C for 24 h, ground into powder, and stored in desiccator until used .

7. Plant extraction

20 g of leaf powder was soaked in 80% ethanol (200 ml; RCL Labcan) under orbital shaking at 150 rpm, room temperature, for 24 h. The solvent was filtered using Whatman® No. 1 filter paper. This extraction was repeated three times. The collected filtrate was combined and concentrated to about 40 ml using rotary evaporator (Hei-VAP, Heidolph, Germany) at 40 rpm, 37°C. This concentrated filtrate was lyophilized (FTS Systems FlexiDry, USA.) until a semi-solid extract was obtained. This extraction gave the yield for young and old leaves of 25.68 and 29.87 %, respectively. Then, the semi-solid ethanolic extract was stored in desiccator until further use.

8. Estimation of total phenolic content

Total phenolic content of the extracts was determined according to Ainsworth and Gillespie [15]. Briefly, diluted extract (100 µl) was mixed with Folin-Ciocalteau reagent (10% v/v, 200 µl; Loba Chemie). Sodium carbonate solution (700 mM, 800 µl; Rankem) was added, and the mixture was incubated in the dark at room temperature for 2 h. This mixture was centrifuged at 15,000 rpm for 10 min. Absorbance of the supernatant was measured at 765 nm using a Spectrophotometer (Evolution 201, Thermo Fisher Scientific, China). Total phenolic content was calculated from a standard curve prepared with various concentrations of gallic acid (2.5 – 50 µg/ml; Sigma), with y = 0.07x – 0.0688 (R^2 = 0.9336). The results are expressed as milligrams of gallic acid equivalent (GAE) per gram of the extract.

9. DPPH radical scavenging assay

The free radical scavenging activity of the extracts was measured using 2,2- Diphenyl- 1-picrylhydrazyl (DPPH, Sigma) as described in a previous publication [16]. The dried ethanolic extract of *A. excelsa* was dissolved with 80% ethanol to prepare a stock solution at the concentration of 1 mg/ml. Various concentrations of *A. excelsa* (5, 10, 25, 50, 75, 100 and 150 µg/ml; 470 µl) were incubated in the dark with DPPH solution (0.1 mM, 940 µl) at room temperature for 30 min. This mixture was centrifuged at 15,000 rpm for 10 min. Absorbance was detected at 517 nm using a Spectrophotometer (Evolution 201, Thermo Fisher Scientific, China). The DPPH radical scavenging activity (%) of *A. excelsa* was calculated as follows:

$$\text{DPPH radical scavenging activity } (\%) = \frac{(\text{Absorbance of the control} - \text{Absorbance of the sample})}{\text{Absorbance of the control}} \times 100$$

Three replicates were performed, and means ± S.D. are presented. IC_{50} values were calculated from the graph plotted between DPPH radical scavenging activity and concentrations of the extracts. The IC_{50} value is defined as the concentration of the extract which is able to reduce the DPPH radical concentration by 50% [17].

10. Evaluation of antibacterial activity

The antibacterial activity of young and old leaf extract of *A. excelsa* was determined using agar well diffusion assay[11]. Briefly, *E. coli* ATCC 25922 were grown overnight in Mueller Hinton broth (MHB) under orbital shaking at room temperature. The *E. coli* inoculum was adjusted to an optical density of 0.1 at 600 nm (OD_{600}), or 1 x 10^8 cells/ml.[18] Then, *E. coli* at this concentration was spread with a sterile swab onto Mueller Hinton agar (MHA) plates, and the wells were subsequently made using a 6 mm cork borer. A 100 µl sample of the extracts (5, 10, 50 and 100 mg/ml (in 80 % ethanol was added into the wells, and the plates were incubated at 37°C for 24 h. Gentamicin at the concentration of 0.1 mg/ml) 100 µl (was employed as a positive control, and 80 % ethanol was used as a negative control. The clear zone around each well was measured, and the antibacterial activity of young and old leaf extract of *A. excelsa* is expressed as the average diameter of the inhibition zone in millimeters) mm .(Three replicates were performed, and mean ± S.D. was presented.

11. Statistical analysis

All data were analyzed by independent t-test using SPSS statistics version 22, and a P value of less than 0.05 was considered as a significant difference.

Results

1. Total phenolic content

Total phenolic contents determined in young and old leaf extracts of *A. excelsa* are shown in Figure 2. Significantly higher phenolic contents were detected in old leaf extracts when compared to young leaf extracts (p<0.05). The total phenolic contents found in young and old leaf extracts were 8.72 ± 1.72 and 16.63 ± 3.30 mg GAE/g extract, respectively. This also showed that old leaf extracts had about 8 mg GAE/g extract more than that observed in young leaf extracts.

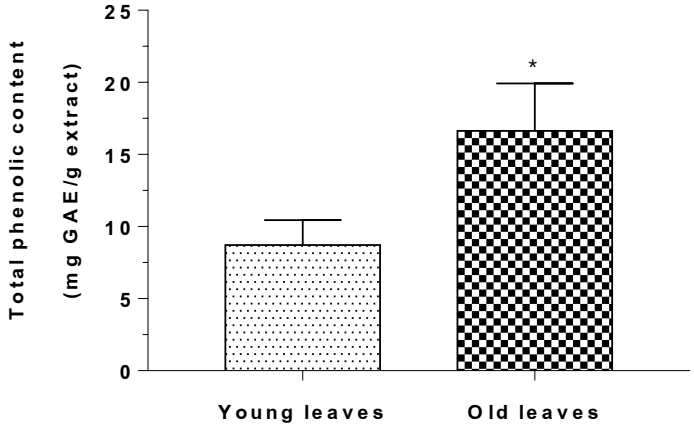

Figure 2. Total phenolic contents in young and old leaf extracts of *A. excelsa*. Young and old leaf extracts were detected for total phenolic contents using the Folin-Ciocalteau assay. The diluted extract was incubated with Folin-Ciocalteau reagent and sodium carbonate solution in the dark at room temperature for 2 h. The results were expressed as milligrams of gallic acid equivalent (GAE) per gram of the extract (n = 3). Asterisks indicate a significant difference at $p<0.05$, when compared to young leaf extracts of *A. excelsa*.

3. DPPH radical scavenging activity

The DPPH radical scavenging activity of ethanolic extracts of young and old *A. excelsa* leaves is presented in Figure 3. The DPPH radical scavenging activity of these extracts increased in a dose-dependent manner (5-150 µg/ml). At 25, 50, 75, 100 and 150 µg/ml, DPPH radical scavenging activity of young leaf extracts was 23.84 ± 5.0, 38.06 ± 14.79, 50.34 ± 2.40, 69.71 ± 9.54 and 89.71 ± 3.58%, respectively (Figure 3A), whereas that for old leaf extracts was 16.12 ± 2.35, 41.71 ± 6.31, 58.81 ± 6.96, 74.18 ± 6.26 and 90.59 ± 0.87%, respectively (Figure 3B). Moreover, IC_{50} values for young and old leaf extracts were 75.00 ± 4.82 and 74.59 ± 2.05 µg/ml, respectively, and the IC_{50} value for ascorbic acid was 9.76 ± 0.88 µg/ml (Figure 4). No significant difference between the IC_{50} values of young and old leaf extracts was observed ($p>0.05$).

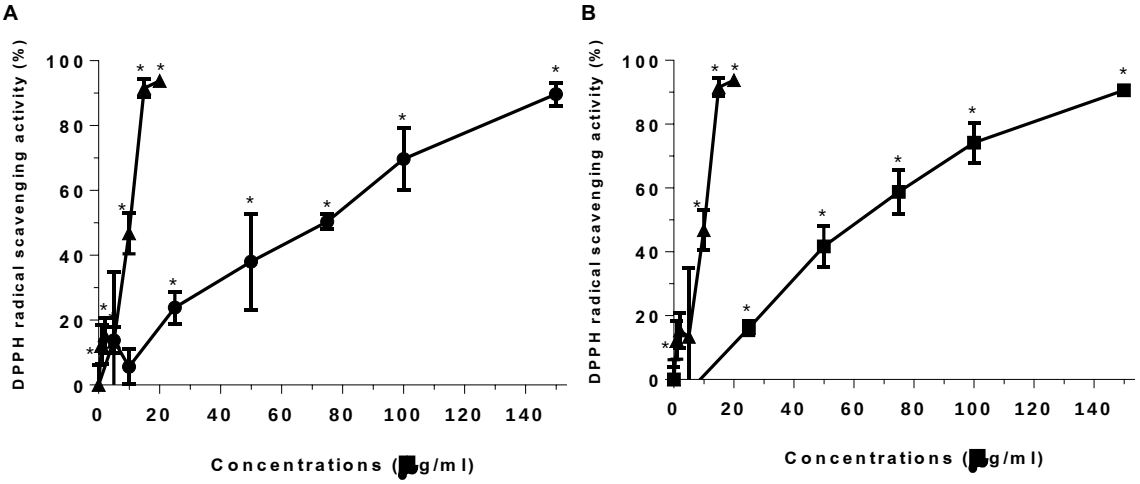

Figure 3. DPPH radical scavenging activity of young and old leaf extracts from *A. excelsa*. The antioxidant activity of ethanolic leaf extracts of young (A; ●) and old (B; ■) leaves from *A. excelsa*, was determined using the DPPH radical scavenging assay. The extracts with various concentrations from 5 to 150 µg/ml were incubated with DPPH radical solution in the dark at room temperature for 2 h, and their absorbance were detected at 517 nm. Ascorbic acid was used as a positive control (▲). Data were presented as mean ± S.D. (n = 3). Asterisks indicate a significant difference at $P<0.05$, when compared with no extract.

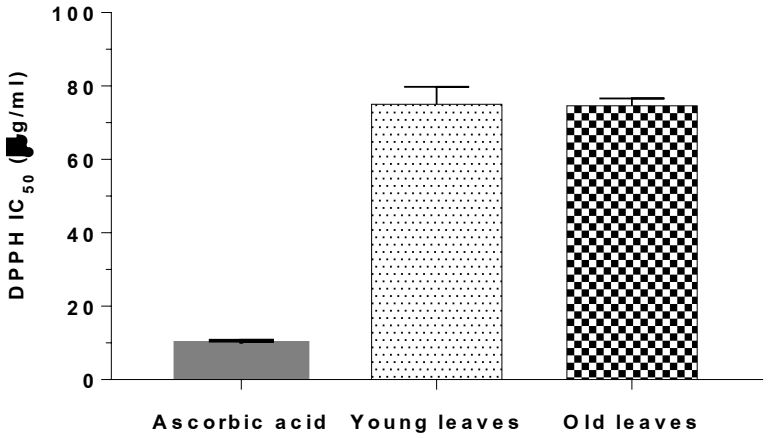

Figure 4. IC_{50} values determined from DPPH radical scavenging assay. IC_{50} values were calculated from the graph plotted between DPPH radical scavenging activity and concentrations of the extracts. No significant difference between IC_{50} values (mean ± S.D.) of ethanolic leaf extracts from young and old leaves of *A. excelsa* was observed.

4. Antibacterial activity

Antibacterial activity of young and old leaf extracts from *A. excelsa* against *E. coli* was performed using the agar well diffusion method, and representative results are presented in Figure 5. Inhibition zones increased in a dose-dependent manner for both young (Figure 5A) and old (Figure 5B) leaf extracts. Significantly greater inhibition zones were detected in old leaf extracts than in young leaf extracts (Figure 6; p<0.05). The inhibition zones of young leaf extracts were in a range from 7 to 11 mm, while these for old leaf extracts were from 10 to 15 mm. Indeed, the inhibition zones for young leaf extracts at concentrations of 5, 10, 50 and 100 mg/ml were 7.00 ± 0.47, 7.67 ± 0.58, 9.67 ± 1.20 and 11.00 ± 0.67 mm, respectively, whereas these for old leaf extracts were 10.00 ± 1.33, 12.78 ± 3.47, 16.00 ± 3.53 and 15.56 ± 0.77 mm, respectively.

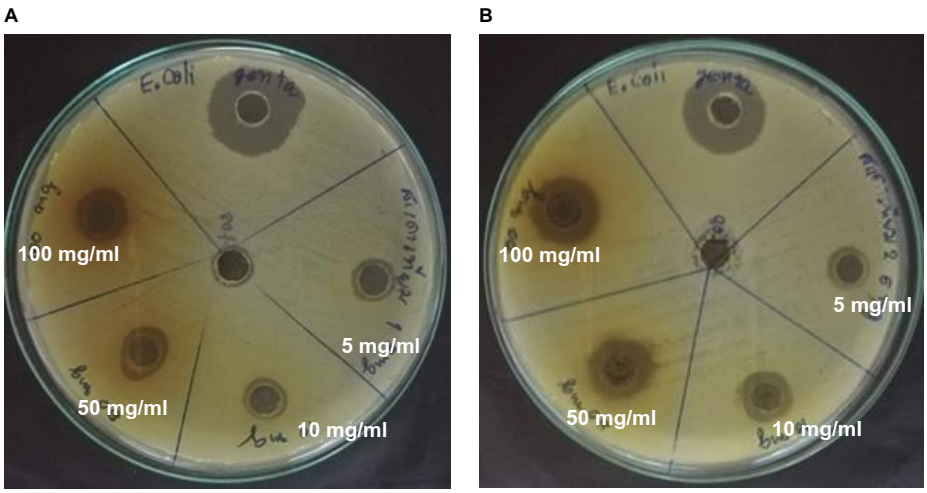

Figure 5. Antibacterial activity of the leaf extracts from *A. excelsa*. Antibacterial activity of young (A) and old (B) leaf extract at various concentrations (100 µl), including 5, 10, 50 and 100 mg/ml, against *E. coli* was assessed by the agar well diffusion method. Gentamicin (100 µl) at the concentration of 0.1 mg/ml was employed as a positive control, and 80% ethanol was used as a negative control. *E. coli* was incubated with the extracts at 37°C for 24 h.

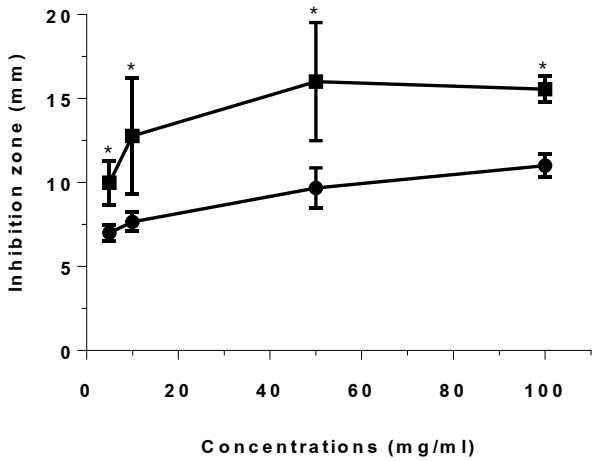

Figure 6. Inhibition zones against *E. coli* of the leaf extracts from *A. excelsa*. Inhibition zones (mm) of young (●) and old (■) leaf extract at various concentrations (100 µl), including 5, 10, 50 and 100 mg/ml, against *E. coli* was measured after 24 h incubation. Asterisks indicate a significant difference at $p<0.05$, when compared to young leaf extracts of *A. excelsa*.

Discussion and conclusion

Nowadays, patients desire more natural ways to reduce symptoms from diseases than chemically synthetic medicines, resulting in the extensive use of folk medicines. In this study, the total phenolic content, antioxidant and antibacterial activity of *A. excelsa* leaves were evaluated. Previously, leaf age has been reported to influence the amount of active ingredients [19], therefore, young and old leaves of *A. excelsa* were compared for their total phenolic content, antioxidant and antibacterial activity.

According to the total phenolic content results, leaf age showed a direct influence on the amount of phenolic compounds . Elevated amounts of phenolic compounds were observed in old leaves of *A . excelsa*. A previous study revealed the similar results .Old leaves of *Vitis vinifera* L. had a higher phenolic content than young leaves . [19] Phenolic compounds have been found to be responsible for many biological activities, such as anti-inflammatory, antioxidant and antibacterial activity[1] . Moreover, they can also be involved in plant defense mechanisms to prevent oxidative damage from reactive oxygen species .[2] In this present study, the *A . excelsa* leaves were extracted with 80 % ethanol, and the total phenolic contents found in young and old leaf extracts (8.72 ± 1.72 and 16.63 ± 3.30 mg GAE/g extract, respectively) were much lower than those observed by a previous study .[13]The *A .excelsa* leaves from Kepong in Selagor, extracted by 70 %ethanol, had total phenolic content of 202 ± 0.42 mg GAE/g extract .[13] This might be from the different locations where the *A . excelsa* leaves were collected .Ghasemzadeh and Jaafar) 2013 (reported that *Pandanus amaryllifolius* leaves collected from different locations could result in different amount of phenolic compounds .[20] The *P. amaryllifolius* leaves collected from Bachok in the North had higher total phenolic contents (6.72 ± 0.36 mg GAE/g extract) than those from Pontian in the South)4.88 ± 0.48 mg GAE/g extract)

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Table 1. Antibacterial activity against *E. coli*, total phenolic contents and antioxidant activity of various plant extracts

Plants	Solvents used in extraction	Solvents used for dissolving	Parts	Antibacterial activity				Total phenolic contents (mg GAE/g extract)	DPPH IC_{50} (µg/ml)	References
				Methods	Inoculum number of *E. coli* (per ml)	Concentrations (mg/ml)	Inhibition zones (mm)			
A. excelsa	80% ethanol in water	80% ethanol	Young leaf	Agar well diffusion	10^8 cells	5	7.00 ± 0.47	8.72 ± 1.72	75.00 ± 4.82	-[a]
						10	7.67 ± 0.58			
						50	9.67 ± 1.20			
						100	11.00 ± 0.67			
			Old leaf			5	10.00 ± 1.33	16.63 ± 3.30	74.59 ± 2.05	
						10	12.78 ± 3.47			
						50	16.00 ± 3.53			
						100	15.56 ± 0.77			
Thymus vulgaris	50% ethanol in water	20% Tween 20 in water	Arial part	Agar well diffusion	10^8 CFU	12.5	18 ± 0	83.37	ND	[2]
						25	18 ± 0			
						50	20.5 ± 0.5			
						100	22.5 ± 0.5			
						200	26.5 ± 1.5			
T. caramanicus						12.5	8 ± 0	102.47	ND	
						25	8 ± 0			
						50	14.5 ± 1.5			
						100	20 ± 0			
						200	28 ± 2			
Zataria multiflora						12.5	10 ± 1	101.92	ND	
						25	12 ± 1			
						50	13.5 ± 0.5			
						100	16 ± 1			
						200	18 ± 2			
Ziziphora clinopodioides						12.5	8 ± 0	37.85	ND	
						25	8 ± 0			
						50	9.5 ± 0.5			
						100	11 ± 1			
						200	12 ± 1			

Plants	Solvents used in extraction	Solvents used for dissolving	Parts	Antibacterial activity				Total phenolic contents (mg GAE/g extract)	DPPH IC_{50} (µg/ml)	References
				Methods	Inoculum number of *E. coli* (per ml)	Concentrations (mg/ml)	Inhibition zones (mm)			
Z. tenuior						12.5	8 ± 0	40.07	ND	
						25	8 ± 0			
						50	9.5 ± 0.5			
						100	10 ± 1			
						200	11.5 ± 0.5			
A. indica	Dichloromethane	20% DMSO	Mature leaf	Disc diffusion	10^5 CFU	5	15	ND	ND	[10]
	Ethanol						13			
	Aqueous						10			
Clausena heptaphylla	Ethanol	Ethanol	Stem bark	Disc diffusion	10^8 CFU	20 and 30	~3.0-4.5	13.42	3.11	[21]
Withania somnifera	80% Methanol	80% Methanol	Leaf	Agar well diffusion	5 x 10^5 CFU	5	28.00 ± 0.56	ND	101.73 ± 8.96	[22]
			Fruit				12.00 ± 1.50		345.68 ± 8.98	
			Root				15.00 ± 0.96		801.93 ± 7.92	
Commelina nudiflora	Water	Water	Whole plant	Disc diffusion	-	50	~9	63.4 ± 0.05	ND	[23]
						75	~11			
						100	~12			
Etlingera elatior	Water	DMSO:water (1:9)	Flower (from Kelantan)	Agar well diffusion	-	10	4.6 ± 0.17	6.19 ± 0.16	34.5 ± 1.42	[24]
			Flower (from Pahang)				2.6 ± 0.22	5.45 ± 0.15	44.6 ± 2.41	
			Flower (from Johor)				NO	5.16 ± 0.15	52.9 ± 2.88	

[a] is the results obtained from this present study. ND is “Not Detection”. – is “not reported in the articles”. NO is “Not Observed”.

Among the biological activities of plant extracts, antioxidant activity has been extensively reported [3, 5, 11] . The IC_{50} values of ethanolic young and old leaf extracts from *A .excelsa* in this present study were 75.00 ± 4.82 and 74.59 ± 2.05 µg/ml, respectively, when determined using the DPPH assay . No significant difference between the DPPH radical scavenging IC_{50} values between young and old leaf extracts was observed . The DPPH radical scavenging IC_{50} values in this present study were much lower than those observed in a previous study .[13]Nur *et al*) .2016 (found that *A . excelsa* leaves extracted by 70 % ethanol had DPPH radical scavenging IC_{50} value of 308 µg/ml .[13]Although we detected lesser amounts of phenolic compounds in this current study, our extracts showed higher or more efficient antioxidant properties when compared to the study of Nur *et al*) . 2016 . (Ghasemzadeh and Jaafar) 2013 (reported that extracts prepared from *P . amaryllifolius* leaves collected from Bachok in the North appeared to have more gallic acid) 0. 42 ± 0. 05 mg/ g extract(than extracts prepared from leaves collected from Klang in the Central region and Pontian in the South (0. 33 ± 0. 04 and 0. 21 ± 0. 02 mg/ g extract, respectively) . Moreover, extracts from Bachok and Klang contained cinnamic acid, while ferulic acid was only found in extracts from Bachok .[20]This reveals that plants of the same species collected from different locations can have different types of phenolic compounds . Thus, further experiments need to be carried out to detect what phenolic compounds are in the *A . excelsa* leaves collected in Songkhla province, Thailand, to determine their contribution to the differences in antioxidant activity.

In this study, leaf age showed affected the antibacterial activity of *A . excelsa* leaf extracts against *E . coli* . A number of studies have reported the link between total phenolic contents and antibacterial activity .[11 ,3-2] The different concentrations of phenolic compounds in wines have been shown to result in different levels of bacterial growth inhibition [6] . *E . coli* was the most sensitive bacteria to all tested phenolic compounds)gallic acid, vanillic acid, protocatechuic acid and caffeic acid(, whereas *Flavobacterium* sp . was the most resistant bacteria [6] . Anti *E. coli* activity, total phenolic contents and antioxidant activity have been reported to be plant-dependent (Table 1). The anti-*E. coli* activity of *Etlingera elatior* flower extracts was dependent on the phenolic content as well as the DPPH scavenging activity. Higher inhibition zones against *E. coli* were observed for extracts with greater amounts of phenolics and with lower DPPH IC_{50} values [24]. Even though the DPPH IC_{50} values observed in this present study are higher, when compared to a previous study (Table 1) [24], greater amounts of phenolics were detected. These phenolics might lead to better inhibition zones against *E. coli* of *A. excelsa* leaf extracts (7.67 ± 0.58 and 12.78 ± 3.47 mm for young and old leaves, respectively), observed in this present study, when compared to *E. elatior* flower extracts (4.6 ± 0.17 and 2.6 ± 0.22 mm for *E. elatior* flower extracts from Kelantan and Pahang, respectively) at the same concentration (10 mg/ml; Table 1) [24]. As well, *Ziziphora clinopodioides* and *Z. tenuior* exhibited higher total phenolic contents but smaller inhibition zones against *E. coli* at the same concentrations (50 and 100 mg/ml; Table 1) [2]. This difference was observed only when compared with old leaf extract of *A. excelsa*, as determined in this present study. However, some plants have shown the stronger anti *E. coli* activity when compared to the extracts presented in this current study (Table 1) [2, 22]. There has been a study reported that the antibacterial effect of phenolic compounds might be initially from the adsorption of phenolics by the cell membrane, which could be subsequently interac with enzymes and metal ions in the bacterial cells .[6]

In conclusion, this study revealed that the leaf age of *A . excelsa* had a noticeable effect on total phenolic contents and antibacterial activity, but not on antioxidant activity . Moreover, the remarkably higher antibacterial activity of old leaf extracts might result from the higher total phenolic content found in old leaves of *A .excelsa*, when compared to young leaves.

Acknowledgements

The authors would like to sincerely thank Faculty of Science and Technology, Songkhla Rajabhat University for providing the financial supports.

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Microscopic Characterization of *Erythrina* Species Distributed in Thailand

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Abstract

Plants in the genus *Erythrina* belong to the family Fabaceae. There are six species distributed in Thailand (*Erythrina variegata* L., *Erythrina fusca* Lour., *Erythrina stricta* Roxb., *Erythrina indica* Lam., *Erythrina subumbrans* (Hassk) Merr. and *Erythrina crista-galli* L.). Due to the similarity of the morphology and synonymous *vernacular names*, the identification of these species is ambiguous. Therefore, an accurate investigation on their identities is essential. This research aimed to study the anatomical characteristics of each species (cross section of midrib) and to determine the constant values of leaves including stomatal number, epidermal cell number, stomatal index, epidermal cell area, vein islet number and palisade ratio. The results indicated that the stomatal type of all six species was paracytic type which was consistent with unique characteristics of plants in this family. In terms of leaf constant numbers, the stomatal number and stomatal indices in the lower epidermis among these six species overlapped, whereas stomata were only found in the upper epidermis of *E. variegata*, *E. crista-galli* and *E. subumbrans* (4-28, 60-136 and 12-44 stomata/mm^2, respectively). *E. crista-galli* demonstrated the highest number of upper stomata, which could be used as a characteristic for identification. In addition *E. variegata* and *E. subumbrans* exhibited distinct upper epidermal cell numbers (404-532 and 1080-1820 cell/mm^2 respectively). This study also revealed overlapping palisade ratios and vein islet numbers among the six *Erythrina* species. Nevertheless, both values could be used to differentiate *E. subumbrans* from other species. Moreover, the cross sections of the midrib of the six investigated *Erythrina* species revealed arrangements of tissue, especially the vascular bundle, that could be used to distinguish each species. No trichomes were found in these species. In conclusion, this study established the qualitative and quantitative microscopic characteristics of six *Erythrina* species in Thailand that can be used as a tool to authenticate these plants.

Keywords: *Erythrina*, Stomatal Index, Epidermal Cell Area, Vein Islet Number, Palisade Ratio

Introduction

Erythrina (coral tree) belongs to the family Fabaceae. The origin of the name *Erythrina* comes from the Greek word "*erythros*" which means red, alluding to the bright red flowers of the trees in the genus [1]. The genus *Erythrina* consists of 110 species of trees and shrubs. This genus is indigenous to the tropics and possibly originated from India and Malaysia [2]. There have been six species of the genus *Erythrina* recorded in Thailand [3]. Five of them are considered to be native to Thailand including *E. fusca* Lour. (= *E. glauca* and *E. ovalifolia*), *E. stricta* Roxb. (= *E. suberosa*), *E. variegata* L., *E. indica* Lam. and *E. subumbrans* (Hassk) Merr. whereas *E. crista-galli* L., is considered to be exotic [3,4].

Plants in *Erythrina* species have been used in traditional systems for the treatment of various ailments and have been found to have many biological activities such as anti-inflammation, antipyretic, nervine sedative, anti-asthmatic, broken bone healing, antiepileptic, hypotensive, uterine stimulant, diuretic, antibacterial, anti-fungal and anti-yeast activities [1]. According to the similarity in morphology and synonymous vernacular names of plants in this genus, the identification of the *Erythrina* species is controversial.

Plant authentication plays a key role in medicinal plant standardization and quality assurance. In addition to macroscopic characters, microscopic examination is a conventional, rapid and inexpensive method to identify plant anatomical structures, such as midrib cross section of mature leaf, and the histological characters of powdered crude drugs. Leaf constant numbers are quantitative microscopic evaluations, which can be effectively used to distinguish closely related species that can only be poorly characterized by qualitative microscopic evaluation [5].

The quantitative microscopic evaluation of *E. variegata, E. fusca, E. stricta, E. indica, E. subumbrans* and *E. crista-galli* has never been established. Therefore,

this research aimed to study the anatomical characteristics of these six Thai *Erythrina* species using microscopic analysis of cross section of midrib to investigate the constant values of leaves including stomatal number, epidermal cell number, stomatal index, epidermal cell area, vein islet number and palisade ratio.

Methodology

Plant materials

Fresh mature leaves of *E. variegata, E. fusca, E. stricta, E. indica, E. subumbrans,* and *E. crista-galli* were collected from 3 different locations in Thailand. Plant specimens were authenticated by Associate Professor Nijsiri Ruangrungsi, Ph.D. The voucher specimens were deposited at the College of Public Health Sciences, Chulalongkorn University, Thailand. Their locations and collection data are shown in Table 1.

Table 1 List of six distributed *Erythrina* species and their different collecting localities

Sample no.	Species	Place of collection (Thailand)	Collecting date (Month, Year)
1.	*E. variegata*	Bangkok	September, 2015
2.		Pathum Thani	October, 2015
3.		Prachin Buri	August, 2015
4.	*E. fusca*	Chaiyaphum	September, 2015
5.		Nakhon Pathom	February, 2016
6.		Rayong	February, 2016
7.	*E. stricta*	Nakhon Ratchasima	April, 2016
8.		Prachin Buri	April, 2016
9.		Saraburi	April, 2016
10.	*E. crista-galli*	Bangkok	November, 2015
11.		Nakhon Pathom	December, 2015
12.		Nakhon Pathom	December, 2015
13.	*E. indica*	Chiang Rai	November, 2015
14.		Chaiyaphum	February, 2016
15.		Nakhon Ratchasima	February, 2016
16.	*E. subumbrans*	Chiang Mai	February, 2016
17.		Chiang Mai	February, 2016
18.		Chiang Rai	February, 2016

Transverse section of the midrib

The fresh mature leaves from six *Erythrina* species (n=3) were cleaned. Cross sections of the midrib were prepared as thin as possible and these tissue sections were then transferred by a brush moistened with water. The sections were mounted onto a slide in water and examined under a photomicroscope attached to a digital camera. Anatomical characters of midrib cross section were illustrated in the proportion related to the original size.

Determination of leaf constant numbers

Leaf constant numbers including stomatal number, epidermal cell number, stomatal index, epidermal cell area, vein islet number and palisade ratio were examined according to Mukherjee PK [6].

Fresh mature leaves were cleaned, cut into small pieces (1 cm x 1 cm), midway between midrib of the leaf and its margin. The cut sections leaves were immersed in Haiter™ solution (containing 6% sodium hypochlorite) diluted with water (1: 1), to remove chlorophyll. The chlorophyll-less leaf sections were gently warmed with chloral hydrate solution (4 g/ml) until transparent [6]. After being washed with water 2-3 times, leaf samples were mounted onto slides in water and observed under a photomicroscope attached to a digital camera. Thirty fields of each species from 3 different locations were examined using AxioVision program.

1) Stomatal number and stomatal index

Both sides of epidermis were observed under a microscope with a 20X objective lens magnification. The stomatal number and the epidermal cell were counted per square millimeter of epidermis. The stomatal index (SI) was determined as a percentage ratio of stomata number (S) to the epidermal cell numbers (E) in the same unit area of epidermis. Using the formula:

$$SI = (S / E + S) \times 100$$

2) *Upper epidermal cell area*

The upper epidermis was observed under a microscope with a 20X objective lens magnification. The epidermal cell area (EA) was evaluated using formula:

$$EA = (1 / E + S) \times 10^6\ \mu m^2$$

Where, E = number of epidermal cells per square millimeter (mm^2) of epidermis

S = the number of stomata in a same area of epidermis

3) Palisade ratio

Groups of four epidermal cells were traced under a microscope with a 40x objective lens magnification and 10x eyepiece lens. The palisade cells lying under the four epidermal cells were counted. The number of palisade cells obtained in each group divided by 4 gave the palisade ratio.

4) Vein islet number

The lower epidermis was observed under a microscope with a 20X objective lens magnification. The number of vein islets per 1 mm^2 of epidermis were counted.

Data analysis

All leaf constant numbers were determined in thirty fields of each species from 3 different locations (ninety fields per each species) and the results were expressed as mean ± SD.

Results

The stomata in six *Erythrina* species was classified as paracytic type, in which the stoma is surrounded by two subsidiary cells parallel to the long axis of guard cells (Figure 1). The epidermal cells and palisade cells of six *Erythrina* species are shown in Figures 2 and 3, respectively. The leaf constant numbers from the microscopic analysis are shown in Table 2.

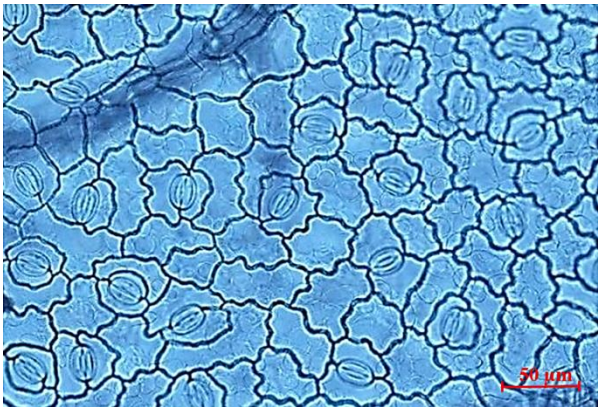


Figure 1 The photograph of paracytic stomata from *E. variegata* represented *Erythrina* species

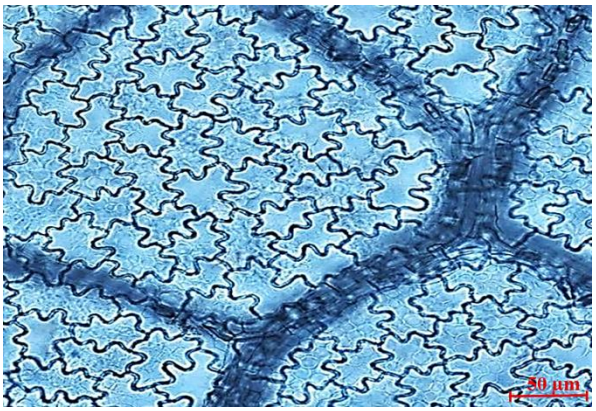


Figure 2 The photograph of upper epidermal cells from *E. variegata* represented *Erythrina* species

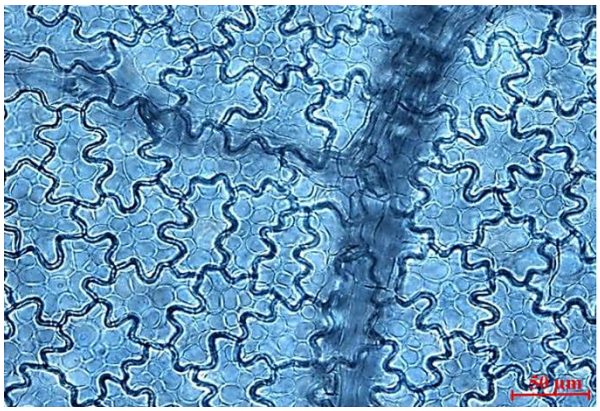


Figure 3 The photograph of palisade cells from *E. variegata* represented *Erythrina* species

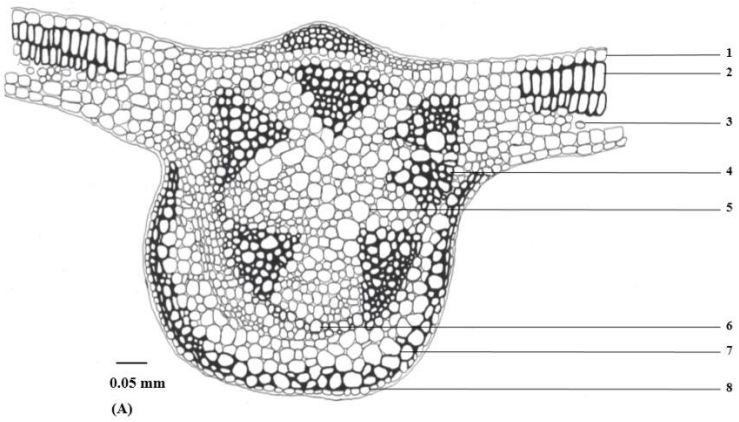
Table 2 Leaf constant numbers of six *Erythrina* species distributed in Thailand

***Erythrina* species**	**Stomatal number* mean ± SD (min - max)**		**Epidermal cell number* mean ± SD (min - max)**		**Stomatal index mean ± SD (min - max)**	
	Upper epidermis	Lower epidermis	Upper epidermis	Lower epidermis	Upper	Lower
E. variegata	10.13 ± 4.95 (4-28)	468.18 ± 30.80 (404-532)	468.18 ± 30.80 (404-532)	839.16 ± 86.80 (496-952)	2.09 ± 0.94 (0.76-5.30)	14.37 ± 1.64 (10.90-19.72)
E. fusca	-	1213.78 ± 166.32 (880-1604)	1213.78 ± 166.32 (880-1604)	1336.98 ± 198.41 (1000-1808)	-	10.80 ± 1.83 (6.22-14.29)
E. stricta	-	693.82 ± 37.97 (624-792)	693.82 ± 37.97 (624-792)	657.73 ± 64.51 (540-776)	-	18.50 ± 2.23 (11.29-23.33)
E. crista-galli	92.44 ± 14.60 (60-136)	1095.38 ± 105.24 (792-1432)	1095.38 ± 105.24 (792-1432)	1369.16 ± 152.06 (1060-1820)	7.82 ± 1.26 (5.38-11.63)	10.47 ± 1.35 (6.62-13.68)
E. indica	-	553.82 ± 76.30 (404-708)	553.82 ± 76.30 (404-708)	648.75 ± 143.99 (376-876)	-	22.41 ± 8.28 (13.74-39.87)
E. subumbrans	24.53 ± 6.76 (12-44)	1405.25 ± 171.81 (1080-1820)	1405.25 ± 171.81 (1080-1820)	1636.18 ± 336.37 (1060-2340)	1.74 ± 0.51 (0.78-3.06)	7.93 ± 1.88 (4.68-11.93)

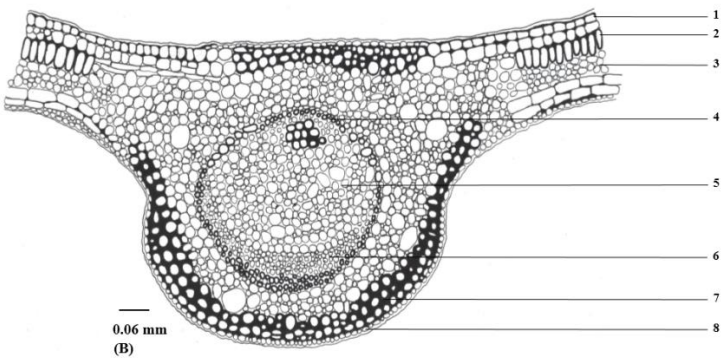
Erythrina species	Upper epidermal cell area (μm^2) mean ± SD (min - max)	Palisade ratio mean ± SD (min - max)	Vein islet number* mean ± SD (min - max)
E. variegata	2100.57±145.41 (1824.82-2118.64)	9.74 ± 1.34 (7.25-13.50)	6.68 ± 1.17 (3.80-11.00)
E. fusca	839.99 ± 112.52 (623.44-1136.36)	7.69 ± 1.11 (4.00-10.00)	6.24 ± 0.77 (4.00-7.75)
E. stricta	1445.52 ± 78.32 (1262.63-1602.56)	6.21 ± 6.21 (3.50-8.75)	6.45 ± 0.76 (4.50-8.25)
E. crista-galli	849.13±81.98 (652.74-1152.07)	6.00 ± 1.15 (3.75-9.25)	6.57 ± 0.94 (5.00-8.50)
E. indica	1841.99 ± 269.36 (1412.43-2475.25)	8.02 ± 1.68 (4.50-12.50)	6.16 ± 1.61 (3.50-9.75)
E. subumbrans	711.94 ± 89.08 (538.79-915.75)	5.13 ± 0.85 (3.50-7.25)	16.58 ± 1.82 (12.75-20.75)

- = absent, * = number per mm^2

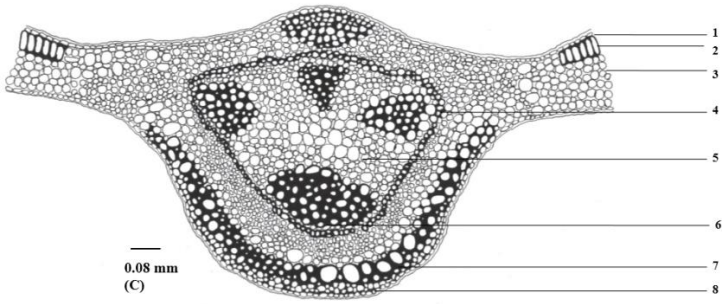
The anatomical characteristics of the midrib of six investigated *Erythrina* species are illustrated (Figure 4). The results revealed an arrangement of tissue, especially the vascular bundle (xylem and phloem tissue), that could be used to distinguish each species. No trichomes were found in these species.



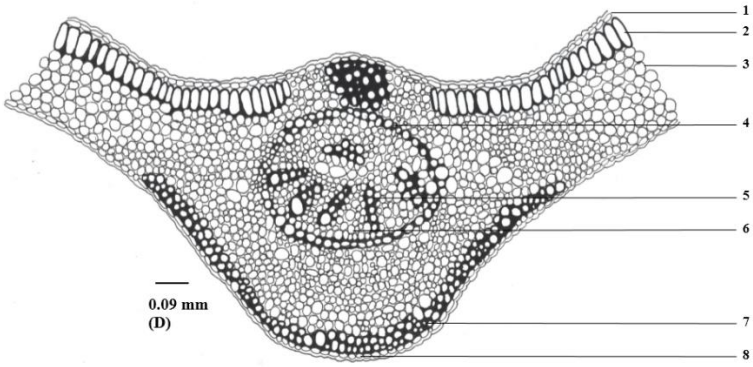
***E. variegata* (A)**



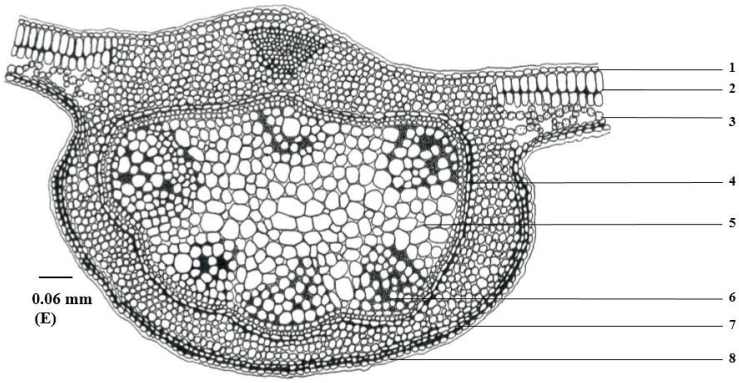
***E. fusca* (B)**



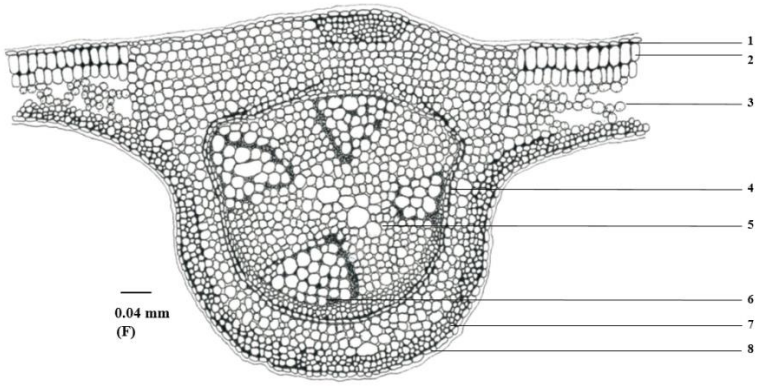
E. stricta **(C)**



E. crista-galli **(D)**



E. indica **(E)**



***E. subumbrans* (F)**

Figure 4 Midrib cross section of the leaf of 1. Upper epidermis, 2. Palisade cell, 3. Spongy cell, 4. Group of fiber, 5. Phloem tissue, 6. Xylem tissue, 7. Collenchyma and 8. Lower epidermis

Discussion and conclusion

The type of stomata in six *Erythrina* species was classified as the paracytic type, in which the stoma is surrounded by two subsidiary cells parallel to the long axis of guard cells (Figure 1). Determination of stomatal number is one of the useful parameters used to distinguish plants at species level [7]. Stomata were found in the upper epidermis only in *E. variegata*, *E. crista-galli* and *E. subumbrans*. (4-28, 60-136 and 12-44 stomata/mm^2, respectively). *E. crista-galli* demonstrated the highest number of upper epidermal stomata, which could be used as a characteristic for identification. The stomatal numbers and stomatal indices in the lower epidermis determined among these six species were overlapping. The stomatal numbers in the lower epidermis among *Erythrina* species in Thailand were found to be less than in *E. velutina* in Brazil (264.60±16.83) [8]. *E. variegata* and *E. subumbrans* exhibited distinct upper epidermal cell numbers (404-532 and 1080-1820 cell/mm^2 respectively). *E. fusca, E. stricta* and *E. indica* could be distinguished by their lower epidermal cell numbers (1000-1808, 540-776 and 376-876 cell/mm^2 respectively).

Epidermal cell area was relatively constant and within a narrow range for each species, which allows for identification although there was some overlapping with closely related species. This value has been used as a taxonomic tool for identification of plant materials [9]. In this study, the epidermal cell areas among *E. fusca, E. crista-galli* and *E. subumbrans* were found to be less than 1200 μm^2 whereas the epidermal cell areas among *E. variegata, E. stricta* and *E. indica* were more than 1200 μm^2.

The other important leaf constant parameters are palisade ratio and vein islet number. The palisade ratio has been used as a diagnostic value for differentiating plant species. Both values can be affected by geographical variation, but differ from species to species. However, our study revealed overlapping of the palisade ratios and vein islet numbers among six *Erythrina* species. Nevertheless, both values could be used to differentiate *E. subumbrans* from other species. In this study, *E. subumbrans* showed the highest vein islet number (12.75-20.75 cell/mm^2) which was less than previously reported of 23.2 ± 1.16 cell/mm^2 in India [10].

Qualitative microscopic investigation provides the supporting evidence for plant identification [11]. The cross sections of midrib of six investigated *Erythrina* species revealed distinguishing characteristics (Figure 4).

In conclusion, this study established the qualitative and quantitative microscopic characteristics for authentication of six *Erythrina* species in Thailand.

Acknowledgements

This research was scholarly supported by Teaching Assistant Scholarship from the Graduate School. The authors would like to thank College of Public Health Sciences, Chulalongkorn University for facility support.

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Pharmacognostic Specification and Chrysin Content of *Oroxylum indicum* Seeds in Thailand

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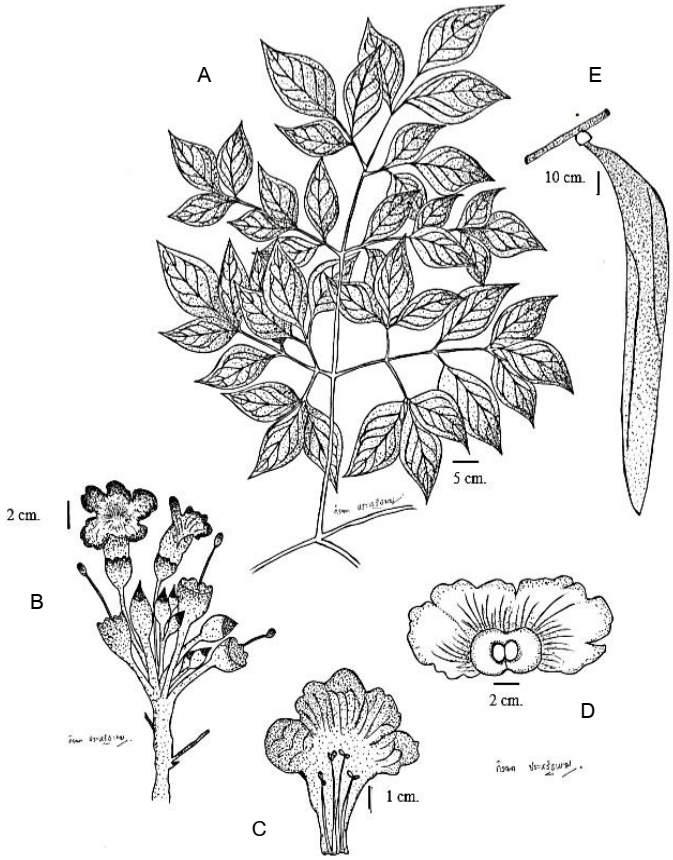
Abstract

Oroxylum indicum (L.) Kurz belongs to the Bignoniaceae family and is known by its common name as the Broken Bones tree. This plant is a medium-sized tree that grows in Asian tropical and subtropical low-altitude open forests. The seed has been widely used in traditional Chinese medicine to treat cough, acute or chronic bronchitis and other respiratory disorders. In Thailand, it has been used in the treatment of cough, throat infection and as a purgative. However, the pharmacognostic specifications of this crude drug and its chrysin content have never been established in Thailand. Consequently, this study aimed to determine the pharmacognostic specifications and chrysin content of *O. indicum* seeds collected from different sources throughout Thailand. The results indicated that loss on drying, water content, total ash, acid-insoluble ash, ethanol soluble extractive value and water soluble extractive value among *O. indicum* seeds in Thailand were 3.32 ± 0.12, 6.89 ± 0.80, 4.40 ± 0.08, 0.47 ± 0.05, 9.74 ± 0.68 and 12.11 ± 0.80 % by dry weight, respectively. The ethanolic extracts were analyzed by thin layer chromatography (TLC) using silica gel 60 GF_{254} as stationary phase, and solvent system of toluene: chloroform: acetone: formic acid (5:4:1:0.2) as mobile phase, The chrysin content of the extracts was found to be 0.17 ± 0.05 and 0.20 ± 0.07 % by dry weight when evaluated by TLC-densitometry and TLC image analysis, respectively. In conclusion the pharmacognostic specification of *O. indicum* seed in Thailand is established. This standard can now be used for quality control of this crude drug. TLC densitometry and TLC image analysis were demonstrated to be a precise, specific and reliable technique for quantification of chrysin in this crude drug.

Keywords: *Oroxylum indicum* seeds, chrysin, pharmacognostic specification, TLC densitometry, TLC image analysis

Introduction

Oroxylum indicum (L.) Kurz is a medium-sized tree growing in southern China, South and South East Asia [2]. It is a plant belonging to the Bignoniaceae family. The plant is easily recognized because of its typical appearance, i.e. flat, kidney-shaped, yellow-green seeds that are surrounded by a light-brown papery wing with a diameter of about five to eight centimeters (Figure 1). The seed of this plant, a traditional Chinese medicine named as Mu Hu Die, has been widely used in the treatment of cough, acute or chronic bronchitis, pharyngitis, pertussis and other respiratory disorders [3]. In Thailand, this seed has been used for as a purgative and in the treatment of throat infections and hypertension. In Nepal, it is used as a digestive. A seed paste is applied to treat abscesses and wounds [4]. The flavonoids found in the seed of *O. indicum* were reported to be baicalein-7-*O*-glucoside, chrysin, baicalein, baicalein-7-*O*-diglucoside and chrysin-7-*O*-diglucoside [5]. Chrysin is a flavone, which is a subgroup of flavonoids. Many polyphenolic compounds, including chrysin, are known to have multiple biological activities, such as anti-inflammation [6], anti-cancer [7, 8] and anti-oxidation [9]. There have been pharmacognostic studies on *O. indicum* stem bark in India [10] and *O. indicum* root, which is one of the ingredients in Ben-Cha-Moon-Yai remedy in Thailand [11]. However, the standardization of *O. indicum* seed crude drug and its chrysin content have never been established in Thailand. This study aimed to investigate the quality parameters of *O. indicum* seed in Thailand and to establish the pharmacognostic specification of this crude drug with reference to the chrysin content as determined by TLC densitometry and TLC image analysis.



Plant Description: A medium tree, 4-12 m tall, branched at top; bark light-brown; leaves, 3-7 cm long, 2-3 pinnate with opposite pinnae, rachis very stout, cylindrical, leaflets 2-4 pairs, 6-12 cm long and 4-10 cm broad, ovate or elliptic, acuminate, glabrous or base rounded; petioles of the lateral leaflets 6-15 mm long; flowers numerous, foetid, in large erect racemes, 0.3-0.6 meter long or even more pedicels 6-30 mm long; corolla usually lurid-purple, reaching 10 cm long, fleshy lobes about 4 cm long with crisped margins; stamens 4, slightly exerted beyond the corolla tube, one of them little shorter than the 4, filaments cottony at the base; seed pods hang down from bare branches; those long fruits curve downward 10-20 cm and resemble the wings of a large bird or dangling sickles or swords in the night; seeds numerous, 6-8 cm long, winged all round except at the base.

Figure 1 *Oroxylum indicum* (L.) Vent. (A) Tripinnately compound leaf, (B) Inflorescence, (C) Petal and stamen, (D) Seed and (E) Pod

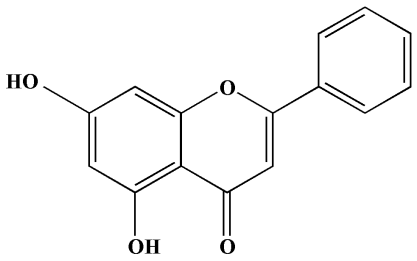


Figure 2 Chrysin structure

Methodology

Plant materials

The seeds of *O. indicum* were collected from 15 sources throughout Thailand and authenticated by Assoc. Prof. Dr. Nijsiri Ruangrungsi. The voucher specimens were deposited at the College of Public Health Sciences, Chulalongkorn University. Any foreign matter was removed and each authentic sample was air dried.

Pharmacognostic evaluations

According to the World Health Organization guidelines [12], we determined the following quality parameters of *O. indicum* seeds: water content; loss on drying; total ash and acid insoluble ash contents; ethanol and water soluble extractive values. The ethanolic extracts prepared by maceration were subjected to thin layer chromatography on TLC silica gel 60 GF_{254} plate (20×10 cm, Merck). The mobile phase was toluene: chloroform: acetone: formic acid (5:4:1:0.2). The plate was observed under ultraviolet light (at 254 and 365 nm) and dipped with 10 % sulfuric acid in ethanol then heated at 100 °C for 15 minutes to obtain the TLC fingerprint.

Quantitative analysis of chrysin

The powder of *O. indicum* seeds (5.0 g) was extracted with 95% ethanol by Soxhlet apparatus until exhaustion. The extracts were filtered, evaporated to dryness and the yields were recorded. The extracts were dissolved in 95% ethanol for quantification. Three microliters of each extract (1 mg/ml) and chrysin standards (0.05, 0.1, 0.2, 0.3 and 0.4 mg/ml) were

applied to the same TLC plate (silica gel 60 GF_{254}, 20x10cm). The TLC plate was developed with a mobile phase of toluene: chloroform: acetone: formic acid (5:4:1:0.2). After development, the plates were scanned under 269 nm UV light to identify chrysin bands (Camag scanner IV). Densitograms with peak areas were obtained by WinCATS software (Camag, Switzerland). The developed TLC plate was also photographed under254 UV light in an ultraviolet viewing cabinet (Spectronics corp., USA) and images were saved as tiff files. The color intensity of chrysin bands were transformed to chromatographic peak areas by Image J software. The contents of chrysin in *O. indicum* extracts and crude drugs were calculated. Each determination was performed in triplicate.

Method validation

The TLC-densitometry and TLC image analysis methods for determination of chrysin in *O. indicum* seeds were validated. Calibration range, accuracy, repeatability, intermediate precision, limit of detection (LOD), limit of quantitation (LOQ), specificity and robustness were performed according to ICH guideline [13].

Results

Macroscopic evaluation

The typical appearance of the dried seed (crude drug) is shown in Figure 3. The typical appearance is a flat, kidney-shaped, yellow-green seed surrounded by a light-brown papery wing with a diameter of about 5-8 centimeters.

Figure 3 Dried seed of *O. indicum*

Microscopic evaluation

The anatomical characteristics of *O. indicum* seed in transverse section view are shown in Figure 4. The histological characteristics of the powdered form are shown in Figure 5.

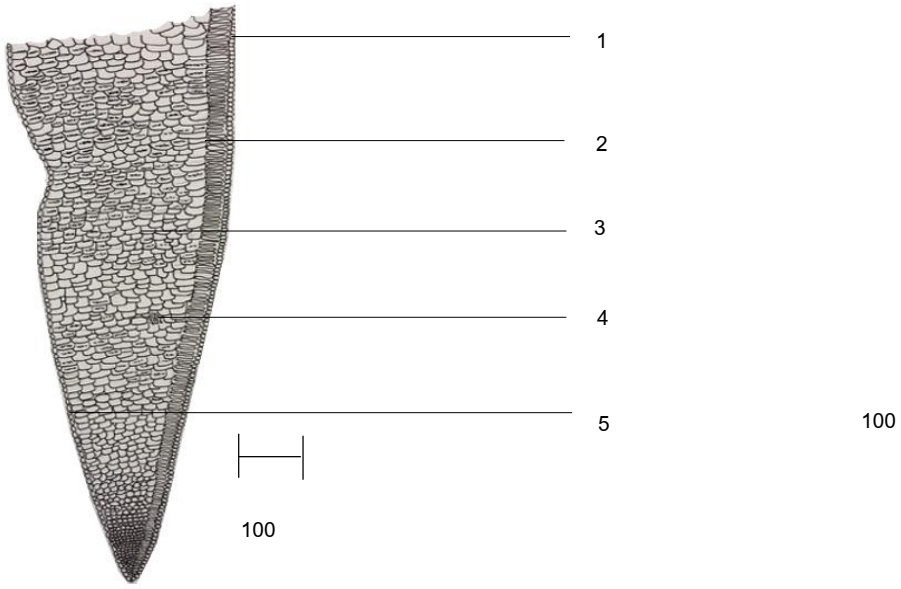


Figure 4 Anatomical character of *O. indicum* seed in transverse section view

1. Endodermis, 2. Endosperm, 3. Cortical parenchyma, 4. Cortical fiber and 5. Epidermis

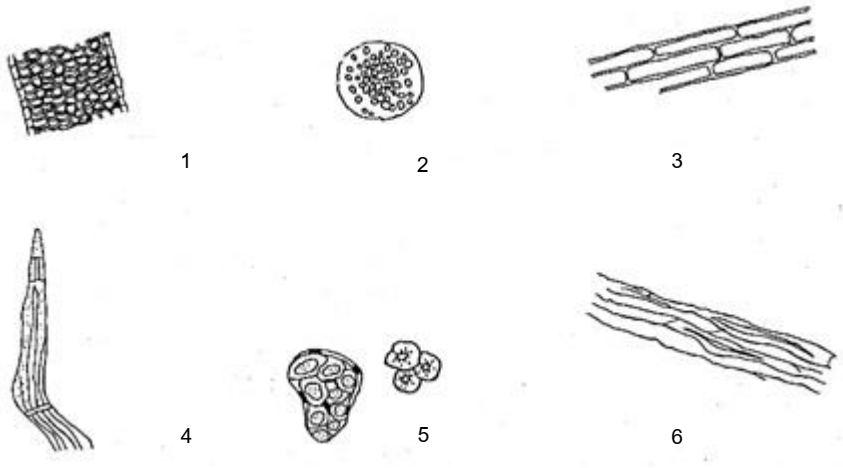


Figure 5 Histological characters of *O. indicum* seed in powdered form 1. Parenchyma in transverse view, 2. Aleurone grains, 3. Parenchyma in longitudinal view, 4. Septate fibre, 5. Stone cells and 6. Fibre

Physico-chemical parameters

The physico-chemical parameters of dried *O. indicum* seed are shown in Table 1. Thin layer chromatographic fingerprint is shown in Figure 6.

Quantitative analysis of chrysin

Reprasentative TLC plates of chrysin solutions and *O. indicum* seed extracts, developed with toluene: chloroform: acetone: formic acid (5:4:1:0.2), are shown in Figure 7. The chrysin contents were determined by TLC-densitometry and TLC image analysis, and the results are shown in Table 2. The average amount of chrysin in seed was found to be 0.167 ± 0.053 (range 0.088-0.299) and 0.20 ± 0.07 (range 0.107-0.389) % by weight of dried seed, by TLC-densitometry and TLC image analysis respectively. Reprasentative TLC–densitograms of chrysin in *O. indicum* seed extracts are shown in Figure 8.

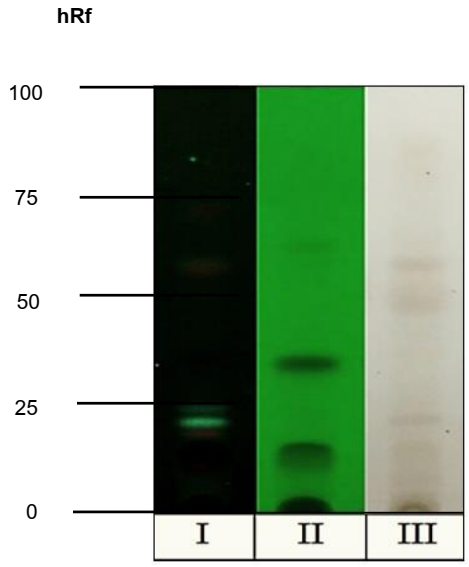
Method validation

The method was validated for accuracy, precision, LOD, LOQ and robustness (by varying mobile phase ratio) as shown in Table 3. The calibration curves are shown in Figure 9. The specificity of the method was validated by checking peak identity and peak purity. The identical absorbance spectra of chrysin standards and samples is illustrated at the peak apex in figure 10(a) and at the up-slope, apex and down-slope of the sample peak in figure 10(b).

Table 1 The quality parameters of *O. indicum* seed

Parameter	Content (% dry weight)*
Loss on drying	3.32 ± 0.12
Water	6.89 ± 0.80
Total ash	4.40 ± 0.08
Acid insoluble ash	0.47 ± 0.05
Ethanol soluble extractive value	9.74 ± 0.68
Water soluble extractive value	12.11 ± 0.80

*The parameters were shown as grand mean ± pooled SD. Samples were collected from 15 different sources in Thailand. Each sample was tested in triplicate.



Stationary phase: Silica gel 60 GF254

Solvent system

Toluene : Chloroform : Acetone Formic acid (5:4:1:0.2)

Detection I = detection under UV 365 nm

II = detection under UV 254 nm

III = detection with 10% sulfuric acid in ethanol and heated

Figure 6 Thin layer chromatographic fingerprint of ethanolic extract of *O. indicum* seed

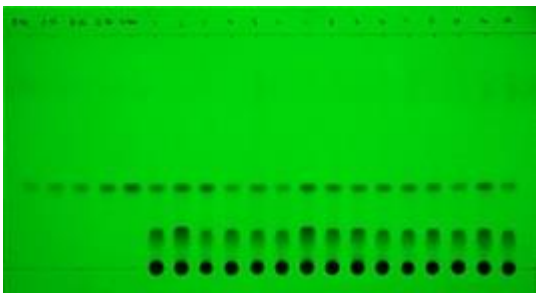


Figure 7 TLC chromatograms under ultraviolet 254 nm developed with toluene: chloroform: acetone: formic acid (5:4:1:0.2); standard chrysin (track 1-5) and *O. indicum* seed extracts from 15 different sources.

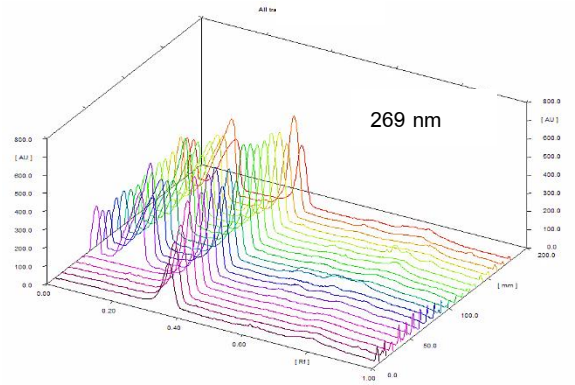


Figure 8 TLC – densitogram of chrysin in *O. indicum* seed extracts from 15 different sources and standard chrysin

Table 2 The chrysin content in *O. indicum* seed from 15 different sources by TLC-densitometry and TLC-image analysis

Sources	Chrysin content (% by dry weight)	
	TLC-densitometry	TLC image analysis
1	0.13	0.16
2	0.18	0.22
3	0.22	0.24
4	0.12	0.15
5	0.18	0.21
6	0.09	0.11

Sources	Chrysin content (% by dry weight)	
	TLC-densitometry	TLC image analysis
7	0.19	0.22
8	0.17	0.19
9	0.17	0.22
10	0.15	0.18
11	0.30	0.39
12	0.19	0.24
13	0.14	0.18
14	0.19	0.23
15	0.09	0.12
Average	**0.17 ± 0.05**	**0.20 ± 0.07**

Table 3 Method validation of TLC-densitometry and TLC Image analysis of chrysin in *O. indicum* seed

Parameter	Validity of TLC-densitometry	Validity of TLC Image analysis
Calibration curve	$y = -5166.x^2 + 21873x + 3812$	y =778- 6x3462 + ^{2}9x -3278
	$R^2 = 0.9998$	R0.9998 = ²
Range (µg/spot)	0.3-1.2	0.3-1.2
Accuracy (% Recovery)	109.55 ± 0.31	107.71 ± 9.94
Repeatability (% RSD)	1.982 ± 0.782	2.997 ± 2.231
Intermediate precision (% RSD)	4.297 ± 2.242	5.122 ± 3.709
Limit of detection (µg/spot)	0.015	0.016
Limit of quantitation (µg/spot)	0.046	0.048
Robustness (% RSD)	2.05	4.07

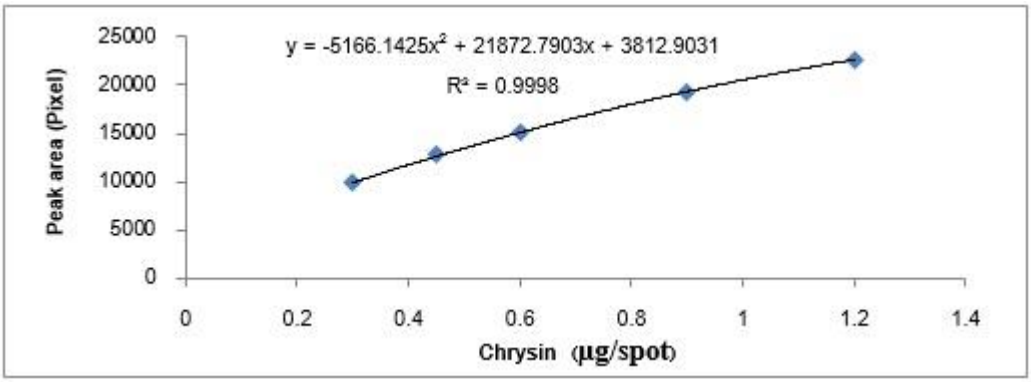


Figure 9 (a) The calibration curve of chrysin standard of TLC-densitometry

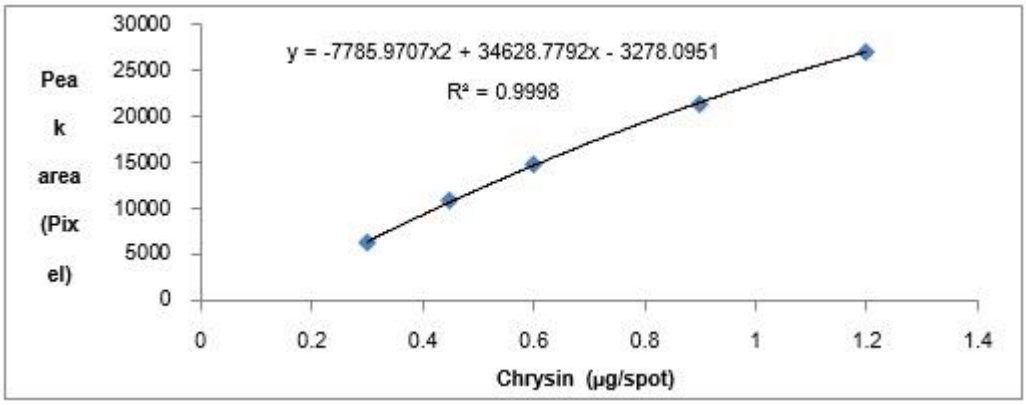
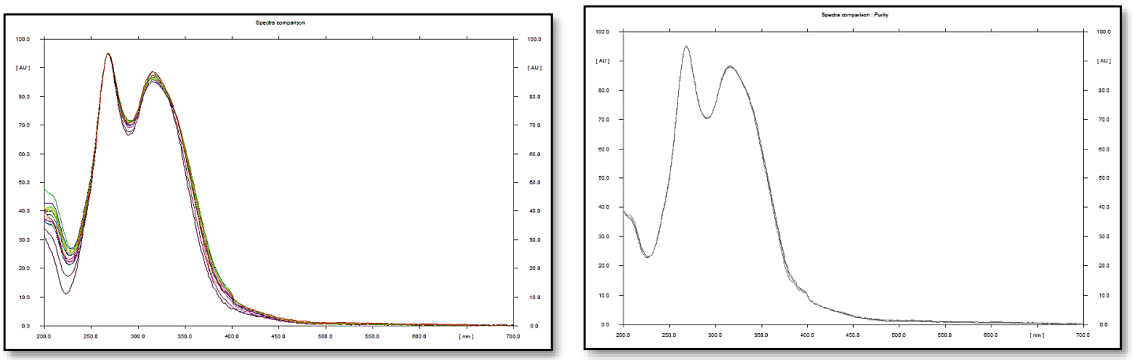


Figure 9 (b) The calibration curve of chrysin standard of TLC-image analysi



(a) **(b)**

Figure 10 The absorbance spectra of chrysin in *O. indicum* seed extracts from 15 different sources and standard chrysin representing peak identity (a) and absorbance spectra of chrysin in the extract using up-slope, apex and down-slope representing peak purity (b)

Discussion and conclusion

Determining the physico-chemical parameters of *O. indicum* is part of the quality control for this herbal drug. The data from this current study form the basis of quality criteria of this crude drug. From our analysis, loss on drying, water content, total ash and acid-insoluble ash should be not more than 3.4, 6.9, 4.4 and 0.5 % by dry weight, respectively. The ethanol soluble extractive matter and water soluble extractive matter should be not less than 9.7 and 12.1 % by dry weight, respectively.

For quantitative analysis, the chrysin content in *O. indicum* seed was found to be 0.17 ± 0.05 and 0.20 ± 0.07 % dry weight of crude drugs by TLC-densitometry and TLC image analysis, respectively. TLC image analysis by image J software can be used as an alternative method to TLC densitometry. However, it was found that the results from TLC image analysis tended to be higher than the results from TLC densitometry (P < 0.01 by pared t-test). In a previous study, Kruger and Ganzera analyzed chrysin in the methanolic extracts of *O. indicum* seeds from four different areas in India by HPLC, and the content was 0.03, 0.10, 0.14 and 0.14 % dry weight [14]. Srinivas and Aparna analyzed chrysin in other plant parts by HPTLC and found that the amounts of chrysin in the ethanolic extracts of *O. indicum* in root, stem and leaf were 0.011-0.014, 0.002-0.004 and 0.006-0.007 % dry weight, respectively [15]. Thus, *O. indicum* seed contained higher amounts of chrysin compared to that of the roots, stem and leaf. Zaveri *et al*. previously reported that baicalein, chrysin, scutellarine and oroxylin-A were present in stem bark and leaves of *O. indicum* using a validated HPLC method for quantification of their compounds in *O. indicum* stem bark and leaves [16].

The accuracy of our method was evaluated by determining the percentage recovery of spiked concentrations of standard chrysin in the sample matrix. The recovery values by TLC-densitometry and TLC image analysis were within acceptable limits (109.55 and 107.71%, respectively) [17]. The precision testing of the chrysin quantitative analysis was conducted by determining the chrysin content of 4 concentrations by 3 replicates on the same day and on different days. The repeatability or intra-day precision and the intermediate precision or inter-day precision were not more than 10%.

The LOD and LOQ were calculated based on the residual standard deviation of a regression line. The LOD values of TLC-densitometry and TLC image analysis, regarded as the lowest concentration of analyte in a sample which could be detected, were found to be 0.015, 0.016 mg/spot, respectively. The LOQ values of TLC-densitometry and TLC image analysis, regarded as the lowest concentration of analyte in a sample which could be quantitatively determined, were 0.046, 0.048 mg/spot, respectively. The robustness, estimated by analysis of the peak area after deliberate variation of mobile phase ratio (9:7:1:0.2, 10:8:2:0.4, 11:9:3:0.6(, showed < 5% RSD of peak area. The calibration curves were polynomial with the range of 0.3 - 1.2 mg/spot. Method validity testing indicated that TLC-densitometry and TLC image analysis were efficient, reliable and suitable techniques for quantitative analysis of chrysin in *O. indicum* seed.

In conclusion, the pharmacognostic specification of *O. indicum* seed in Thailand is established. This standard can now be used for quality control of this crude drug. Quantitative TLC can be used as a precise specific and reliable technique for analysis of chrysin content in this crude drug.

Acknowledgments

The authors are grateful to the College of Public Health Sciences, Chulalongkorn University and all staff members for facility supports.

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Antibacterial Activity Of Essential Oil From *Zanthoxylum Rhetsa* (Roxb.) Dc. Fruit

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Abstract

The essential oil from the fruit of *Zanthoxylum rhetsa* (Roxb.) DC. contains a number of compounds, including monoterpenes, which have many bioactivities such as insect repellent, antidiarrheal, and antioxidant. In this research, we studied the biological potential of the fruit, which is used in the northern part of Thailand as a spice for cooking and in folk medicine. The oil was isolated by distillation and analyzed by gas chromatography-mass spectrometry and the highest percentage constituent was identified to be alpha-limonene (59.68%). Oils were investigated for antibacterial activity against *Staphylococcus aureus, Bacillus cereus, Enterococcus faecalis, Salmonella enteritidis, Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa.* The results showed an inhibitory effect against *E. faecalis, E. coli,* and *K. pneumoniae* (MIC value of 6.25% v/v) and a bactericidal effect against *B. cereus* and *S. aureus* (MBC value of 25% v/v). Thus the *Z. rhetsa* essential oil exhibited antibacterial activity against both Gram positive and Gram negative bacteria, which should be useful for the pharmacological management and pharmaceutical cosmetics in the future.

Keywords : Antibacterial, Essential oil, *Zanthoxylum rhetsa* (Roxb.) DC.

Introduction

Zanthoxylum rhetsa (Roxb.) DC. belongs to the family Rutaceae and is commonly known as Makhwaen (*Zanthoxylum limonella* (Dennst.) Alston). It is a small or medium-sized tree with spines. Leaves are pinnately compound, particularly at the base, with a tapering, pointed tip. Flowers are numerous, fairly small, yellowish-white, with a red center, and 4-parted. Fruit is solitary and finely tubercle. Seeds are somewhat rounded and bluish-black. It is a well-known traditional plant used in the northern part of Thailand such as Chiang Mai, Chiang Rai, Phayao, Lamphun, Lampang, Phrae and especially Nan. It is commonly used for cooking and in many other areas, including as an antimicrobial, antioxidant, anti-inflammatory, and antidiarrheal It is also used for toothache, and for its skin and digestive properties [1-6]. According to a previous study, it is noticeable that the essential oil has antimicrobial activity against Gram positive and Gram negative bacteria, which are the causes of skin and gastrointestinal tract disorder.s The oil showed the strongest killing effect against *Staphylococcus aureus* and *Escherichia coli* (within 9 minutes) while the multi-drug resistant bacteria, methicillin-resistant *S. aureus* (MRSA) and the extended-spectrum β-lactamase-producing *E. coli* (ESBL-EC) were completely eradicated within 90 minutes [7-8]. Furthermore, it could prohibit *Bacillus subtilis, E. coli, Klebsiella pneumonia* and *Vibrio cholera* [9]. and the 80% ethanol extract from *Z. rhetsa* fruit inhibited *Proteus mirabilis*, *E. faecalis, S. epidermidis* and *Streptococcus mitis* [10]. Nowadays, there are many uses of plants for controlling microbial infections instead of chemical substances. The essential oil from *Z. rhetsa* was found to containt alpha-pinene, limonene and sabinene, which were the active compounds for controlling microbial activity [11]. However, the activity against microbes and constituents of the oil have been reported to vary from place to place. This study was conducted to evaluate the antibacterial activity and the constituents of the essential oil from *Z. rhetsa* fruits from Nan province.

Methodology

(i) Preparation of the essential oil

The mature fruit of *Z. rhetsa* was collected from Na Muen district, Nan province, authenticated by the taxonomist and a voucher specimen 039023 has been deposited in the CMU herbarium, faculty of Science, Chiang Mai University. The fruits were dried and pulverized to a powder in a mechanical grinder. Four

hundred grams of the powder was weighed and distilled by hydrodistillation in 1,200 mL of water at 100 °C for 4 hours. The distillate was collected and dried with sodium sulphate anhydrous. The essential oil was kept at 4 °C before use.

(ii) GC-MS analysis

GC-MS was carried out on GC 7890A (Agilent Technology, USA) equipped with a HP-5MS (30 mx 0.25 mm ID x 0.25 μm film thickness). Samples were injected for 0.5 μl with a split ratio of 50:1 with a flow rate of helium 1 ml/min as a carrier gas. The oven temperature was 60 to 240 °C at a rate of 3 °C min^{-1}. The inlet was 250 °C with 60 min total runtime. In term of MS, The MS combined with GC used in MSD 5975C (EI) (Agilent Technology, USA). The MS quadrupole system temperature was 150°C and the MS source was 230 °C. The ionization energy was 70 eV and mass range was 30 to 500 amu. The constituents were identified by comparison of their mass spectra with those in the mass spectra of references of Wile libraries or database of National Institute Standard and Technology (NIST) attached to the GC-MS instrument and reported.

(iii) Microorganisms

The essential oil from *Z. rhetsa* fruit was examined for antibacterial activity against Gram positive bacteria *Staphylococcus aureus, Bacillus cereus, Enterococcus faecalis* and Gram negative bacteria *Salmonella enteritidis, Escherichia coli, Klebsiella pneumoniae* and *Pseudomonas aeruginosa*. All of them are common causes of gastrointestinal and skin infection diseases. The microbials were obtained from School of Medical Sciences, University of Phayao.

(iv) Antibacterial activity by disk diffusion method

At least four morphologically similar colonies were transferred with a sterile loop into tryptic soy broth (TSB) and incubated with shaking at 35-37 °C until the visible turbidity was equal to 0.5 McFarland. The inocula were uniformly spread with sterile cotton swabs onto Mueller Hinton agar plates. Sterile filter paper was made into 6 mm discs and dipped with 20 μl of the essential oil at various concentrations (6.25-100% v/v in DMSO). Ten micrograms of gentamicin and imipenem were used as positive control and DMSO was used as negative control. The plates were incubated at 37 °C for 24 hours. The antibacterial activity was expressed as zone of inhibition.

(v) Determination of antibacterial activity with minimal inhibitory concentration (MIC)

Inocula were prepared bt the same method as disk diffusion. A 100 microliter inoculum was added to each well in 96 well plates and 100 microliters of essential oils were added with 2-fold serial dilution, in triplicate. The negative control was DMSO. The plates were incubated at 37 °C for 24 hours. The MIC was the lowest concentration of test compound that could completely inhibit the growth of bacteria.

(vi) Determination of antibacterial activity with minimal bactericidal concentration (MBC)

MBC was determined by subculturing from the broth dilution MIC tests onto tryptic soy agar (TSA) plates. The plates were incubated at 37 °C for 24 hours. After the incubation period, the plate with the lowest concentration of the antibacterial that showed no bacterial growth was the MBC.

Results and Discussion

1. Essential oil

The fruits (400 g) were distilled and yielded 19.35% v/w of essential oil which was clear and colorless. The essential oil was analyzed by GC-MS. It was injected into the GC-MS and the mass spectra was compared with the database from the library and the results are shown in Figures 1 and 2 and Table 1. The results show the total ion current (TIC) chromatogram, retention time (minute), the percentage of match qualities and the percentage of relative area that calculated by area under the curve of chromatogram. The essential oil had 12 compounds including monoterpenes, sesquiterpenes and oxygenated sesquiterpenes but the main constituents were monoterpenes. The major components of the essential oil were alpha-limonene (59.68%), (E)-beta-ocimene (11.30%) and alpha-phellandrene (10.88%) at the retention time of 7.053, 7.587 and 6.237 minutes, respectively.

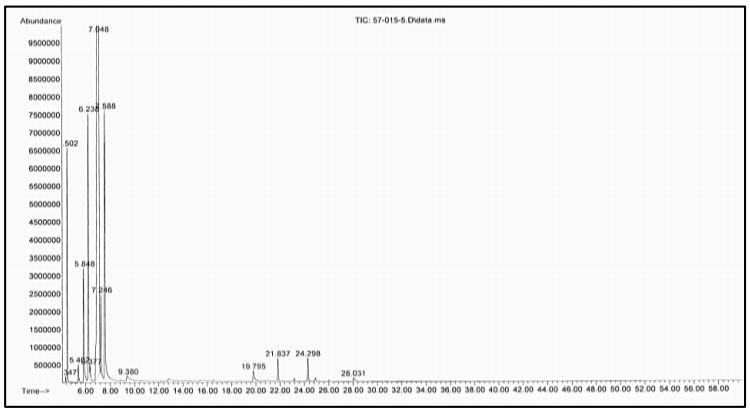


Figure 1 TICs of the GC-MS analyses of essential oil from *Z. rhetsa*

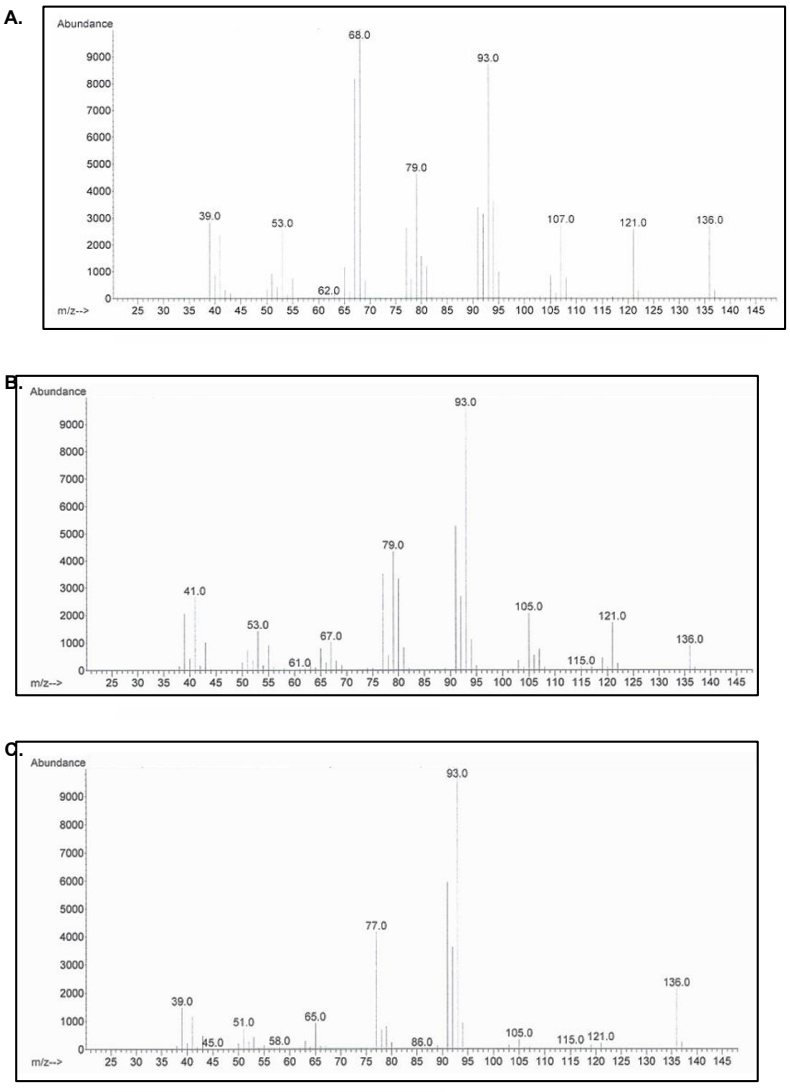
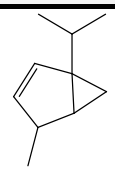
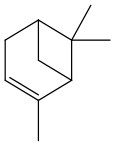
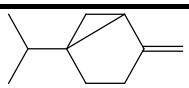
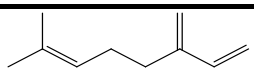
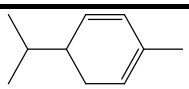
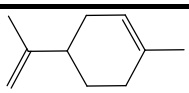
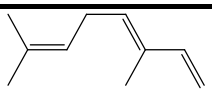
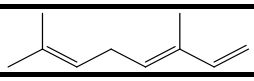
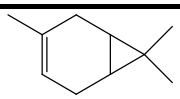
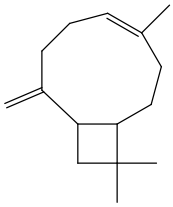
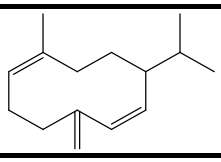
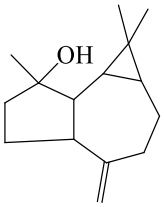


Figure 2 Mass spectra of (A): alpha-limonene, (B): (E)-beta-ocimene and (C): alpha-phellandrene

Table 1 Constituents of essential oil from *Z. rhetsa* by GC-MS

Structure	Compound	Formula	Retention time (minute)	%Relative area	%Match qualities
	1-isopropyl-4-methylbicyclo[3.1.0]hex-2-ene	$C_{10}H_{16}$	4.352	0.14	94
	alpha-(+)-pinene	$C_{10}H_{16}$	4.503	5.88	96
	(+)-sabinene	$C_{10}H_{16}$	5.405	0.42	97
	beta-myrcene	$C_{10}H_{16}$	5.848	4.56	96
	alpha-phellandrene	$C_{10}H_{16}$	6.237	10.88	96
	alpha-limonene	$C_{10}H_{16}$	7.053	59.68	99
	trans-alpha-ocimene	$C_{10}H_{16}$	7.247	3.03	96
	(E)-beta-ocimene	$C_{10}H_{16}$	7.587	11.30	98
	(+)-3-caren	$C_{10}H_{16}$	9.381	0.46	90
	caryophyllene	$C_{15}H_{24}$	21.838	1.01	99
	(-)-germacrene d	$C_{15}H_{24}$	24.301	1.09	99
	spathulenol	$C_{15}H_{24}O$	28.034	0.14	98

2. Antibacterial Activity

We investigated the antibacterial study activity of the essential oil from *Z. rhetsa* on Gram positive bacteria *S. aureus, B. cereus, E. faecalis,* and Gram negative bacteria *S. enteritidis, E. coli, K. pneumoniae* and *P. aeruginosa* which were pathogens of skin disease and gastrointestinal tract disorders. In general, the essential oil showed less activity than the positive controls; gentamicin (aminoglycoside antibiotic) and imipenem (beta-lactam antibiotic). Nevertheless, the oil showed slightly higher activity against *S. aureus* and *S. enteritidis* compared to gentamicin, although it didn't

have activity against *P. aeruginosa,* as shown in Table 2. In addition, the oil prohibited the growth of *E. faecalis, E. coli and K. pneumonia* at a concentration of 6.25% v/v and *B. cereus*, *S. aureus* and *S. enteritidis* at 12.5, 25 and 50% v/v, respectively. Moreover, all bacteria except *P. aeruginosa* were inhibited (MIC) at 50% v/v as shown in Table 3. The minimum bactericidal concentration of essential oil was 25% v/v for *B. cereus* and *S. aureus* and 50% v/v for *S. enteritidis, E. coli and K. pneumonia*, with no bactericidal activity against *E. faecalis* and *P. aeruginosa* at 50% v/v, as shown in Table 4. Thus, the essential oil from fruits of *Z. rhetsa* with the main constituents monoterpenes exhibited antibacterial activity against both Gram positive and Gram negative bacteria, except *P. aeruginosa*. According to the previous study, Rout *et, al*. (2007) reported the four major monoterpenes of pericarp from *Z. rhetsa* were sabinene, terpinen-4-ol, beta-phellandrene and alpha-terpineol [12]. Moreover in 2012, Tangjitjaroenkun *et, al*. reported the three major monoterpenes from *Z. limonella* (Dennst.) Alston (sabinene, limonene and terpinen-4-ol) inhibited bacteria and multi-drug resistant bacteria [8]. In an animal-skin irritation studies in 2012, Dhiman *et, al*. reported that a mixture of *Curcuma longa*, *Z. limonella* and *Pogostemon heyneanus* essential oils in a 1:1:2 ratio at 5%, 10% and 20% (v/v) concentration was effective against blackflies in northeastern India and no appreciable clinical and behavioral signs were observed in an acute dermal toxicity model using rats [13].

Table 2 Antibacterial activity of the essential oil from *Z. rhetsa* by disk diffusion method

Bacteria	Zone of Inhibition (mm)							
	Concentration of essential oil (%v/v)					Gentamicin (10 µg)	Imipenem (10 µg)	DMSO
	6.25	12.5	25	50	100			
S. aureus	-	-	+	+	2.0±0.0	+	19.0±0.0	-
E. faecalis	+	+	+	1.7±0.6	1.7±0.6	6.0±0.0	11.0±0.0	-
B. cereus	-	+	+	2.3±0.6	4.0±0.0	9.0±0.0	18.0±0.0	-
S. enteritidis	-	-	+	+	2.0±0.0	+	14.0±0.0	-
E. coli	+	+	+	+	+	6.0±0.0	12.0±0.0	-
K. pneumoniae	+	+	+	+	+	6.0±0.0	11.0±0.0	-
P. aeruginosa	-	-	-	-	-	7.0±0.0	7.0±0.0	-

Value shown are mean ± SD (n=3) and represent an inhibition zone in millimeters (mm) after incubated at 37 °C for 24 hours. +; zone of inhibition <1 mm. -; no activity.

Table 3 Minimal inhibitory concentration (MIC) of the essential oil from *Z. rhetsa*

Bacteria	Concentration of essential oil (%v/v)				
	50	25	12.5	6.25	3.13
S. aureus	+	+	-	-	-
E. faecalis	+	+	+	+	-
B. cereus	+	+	+	-	-
S. enteritidis	+	-	-	-	-
E. coli	+	+	+	+	-
K. pneumoniae	+	+	+	+	-
P. aeruginosa	-	-	-	-	-

+; no growth of the organism. -; growth of the organism.

Table 4 Minimal bactericidal concentration (MBC) of the essential oil from *Z. rhetsa*

Bacteria	Concentration of essential oil (%v/v)				
	50	25	12.5	6.25	3.13
S. aureus	+	+	-	-	-
En. faecalis	-	-	-	-	-
B. cereus	+	+	-	-	-
S. Enteritidis	+	-	-	-	-
E. coli	+	-	-	-	-
K. pneumoniae	+	-	-	-	-
Ps. aeruginosa	-	-	-	-	-

+; no growth of the organism. -; growth of the organism.

Conclusion

From this study it can be concluded that the essential oil from *Z. rhetsa* fruit had antimicrobial activity against *S. aureus*, *E. faecalis, B. cereus*, *S. enteritidis*, *E. coli* and *K. pneumonia* and can potentially be used as an antibacterial supplement in the development of new therapeutic agents. Nevertheless, the limitation of this study was the presence of a complex mixture of compounds in the oil. Isolation of individual phytochemical constituents and testing them for their biological activity is likely to give fruitful results. Additional *in vivo* studies and clinical trials are needed to evaluate the potential of the oil as an anti-bacterial agent for use in food and cosmetics, and as a gastrointestinal drug and treatment for skin disease.

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Acknowledgements

The authors gratefully acknowledge University of Phayao for financial support and we would like to thanks Dr.Rodney Harris for checking English.

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Interaction of *Tiliacora triandra* (Colebr.) Diels with Conventional Digoxin in Everted Rat Intestine

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Abstract

The increasing use of herbs, which are often administered in combination with conventional drugs, may result in the potential for herb-drug interaction. *Tiliacora triandra* (Colebr.) Diels has long been used in traditional medicine, as well as in Thai cuisine, but there remains insufficient understanding of the herb-drug interaction. This study aimed to investigate the influence of aqueous extracts of *T. triandra* (TTA) on the efflux of digoxin, studied in the everted intestines of rats. Male Sprague-Dawley rats were orally administered with TTA (0.5, 1.0, and 2.0 g/kg BW) once daily, for seven days; after which, the digoxin efflux was examined in the everted intestines. Our results found that at low dosage of TTA modulated the efflux of digoxin. Administration of TTA (0.5 and 1.0 g/kg BW) significantly increased the efflux rate of digoxin to 1.16; 1.51 times higher than the control. Moreover, when the concentration of TTA was increased (2.0 g/kg BW), a significant decrease in the digoxin efflux was observed. The efflux rate was dramatically reduced to 0.34, 0.29, and 0.22 times lower than control and TTA (0.5 and 1.0 g/kg BW) treated groups, respectively. This study represents the first insight into the interaction of *T. triandra* with the conventional drug digoxin. The interaction may involve a biphasic effect, involving the induction or inhibition of the drug transporter P-gp. However, the mechanism of interaction and the interference with the pharmacokinetics of digoxin require further study.

Keywords: Herb-Drug interaction, Digoxin, *Tiliacora triandra* (Colebr.) Diels, P-glycoprotein

Introduction

The increasing uses of herbs or dietary supplements, which are often administered in combination with conventional drugs, results in the potential for herb-drug interaction. The interaction can affect either the pharmacodynamics or pharmacokinetics of other drugs. Pharmacodynamic interactions can cause alterations in the way herbal medicines affect tissue and organ systems, by producing synergistic or antagonistic activities; whereas pharmacokinetic interactions can cause alterations of the absorption, metabolism, and disposition of other drugs [1]. Available data indicates that the mechanisms involved are the induction or inhibition of metabolizing enzymes cytochrome P450 (CYP) and the drug transporter P-glycoprotein (P-gp). P-gp is a member of the ATP-binding cassette (ABC) super family; located mainly in the epithelial cells, including the apical surface of epithelial cell linings, such as the small intestine and blood brain barrier (BBB) [2]. P-gp functions as an efflux pump that actively extrudes a diverse range of substrates, including drugs. Today's established herbal medicines; such as *Hypericum perforatum* L., *Ginkgo biloba* L., *Allium sativum* L., *Camellia sinensis* L., *Zingiber officinale* Roscoe, *Piper nigrum* L. and *Tiliacora triandra* (Colebr.) [3,4], can all effectively alter P-gp activity.

Tiliacora triandra (Colebr.) Diels or *Yanang* (in Thai) is an edible plant belonging to the Menispermaceae family [5], and is used particularly in Northeastern Thai cuisine, as well as an herbal remedy in folklore medicine. *T. triandra* has been consumed as an antioxidant, antipyretic, anti-malarial, anti-inflammatory, anticancer, and as an antidote for poisoning and reducing toxicity of various toxic compounds. The commonly edible leaf shows no toxicity (5000 mg/kg), and no adverse effects, as observed in sub-chronic toxicity tests (300-1200 mg/kg) [6, 7, 8]. Screening of methanol extract found phenolics, alkaloids, flavonoids, terpenoids, saponins and cardiac glycosides [9]. Phytochemical profile showed three bisbenzylisoquinoline alkaloids; tiliacorinine, tiliacorine and nortiliacorinine and the main compound

oxoanolobine [10,11]. Despite the long time use of *T. triandra* as a dietary supplement, and as a herbal medicine with respective benefits for health, the interaction between *T. triandra* and conventional drugs, namely digoxin, which is a cardiac glycoside for heart failure treatment, have not been reported. Thus, this study investigates the influence of aqueous extracts of *T. triandra* on the efflux of digoxin, employing an everted rat intestine as a model.

Methodology

2.1. Chemicals

The digoxin was purchased from Fluka (Poland), the ethanol (95%) and the chemicals for Dulbecco's phosphate buffer were procured from BDH Prolabo (UK), and the methanol and acetonitrile were purchased from Honey Burdick & Jackson (USA). All other chemicals are analytical grade, procured from commercial suppliers.

2.2 Plant collection and extraction

Fresh leaves of *Tiliacora triandra* (Colebr.) Diels were obtained from Thailand's northeastern province of Khon Kaen. A mixture of 250g of fresh leaves were cut in to small pieces, crushed in a blender (OTTO, Thailand), and mixed with water at a ratio of 1:4 (w/v). The extract was then filtered through a straining cloth. The filtrate was collected and dried in a freeze dryer (Labogene^{TM} Denmark) in order to create aqueous extracts of *T. triandra* (TTA.), at a percentage yield of 4.24, and stored until usage at 4˚C.

2.3 Experimental animals and preparation of the everted intestines

Male Sprague-Dawley rats at eight weeks old (250-300g) were purchased form Nomura Siam International Co., Ltd., Thailand. The animals were maintained at a temperature of 25±2 °C, provided 12 hour light and dark cycles, fed with a standard laboratory animal diet, and given water *ad libitum*. Experiments were designed and conducted in accordance with the ethical norms approved by the Animal Ethics Committee of Khon Kean University, Khon Kaen, Thailand (ACUC-KKU_21/2559).

The rats were randomly divided into four groups (n=6).

Group 1, the negative control group, consisted of rats orally administered with 3 ml distilled water, once daily for seven days.

Groups 2-4, the treated groups, contained rats orally administered with TTA, in a single daily dose of 0.5, 1.0, and 2.0 g/kg BW, respectively for seven days.

The animals were observed for behavioral changes over the experimental period. After the last treatment dose, at 24 hours, the rats were anesthetized with pentobarbital (30 mg/kg, ip). The animals were then sacrificed, and a 30 cm portion of the ileum was immediately removed. The luminal contents were flushed with ice-cold normal saline, and each 10 cm long segment was inverted [12].

2.4 Efflux of digoxin

One milliliter of digoxin (36 mM) was introduced into the serosal side (inside) of the inverted sac, and incubated in 8 ml of Dulbecco's phosphate buffer pH7.4 (D-PBS), which was saturated with a 95/5% O_2/CO_2 gas at 37°C. Periodic sampling of the mucosal-side medium (0.2 ml) occurred over a period of 60 minutes and each 0.2 ml sample was substituted with an equal volume of fresh D-PBS.

2.5 Analytical method of digoxin

The mucosal-side samples were extracted with 0.4 ml of methanol, and then centrifuged at 10,000 g for 10 minutes. The supernatant containing digoxin was analyzed using high performance liquid chromatography (HPLC, Shimadzu LC-20A). The digoxin was separated as follows [13]:

Column: C18 (Hydro-RP 80A 250 x 4.5 mm)

Mobile phase: acetonitrile and DI water (40:60 %V/V)

Flow rate: 1ml/min

Injection: 20µl

Detector: UV-Visible (220nm)

Data was expressed as the mean ±SD, and the statistical significance was performed using the analysis of the variance (ANOVA).

Results

The effects of the oral administration of the aqueous extracts of *T. triandra* (0.5-2.0 mg/kg BW) were observed over a seven day period. No unanticipated deaths were recorded in either the control or treated animal groups, and no detectable changes in behavioral or physiological activities occurred within the TTA treated groups. There were no significant differences in the initial and final weights and the liver, kidney, and testis weight; in both the control and treated groups (table 1); nor did

the internal organs show any signs of abnormality, as observed with the naked eye.

Table 1. Body and organ weights of rats treated with aqueous extracts of *T. triandra* (TTA) over a period of seven days, (n=6).

Treatment	Body weight (g)		Organ weight (g)		
	Initial weight	Final weight	Liver	Kidney	Testis
Control	273.00±2.83	373.50±0.39	4.61±1.46	0.89±0.01	0.99±0.09
TTA (0.5 g/kg)	273.00±12.02	373.50±0.97	3.88±3.64	0.82±0.10	0.93±0.12
TTA (1.0 g/kg)	290.50±21.92	358.50±0.15	4.59±0.52	0.92±0.13	0.94±0.11
TTA (2.0 g/kg)	280.00±8.49	370.50±0.17	4.81±0.62	0.84±0.06	0.99±0.02

The effects of the aqueous extracts of *T. triandra* (TTA) on the efflux of the cardiac glycoside digoxin was investigated using HPLC to detect digoxin at a retention time of 4.12 minutes (Fig. 1A), 1B) in mucosal-side samples of inverted rat intestines (Fig. 1B). The results show that low dosages of TTA modulated the efflux of digoxin. Administration of TTA at 0.5 and 1.0 g/kg BW increased the efflux rate of digoxin in each inverted intestine, at times ranging from ten to 60 minutes. Significant increases were observed at TTA treatments of 1.0 g/kg BW, compared with both the control and 0.5 g/kg BW TTA treated groups, respectively. In 60 minutes of observation, the induction of digoxin efflux was 1.16 and 1.51 times higher than those of the control and 0.5 g/kg BW TTA treated groups, respectively. Conversely, when the TTA increased to 2.0 g/kg BW, the efflux of digoxin significantly decreased. At 60 minutes, the high dose TTA (2.0 g/kg BW) treated group, the efflux rate was reduced. The efflux rate was lower than the control, and the low-dose treated groups (0.5 and 1.0 g/kg BW) by 0.34, 0.29, and 0.22 time respectively. The results demonstrated the interaction of TTA with the conventional drug digoxin in inverted rat intestines.

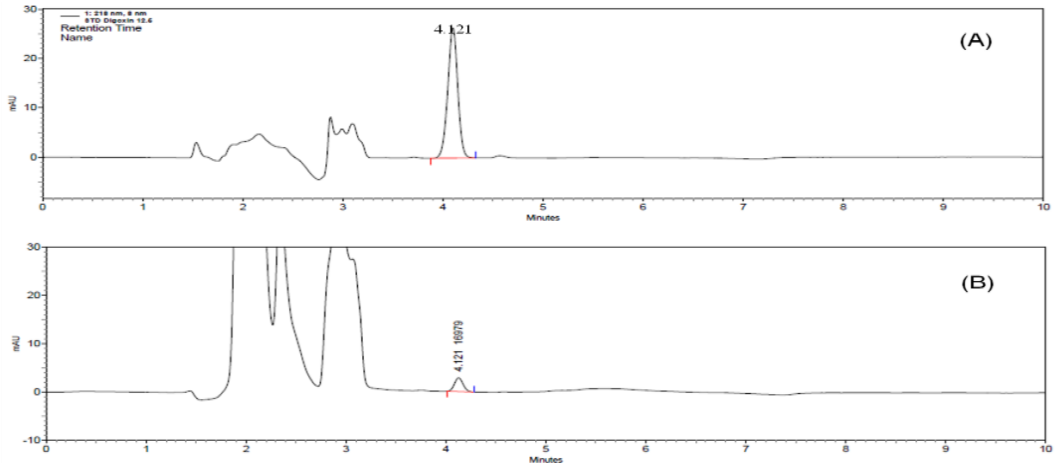

Figure 1. Chromatograms of the standard digoxin (A) and digoxin efflux from everted intestines under the described conditions.

Table 2. Effects of *Tiliacora triandra* aqueous extracts (TTA) on digoxin efflux in everted intestines.

Time (minutes)	Digoxin efflux (mM/cm^2)			
	Control	TTA 0.5 g/kg BW	TTA 1.0 g/kg BW	TTA 2.0 g/kg BW
0	5.46±3.25	7.86±1.02^{a}	8.72±1.04^{a}	0.87±0.07^{abc}
10	7.55±1.29	8.48±0.73	10.59±1.57^{ab}	0.92±0.08^{abc}
20	7.03±0.69	8.40±0.78^{a}	10.57±1.74^{ab}	1.03±0.05^{abc}
30	7.61±0.73	8.43±1.00	10.61±1.26^{ab}	1.36±0.16^{abc}
40	7.26±0.29	8.30±1.01	10.64±0.95^{ab}	1.65±0.20^{abc}

Time (minutes)	Digoxin efflux (mM/cm^2)			
	Control	TTA 0.5 g/kg BW	TTA 1.0 g/kg BW	TTA 2.0 g/kg BW
50	7.73±0.34	8.47±0.77	11.11±1.17^{ab}	2.04±0.24^{abc}
60	7.29±0.30	8.46±0.75^{a}	11.01±0.76^{ab}	2.46±0.49^{abc}

$^{a, b, c}$ Significant difference (P<0.05) in the control, TTA (0.5 g/kg BW), and TTA (1.0 g/kg BW) in each time of treatment, respectively.

Conclusion and discussion

There is a widespread public perception in Thailand that long term dietary and herbal remedies of botanical ingredients, such as *Yanang* are a safe form of alternative medicine. Unlike conventional drugs, natural botanical products provide a complex mixture of bioactive constituents, which may or may not provide therapeutic benefits. Further benefits may be found through the incorporation of drug interaction, such as with digoxin. Digoxin is a cardiac glycoside prescribed for heart failure treatment, and may also be used for atrial fibrillation to control ventricular rates. However, toxicity may develop over extended dosages during long-term therapy. Due to their narrow therapeutic index, any slight changes in the alteration of serum concentrations of digoxin may cause serious side effects in chronically ill patients; such as anorexia, vomiting, neurological symptoms, and may trigger fatal arrhythmias [14,15].

This study presents the effects of the interaction of *Tiliacora triandra* (Colebr.) Diels (TTA) with the conventional drug digoxin, in which the biphasic effects were observed. At low doses, TTA (0.5 and 1.0 g/kg BW) acted as an inducer, which modulated the efflux of the digoxin. At a higher concentration of TTA (2.0 g/kg BW), it acted as an inhibitor in inverted rat intestines. Studies of volunteer subjects found that a co-administration of a *Hypericum perforatum* extract decreased digoxin AUC (0-24) levels, as well as the drug bioavailability; and that the mechanism involved may be an induction of P-gp [16]. Concordance with intestinal absorption, and the distribution and excretion mediated by the multiple-drug-resistance gene (MDR1), produced by P-glycoprotein (P-gp) and other ABC transporters, are widely accepted as the reasons for low or variable oral absorption of digoxin [17]. Several herbal constituents, such as flavonoids, have been reported to effectively modulate P-gp activity by directly interacting with the ATP-binding site, or the substrate binding site. Moreover, some herbs were activated as a key regulator of MDR1 gene; pregnane X-receptor, or orphan receptors. We therefore concluded that TTA containing flavone may successfully interact with conventional drugs due to its biphasic effect, or the induction or inhibition of the drug transport P-gp. This interaction may further alter the pharmacokinetics of the drug, and increase the risk of toxicity. Conversely, the over-expression of P-gp in cancer cells is a reason for the extruding of chemotherapeutic agents and the development of transporter mediated resistance [2]. TTA may prove to be an effective therapy to overcome the difficulty associated with the P-gp and the co-administration of chemotherapeutic agents and the P-gp inhibitor, however, the action mechanism requires further study.

Acknowledgements

Graduate School, KhonKaen University, KhonKaen, Thailand is acknowledged for grant support.

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Effects of Auricular Acupressure and Moxibustion on Pain Relief in Primary Dysmenorrhea

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Abstract

Dysmenorrhea is a common gynecologic disorder. In Chinese medicine acupuncture and moxibustion are the standard treatments for dysmenorrhea. As the smoke produced by moxibustion may be harmful the human body, other approaches such as auricular acupressure have been considered . This study compared the effects of auricular acupressure and moxibustion on the reduction of pain in primary dysmenorrhea. 61 female students with the diagnosis of primary dysmenorrhea were rated for their dysmenorrhea severity by visual analogue scale (VAS) immediately prior to entry into the study. Patients were randomly divided into two treatment groups to receive either auricular acupressure (experimental group, n=31) or moxibustion (control group, n=30), which was commenced at the next menstrual cycle. Auricular acupressure was applied to Uterus, Liver and Shenmen point for 7 days before menstruation and moxibustion was applied to Guanyuan (GV4) for 15 min, once daily for 7 days before menstruation. After treatment VAS score was rated again during menstruation in both groups. There was no statistically significant difference in VAS score between two groups (P-value=0.590) before treatment. After one menstrual cycle treatment, VAS score were significantly decreased in both groups (P-value <0.001). Mean pain scores decreased by 59.5% and 44.1% in auricular acupressure group and moxibustion group respectively. Between two groups there was no statistically significant difference in the VAS score (P-value=0.399) after treatment. Auricular acupressure was as effective as moxibustion for pain relief in patients with primary dysmenorrhea. We recommend auricular acupressure as an effective alternative treatment to moxibustion for primary dysmenorrhea.

Keywords: acupressure, moxibustion, primary dysmenorrheal

Introduction

Primary dysmenorrhea is painful menstruation without pathology. This type is widely prevalent among young women and is highest in the 20 to 24 year old age group. Primary dysmenorrhea often occurs after having a period or 1-2 hours before and the duration of suffering is approximately 48-72 hours. There is some evidence that an increase or imbalance in prostaglandins can lead to primary dysmenorrhea. Thus, non-steroidal anti-inflammatory drugs (NSAIDs) are the first-line treatment option[1]. Nevertheless, these drugs have some side effects such as gastrointestinal problems and cutaneous adverse drug reactions. Therefore, alternative treatments such as acupuncture and moxibustion have been proposed to reduce dysmenorrhea. Scientific studies find acupuncture can effectively increase the number and diameter of uterine microvessels and capillaries in PD rats, which may contribute to its effect in relieving dysmenorrhea by improving uterine microcirculation[2]. Besides acupuncture, the standard treatment for dysmenorrhea in Chinese medicine is moxibustion[3], in which the hotness from moxibustion can decrease prostaglandins, blood viscosity, and erythrocytic electrophoresis time so it can increase blood circulation to the pelvic area and uterus[4]. A previous study found the isolated herbal moxibustion on Shenque point (CV 8=umbilicus) is more effective than oral medication of analgesic for primary dysmenorrhea [5]. But there is still debate on the safety of moxa smoke. Some reports have shown that moxa smoke may be harmful to the human body, such as causing allergic reactions. The mugwort leaf contains terpenes; it may produce polycyclic aromatic carcinogens in the process of combustion[6]. To avoid the controversy of the smoke effects of moxibustion. other treatments for dysmenorrhea therefore need to be explored. A recent study found that alternative medicines such as aromatherapy, auricular acupressure, electrotherapy and herbal therapy are effective [7][8][9].

Auricular acupressure or AA has been applied for pain relief, epilepsy, anxiety, and obesity[7]. A study of ear taping by adhesive plaster pressed with Vaccaria

seeds in primary dysmenorrhea patients found that the treatment had a better effective rate than the western medication group [8]. AA therefore appeared to be an attractive alternative to moxibustion in primary dysmenorrhea. As there was no previous study which compared the efficacy between AA and moxibustion, this study therefore had the objective of studying and comparing the efficacy of both methods.

Methods

An experimental, randomized, controlled clinical trial.

The instruments for data collection

1. Screening form for participant's case record including symptoms and signs.
2. The Visual Analogue Scale (VAS) for pain assessment using a 10 cm line representing the continuum of the participant's opinion towards the degree of pain. The most right end of the line represents "unbearable pain" and the most left end represents "no pain at all". The participants were asked to rate the degree of pain by making a mark on the line. The scores 0-3 points were classified as mild dysmenorrheal, 3.1-6 points moderate, and 6.1-10 points severe. Patients were evaluated by the same physician (not blinded) for VAS before and after treatment.

Subjects

Female patients diagnosed with primary dysmenorrhea. Inclusion criteria were: (1) women aged between 17-25 years old. (2) Patients having history of regular menstrual cycles. (3) Patients were diagnosed with primary dysmenorrhea by a gynecologist based on the patient's history, physical and gynecological examination. Exclusion criteria were: (1) participation in other clinical trials. (2) Patients were sensitive to herbal therapy. Discontinuation criteria were: (1) Patients could not tolerate to the smell of moxa. (2) Patients required withdrawing herself (3) Patients could not follow the protocol. The study was approved by the School of Health Science ethical committee (Mae Fah Luang University). The ethical number was 1/2557.

Intervention

Sixty one patients with a diagnosis of primary dysmenorrhea were recruited for the study. Their dysmenorrhea severity was rated by visual analogue scale (VAS) immediately prior to entry into the study. Patients were numbered in order of admittance to our out-patient clinic and randomly separated in two groups: auricular acupressure, and moxibustion. On their following menstrual cycle thirty one patients were given auricular acupressure treatment and thirty patients were given moxibustion treatment according to the randomly assignation.

After the first day of treatment eight patients in moxibustion group required to withdraw due to they could not tolerate with the smell of moxa. So there were 31 patients in AA group (experiment group, n=31) and 22 patients in moxibustion group (control group, n=22). After treatment VAS score was rated again during menstruation in both groups. In auricular acupressure group, the acupuncturist used ear seeds tape and press over the auricular points of Uterus, Shenmen, Liver. The patients have to press four times a day, each point press lasting for 1 minute. In moxibustion group, the acupuncturist used warmed moxa stick at Guanyuan(GV4), located 3 inch below the umbilicus, lasting for 15 minutes. Both groups received the treatment every day for 7 days until the first day of the period.

Outcome Assessment

Statistical Analysis

Descriptive statistics were given as mean ± standard deviation (SD). Paired sample t test was used in comparing parameters of before and after treatment. Differences between the groups were investigated using the independent sample t test. The level of significance was set at P-value<0.05.

Results

The procedure in auricular acupressure group was well tolerated by all patients. In moxibustion group, 8 patients could not tolerate to the smell of the moxa. None of the subjects in both groups declared adverse events or complications of applications. Demographic characteristics of patients in auricular acupuncture and moxibustion groups were compared and are shown in Tablem1.

Table 1 Patients'demographics characteristics in auricular acupressure and moxibustion group.

Characteristics	AA (N = 31) Mean± SD	Moxibustion (N=22) Mean± SD	P-value
Age (years)	19.95±1.11	20.24±1.05	0.301
Menarche (Age in year)	12.46±1.20	12.44±0.97	0.297
Age of menstrual pain (Age in year)	13.71±1.86	13.74±1.40	0.414
Duration of mense (days)	4.20±0.95	4.25±1.20	0.461

There was no statistically significant difference in VAS scores between the two groups (P-value=0.590) before treatment. After one menstrual cycle treatment, VAS scores were significantly decreased in both groups (P-value=0.05). Mean pain scores decreased by 59.5% and 44.1% in the auricular acupressure group and moxibustion group respectively. Between two groups, there was no statistically significant difference in the VAS score (P-value=0.399) after treatment [Table 2].

Variable	AA Mean± SD	Moxibustion Mean± SD	P-value†
VAS (before)	7.81±1.78	7.41±1.59	0.590
VAS (after)	3.16±2.00	4.14±2.12	0.399
P-value††	<.001*	<.001*	

*Significant at p-value <0.05

†Pair-t-test

†† Independent t-test

Table 2 Comparison of pain assessment by VAS between auricular acupressure and moxibustion group at before and after treatment.

Discussion

The present study's findings indicate immediate improvement in pain outcome measures by using VAS after both auricular acupressure and moxibustion application. One menstrual cycle after treatment, measurements were not significantly different between the two groups. The inhibition of the pain with auricular acupressure maybe due to the mechanism of AA's close relationship with the autonomic nervous system [7]. Moxibustion effect is most likely due to increased circulation in the uterus [4]. This study indicated that the auricular acupressure was as effective as the moxibustion in pain relief. VAS scores were significantly improved in both groups after treatment. The effectiveness of auricular acupressure and moxibustion given for one cycle of menstruation, as demonstrated in our study, was lower than results of previous studies which treated for 3 cycles of menstruation[5][8]. Their higher number of given treatment is likely to be the reason of a superior outcome to ours.

One advantage of auricular acupressure treatment is that it produces far fewer adverse side-effects compared with moxibustion. Moxibustion treatments were not well tolerated by patients because the smoke. The present study does have some limitations namely: treatment was for only one period of menstruation, relatively small number of patients, lack of longer term follow-up, lack of observation of the menstruation characteristics in both groups and not being a double-blind design. Small patient group may cause a type 2 statistical error. Additionally, a potential bias could have ensued due to the fact that the physician who performed the assessments was not blinded. Future studies are being planned to take into account these weaknesses. Nevertheless our results are noteworthy in that they confirmed that both auricular acupressure and moxibustion significantly relieved the pain of primary dysmenorrhea in young Thai women. Auricular acupressure was advantageous above moxibustion due to its ease of use, tolerability, lack of side effects, and because patients could apply the treatment themselves.

Acknowledgements

This work was supported by Mae Fah Luang University Thailand.

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Screening of Antibacterial Activities of 53 Thai Herbal Extracts Against Acne-Involved Bacteria

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Abstract

Acne vulgaris is a common chronic skin disease caused by blockage and/or inflammation of the pilosebaceous unit. Bacterial hypercolonization is involved in the pathogenesis of inflammatory acnes. This study aimed to evaluate *in vitro* antibacterial activities of 53 ethanolic herbal extracts against acne-involved bacteria. The activities were preliminarily investigated by disc diffusion method. Then the herbal extracts that exhibited large inhibition zones were further investigated for their minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) values by broth dilution method. The results showed that 19 out of 53 tested extracts inhibited the growth of *Propionibacterium acnes*, of which 8 extracts showed inhibition zone greater than or equal to 10 mm and could also inhibit the growth of *Staphylococcus aureus* and *Staphylococcus epidermidis*. Further investigations showed that *Phyllanthus emblica* and *Piper betle* leaf extracts exhibited the strongest activity with MIC values of 1 mg/ml against *P. acne* and more than 1 mg/ml against *S. aureus* and *S. epidermidis*. The MBC values against all bacterial species of both herbal extracts were more than 1 mg/ml. These results suggest that *P. emblica* and *P. betle* leaf extracts are potential natural sources for the development of acne control products.

Keywords: Antibacterial activity, Acne, Herbal extracts, *Phyllanthus emblica*, *Piper betle*

Introduction

Acne vulgaris is a chronic skin disease that most commonly occurs in teenagers [1]. It is caused by blockage and/or inflammation of the pilosebaceous unit consisting of the sebaceous glands and the hair follicles. Thus the areas susceptible to acne are the areas with high concentrations of sebaceous glands such as face, chest, upper back, and upper arms. Acne vulgaris can be divided into non-inflammatory acne and inflammatory acne. Non-inflammatory acne involves excess sebum production by sebaceous glands mediated by androgen hormones and follicular hyperkeratinization, resulting in the formation of comedones [1, 2]. The blockage of the pilosebaceous unit leads to inflammation and bacterial hypercolonization [1, 2]. Inflammatory acne lesions include papules, pustules and nodules which often leave scars and hyperpigmentation [3]. In addition, acne can be the cause of psychosocial issues and can affect the quality of life [4]. Skin microbiota isolated from subjects with acne that are believed to be related to acne pathogenesis are *Propionibacterium acnes*, *Staphylococcus aureus* and *Staphylococcus epidermidis* [5, 6]. *P. acne*s plays an important role in the progression of inflammatory acne. It secretes lipases that digest sebum triglycerides into free fatty acids. These free fatty acids and *P. acnes* itself induce inflammatory responses. These responses are the causes of follicle wall rupture. Moreover, *P. acnes* can also promote follicular hyperkeratinization by inducing integrin and filaggrin [3]. It is also able to form biofilms, which improve adherence to the follicle and increase its resistance to antibiotics [3]. *S. aureus* plays a prominent role in folliculitis and is found in acne lesions [5]. It can produce several enzymes that invade deeper skin tissue such as proteases, lipases, hyaluronidases and collagenases [5]. *S. epidermidis* is a human commensal bacterial strain which has been reported to counteract the infection of *S. aureus* by secreting a serine protease to inhibit the colonization of *S. aureus* [7]. However, its influence on the growth of *P. acnes* is still controversial [8, 9]. *S. epidermidis* can also become an infectious agent in some conditions such as immune system deficiency [5].

Herbal medicines have long been used in various countries including Thailand for the treatment of various symptoms and diseases as well as for health promotion. In the cosmetic field, the use of herbal medicines is rapidly gaining interest and is a modern trend in beauty products [10]. Thai traditional medicine teaches that skin disease and abscess are caused by an imbalance of four elements, i.e. earth, water, air and fire,

which are the building blocks of life. The imbalance may be caused by exogenous and/or endogenous factors, such as climate, environment, pollution, age and food. In general, there are a lot of medicinal “tastes” that act to heal various diseases based on this imbalance of the elements. In this study, 53 Thai herbs were chosen for their cool, abashed (maobua), sour, and astringent tastes, which have properties of counteracting fire (or heat), counteracting poison/toxin, cleansing and wound healing, respectively. Herbal ingredients in Thai medicine recipes used to treat abscesses and other skin diseases, were also included [11-26]. In addition, Thai herbs reported to have antibacterial activities against other bacteria [27-31] were investigated for their effects on *P. acnes*, *S. aureus* and *S. epidermidis*. The *in vitro* antibacterial activities of the 53 herbal extracts against acne-involved bacteria were evaluated. The scientific names of these 53 Thai herbs and their parts used are shown in Table 1.

Methodology

1. Preparation of extracts

Plant materials were collected from four different locations in Thailand including Amphoe Mueang and Amphoe Bang-Krathum of Phitsanulok Province; Chao Krom Poe Dispensary in Bangkok, Chaophraya Abhaibhubejhr Hospital in Prachin Buri, and The Queen Sirikit Department of Sericulture in Phrae. These plants were confirmed and kept as herbarium specimens at Phitsanulok Naresuan University (PNU) Herbarium, Department of Biology, Faculty of Science, Naresuan University. Dried powders of plant materials were macerated with 95% ethanolic solution with shaking for 2 days. The maceration was repeated two times. The maceration extracts were combined, filtered and evaporated by using vacuum rotary evaporator (Buchi Ltd., Bangkok Thailand). The extracts were further evaporated to dryness on a water bath (M25 Lauda, Deutschland, Germany). Percentage yield was calculated by the following equation.

$$\text{Percentage yield} = \frac{\text{Weight of extract}}{\text{Weight of dried powdered plant}} \times 100$$

2. Anti-bacterial activity assay of extracts

P. acnes DMST 14916, *S. aureus* ATCC 25923 and *S. epidermidis* DMST 518 were used in this study. The study was approved by Naresuan University Institutional Biosafety Committee (NUIBC). The inocula of tested organisms were prepared by direct colony suspension method. Then bacterial suspensions were prepared in saline solution (0.85% NaCl). To prepare the inoculum of approximately $1x10^8$ CFU/ml, the turbidity of *S. epidermidis* and *S. aureus* suspensions were adjusted to obtained turbidity (OD_{600}) in the range of 0.08-0.10 by using spectrophotometer (BioTek Instruments, USA), while the density of *P. acnes* suspension was adjusted to 0.5 McFarland standard turbidity.

2.1 Screening of anti-bacterial activities of extracts by disc diffusion method

The sterile paper discs, 6 mm in diameter, were prepared by absorbing with 20 µl of each tested extract (100 mg/ml in ethanol) and then the disc was dried at 50 °C for 2 h. The standardized suspensions of *S. aureus*, and *S. epidermidis* were spread on the surface of Mueller Hinton agar plates by using sterile cotton swabs. Then the sample discs were placed on the agar plates. Gentamicin (10 µg/disc) was used as a positive control. Solvent control disc (i.e. disc impregnated with ethanol) was also included. The plates were incubated at 37 ºC for 24 h under aerobic condition. The standardized suspension of *P. acnes* was spread on the surface of Brain Heart Infusion agar plates. Clindamycin (2 µg/disc) was used as a positive control. The plates were incubated at 37 ºC for 72 h under anaerobic conditions. Antibacterial activity was assessed by measuring the diameter of the inhibition zone (mm) using a vernier caliper. The experimenst were performed in triplicate. The extracts which showed inhibition zone against *P. acne* greater than or equal to 10 mm and could also inhibit the growth of *S. aureus* and *S. epidermidis* were further investigated for minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) by broth dilution method.

2.2 Determination of minimal inhibitory concentrations and minimal bactericidal concentrations

The MIC values of the extracts against *S. aureus* and *S. epidermidis* were determined by broth micro-dilution method, and against *P. acnes* by broth macro-dilution method. A stock solution of each extract (1 mg/ml) was prepared by dissolving an extract in a co-solvent, which composed of 95% ethanol, methanol,

tween 80 and broth medium in ratio 1:2:1:96 %v/v. Then the stock solution was further diluted with broth medium to cover the concentration range between 0.25 – 1 mg/ml. The suspensions of *S. aureus, S. epidermidis*, and *P. acnes* were prepared as described previously and then diluted with Mueller-Hinton broth for *S. aureus* and *S. epidermidis* and with Brain Heart Infusion broth for *P. acnes* to obtain density at approximately 1 x 10^6 CFU/ml. The bacterial suspensions of *S. aureus* and *S. epidermidis* (10 µl) were inoculated into 100 µl of the sample solution in a 96-well microplate while the bacterial suspension of *P. acne* (70 µl) was inoculated into 700 µl of the sample solution in a test tube. In these experiments, the positive growth control wells/tubes consisted of bacterial inoculum in broth medium, while the solvent control sample wells/tubes consisted of bacterial inoculum in broth medium and the co-solvent. The negative growth control wells/tubes contained broth medium only. Microplates were incubated at 37 ºC for 24 h under aerobic conditions for *S. aureus* and *S. epidermidis* and tubes were incubated at 37 °C for 72 h under anaerobic conditions for *P. acnes*. Turbidity of the suspension was measured at OD_{600} before and after incubation. The MIC was defined as the lowest concentration that exhibited greater than or equal to 90% growth inhibition. Then, the MBC values were determined by transfer of the suspensions to nutrient agar plates for *S. aureus* and *S. epidermidis* or Brain Heart Infusion agar plates for *P. acnes*. The MBC was defined as the lowest concentration that exhibited no bacterial colonies after incubation. The experiments were performed in triplicate.

Results

1. Preparation of plant extracts

The percentage yield of the 53 Thai herbal extracts was 0.99-35.19% (Table 1). *Coccinia grandis* leaf extract and *Morus alba* (Cultivar Nakhon Ratchasima 60) wood extract showed the highest and the lowest percentage yield, respectively.

2. Antibacterial activities assay of extracts

2.1 Screening of antibacterial activities of extracts by disc diffusion method

Firstly, the extracts were screened for their antibacterial activities by disc diffusion method. The results showed that 19, 19 and 12 extracts of the 53 tested extracts exhibited inhibition zones against *P. acnes*, *S. aureus* and *S. epidermidis*, respectively (Table 1). Among these, 8 extracts showed inhibition zone greater than or equal to 10 mm against *P. acnes* and also inhibited growth of *S. aureus* and *S. epidermidis* (Table 1). These extracts were *Blumea balsamifera* leaf extract, *Boesenbergia rotunda* rhizome extract, *M. alba* (cultivar Nakhon Ratchasima 60) wood extract, *M. alba* (cultivar Chiang Mai 60) wood extract, *M. alba* (cultivar Khun-phi) wood extract, *M. alba* (cultivar Sakon Nakhon) wood extract, *Phyllanthus emblica* leaf extract and *Piper. betle* leaf extract. These plant extracts were further investigated for MIC and MBC values using broth dilution method.

2.2 Investigation of minimal inhibitory concentrations and minimal bactericidal concentrations

The MIC values of the extracts against *S. aureus* and *S. epidermidis* were determined by broth microdilution method and against *P. acnes* by broth macro-dilution method. The co-solvent used in this study was composed of 95% ethanol, methanol, tween 80 and medium broth in ratio 1:2:1:96 %v/v. After incubation, the solvent control sample showed the same turbidity as the positive growth control sample, suggesting that the co-solvent had no effect on the growth of bacterial cells. Most of the extracts exhibited less than 90% growth inhibition at the highest concentration tested, suggesting these extracts had MICs and MBCs more than 1 mg/ml (Table 2). Only *P. emblica* leaf extract and *P. betle* leaf extract exhibited more than 90% growth inhibition of *P. acnes,* indicating that their MICs against *P. acnes* were 1 mg/ml (Table 2).

Table 1 Percentage yields and Zone of inhibition against *P. acnes*, *S. aureus* and *S. epidermidis* of fifty three extracts.

Scientific name	Part used	% yield	Zone of inhibition (mm)		
			P. acnes	*S. aureus*	*S. epidermidis*
Acacia concinna	leaf	1.36	6.33±0.29	-	-
Acanthus ebracteatus	leaf	13.67	-	-	-
Alpinia siamensis	leaf	10.96	-	-	-
Annona squamosal	leaf	5.97	-	-	-
Aquilaria malaccensis	leaf	4.08	6.33±0.58	-	-
Averrhoa bilimbi	fruit	22.74	-	-	-
Averrhoa bilimbi	leaf	14.39	-	-	-
Averrhoa carambola	leaf	9.93	-	-	-
Azadirachta indica	leaf	7.52	9.50±1.32	-	-
Barleria lupulina	leaf	13.57	-	-	-
Bauhinia acuminate	leaf	8.98	-	7.40±0.32	7.12±0.71
Blumea balsamifera	leaf	3.01	9.67±0.58	8.05±1.22	9.08±0.66
Boesenbergia rotunda	rhizome	5.70	11.00±1.00	8.34±0.16	7.76±0.44
Bridelia ovate	leaf	2.43	6.17±0.29	7.06±0.16	-
Butea monosperma	leaf	4.40	-	-	-
Carissa carandas	leaf	13.08	-	-	-
Citrus aurantifolia	leaf	4.85	7.00±0.50	-	-
Citrus hystrix	leaf	5.05	13.33±1.53	7.99±0.88	-
Citrus maxima	leaf	2.99	6.67±0.29	-	-
Cleome viscosa	aerial part	1.58	10.67±1.53	6.17±0.29	-
Clerodendrum petasites	leaf	11.92	-	-	-
Clinacanthus nutans	leaf	11.30	-	-	-
Coccinia grandis	fruit	4.90	-	-	-
Coccinia grandis	leaf	35.19	-	-	-
Garcinia schomburgkiana	leaf	8.84	6.67±0.29	7.22±0.44	-
Houttuynia cordata	leaf	6.17	-	-	-
Justicia fragilis	leaf	7.32	-	-	-
Kalanchoe pinnata	leaf	1.65	-	-	-
Lawsonia inermis	leaf	17.33	-	8.62±0.55	-
Lindenbergia philippensis	leaf	21.79	-	-	-
Morinda citrifolia	fruit	6.64	-	-	-
Morus alba (Cultivar Nakhon Ratchasima 60)	twig wood	0.99	12.50±1.80	9.40±0.26	10.27±0.64
Morus alba (Cultivar Buriram 60)	twig wood	1.43	8.83±0.29	7.46±0.47	7.29±0.42
Morus alba (Cultivar Chiang Mai 60)	twig wood	1.30	10.67±1.15	8.97±0.64	8.43±0.83
Morus alba (Cultivar Khun-phi)	twig wood	1.14	11.00±1.73	7.43±0.45	8.15±0.88
Morus alba (Cultivar Sakon Nakhon)	twig wood	1.12	18.67±1.15	10.62±0.36	10.72±0.70
Ocimum sanctum	leaf	5.61	-	-	-
Oxystelma Esculentum	leaf	3.23	-	-	-
Phyllanthus acidus	leaf	3.11	-	-	-
Phyllanthus emblica	fruit	24.66	-	6.33±0.58	-
Phyllanthus emblica	leaf	13.59	17.67±0.58	9.57±0.47	7.45±0.68
Phyllanthus urinaria	leaf	2.91	8.33±0.58	-	-
Piper betle	leaf	6.96	11.50±1.32	14.72±1.20	11.98±0.45
Rhinacanthus nasutus	leaf	11.22	-	6.62±0.54	-
Senna alata	flower	11.67	-	10.22±3.66	11.93±0.39

Note: - = non-activity

Table 1 Percentage yields and Zone of inhibition against *P. acnes*, *S. aureus* and *S. epidermidis* of fifty-three extracts.

Scientific name	Part used	% yield	Zone of inhibition (mm)		
			P. acnes	*S. aureus*	*S. epidermidis*
Senna alata	leaf	7.68	-	6.19±0.32	8.49±1.60
Senna siamea	leaf	22.43	-	-	-
Sesbania grandiflora	flower	22.87	-	-	-
Thunbergia laurifolia	leaf	3.92	-	-	-
Tiliacora triandra	leaf	5.80	-	-	-
Tinospora crispa	vine	9.22	-	-	-
Tradescantia spathacea	leaf	6.90	-	-	-
Vitex trifolia	leaf	5.33	-	-	-
Gentamicin (10 µg/disc)			ND	26.72±0.73	8.70±0.37
Clindamycin (2 µg/disc)			48.10±0.21	ND	ND
95% Ethanol (20 µg/disc)			-	-	-

Note: - = non-activity, ND = not determined

Table 2 Minimal Inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of the extracts against *P. acnes S. aureus* and *S. epidermidis*.

Extracts	MIC (mg/ml)			MBC (mg/ml)		
	P.acnes	*S.aureus*	*S.epidermidis*	*P.acnes*	*S.aureus*	*S.epidermidis*
Blumea balsamifera, leaf	>1	>1	>1	>1	>1	>1
Boesenbergia rotunda, rhizome	>1	>1	>1	>1	>1	>1
Morus alba, twig wood (Cultivar Nakhon Ratchasima 60)	>1	>1	>1	>1	>1	>1
Morus alba, twig wood (Cultivar Chiang Mai 60)	>1	>1	>1	>1	>1	>1
Morus alba, twig wood (Cultivar Khun-phi)	>1	>1	>1	>1	>1	>1
Morus alba, twig wood (Cultivar Sakon Nakhon)	>1	>1	>1	>1	>1	>1
Phyllanthus emblica, leaf	1	>1	>1	>1	>1	>1
Piper betle, leaf	1	>1	>1	>1	>1	>1

Discussion and conclusion

The screening of antibacterial activity of 53 Thai herbal ethanolic extracts showed that *P. emblica* and *P. betle* leaf extracts exhibited the strongest antibacterial activity against *P. acnes*, *S. aureus* and *S. epidermidis*. *P. emblica* is known as “Makhampom”. Its main use in cosmetics are from the fruit parts and leaves are rarely used. However, in Thai traditional medicine, *P. emblica* leaves are boiled in water and the decoction is used in a bath for the treatment of fever, itching, rash, pustule and wounds [15, 22]. From the literature search, the antibacterial activities of *P. emblica* leaf extracts against *P. acnes*, *S. aureus* and *S. epidermidis* have been previously reported by Niyomkam et al. [32]. The *P. emblica* leaf extracts in the study of Niyomkam et al. [32] were prepared using ethyl acetate under reflux conditions. The resulting marc was further extracted using methanol under reflux conditions. Both of the ethyl acetate and methanol extracts showed antibacterial activity with MIC values between 0.3 – 2.5 mg/ml. In general, most herbs used in human treatment are prepared by decoction and thus water is the best solvent for herbal extraction. However, water can extract only hydrophilic compound from the plant materials. Ethanol is an alternative solvent which is known to be a good solvent for polyphenol extraction and safe for

consumption [33]. Our study prepared *P. emblica* leaf extract using ethanol 95% as the extraction solvent by simple maceration technique. The ethanolic *P. emblica* leaf extract still showed good antibacterial activity against *P. acnes*, *S. aureus* and *S. epidermidis* (Table 1 and 2).

P. betle is called "Phlu" in Thai. In Thai traditional medicine, leaves of *P. betle* are mashed and alcohol is added to make a paste before applying to affected areas for treatment of swelling, bruise, urticaria, ringworm and athlete's foot [15]. Previously, Wardhani et al. [34] prepared *P. betle* leaf extract using 70% ethanol as the extraction solvent and investigated antibacterial activity against *P. acnes*. They reported the MIC and MBC values of 25 and 50 mg/ml. Recently, the antibacterial activities of *P. betle* l e a f ethanolic extract have also been reported by Taukoorah et al. [35]. The MIC values of *P. betle* l e a f ethanolic extract against *P. acnes* and *S. aureus* were 4 and 1 mg/ml and no inhibition of *S. epidermidis* was observed. However, the MBC value was not reported. The current study confirmed the antibacterial activity of *P. betle* l e a f ethanolic extract against *P. acnes*.

The phytochemical constituents of ethanolic extract of *P. betle* leaves have been reported to be flavonoids, tannins, saponins, phenols, glycosides, alkaloids, steroids, terpenoids, proteins and carbohydrates [36]. For ethanol extract of *P. emblica* leaves, it was reported to contain alkaloids, simple phenolics, tannins, lignin, saponins, flavonoids, vitamin C, carbohydrates, glyceroids, oil and fats [37, 38]. The phenols [38], phenolic compounds [39], polyphenolic compounds, flavonoids, saponins [34] and sterols [40] are all known to have antibacterial activity. The mechanism of phenolic compounds antibacterial activity involves in the promotion of K^+, H^+ across bacteria membrane that affects proton motive force and inhibits ATP synthesis [39]. Polyphenolic compounds, flavonoids and saponins cause damage to bacterial cell membrane [34]. On the other hand, sterols disrupt the cell wall, affecting permeability [40]. Moreover, both *P. emblica* and *P. betle* leaf extracts have also been reported to possesses antioxidant and anti-inflammatory activities which should affect their anti-acne properties [41, 42, 43]. Therefore, *P. emblica* and *P. betle* leaf extracts are suitable for developing as anti-acne formulations.

Acknowledgements

The financial support from Thai Traditional Medical Knowledge Fund, Department for Development of Thai Traditional and Alternative Medicine, Ministry of Public Health, Thailand

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Development of Plastic Hollow Microneedles

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Abstract

Microneedles are currently attracting great attention because they can deliver drug without pain, unlike typical hypodermic needles. Hollow microneedles are of particular interest since they can deliver a large dose of liquid formulations. Furthermore such devices are safe for self-administration. However, hollow microneedles suffer from complicated shapes as well as difficult fabrication, preventing their commercialization. Here, we describe the development of hollow microneedles which consist of two components, microneedle patch and cap, which were specifically designed for simple assembly and easy drug incorporation. For the fabrication process, a novel polymer mold was introduced enabling us to fabricate the microneedle patch by using single-step injection molding. The mechanical properties of the fabricated hollow microneedles were characterized in failure test, and penetration test. The results revealed very good mechanical properties of the fabricated hollow microneedles making them a promising candidate for commercial applications.

Keywords: Microneedles, Hollow Microneedles, Injection Molding, Drug Delivery.

Introduction

Injection is a highly effective method for drug delivery into the body. However, some people suffer from pain associated with the injection. To circumvent this issue, microneedles have recently been developed [1-3]. Microneedles can not only deliver drugs, vaccines and hormones into the body without pain, but are also safe for self-injection and disposal. Among several types of microneedles, hollow microneedles have several advantages since they can deliver a large dose of liquid formulations resulting in a faster absorption of the drugs. Until now, hollow microneedles have been developed using a variety of materials such as silicon, metals and plastics [4-6]. Plastic hollow microneedles have good mechanical properties since they are not brittle and the fabrication process is relatively easy. However, the fabrication of plastic hollow microneedles requires several processes such as injection molding and laser machining resulting in higher cost. Furthermore, the injection mold is not easy to machine due to the complicated geometry of the hollow microneedles. Therefore, this work aimed to develop plastic hollow microneedles which can be easily fabricated using a novel polymer mold and thus improve the commercialization of hollow microneedles for a wide range of applications.

Methodology

1. Design of Hollow Microneedles

Our proposed hollow microneedles consist of two components: microneedle patch and cap. The microneedle patch is comprised of a number of microneedles with a hole through each microneedle. The cap is designed to have a wing structure where it locks into the microneedle patch. Furthermore, the diaphragm (flat part) of the cap is designed to deflect upon application of a certain force. This structure is used to generate pressure for releasing the drug.

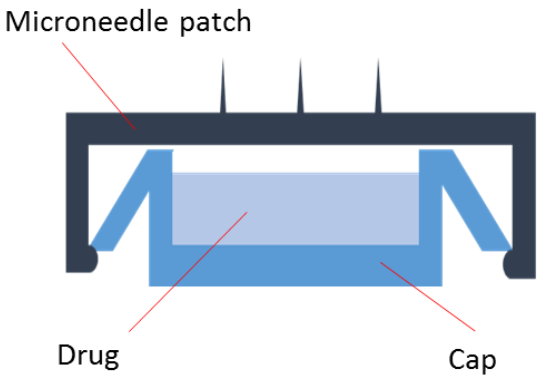

Fig.1. Design of our hollow microneedles which consists of 2 component: microneedle patch and cap

2. Design and Fabrication of Microneedle Patch

The microneedle patch was designed to have 16 microneedles. The shape of microneedles was designed as follows: the tip size is 30 μm, the needle height is 800 μm, the wall angle is 73 degree and the hole diameter is 100 μm as schematically shown in Fig.2. The microneedle patch is designed to be manufactured by injection molding. The molds of the microneedle patch were drawn in a CAD program as shown in Fig. 3. The male mold was fabricated using a CNC machine whereas the female mold was fabricated by casting a polymer mold using a pre-female template. This fabrication step is much easier and cheaper than the conventional fabrication process of a female mold. In the fabrication process, polyethylene and polypropylene were used in the fabrication of microneedle patch by using injection molding. After that, the fabricated microneedle patches were observed in a scanning electron microscope (FEI, Inspect s50).

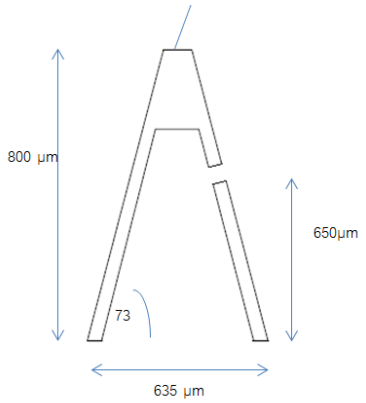

Fig.2 Design of microneedle

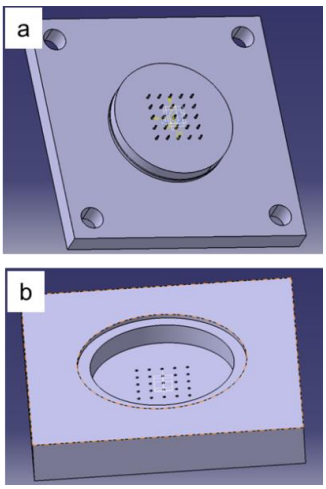

Fig.3. Design of the injection molds of microneedle patch (a) male mold and (b) female mold

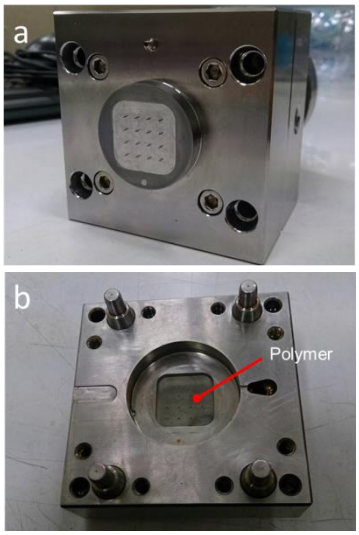

Fig.4. The injection molds of microneedle patch (a) male mold and (b) female mold

3. Design and Fabrication of Cap

The cap is designed to have a wing structure which can lock with the microneedle patch. This wing structure offers a large tolerance of ±3 mm in the assembly step. The thickness of the diaphragm is designed to deflect when a certain force is applied. The cap is also fabricated by injection molding.

4. Failure test

A compressive failure test was performed to characterize the mechanical property of the fabricated plastic hollow microneedles. Each microneedle was compressed in a Universal Test Machine and the microneedles were observed under a microscope.

5. Penetration test

Penetration test was performed to confirm that the fabricated microneedles could penetrate into skin. Parafilm is used as a skin model in the penetration test. Applied force is increased gradually until all 9 microneedles penetrate the parafilm. The parafilm was then observed under a microscope.

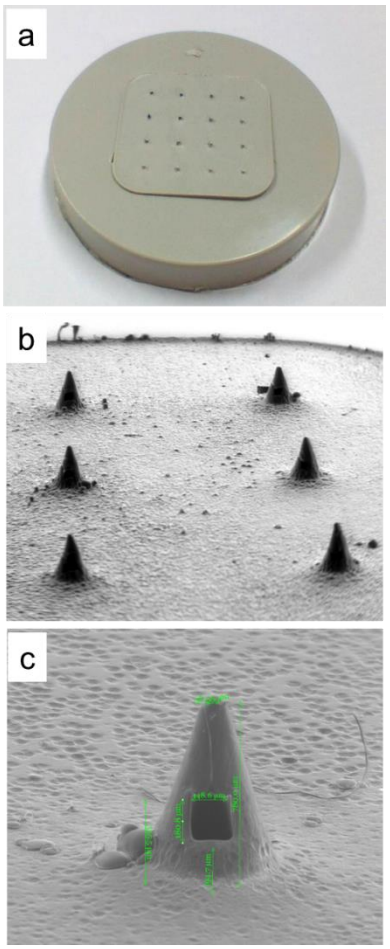

Fig.5. (a) an image of the fabricated microneedle patch, (b) a SEM image of the fabricated microneedle patch and (c) a zoom-in SEM image

Fig.6. The fabricated cap

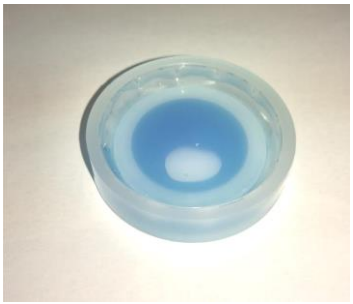

Fig.7. The assembled plastic hollow microneedles

Results and discussion

1. Fabrication results

The SEM images of the fabricated hollow microneedles are shown in Fig. 5. The needle height, tip size and hole size were approximately 760, 50 and 150 μm, respectively. The fabricated cap is shown in Fig. 6. In the assembly step, the cap is filled with drug and the microneedle patch was pressed against the cap. Fig. 7 shows the image of the assembled plastic hollow microneedles.

2. Failure test

As the force increased, the tips of the plastic microneedles gradually deformed which is a typical plastic deformation. The polypropylene microneedles could withstand a large force up to 1.5 N/needle without fracturing or breaking into pieces suggesting their safety for practical use. The average deformation of the microneedle was approximately 70 μm when the applied force was 1 N.

3. Penetration test

The result of penetration test is summarized in table 1. It was found that all 9 microneedles were able to pierce through the parafilm when the applied force is more than 15 N. This result revealed that the microneedles could pierce through the skin model. Interestingly, no significant damages were found on all the microneedles after the penetration test suggesting a very good mechanical property of the microneedles.

Fig. 8. The parafilm after penetration test using an applied force of 15 N

Table 1 Number of microneedles that pierced through parafilm at various applied forces

Force	Number of microneedles that pierced through parafilm
9 N	1
11 N	3
13 N	7
15 N	9

Conclusion

In this work, plastic hollow microneedles were successfully developed. They consists of two components, microneedle patch and cap, which were specifically designed for simple assembly and easy drug incorporation. For the fabrication process, a novel polymer mold was introduced enabling us to fabricate the microneedle patch by using single-step injection molding. The mechanical properties of the fabricated hollow microneedles were characterized in failure test, and penetration test. The results revealed very good mechanical properties of the fabricated hollow microneedles making them a promising candidate for commercial applications.

Acknowledgments

This research was supported by Chulalongkorn University through Chulalongkorn Academic Advancement into Its 2nd Century Project (Smart Medical Device).

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Isoliquiritigenin from *Butea monosperma* Suppresses Inflammatory Mediators in Lipopolysaccharide-induced RAW 264.7 Cells

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Abstract

Overproduction of inflammatory mediators are harmful and also contribute to the development of various types of inflammatory diseases. Plants are the main sources of novel lead compounds for their anti-inflammatory activity. The objective of this study was to evaluate the anti-inflammatory effects of liquiritigenin (LQ) and isoliquiritigenin (ILQ) isolated from the *Butea monosperma* (Lam.) Taub. on lipopolysaccharide (LPS)-induced RAW 264.7 cells. The cytotoxic effects of these compounds in RAW 264.7 cells were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The nitric oxide (NO) inhibitory effects of LQ and ILQ were determined by Griess assay in LPS-induced RAW 264.7 cells. We found that pre-treatment with ILQ suppressed NO secretion with the half-maximal inhibitory concentration (IC_{50}) value of 13.00 $\pm$ 0.60 μM, which was dramatically better than LQ (IC_{50} value >128.00 μM). We further examined the effects of ILQ on the secretion of pro-inflammatory cytokines by enzyme-linked immunosorbent assay. Pre-treatment with ILQ inhibited the secretion of tumor necrosis factor alpha (TNF-α), interleukin (IL)-1β and IL-6 with IC_{50} values of 13.00 $\pm$ 1.15, 15.66 $\pm$ 0.67, and 10.93 $\pm$ 0.64 μM, respectively. These results were consistent with the anti-inflammatory effect of ILQ from other plants. Thus, ILQ is an effective candidate for ameliorating inflammatory conditions and also deserving of further investigation on modulating inflammatory responses.

Keywords: Chalcone, Inflammation, Cytokines, Nitric Oxide

Introduction

Inflammation is a biological process that occurs in damaged areas of the body and involves various types of pro-inflammatory mediators [1]. Macrophages are one type of immune cell that play a key role during inflammation. In the presence of bacterial product, lipopolysaccharide (LPS) binds with the toll like receptor-4 (TLR4), which leads to the activation of inflammatory pathways, and consequently causes the secretion of inflammatory mediators such as nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, IL-6, and other inflammatory mediators [2]. These mediators play a role in various types of diseases including gastrointestinal, renal, and cardiovascular effects [3-5]. NO, an inflammatory mediator, plays a crucial role in inflammation. Increased expression of inducible nitric oxide synthesis (iNOS) has been linked with the severe inflammatory diseases [6]. Although there are many naturally occurring bioactive compounds that can inhibit these inflammatory mediators; there is still great demand to find novel drug candidates with high effectiveness for the inhibition of inflammation, and low cytotoxicity.

Traditionally, medicinal plants have been used to suppress inflammation. *Butea monosperma* is commonly known as the 'Palash' or 'Flame of forest' and belongs to the Fabaceae family. This plant is available in South and South East Asian countries [7]. There is evidence that the bark of *B. monosperma* can be used as anti-diarrhoeal agent [8]. The n-butanol fraction of *B. monosperma* flower extract has been found to exert anti-cancer activities against Huh7 and HepG2 human hepatoma cells and mouse liver AML12 cells [9].

Hepatoprotective activity of aqueous extract from *B. monosperma* flowers has already been reported [10].

Liquiritigenin (LQ) and isoliquiritigenin (ILQ) have been isolated from *B. monosperma* [7]. Numerous evidence suggests that these two compounds possess a variety of pharmacological effects, such as an improvement of liver injury, anti-tumor, anti-diabetic, anti-platelet, and anti-cancer effects in both *in vitro* and *in vivo* models [11–15]. Liquiritigenin (LQ) has been shown to have anti-inflammatory activity in LPS-stimulated RAW 264.7 cells [16]. ILQ isolated from *Dalbergia odorifera* has been shown to exert anti-inflammatory activity by suppressing the production of NO, TNF-α, IL-1β and also up-regulates heme oxygenase-1 expression in LPS-induced RAW 264.7 cells [17]. However, LQ and ILQ isolated from *B. monosperma* have not yet been reported for their anti-inflammatory roles in LPS-induced RAW 264.7 cells. Thus, to clarify the anti-inflammatory effects of these two isolated compounds, we focused on NO production and pro-inflammatory cytokine secretion in LPS-induced RAW 264.7 cells.

Methods

Chemicals

LPS from *Escherichia coli* 0111:B4, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), dexamethasone (Dex), sulfanilamide, and naphthylethylene were purchased from Sigma-Aldrich (St. Louis, MO, USA). Enzyme-linked immunosorbent assay (ELISA) kits were obtained from BioLegend (San Diego, CA, USA).

Plant materials

The flowers of *B. monosperma* were purchased from Tai-un-jan herbal store, Bangkok in 2006. A voucher specimen is deposited at the Faculty of Science, Ramkhamhaeng University (Apichart Suksamrarn, No. 054). LQ and ILQ from *B. monosperma* were obtained from ethyl actetate extract as previously described by Chokchaisiri et al., 2009 [18].

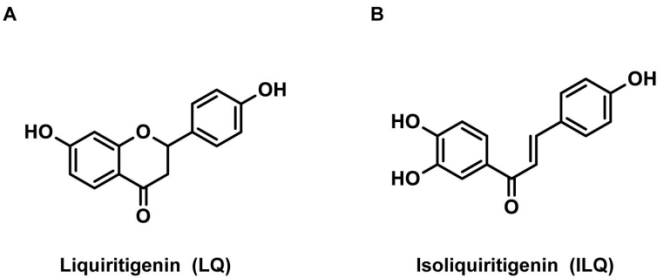

Figure 1 Structure of liquiritigenin (LQ) and Isoliquiritigenin (ILQ)

Cell culture

RAW 264.7 cells (ATCC, Rockville, MD, USA) were cultured in RPMI-1640 (Corning, New York, USA) with 10% fetal bovine serum (Biochrom GmbH, Berlin, Germany), 1% penicillin/streptomycin (Gibco, Gaithersburg, USA) and 2 mM stable glutamine (Gibco, Gaithersburg, USA). The cultures of the RAW 264.7 cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C.

Cytotoxicity assay

RAW 264.7 cells were seeded on 96-well plate at a density of 3.0 × 10^5 cells/cm^2. Cells were treated with LQ and ILQ with 4–128 μM for 24 h followed by incubation in a 5% CO_2 incubator at 37°C. MTT (0.5 mg/mL) solution was added. After 3 h incubation, the formazan crystals were dissolved by 200 μL of DMSO. The absorbance was measured using the microplate plate reader at 560 nm and 670 nm. The following equation was used for the calculation of the percentage of cell viability.

Cell viability = [(Abs $_{\text{treated sample}}$/ Abs $_{\text{untreated sample}}$) × 100].

Nitrite assay

RAW 264.7 cells were grown at a density of 3.0 × 10^5 cells/cm^2 in a 96 well-plate. Cells were pre-incubated with different concentrations (4–128 μM) of LQ and ILQ for 1 h followed by LPS (5 ng/mL) treatment for 24 h. Culture media was collected and subjected to the Griess assay [19]. Briefly, 150 μL of each sample

was mixed with 130 μL distilled water and 20 μL Griess reagent (1% sulfanilamide and 0.1% naphthylethylene in 2.5% phosphoric acid solution) in a 96-well plate. After 30 min incubation at room temperature, absorbance was measured at 540 nm using a microplate reader (Thermo Fisher Scientific, MA, USA). Concentration of nitrite was determined using a $NaNO_2$ serial dilution standard curve with $r^2 > 0.9990$.

Selectivity index

Calculation of the selectivity index (SI) value was performed in order to determine the selectivity of LQ and ILQ on NO inhibition rather than inhibition of NO due to general toxicity. SI was obtained by dividing the CC_{50} value for cytotoxicity by the IC_{50} value for NO inhibition.

Enzyme-linked immunosorbent assay (ELISA)

RAW 264.7 cells were plated in 24-well plates at a density of 3.0×10^5 cells/cm^2 and incubated for 24 h. Cells were pre-treated with ILQ at different concentrations (8–32 μM) for 1 h prior to LPS (5 ng/ml) treatment for an additional 24 h. Culture supernatant was collected and evaluated for the pro-inflammatory cytokines TNF-α, IL-1β and IL-6 using a commercial ELISA kit (San Diego, CA, USA) following the manufacturer's instructions.

Statistical analysis

The results are expressed as mean ± SEM of the three independent experiments. Significant differences between the different groups were ascertained at a *p*-value < 0.05 using Bonferroni's multiple comparison tests. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA, USA).

Results

Effects of LQ and ILQ on cell viability

The MTT reduction assay was used to evaluate the cytotoxic effect of the two isolated compounds LQ (Figure 1A) and ILQ (Figure 1B) in LPS-induced RAW 264.7 cells. LQ and ILQ did not exhibit any cytotoxicity at concentrations of 4–32 μM (Figure 2). ILQ caused cytotoxicity at concentrations of 64 μM and 128 μM (Figure 2B). The half-maximal cytotoxic concentrations (CC_{50}) of LQ and ILQ were $>$ 128.00 μM and 81.00 ± 1.0 μM, respectively (Figure 3C).

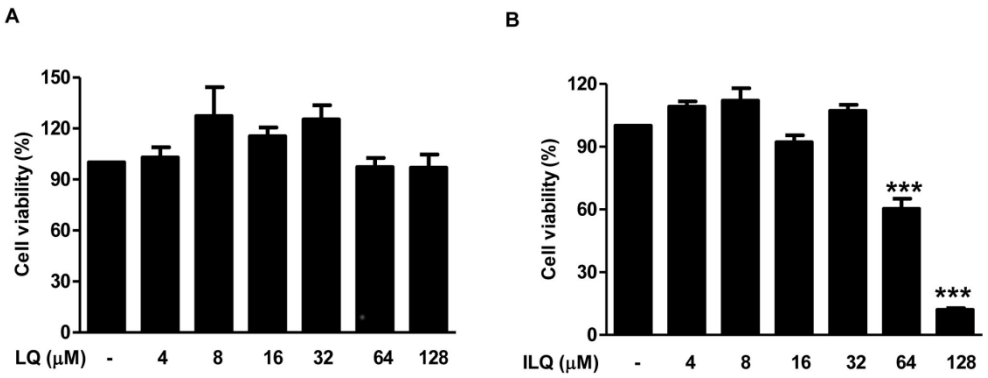

Figure 2 Effects of LQ and ILQ on cell viability in RAW 264.7 cells. Cells were treated with various concentrations (4–128 μM) of (A) LQ and (B) ILQ for 24 h followed by MTT assay. Values are expressed as means ± SEM of three-independent experiments in triplicate. The symbol " – " is denoted as the absence of the compounds.

***$p < 0.001$ versus control group without any treatment.

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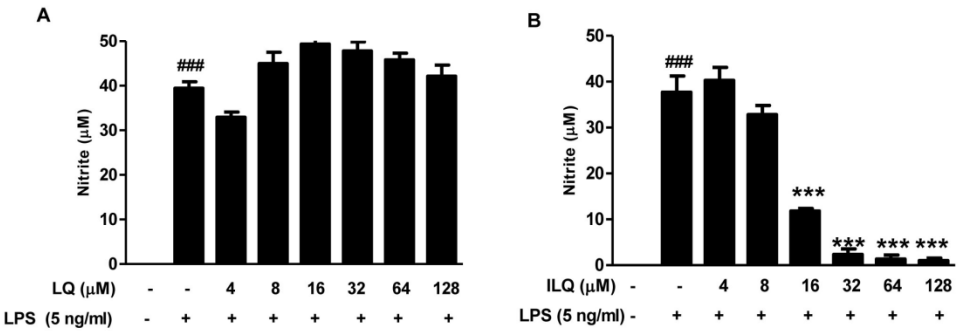
Effects of LQ and ILQ on NO production

NO production was significantly increased after LPS (5 ng/ml) stimulation (Figure 3). Pre-treatment with ILQ suppressed the production of NO in a dose dependent manner (Figure 3B). LQ and ILQ were found to have IC_{50} values of $>$128.00 μM and 13.00 ± 0.60 μM, respectively (Figure 3C). To assess the selective NO inhibition, SI values were calculated. LQ and ILQ had SI values of 1.00 and 6.23, respectively. The higher SI value obtained for ILQ represents high selectivity of ILQ inhibition of NO secretion rather than any inhibition caused by toxicity. The anti-inflammatory drug

dexamethasone (Dex) had a IC_{50} value of 4.83 ± 0.33 μM, as shown in Figure 3C.



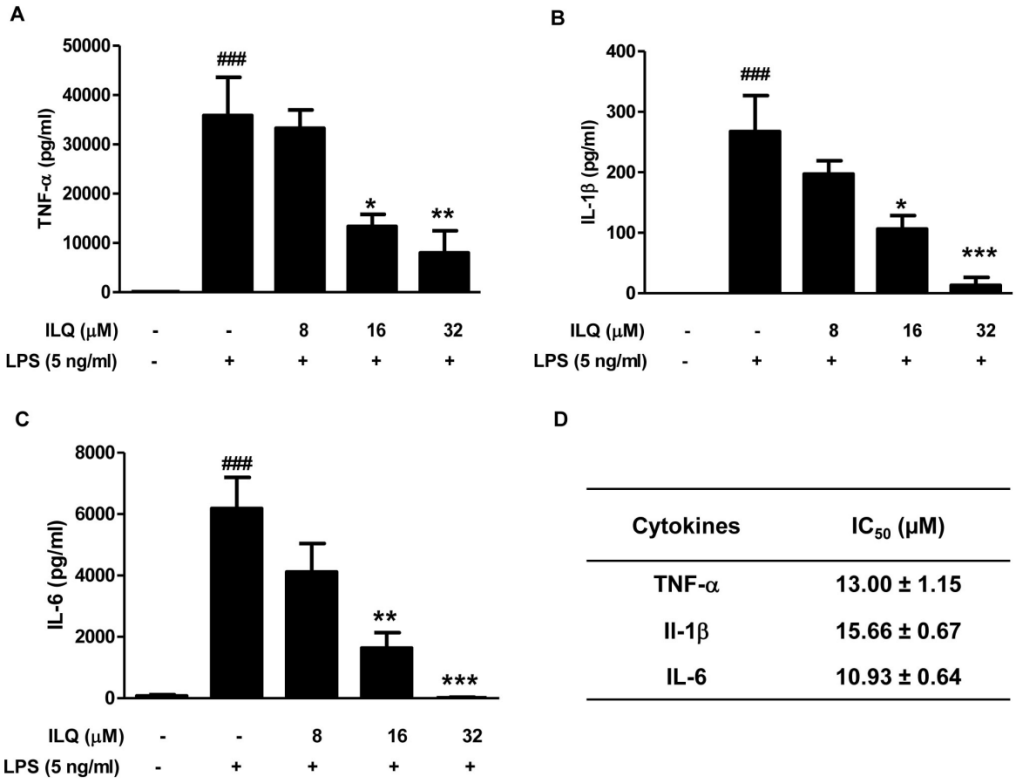
C

Compounds	CC_{50} (μM) of MTT	IC_{50} (μM) of NO
LQ	>128.00	>128.00
ILQ	81.00 ± 1.0	13.00 ± 0.60
Dex	>128.00	4.83 ± 0.33

Figure 3 Effects of LQ and ILQ on NO production in LPS-activated RAW 264.7 cells. Cells were pre-treated with different concentrations of (A) LQ and (B) ILQ for 1 h followed by 5 ng/mL of LPS treatment. Culture supernatant was then subjected to Griess assay. (C) The half-maximal cytotoxic concentration (CC_{50}) and the half-maximal NO inhibitory concentration (IC_{50}) of LQ and ILQ were determined from dose response curves of 24 h treatment. Values are expressed as means ± SEM of three-independent experiment in triplicate. The symbols " – " and " + " are denoted as the absence and presence of the compounds, respectively. $^{###}p < 0.001$ versus control group without any treatment; $^{***}p < 0.001$ versus LPS-stimulated RAW 264.7 macrophages.

Effects of ILQ on the secretion of pro-inflammatory cytokines

We further investigated whether ILQ suppresses the level of pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) in LPS-stimulated RAW 264.7 cells. Our results showed that levels of pro-inflammatory cytokines TNF-α (Figure 4A), IL-1β (Figure 4B) and IL-6 (Figure 4C) were significantly increased upon induction by LPS (5 ng/ml). ILQ significantly suppressed these cytokines (Figure 4). The half-maximal inhibitory concentrations (IC_{50}) of TNF-α, IL-1β and IL-6 were 13.00 ± 1.15, 15.66 ± 0.67 and 10.93 ± 0.64 μM, respectively (Figure 4D).



Cytokines	IC_{50} (μM)
TNF-α	13.00 ± 1.15
Il-1β	15.66 ± 0.67
IL-6	10.93 ± 0.64

Figure 4 Effects of ILQ on the secretion of pro-inflammatory cytokines in LPS-induced RAW 264.7 cells. Cells were pre-treated with ILQ (8–32 μM) for 1 h followed by LPS (5 ng/mL) treatment for 24 h. The levels of pro-inflammatory cytokine (A) TNF-α, (B) IL-1β and (C) IL-6 in LPS-activated RAW 264.7 cells were examined using ELISA assay. (D) The half-maximal inhibitory concentration (IC_{50}) of cytokines were determined from dose response curves of 24 h treatment of ILQ. The results were obtained from three- independent experiments. Values are expressed as means ± SEM. The symbols " – " and " + " are denoted as the absence and presence of the compounds, respectively. $^{###}p < 0.001$ versus control group without any treatment; $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus LPS-stimulated RAW 264.7 macrophages.

Discussion and Conclusion

Accumulating evidence has shown that overproduction of inflammatory cytokines in an injured area is responsible for acute and chronic pathological disorders [20–21]. Herein, ILQ isolated from the flowers of *B. monosperma* suppressed inflammatory mediators NO and pro-inflammatory cytokines in LPS-induced RAW 264.7 macrophages.

Activated macrophages play an important role in inflammation. After induction by LPS or other bacterial endotoxin, they secrete large amounts of NO, TNF-α, IL-1β, and IL-6 via numerous signaling cascades [22]. The NO production in the inflamed area can destroy foreign organisms and save ithe njured area from chronic inflammation, but over production of NO plays an important role in the destruction of tissues [23–24]. It has been reported that TNF-α and IL-1β promote secretion of NO in mice and in macrophages cells [25–26]. Several studies suggested that prolonged secretion of TNF-α, IL-6, and IL-1β in macrophages causes many diseases, including septic shock, weight loss, and inflammatory bowel disease [27–28]. ILQ is a chalcone belonging to the flavonoid family. Clacones are available in many natural plants and has great value due to their various pharmacological activities. It has been reported that ILQ significantly inhibited the protein expression of iNOS and COX-2 by preventing the degradation and phosphorylation of IκB-α, and by blocking the activation of NF-κB in LPS-stimulated RAW 264.7 macrophages [29]. A Previous study reported that 2′,4′-dihydroxy-6′-methoxychalcone elicited inhibitory effect on LPS-induced TNF-α, IL-1β, and IL-6 release in IFN-γ/LPS-stimulated BV2 cells by blocking the NF-κB signaling pathway [30]. Moreover, ILQ suppressed TNF-α-induced IκB kinase (IKK) activation in endothelial cells [31].

Activation of NF-κB plays a crucial role in inflammation. NF-κB is inactivated in the cytoplasm area of the cells where it is complexed with IκB-α protein. Phosphorylation of IκB-α by IKK leads to its subsequent ubiquitination and degradation and this leads to the translocation of NF-κB into the nucleus [32]. It has been revealed that the cysteine-179 residue that lies between the two serine residues in the activation loop of IKK can be a site for the modification by IKK inhibitors [33]. Chalcone butein (with four hydroxyl-groups) has been shown to attenuate IκB-α phosphorylation by direct inactivation of IKK through the cysteine 179 residue [34]. Reports have also suggested that

altering the position of functional groups in the A and B ring of chalcone compounds could increase their anti-microbial and anti-inflammatory activities [18, 30]. However, an NO inhibitory effect was not observed with LQ because of their structural modification, incorporation of functional group, and disruption of the α,β-unsaturated carbonyl moiety [35]. In summary, this study showed that ILQ from *B. monosperma* offers a remarkable potentiality in anti-inflammatory activity by reducing NO production and secretion of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 in LPS induced RAW 264.7 cells. ILQ is expected to be a good candidate for use in the reduction of inflammatory diseases.

Acknowledgements

This study was financially granted by Walailak University fund for graduate studentships to Rana Dhar and Rungruedee Kimseng and The Thailand Research Fund [DBG5980003].

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Novel Dissolving Microneedles Made of Maltose Solution for Transdermal Drug delivery

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Abstract

Currently, microneedles are attracting great attention because they offer painless injection as opposed to typical hypodermic needles. Dissolving microneedles made of a biodegradable material mixed with drug are of particular interest since they can dissolve after insertion into the skin leaving no infectious waste. In this work, dissolving microneedles made of maltose solution have been demonstrated for the first time. The proposed microneedles offer several benefits over other dissolving microneedles including low cost, fast release, and low temperature processing. According to the experimental results, the developed maltose microneedles show a very good mechanical property and high solubility. This opens up great potential towards the commercialization of dissolving microneedles for a wide range of applications.

Keywords: Microneedles, Dissolving Microneedles, Maltose.

Introduction

Injection is a drug delivery method that is widely used due to the ease of operation, low cost and drug compatibility. However, injection generally utilizes a long hypodermic needle to pierce though skin to deliver a drug. This causes pain to some patients. According to one study, more than 10% of the US population has needle phobia, which prevents some patients from receiving medical treatment [1]. This may lead to problems in the healthcare system over a long term [2]. Moreover, used hypodermic needles are infectious waste that causes serious problems in waste containment and disposal.

Recently microneedles have emerged as an interesting alternative for transdermal drug delivery because the needle sizes are in micron-scale, enabling them to penetrate through human skin without stimulating pain nerves [3-7]. Among several types of microneedles, dissolving microneedles are of particular interest since the dissolving microneedles can deliver drug after piercing through skin, and then dissolve without leaving any infectious waste. In addition, they can be produced by a casting process that is applicable to various materials, and are suitable for mass production [4, 5].

Various types of degradable materials, such as silk protein, sugars and polymers have been used to fabricate dissolving microneedles [8-11]. Among all of these materials, sugars are the most interesting materials due to their great mechanical property and high solubility. However, there is a critical problem in the fabrication of sugar microneedles, since the fabrication process requires temperatures above the melting point of the sugar, typically more than 140°C. Such high temperatures may affect the quality of the drug contained in the sugar microneedles. Recently, sugar microneedles made of a mixture of sugar solutions were fabricated at a relatively low temperature of 50°C [4]. Although, this could circumvent the issue of high temperature during the fabrication process, the cost of those sugars is very expensive, hindering the potential of this technique for commercial applications. Therefore, this research aimed to develop novel dissolving microneedles from a low-cost sugar solution and characterize their mechanical properties.

Methodology

1. Materials

Maltose monohydrate powder was purchased from Acros Organics and used as the matrix material of the dissolving microneedles. Polydimethylsiloxane (PDMS) silicone elastomer (Sylgard® 184) was purchased from Dow Corning. Methylene blue ($C_{16}H_{18}N_3SCl$) solution, which was applied in this process, was prepared by mixing methylene blue and deionized (DI) water at a weight ratio of 1: 100.

2. Maltose microneedles fabrication

In a preliminary study, we studied the formation of sugar sheets on glass slides by evaporating several sugar solutions at 50°C. It was found that maltose solution could form a sugar sheet with good mechanical properties without crystallization (result not shown).

Therefore maltose solution was used in the fabrication of dissolving microneedles. The fabrication process of microneedles made of maltose sugar solution is shown in Fig. 1. First an aluminum workpiece was machined using micro milling method to fabricate an 8 X 8 microneedle array template with a microneedle height of 1.8 mm, a base width of 0.4 mm and a tip diameter of 0.2 mm. Then, the template was chemically etched in Al Etchant Type A at 90°C for 2h to obtain a sharp microneedle master [2]. Next, the master was used to produce a negative Polydimethylsiloxane (PDMS) mold for subsequent microneedle casting.

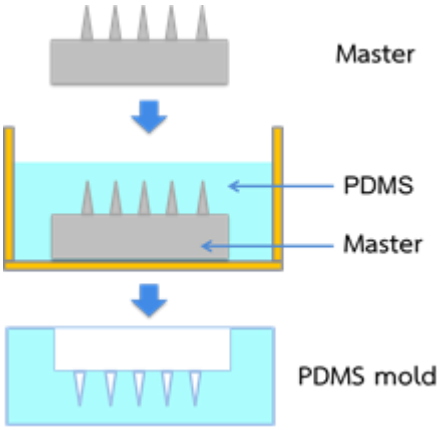

Fig.1. the process of producing microneedles mold from PDMS.

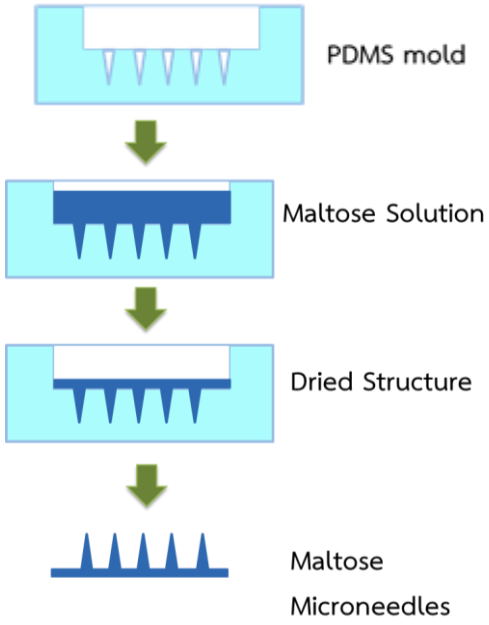

Fig.2. the fabrication process of maltose microneedles from maltose solution.

Maltose solution was prepared by mixing DI water with maltose powder at a weight ratio of 5:1. Then, the solution was mixed with methylene blue solution and stirred by a magnetic stirrer until the solution became homogeneous. Next, the PDMS mold was cleaned by sonication in an ultrasonic bath for at least 15 min to remove impurities that could remain in the mold. After that, maltose solution was poured into the PDMS mold. Vacuum was applied to ensure that the maltose solution fllled the PDMS mold. The sample was then baked at a temperature of 65 °C for 48-60 h. After the sample dried, the microneedles were left to cool down to room temperature for 35-40 min before being removed from the mold. The fabricated maltose microneedles were later examined by a digital microscope (Dino-Lite Am411 Pro).

3. Failure strength test

The failure test has was performed in order to examine the failure strength of the fabricated maltose microneedles. The test was conducted by using a

universal testing machine (Shimadzu EZ-S 500N) to apply an axial load to one microneedle at a time. The displacement and applied force were recorded and plotted in order to examine the stress/strain relation of the maltose microneedles.

4. Dissolving test

The dissolving test was performed to observe the dissolution rate of the fabricated maltose microneedles when applied on a gelatin layer. The gelatin layer was prepared by mixing gelatin powder with DI water at a ratio of 30% by weight. In the test, the fabricated maltose microneedles were applied to the gelatin layer for a certain period of time before being removed and observed under a digital microscope to examine the deformation.

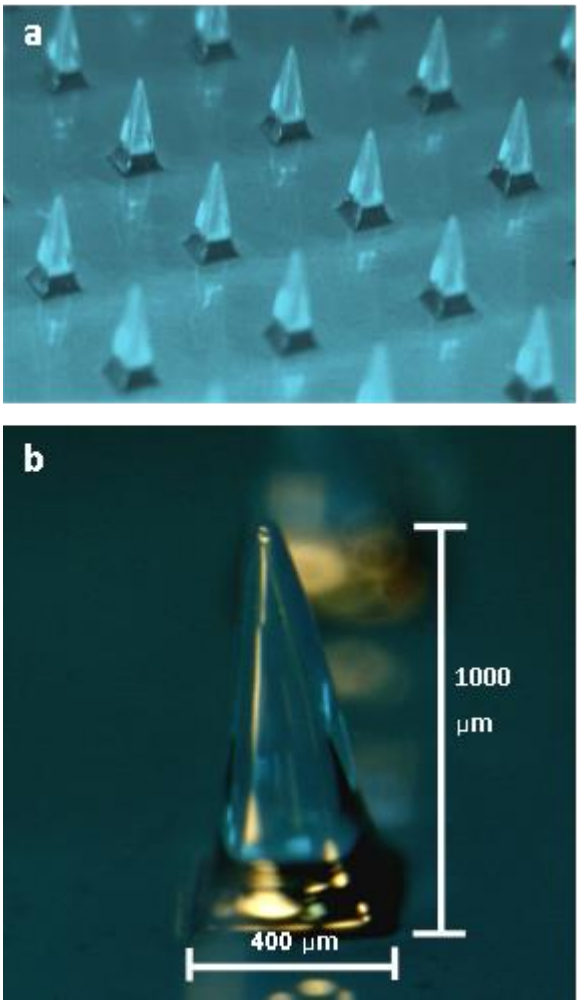

Fig.3. (a) Maltose Microneedles and (b) Zoom-In image of maltose microneedles

Results

The 8x8 maltose microneedle arrays were successfully fabricated as shown in Fig. 3. The needle height, base width and tip were approximately 1,000, 400 and 10 μm, respectively. It is worth noting that no crystallization was observed in the fabricated microneedles suggesting very low crystallinity of the maltose solution. In the failure test, the exert force increased steadily at the displacements between 0.05 and 0.19 mm as shown in Fig. 4. This step revealed a steady deformation of microneedles in the Z-axis, while the microneedles did not fracture. However, when the applied force reached around 4.07 N/needle, the applied force rapidly decreased, indicating failure of the needles. The results showed that the maltose microneedles can sustain an axial force up to 4.07 N/needle before failure.

In the dissolving test, the fabricated microneedles dissolved very quickly after insertion into the gelatin layer. Within 5 seconds following the insertion, all the microneedles completely dissolved and only the base plate remained, as shown in Fig. 5(b). Moreover, the methylene blue inside the microneedles

was released onto the gelatin layer as shown in Fig. 6. This result reveals the great solubility of the fabricated maltose microneedles.

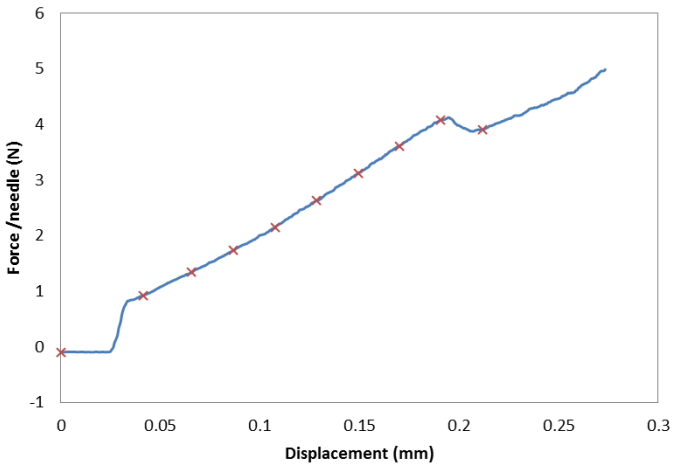

Fig. 4. the exert force per needle (F) vs. the displacement of each needle

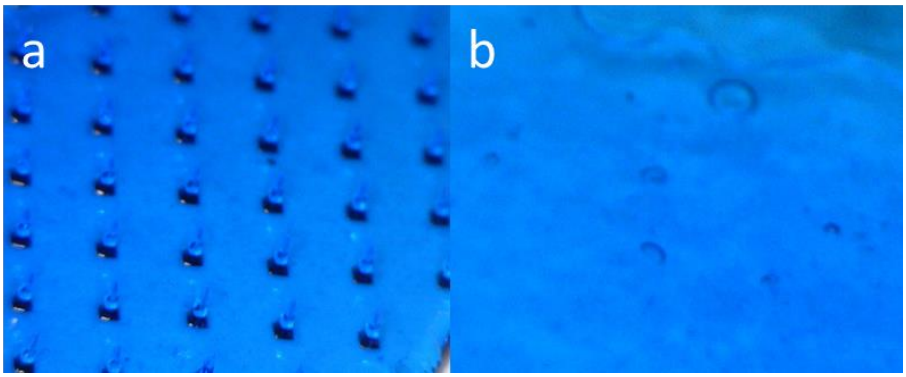

Fig.5. Maltose microneedles (a) before applying on the gelatin and (b) 3 s after the insertion

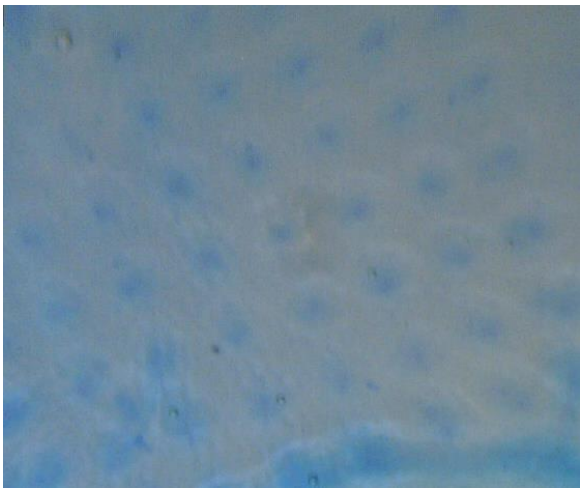

Fig.6. Gelatin layer after being applied by the maltose microneedles for 3 s

Discussion and conclusion

Microneedles made of maltose solution were successfully developed for the first time. The developed microneedles offer several advantages including low cost, fast release, and low temperature processing. Furthermore, the fabricated maltose microneedles showed high dissolvability and very good mechanical strength, sustaining an axial force of up to 4.07 N/needle before fracturing. This opens up great potential towards the commercialization of dissolving microneedles for a wide range of applications.

Acknowledgement

This research has been supported by the Ratchadaphiseksomphot Endowment Fund of Chulalongkorn University (CU-58-004- HR).

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Anti-Cancer Activity of the Curcumin Analog Mono-*O*-Demethylcurcumin on Invasive Multidrug-Resistant Oral Squamous Cell Carcinoma *in vitro*

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Abstract

Metastasis is a major cause of death in cancer pateints. Oral squamous cell carcinoma is the most common head and neck cancer, characterized by a poor prognosis and low survival rate. The average five-year survival rates vary from 50 to 80%, according to the occurrence of invasion and metastasis, which is responsible for determining the stage of the cancer. Curcuminoid is the major curcuminoid compound isolated from turmeric and has been reported to have excellent anti-cancer activity in various tumors in both *in vitro* and *in vivo* models. This study aimed to investigate the anti-cancer activity of the curcumin analog mono-*O*-demethylcurcumin compared with curcumin, in an invasive multidrug-resistant oral squamous cell carcinoma cell line (CLS-354/DX). The anti-proliferation effect was determined by tetrazolium dye MTT assay. The anti-migration and anti-invasion effects were investigated by the transwell migration and invasion chamber assay, respectively. The results showed that both compounds reduced CLS-354/DX viability and migration and invasion ability, but mono-*O*-demethylcurcumin was significantly more potent than curcumin. The half maximal inhibitory concentration (IC_{50}) values of curcumin and mono-*O*-demethylcurcumin were 44.99±2.92 μM and 19.10±0.71 μM, respectively. Application of mono-*O*-demethylcurcumin and curcumin at non-cytotoxic concentrations inhibited cell migration and invasion in a dose-dependent manner. Treatment with 5 μM mono-*O*-demethylcurcumin decreased CLS-354/DX migration and invasion scores by 63.84±3.31% and 11.40±2.82%, respectively. While, treatment with 15 μM of curcumin showed increased CLS-354/DX migration and invasion scores by 72.45±4.17% and 21.70±4.39%, respectively. These results suggest that mono-*O*-demethylcurcumin exhibits excellent anti-proliferation, anti-migration and anti-invasion activity on multidrug-resistant oral squamous cell carcinoma. This compound may thus have a potential as a chemopreventative or therapeutic agent for multidrug-resistant oral cancer.

Keywords: anti-migration, anti-invasion, mono-*O*-demethylcurcumin, invasive multidrug-resistant oral squamous cell carcinoma

Introduction

Head and neck cancer, which is the sixth leading cancer worldwide, describes a group of cancers that originate in the lip, mouth, nose, throat, tonsil, pharynx, salivary gland and larynx [1, 2]. Oral cancer refers to cancers of the lip, tongue, gingiva, floor of the mouth, palate, maxilla, vestibule and retromolar area up to the anterior pillar of the fauces (1). Squamous cell carcinoma (SCC) is the most frequent malignant tumor of the head and neck region (2). Factors that are known to increase oral cancer risk include; use of tobacco, consumption of alcohol and betel quids containing areca nut, poor diet, physical inactivity, infection with high-risk types of human papillomavirus (HPV), and reproductive changes [2, 3]. Over 60% of patients with oral cancers present with either regional or distant spread [4]. Therefore, the five-year survival rates for oral cancer are poor, averaging between 50 to 80% and varying depending on the stage of the disease [5, 6]. The treatment approaches depend upon the stage and location of the cancer. Chemoradiotherapy is the established standard treatment for patients who present with inoperable cancers or patients in whom the operation would be associated with unacceptable morbidity. A pharmaceutical chemotherapy drug that is widely used to treat many solid tumors is cisplatin [7]. Cisplatin is a high responsiveness platinum-based chemotheraputic agent, but its use is associated with multiple severe side effects affecting renal, otologic, and bone marrow function. Furthermore, the majority of oral cancer patients eventually relapse with cisplatin-resistant disease [8]. Therefore, the finding of a novel

chemotheraputic agents and optimal chemotheraputic treatment for oral cancer remains a challenge.

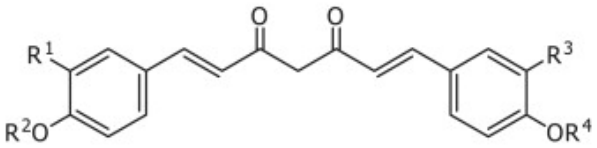
Curcuma longa, or turmeric, is a herbal plant that belongs to the family Zingiberaceae. Turmeric is comprised of a group of three major curcuminoids: curcumin (77%), demethoxycurcumin, (17%) and bisdemethoxycurcumin (3%) [9]. Compounds from *C. longa* have been reported to have several anti-cancer properties and curcumin has been reported to inhibit cell proliferation and promote apoptosis of various cancers, both *in vitro* and *in vivo* [10, 11]. Moreover, several anti-migration and anti-metastasis activities of curcumin have been reported, for example, curcumin inhibits invasiveness and epithelial-mesenchymal transition in oral squamous cell carcinoma by reducing matrix metalloproteinase 2, 9 and modulating the p53-E-cadherin pathway [12], curcumin inhibits the invasion of lung cancer cells by modulating the PKCα/Nox-2/ROS/ATF-2/MMP-9 signaling pathway [13], curcumin inhibits lung cancer invasion and metastasis by attenuating the GLUT1/MT1-MMP/MMP2 pathway [14], curcumin inhibits tumor epithelial‑mesenchymal transition by downregulating the Wnt signaling pathway and upregulating NKD2 expression in colon cancer cells [15], curcumin inhibits the invasion of thyroid cancer cells via down-regulation of the PI3K/Akt signaling pathway [16]. In addition, since the structure of β-diketone of curcumin can be easily catalytically decomposed, several curcumin analogs have been developed that show more potent anti-oxidant, anti-bacterial, anti-mycobacterial, anti-cancer, and anti-metastatic activity [17-21].

The aims of this study were to evaluate the anti-migration and anti-invasion activity of mono-*O*-demethylcurcumin (curcumin analog) in an invasive multidrug-resistant oral squamous cell carcinoma cell line (CLS-354/DX) compared with curcumin. Therefore, the impact on %viabilty, %migration and %invasion were evaluated.

Methodology

Chemicals and reagents

Mono-*O*-demethylcurcumin (curcumin analog) and curcumin compounds from *curcuma longa* were kindly supported by Prof. Dr. Apichart Suksamrarn, Faculty of Science, Ramkhamhaeng, University, Thailand and Dr. Chatchawan Changtam, Division of Physical Science, Faculty of Science and Technology, Huachiew Chalermprakiet University, Thailand. Roswell Park Memorial Institute medium 1640 media (RPMI-1640) was purchased from Ward Medic LTD., PART. (Hyclone Dr, Logan, UT). Fetal bovine serum (FBS) was purchased from Biochrom GmbH (Berlin, Germany). Penicillin/streptomycin, trypsin/EDTA and trypan blue were purchased from Gibco, Life Technologies (Carlsbad, CA, USA). PBS was purchased from Thermo Fisher Scientific Inc., (CA, USA). 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan or MTT was purchased from Sigma–Aldrich Corp., (St. Louis, MO, USA). DMSO was purchased from Sigma-Aldrich Co., (Lezennes, France).



R^1=R^3=OMe, R^2=R^4=H: Curcumin

R^1=OMe, R^2= R^4= H, R^3= OH: Mono-*O*-demethylcurcuin

Figure 1 Chemical structure of curcumin and mono-*O*-demethylcurcumin (18).

Cell line and culture conditions

CLS-354/DX invasive multidrug-resistant oral squamous cell carcinoma cell line was established from CLS-354 cell line (Cell Line Service, Germany) by Dr. Tanyarath Utaipan, Prince of Songkla University, Pattani Campus, Thailand and Asst. Prof. Dr. Warangkana Chunglok, Walailak University, Thailand. CLS-354/DX is fibroblast-like human mouth carcinoma cell line (or EMT-derived phenotype), which is more aggressive, as described previously [22]. This cell was maintained in a monolayer culture in RPMI-1640 medium with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 2 mM L-glutamine at 37°C with 5% CO_2/ 95% air. CLS-354/DX cell line was grown as monolayer and cells were considered ready to treat when they reached 80% confluence.

Cells Viability Assay

CLS-354/DX cell line was seeded onto 96-well plates (Corning®, Sigma-Aldrich Pte Ltd., Singapore) with 1.5×10^4 cells per well in RPMI-1640 media with 10% FBS and cultured for 24h. After that cells were treated with various doses of mono-*O*-demethylcurcumin and curcumin. After 24h of treatment, cell viability was assessed by MTT assay. First, 0.5 mg/ml of MTT reagent was added to each well for at least 3 hours of treatment. Optical density (OD) values were determined at 560 nm and 670 nm using a microplate reader (Multiskan™ GO Microplate Spectrophotometer, Thermo Fisher Scientific Inc., CA, USA). The viable cell number was directly proportional to the production of formazan, which was reflected by the color intensity measured after solubilization with DMSO. All samples were assayed in triplicate in at least 3 independent experiments and results were calculated as half maximal inhibitory concentration (IC_{50}) as mean ± SEM by Graphpad prism 5. Student t-test analysis was used for statistical analysis.

Migration Chamber Assay

CLS-354/DX cells were resuspended to $1x10^4$ cells/well in serum reduced medium containing mono-*O*-demethylcurcumin and curcumin at non-cytotoxic concentrations and were carefully transferred into the upper chambers of a Boyden migration chamber (Cell Biolabs, Inc., CA, USA). The lower chamber was filled with 10% FBS medium. The Boyden migration chamber was incubated at 37°C with 5% CO_2 for 24 h. After that non-migrated cells were removed with a cotton swab and washed twice with PBS. Migrated cells were fixed by methanol for 10 min and stained with crystal violet stain solution for 20 min. The migrated cells at least 15 at the lower were counted in randomly selected microscopic fields (20x). Experiments were performed independently three times and results expressed as the %cell migration ± SEM. Values were calculated by Graphpad prism 5. Student t-test analysis was used for statistical analysis.

Invasion Chamber Assay

CLS-354/DX cells were resuspended to $2.5x10^4$ cells/well in serum reduced medium containing with mono-*O*-demethylcurcumin and curcumin at non-cytotoxic concentrations and were carefully transferred into the upper chambers of invasion chambers (Cell Biolabs, Inc., CA, USA). The lower chamber was filled with 10% FBS medium. The invasion chambers were incubated at 37°C with 5% CO_2 for 22 h. After that non-invading cells were removed with a cotton swab moistened with medium and invested cells were fixed by methanol for 2 min. Invested cells were stained with crystal violet stain solution for 2 min and counted in 10 randomly selected microscopic fields (20x). Experiments were performed three times and results expressed as the invasive ability ± SEM. Values were calculated by Graphpad prism 5 and Student t-test analysis was used for statistical analysis. The invasion chambers were warmed to room temperature and incubated at 37°C with based culture medium for 2 hours after remove the package from -20°C.

Results

Mono-*O*-demethylcurcumin inhibited CLS-354/DX cell proliferation

CLS-354/DX cells were oral squamous cell carcinoma cells line exhibiting elongated (fibroblast-like) shape. The cells are invasive and multidrug resistant [23]. The methylthiazoletetrazolium (MTT) assay was performed to measure the inhibitory effect of mono-*O*-demethylcurcumin on tumor cell proliferation (Figure 2A). CLS-354/DX cell line was seeded onto 96-well plates and treated with various concentrations of mono-*O*-demethylcurcumin and curcumin. A range of 0–60 µM of compounds was used to treat CLS-354/DX cell line for 24 h. After that the half maximal inhibitory concentration (IC_{50}) was calculated. Mono-*O*-demethylcurcumin demonstrated satisfactory inhibitory activities against the CLS-354/DX cell line. The half maximal inhibitory concentration value (IC_{50}) of mono-*O*-demethylcurcumin was 19.10±0.71 µM, while the IC_{50} value of curcumin against this cell line was 44.99±2.92 µM (Table 1). Morphological observation showed that both 19 µM of mono-*O*-demethylcurcumin and 40 µM of curcumin could reduce cell viability by approximately 50% following the 24h treatment (Figure 2B). These results therefore demonstrated that mono-*O*-demethylcurcumin exhibits better anti-proliferation activity of CLS-354/DX than curcumin and cisplatin (Table 1). In addition, the non-cytotoxic and 10% inhibitory concentration (IC_{10}) were calculated for subsequent experiments. Results showed that IC_{10} concentration of mono-*O*-demethylcurcumin was approximately 5 µM and IC_{10} concentration of curcumin was 15 µM. At this concentration, the anti-proliferative effect was not evident (Figure 2A).

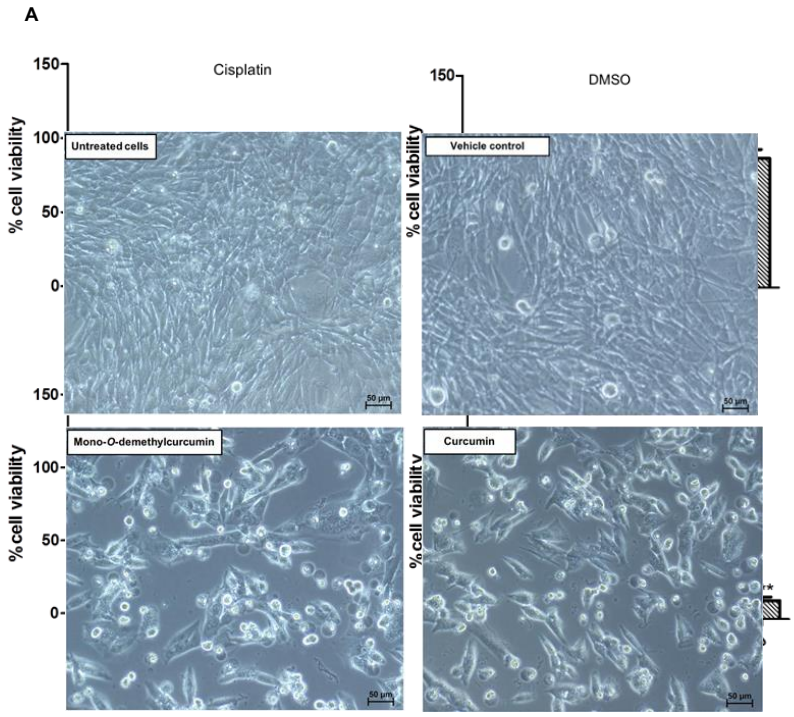


Figure 2 (A) Proliferation effects of mono-*O*-demethylcurcumin, curcumin, cisplatin (positive control) and vehicle control (dimethyl sulfoxide) on CLS-354/DX cell line. CLS-354/DX cell line was incubated with mono-*O*-demethylcurcumin (3.7, 7.5, 15, 30 and 60µM) for 24h and performed by the MTT assay, Optical density values were measured at 560nm and 670nm wavelength. All samples were performed in triplicates at least 3 independent experiments and results were calculated %cell viability as mean ± SEM by Graphpad prism 5. Student t-test analysis was used for statistical analysis. *p< 0.05 compared with control group (Untreated cell), ***p< 0.001 compared with control group (Untreated cell). (B) Cell morphology of CLS-354/DX cell upon treated with IC_{50} of mono-*O*-demethylcurcumin (19µM), curcumin (40µM), vehicle control (1.2µM) and untreated control for 24 h.

Table 1 IC_{50} and IC_{10} of mono-*O*-demethylcurcumin on CLS-354/DX cell line.

Compounds	IC_{50} (µM)	IC_{10} (µM)
Cisplatin	30.21±2.76	10
Curcumin	44.99±2.92[a]	15
Mono-*O*-demethylcurcumin	19.10±0.71[a,b]	5

IC_{50}: half maximal inhibitory concentration.

IC_{10}: 10% inhibitory concentration

Data were expressed as mean ± SEM (n= 5-11). Student t-test analysis was used for statistical analysis. a: differences are statistically significant when compare with cisplatin, b: differences are statistically significant when compare with curcumin

Mono-*O*-demethylcurcumin inhibited CLS-354/DX cell migration

The effect of mono-*O*-demethylcurcumin on cell migration was investigated in the CLS-354/DX cell line by using a transwell migration chamber assay. The CLS-354/DX cell line was seeded onto the upper chamber with IC_{10} quantities of mono-*O*-demethylcurcumin and curcumin in serum reduced medium and the lower chamber contained 10% FBS medium. After incubation at 37°C, 5% CO_2 for 24h, the number of migrated cells were counted and cell migration rate was calculated as percent of control. Moreover, the dose-dependent effects of compounds were also evaluated. The results showed that both mono-*O*-demethylcurcumin and curcumin

inhibited CLS-354/DX cell migration in a dose-dependent manner. Treatment with 5μM and 10μM of mono-*O*-demethylcurcumin decreased CLS-354/DX migration by 63.85±3.3% and 31.20±2.91%, respectively. On the other hand, treatment with 15μM and 30μM of curcumin decreased CLS-354/DX migration by 72.45±4.17% and 33.55±2.12%, respectively (Figure 4). The comparison between mono-*O*-demethylcurcumin and curcumin revealed that mono-*O*-demethylcurcumin exhibited better anti-migration activity in CLS-354/DX than curcumin (Figure 5).

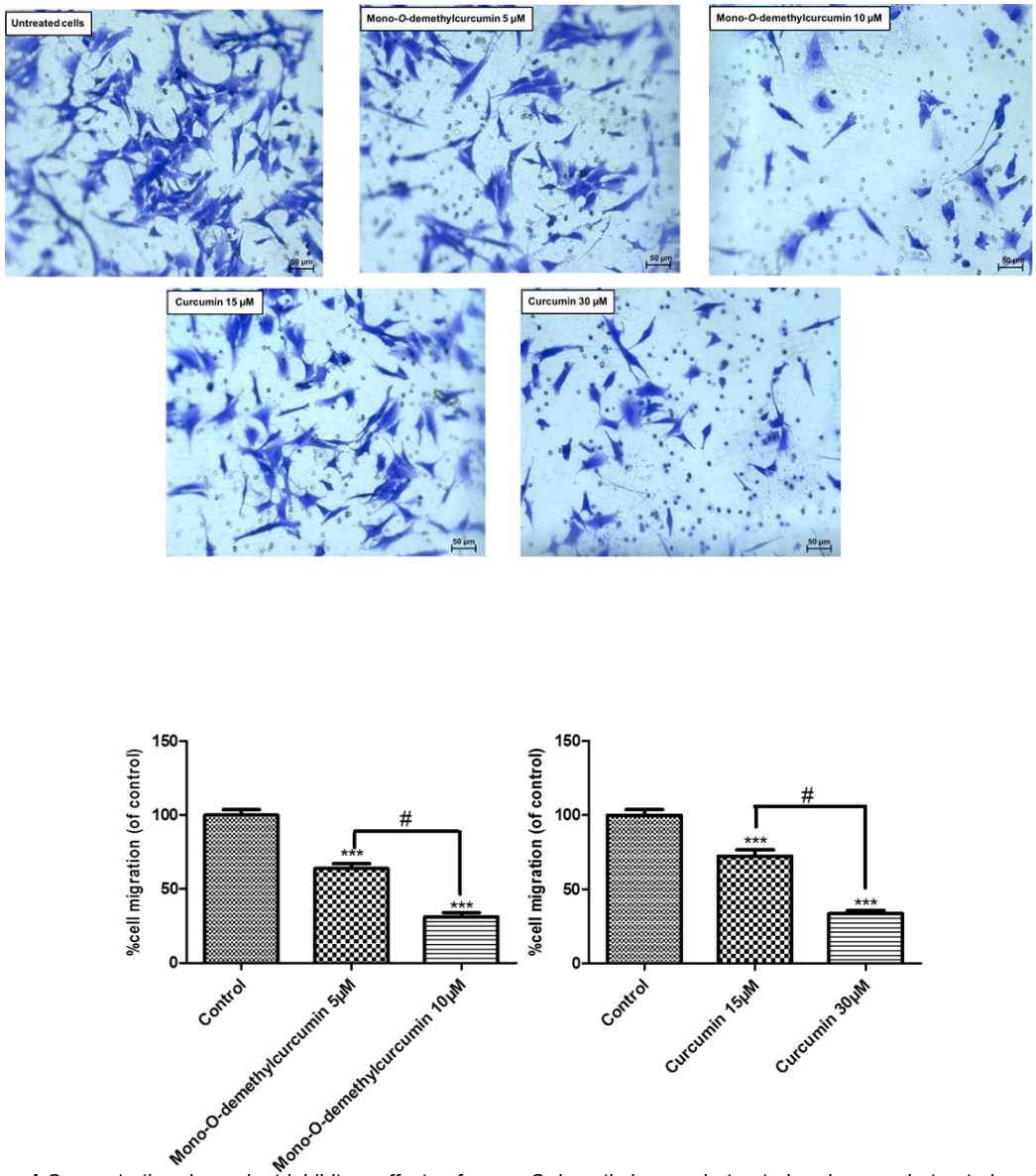


Figure 4 Concentration-dependent inhibitory effects of mono-*O*-demethylcurcumin-treated and curcumin-treated on CLS-354/DX cell migration. The graphs represent CLS-354/DX cell migration ability after 24 h treatment with 5μM and 10μM mono-*O*-demethylcurcumin and 15μM and 30μM curcumin. Values were calculated by Graphpad prism 5. Data are reported as mean ± SEM, n= 3. Student t-test analysis was used for statistical analysis. ***p< 0.001 compared with control group (Untreated cell), #p value< 0.001 compared with lowest concentration of group. The photographic image (20x) represents the number of migrated CLS-354/DX cells.

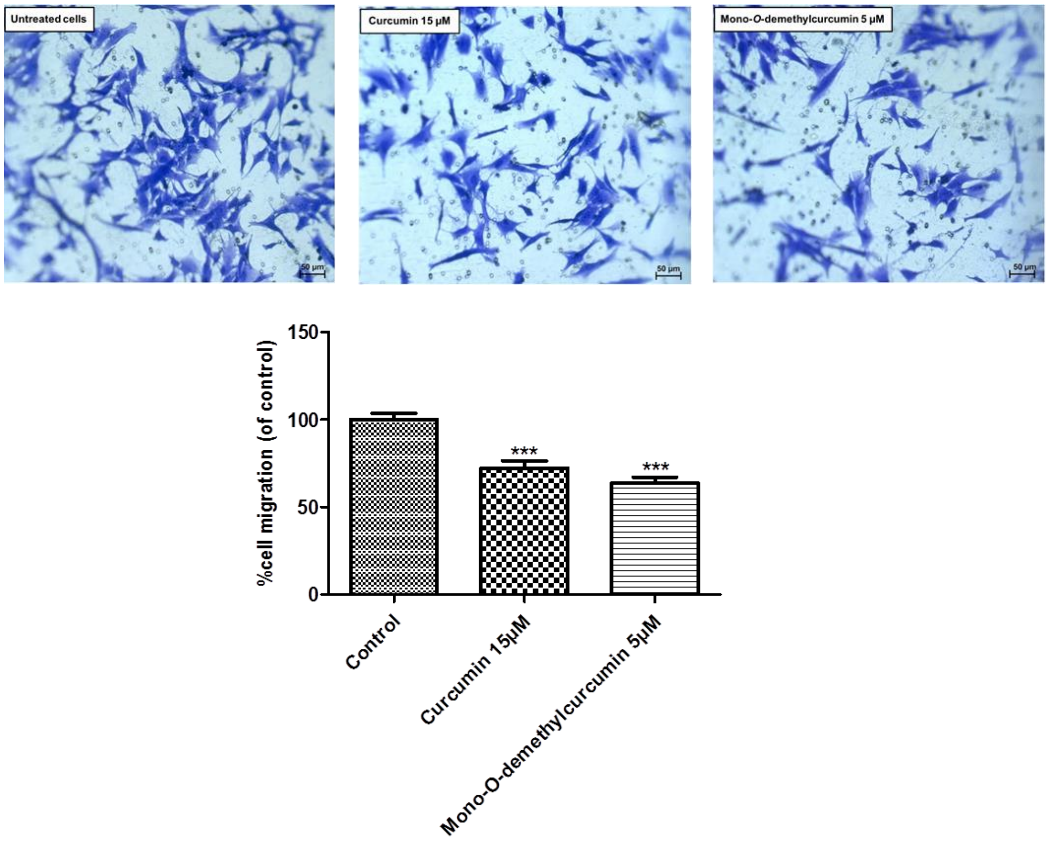


Figure 5 Comparison migration inhibitory effects of mono-*O*-demethylcurcumin and curcumin in CLS-354/DX cell line. The graphs represent CLS-354/DX cell migration ability after 24 h treatment with IC_{10} concentrations of mono-O-demethylcurcumin (5µM) and curcumin (15µM). Values were calculated by Graphpad prism 5. Data are reported as mean ± SEM, n= 3. Student t-test analysis was used for statistical analysis. ***p< 0.001 compared with control group (Untreated cell). The photographic image (20x) represents the number of migrated CLS-354/DX cells.

Mono-*O*-demethylcurcumin inhibited CLS-354/DX cell invasion

The effects of mono-*O*-demethylcurcumin on CLS-354/DX cell invasion ability were investigated by using IC_{10} concentrations with a standard invasion chamber assay. Concentrations of 5µM of Mono-*O*-demethylcurcumin and 15µM of curcumin were added into the upper compartment of the invasion chamber. After incubation at 37°C, 5% CO_2 for 22h, the number of invasive cells was counted and the cell invasion rate was calculated as percent of control. The results showed that mono-*O*-demethylcurcumin decreased number of CLS-354/DX invasion about 11.40±2.82% While, curcumin decreased number of CLS-354/DX invasion about 21.70±4.39% (Figure 6).

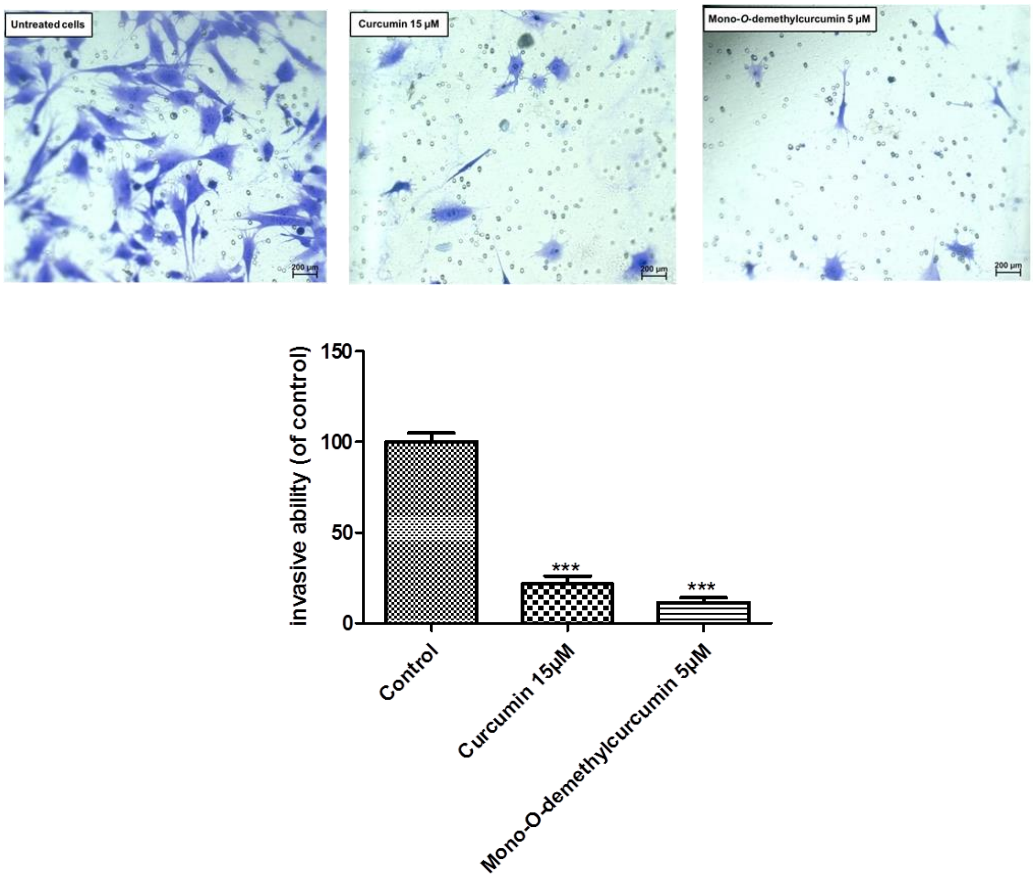


Figure 6 Invasion inhibitory effects of mono-O-demethylcurcumin and curcumin in CLS-354/DX cell line. The graphs represent CLS-354/DX cell invasion ability after 22 h treatment with IC_{10} concentrations of mono-O-demethylcurcumin (5µM) and curcumin (15µM). Results expressed as the mean invasive ability ± SEM microscopic field. Values were calculated by Graphpad prism 5. Student t-test analysis was used for statistical analysis. ***p< 0.001 compared with control group (Untreated cell). The photographic image (20x) represents the number of invasive cells was determined using an invasion chamber assay.

Discussion and Conclusions

Curcuma longa, (*C. longa*) or turmeric, is comprised of a group of three major curcuminoids; curcumin (77%), demethoxycurcumin, (17%) and bisdemethoxycurcumin (3%). Curcumin is the main constituent found in the rhizomes of the plant. Polyphenols are responsible for the medicinal effects of these compounds [9, 24]. Mono-*O*-demethylcurcumin is a very minor natural curcuminoid. However, Mono-*O*-demethylcurcumin can be synthesized by removal of a methyl group from curcumin by demethylation with a 43% yield of synthesis [25]. Numerous previous studies have provided evidence of the anti-carcinogenic properties of curcumin in many cancers, both *in vitro* and *in vivo*, including oral and head and neck cancers [9]. Curcumin inhibits cell proliferation and induces cell cycle arrest and apoptosis in various cancer cells i.e. prostate, breast, pancreatic, lymphoma and oral squamous cell carcinoma, by targeting multiple pathways [26-29]. The half maximal inhibitory concentration values (IC_{50}) of curcumin in invasive cancer cell lines are between 20-40 µM [19, 30, 31]. In this study, the IC_{50} of curcumin in an invasive multidrug-resistant oral squamous cell carcinoma cell line (CLS-354/DX) was about 45 µM. This high inhibitory dose of curcumin limits its use in therapeutic application so curcumin analogs have attracted considerable attention. Curcumin analogs have shown potential anti-cancer effects and have induced cell apoptosis better than parental curcumin [15-19]. Compared with curcumin, curcumin analogs have better metabolic stability and pharmacological activity [17]. In this study, mono-*O*-demethylcurcumin (curcumin anolog) exhibited better anti-proliferation activity, as evaluated by MTT assay, than curcumin. Multidrug resistance (MDR), including cisplatin resistance, is the most critical problem leading to therapeutic failure of head and neck cancer. Tthe IC_{50} of cisplatin in the multidrug resistant CLS-

354/DX cell line in our study was 33.23±0.96μM, which is consistent with previous reports [23]. In the current study, the results also show that mono-*O*-demethylcurcumin exhibited a better anti-proliferative effect than the standard drug cisplatin. Moreover, mono-*O*-demethylcurcumin appears to have limited cytotoxicity to normal cells. Mono-*O*-demethylcurcumin was nontoxic to a human gingival fibroblast cell line at concentrations up to 10μg/mL or 28.22μM [32]. In an immortalized rat microglial cell line, mono-*O*-demethylcurcumin at concentrations of 20–40μM could decrease cell viability significantly in a concentration dependent manner. However, at concentrations of 10μM viability was similar to the untreated control [33].

Multiple organ failure caused by metastasis is a major cause of death in cancer patients. In fact, prognosis of cancer is mainly determined by the invasiveness of the tumor and its ability to metastasize. Although there are several drugs available to control cancer growth in humans, there are no drugs presently available to specifically inhibit the metastasis of cancer cells. Thus, active compounds demonstrating anti-invasive and anti-metastatic properties are defined as a new catalog of chemopreventive agents [34, 35]. Curcumin has been reported to inhibit invasiveness, metastasis and epithelial-mesenchymal transition in many cancer cell lines such as oral, lung, thyroid and colon cancer cell line through a number of different cellular pathways. For example, curcumin inhibits lung cancer invasion and metastasis by attenuating the GLUT1/MT1-MMP/MMP2 pathway [14], curcumin inhibits tumor epithelial‑mesenchymal transition by downregulating the Wnt signaling pathway and upregulating NKD2 expression in colon cancer cells [15], curcumin inhibits the invasion of thyroid cancer cells via down-regulation of the PI3K/Akt signaling pathway [16]. However, There were are few reports about anti-invasiveness and anti-metastasis activity of curcumin analogs, and there are no reports about the anti-invasiveness and anti-metastasis activity of mono-*O*-demethylcurcumin in invasive oral squamous cell carcinoma. In this study, mono-*O*-demethylcurcumin (curcumin analog) was found to inhibit the migration and invasion activity of invasive multidrug-resistant oral squamous cell carcinoma evaluated by migration and invasion chamber assays. However, the underlying mechanism still needs further study to be elucidated.

Taken together, the results in this study suggest that mono-*O*-demethylcurcumin exhibited excellent anti-cancer activity on invasive multidrug-resistant oral squamous cell carcinoma. Mono-*O*-demethylcurcumin could possess anti-proliferation, anti-migration and anti-invasion activity against other cell lines. Although, further study is required to evaluate both the underlying mechanism and to evaluate the anti-cancer activity in an *in vivo* study, these results show that mono-*O*-demethylcurcumin has potential as a chemotherapeutic agent for multidrug-resistant oral cancer.

Acknowledgements

All work was supported by Funding agency: WU57305 and WU58118, Institute of Research and Development, Walailak University and School of Allied Health Sciences, Walailak University.

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Fatty Acid Composition of Sacha Inchi (*Plukenetia volubilis* L.) Oil and Efficacy of Sacha Inchi Lotion

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Abstract

Sacha inchi (*Plukenetia volubilis* L.) or “Inca peanut” is widely cultivated in Northern Thailand provinces such as Phayao, Lampang, Chiang Mai, and Chiang Rai due to the appropriate geographical location and climate. The aims of this study were to determine the fatty acid composition of Sacha inchi oil cultivated in Chiang Rai and evaluate the efficacy of Sacha inchi lotion after topical application to skin. Sacha inchi seed oil was prepared by using soxhlet extraction with *n*-hexane (1:20 w/v). GC-MS analysis was used for analyzing fatty acid composition. Sacha inchi oil was used as an active ingredient in lotion and evaluated by 30 volunteers for its efficacy after application to skin. The main fatty acids in Sacha inchi oil were linoleic acid (45.72%), linolenic acid (42.27%), palmitic acid (6.42%) and stearic acid (4.53%). Sacha inchi lotion containing 2.5% Sacha inchi oil was formulated. The lotion showed no irritation after closed patch test. Sacha inchi lotion increased skin moisture and elasticity significantly ($p<0.001$) after 7 days application and maintained these effects for 28 days. After 28 days application, a significant decrease ($p<0.05$) in melanin value was observed. The Sacha inchi lotion was found to be superior to the placebo control. Sacha inchi oil contains high amounts of linoleic and linolenic acid. The application of Sacha inchi lotion significantly improved skin parameters after 7 days and for 28 days. The result of this study confirm the benefit of Sacha inchi oil for use in cosmetics, which might increase the value of Sacha inchi.

Keywords: *Plukenetia volubilis* L., Sacha inchi, Fatty acid, Lotion, Skin moisturizer

Introduction

Sacha inchi (*Plukenetia volubilis* L.) or “Inca peanut” is a perennial plant of the Euphorbiaceae family. It grows in Amazonian rainforests ranging from the heights of 200 to 1500 m in an environment with water and well-drained acidic soil [1]. The plant is a wild, semi-woody, perennial, oleaginous vine with slightly hairy leaves [2]. It has a star-shaped fruit, which contains dark oval seeds 1.5 - 2.0 cm in diameter (Figure 1). The Amazon natives produce flour and oil from Sacha inchi seeds. They use the products for preparation of different meals and beverages. It has also been used medically in treating rheumatic problems and aching muscles. The oil is also used for cosmetic purposes by women of the native Amazonian tribes [3]. It is traditionally used as everyday skin care oil applied to preserve skin softness and healthiness [1].

According to previous studies, Sacha inchi seed oil has been extracted by various methods such as subcritical fluid extraction with *n*-propane [4], supercritical carbon dioxide extraction [5], hot pressed [6], cold pressed [7] and soxhlet extraction [8]. As reported by those studies, Sacha inchi seed is a good source of oil (35-60%) and proteins (25–27%). The oil contains high levels of essential polyunsaturated fatty acids (PUFAs), namely linolenic acid (C18:3 or omega 3) and linoleic acid (C18:2 or omega 6), representing about 47–51% and 34–37% of total oil content, respectively [2, 7]. Other fatty acid such as oleic acid, palmitic acid, and stearic acid are also present in Sacha inchi seed oil.

PUFAs are not synthesized by the human body. Thus, they must be introduced by food or by cosmetic product application [9]. PUFAs are believed to revitalize the skin and give skin a youthful appearance. A previous report found that PUFAs may restore dry skin conditions as well as have therapeutic effects on skin disorders, such as dry skin, desquamation (scaly skin), wounds failing to heal, loss of humidity, erratic keratinization processes (blocking of follicles), increasing rates of mitosis (disorganized skin layers), tendency towards eczema and itchiness [10].

Recent studies indicate a high content of tocopherols (1500-2000 mg/kg) in Sacha inchi seeds that could act as antioxidant agents. Antioxidant agents that display a strong antioxidant activity are considered to promote human health, since they are responsible for critical biological functions [1, 6]. Furthermore, the amino acid profile of Sacha inchi protein fraction showed relatively high levels of

cysteine, tyrosine, threonine, and tryptophan [11]. Moreover, polyphenolic compounds were also detected in Sacha inchi oil derived from cold pressing of the seed; among them, phenyl alcohol, flavonoid, seicoridoid, and lignan classes [7].

Sacha inchi was first grown in Thailand 5-6 years ago in Nong Khai province and Maejo University in Chiang Mai has found that Sacha inchi is able to grow in every part of Thailand, Sacha inchi is now widely cultivated in Thailand, especially in Chiang Rai province. Sacha inchi oil can be added to many formulations such as moisturizing cream and anti-aging lotion. There are many Sacha inchi creams or lotions sold in the market. However, very few studies have been published examining the efficacy of Sacha inchi cream on skin.

Phytochemical screening of Sacha inchi oil derived from soxhlet extraction (with *n*-hexane) of Sacha inchi grown in Chiang Rai revealed the presence of flavonoids and DPPH free-radical scavenging activity [12]. Accordingly, the aims of this study were to evaluate the fatty acid composition of Sacha inchi oil, formulate Sacha inchi oil lotion and evaluate the product efficacy after applying to skin. The results of this study might support further use of Sacha inchi oil in cosmetics and increase the value of this oil.

Figure 1 Visual appearance of Sacha inchi seeds

Methodology

Plant material

Dried Sacha inchi seed (Figure 1) was purchased from Chiangrai Agriculture Development Co., Ltd., Tambon Nanglae, Meuang, Chiang Rai, Thailand. The seed was dried in a hot air oven (Ontherm/06503) at 55ºC until a constant weight was achieved (about 72 h). The moisture content was measured by using a moisture analyzer (Ohaus/MB45).

Chemical and reagents

All the chemicals used in the formulation were cosmetic grade. *n*-Hexane was purchased from Sigma Chemicals Co., USA.

Sacha inchi seed oil extraction

The dried Sacha inchi seed was ground in a blender (Panasonic/ MX-J210GN) and extracted by the soxhlet extraction method of Sawatpakdee and Wuttisin (2017). Soxhlet extractor capacity of 500 ml was used and the extraction time was 5 h using *n*-hexane at a solid to solvent ratio of 1:20 (w/v). The solvent was evaporated at 50°C under reduced pressure by rotary evaporation (Eyela/CCA-1110) and left under a fume hood (48 h) to evaporate the solvent residue. The percentage yield was calculated. Sacha inchi oil was stored at 4ºC until used.

Determination of fatty acid composition by Gas chromatography-mass spectrometry (GC-MS)

The fatty acid composition was determined by converting all fatty acids of triglycerides into their corresponding methyl esters followed by GC-MS analysis [13]. Sacha inchi oil (200 µl) was methylated with 0.5% NaOH in methanol (10 ml) and then incubated at 60ºC for 10 min. The methyl esters were extracted with *n*-hexane (8 ml) for 1 min. The *n*-hexane layer was washed with 4 ml distilled water and dried over anhydrous sodium sulfate. Analysis was carried out with gas chromatography (Agilent 6890N) equipped with a capillary column (HP-5MS, 0.25 mm × 30 m × 0.25 µm) and connected to mass selective detector (MSD model MS 5973N). Helium was used as carrier gas. Oven temperatures were set at 140ºC (5 min), 200ºC (15 min) and 250ºC (4.5 min). The injector and detector temperatures were set at 220ºC. Data analysis was carried out using the wiley7n.1 and Pest.1 libraries.

Formulation of lotion containing Sacha inchi oil

Lotion containing Sacha inchi oil was prepared as shown in Table 1. Distilled water was heated up to 75°C and Carbomer 934 was added (2% dispersion). The other

water soluble components were added and the mixture was heated to 80°C. The ingredients in part B were mixed together and heated to 80°C before being slowly mixed into the aqueous phase with continuous stirring until cooling down to 30-40°C. Phenoxyethanol and fragrance were added and mixed until uniform. The control lotion (base) was formulated using the same procedure without Sacha inchi oil.

Table 1 Development of Sacha inchi lotion

Ingredients	**%w/w**	
	Control lotion	**Sacha inchi lotion**
Part A		
Distilled water	72.50	72.50
Carbomer 934 (2% dispersion)		
Glycerin	14.00	14.00
Triethanolamine (99%)		
Part B		
Stearic acid		
Cetyl alcohol		
Glycerylmonostearate		
Isopropylpalmitate	8.25	8.25
PEG-40 stearate		
Petrolatum USP		
Mineral oil	5.00	2.50
Sacha inchi oil	-	2.50
Part E		
Phenoxyethanol	0.25	0.25
Fragrance	q.s.	q.s.

Physical properties of Sacha inchi lotion

The pH of Sacha inchi lotion was measured by using a pH meter (Mettler Toledo Seven Easy S20). The viscosity of Sacha inchi lotion was measured by a viscometer (Brookfield RVD-II+P) at 15 rpm under ambient temperature using spindle no.5 (30 sec). The phase separation was tested by centrifugation (Spectrafuge/16M) at 5000 rpm for 30 min.

Patch test

Patch test was performed on inner forearms of 30 volunteers to determine skin irritation. Control lotion and Sacha inchi lotion were put on Finn chambers® and compared with 1% sodium lauryl sulfate (positive control) and distilled water (negative control). After 24 hours occlusion, the patch was removed and observed for any skin redness or irritation. The scores were recorded for the presence of erythema using a score of 5 points, ranging from 0-4; where 0 = absence of erythema, 1 = mild erythema, 2 = redness, 3 = swelling and 4 = severe erythema [14].

Human skin efficacy test

Sacha inchi lotion was evaluated for it efficacy by 30 volunteers, age 21-23 years old who has no history of dermatological diseases or cosmetics allergies. This study was approved by the Ethics in Human Research Committee of Mae Fah Luang University (No. REH-59085).

Efficacy of the Sacha inchi lotion was evaluated using a previously described method [15]. Volunteers were instructed not to use moisturizers, body lotions, and or occlusive cosmetic preparations on the area tested for 12 h prior to the *in vivo* study. All subjects rested in a room maintained at 25±1°C and 40-60% relative humidity for 15 min prior to performing the efficacy test. Skin monitoring was evaluated by using Corneometer® CM 825 (skin moisture), Mexameter® MX 18 (melanin value), and Cutometer® MPA 580 (skin elasticity). Baseline skin data were recorded prior to applying test compounds. The short term skin moisturizing effect was determined after Sacha inchi lotion application for 15, 30 and 45 min compared with control lotion. In addition, volunteers were assigned to apply

control lotion and Sacha inchi lotion for twice a day in 2 areas (1×1 inch/each) at the upper inner arm in randomized single-blind procedure. Skin parameters were recorded before and after using at 7th, 14th, 21st, and 28th day and compared with baseline (initial).

Data analysis

The data were expressed as mean±S.D. of triplicate measurements. The statistical analysis was determined by paired-sample t-test statistics and independent samples test via program IBM SPSS statistics version 21 (Licensed for Mae Fah Luang University).

Results

Sacha inchi seed oil extraction

Sacha inchi oil has an orange-yellow color. The moisture content of Sacha inchi seed before drying at 55ºC was 6.9±0.46% and after drying was 4.3±0.51%, which is similar to a previous reports by Follegatti-Romero *et al*. (2009) [5], Gutiérrez *et al*. (2011) [8] and Chirinos *et al*. (2013) [6]. Thus, the percentage of moisture content was within the range of 0-13% reported to be suitable for storage and processing without microorganism degradation of the triacylglycerol [5]. The percentage yield was 43.55%.

Fatty acid compositions of Sacha inchi oil

Fatty acid composition of Sacha inchi oil was measured by GC-MS. The results in Figure 2 and Table 2 show that the main fatty acids found in Sacha inchi oil were linoleic acid (RT:23.47 min, m/z 279.3, 45.72%), linolenic acid (RT:23.73 min, m/z 277.3, 42.27%), palmitic acid (RT:18.70 min, m/z 255.3, 6.42%), and stearic acid (RT:24.40 min, m/z 283.4, 4.53%).

Physical properties of Sacha inchi lotion

The pH of Sacha inchi lotion was 5.5. The viscosity was 11,506±59 cP. Sacha inchi lotion and control lotion both had a white color as shown in Figure 2. After 1 month storage there was no change in color or pH and no phase separation occurred.

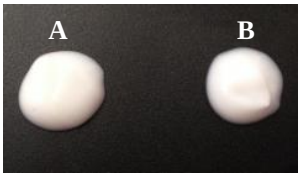

Figure 2 Sacha inchi lotion (A) and control lotion (B)

Patch test

All 30 volunteers reported no irritation and no severe erythema after the closed patch test and during the 1 month use for both control lotion and Sacha inchi lotion. This indicated that Sacha inchi lotion was safe for long-term use.

Human skin efficacy and satisfaction test

A short term skin moisturizing effect was determined after application of Sacha inchi lotion for 15, 30 and 45 min compared with control lotion. The results in Table 3 show that Sacha inchi lotion increased skin moisture after application and prolonged this moisturizing effect for 45 min ($p<0.05$, compared with initial) while base lotion only increased skin moisture after 15 min application ($p<0.05$, compared with initial).

The skin improvement after continuous use was measured as skin moisture, skin elasticity and melanin value. The results in Table 4 show that Sacha inchi lotion significantly increased skin moisture and elasticity after 7 days application ($p<0.001$) and maintained these effects during 28 days use. Moreover, melanin value also significant decreased ($p<0.05$) after 28 days application.

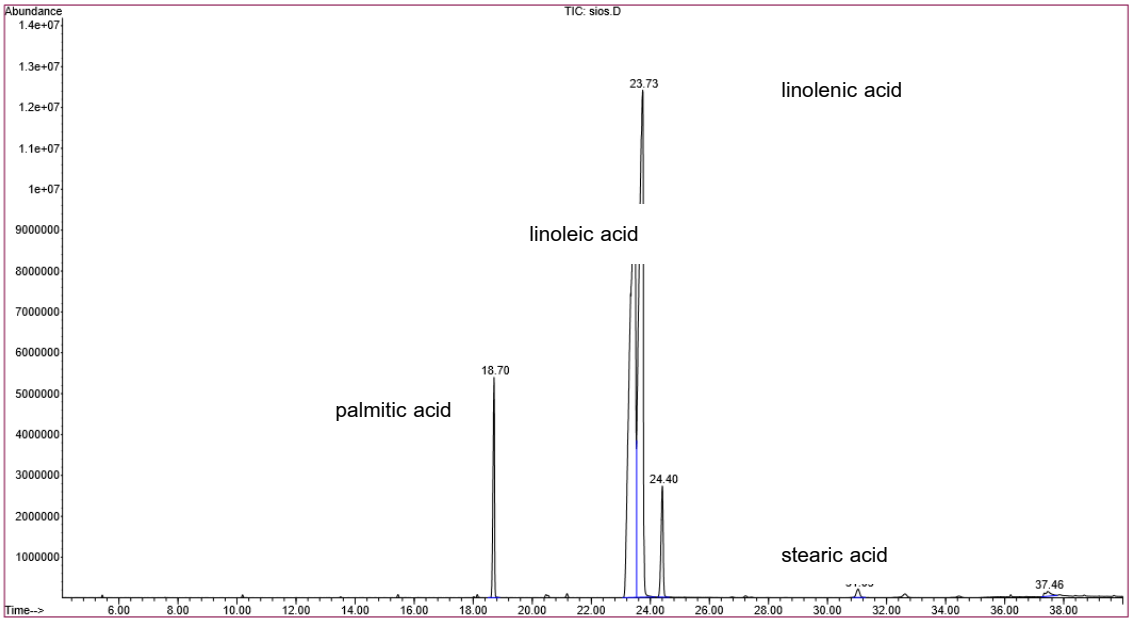

Figure 2: Chromatogram showing fatty acid composition of Sacha inchi oil analyzed by GC-MS

Table 2 Fatty acid composition of Sacha inchi oil.

Linolenic	Linoleic	Palmitic	Stearic	Oleic	Reference
42.27	45.72	6.42	4.53	-	Present study
45.20	36.80	4.50	3.20	9.60	Hamaker *et al*., 1992
50.41	34.08	4.24	2.50	8.41	Follegatti-Romero *et al*., 2009
50.80	33.40	4.40	2.40	9.10	Gutiérrez *et al*., 2011
46.80	36.20	4.30	3.00	9.00	Fanali *et al*., 2011
12.80-16.00	12.40-14.10	1.60-2.00	1.10-1.30	3.50-4.70	Chirinos *et al*., 2013
44.00	40.00	4.00	3.00	9.00	Liu *et al*., 2014
48.32	34.10	4.70	3.30	8.90	Cisneros *et al*., 2014
48.00	35.00	5.00	2.00	9.00	Gonzalez-Aspajo *et al*., 2015
-	40.45	41.29	7.63	-	Sawatpakdee and Wuttisin, 2017

Table 3 Short term skin moisturizing effect after Sacha inchi lotion application.

Time (min)	Control lotion		Sacha inchi lotion	
	Value	ΔChange (Min, Max)	Value	ΔChange (Min, Max)
Initial	68.40±11.22	-	55.48±7.52	-
15	81.80±6.05*	13.41±7.59 (3.99,22.83)	73.37±10.24*	17.89±11.68 (3.38,32.40)
30	78.66±5.41	10.27±12.22 (-4.90,25.44)	77.59±8.32*	22.11±7.34 (12.99,31.23)
45	80.35±8.25	11.95±8.24 (1.72,22.19)	79.11±11.13*	23.63±10.72 (10.33,36.94)

ΔChange = After-Initial. Values are expressed in Mean±SD (n=5). * Significantly difference ($p<0.05$) when compared with initial.

Table 4 Skin efficacy evaluation after Sacha inchi lotion application.

Time (day)	Control lotion		Sacha inchi lotion	
	Value	ΔChange (Min, Max)	Value	ΔChange (Min, Max)
Skin moisture				
Initial	51.43±14.38	-	46.42±13.53	-
7th	56.87±16.88	5.44±8.34 (2.33,8.56)	49.68±13.79**	3.26±1.83 (2.58,3.95)
14th	59.05±14.82	7.62±12.14 (3.09,12.16)	61.48±11.93**	15.06±10.46 (11.16,18.97)
21st	65.16±14.31**	13.73±14.09 (8.47,18.99)	67.11±13.57**	20.68±12.10 (16.16,25.20)
28th	65.04±12.72**	13.60±13.22 (8.67,18.55)	77.08±10.37**	30.66±11.92 (26.21,35.11)
Elasticity				
Initial	1.00±0.13	-	0.91±0.08	-
7th	1.00±0.10	-0.01±0.08 (-0.04,0.02)	1.08±0.16**	0.17±0.19 (0.10,0.24)
14th	0.95±0.09	-0.06±0.08 (-0.09,-0.03)	1.05±0.17**	0.14±0.21 (0.06,0.22)
21st	0.96±0.08	-0.04±0.07 (-0.07,-0.01)	1.12±0.13**	0.21±0.18 (0.15,0.28)
28th	1.02±0.08	0.01±0.10 (-0.02,0.05)	1.40±0.19**	0.50±0.23 (0.41,0.58)
Melanin value				
Initial	175.73±31.13	-	181.42±34.81	-
7th	192.48±38.26	16.75±20.79 (8.99,24.51)	186.41±34.45	4.99±15.65 (-0.85,10.83)
14th	183.01±31.45	7.28±19.58 (-0.02,14.60)	172.34±31.10	-9.08±19.87 (-16.50,-1.66)
21st	176.50±31.25	0.77±12.41 (-3.86,5.40)	185.23±30.60	3.82±23.39 (-4.92,12.55)
28th	167.94±31.67*	-7.79±14.09 (-13.05,-2.52)	165.20±30.37**	-16.21±21.69 (-24.31,-8.11)

ΔChange = After-Initial. Values are expressed in Mean±SD (n=30). ** Significantly difference ($p<0.001$) when compared with initial. * Significantly difference ($p<0.05$) when compared with initial.

Discussion and Conclusion

In Thailand, Sacha inchi is cultivated in many areas for seeds to consume because it is rich in omega-3 or linolenic acid, which is reported to be beneficial to health [17, 18]. In this study, Sacha inchi seed oil was prepared by using soxhlet extraction with *n*-hexane. Fatty acid composition was determined and we found the presence of linoleic (45.72%), linolenic (42.27%), palmitic (6.42%), and stearic acids (4.53%). The fatty acid composition of Sacha inchi oil in this study was quite different to previous studies, as shown in Table 2. The present study shows that Sacha inchi oil cultivated in Chiang Rai contains high levels of linoleic acid and linolenic acid, which makes it a rich source of omega-6 and omega-3 fatty acids. The differences that we saw in the fatty acid content might be due to using different cultivars, geographical effects, changes in climate and growing conditions, the harvesting time of the seeds [19] and the processing (e.g. roasting prior to extraction) as well as differences in the method of oil extraction [6] and the quantification methods used in the analysis.

At present, Sacha inchi oil is added to many cosmetic formulations such as moisturizing creams, anti-aging lotions, and body oils. These products are distributed widely in local markets around Thailand without scientific testing of their efficacy. In the present study, the Sacha inchi lotion containing 2.5% Sacha inchi oil was formulated and tested by applying to volunteers' skin. Topically applied Sacha inchi lotion was found to increase skin moisture and elasticity. Sacha inchi lotion increased skin moisture significantly ($p<0.05$) after application and prolong the moisturizing effect for 45 min while base lotion that contained mineral oil increased skin moisture significantly ($p<0.05$) only after 15min application. This indicated that Sacha inchi lotion could prolong the moisturizing effect for longer than base lotion. The reason for this might be due to the occlusive effect of Sacha inchi seed oil blocking

transepidermal water loss (TEWL) from the skin surface, thereby increasing hydration of the stratum corneum. Additionally, the humectant activity of Sacha inchi lotion is likely to contribute to the moisturizing effect because it helps to retain moisture from the environment and restore water to the skin. Sacha inchi oil contains high levels of linoleic, linolenic and palmitic acids. These fatty acids are the most frequently used fatty acids in cosmetic products. They are emollients and have occlusive properties to reduce TEWL through the skin, mainly by means of making a protective layer on the epidermis. They also soften the stratum corneum and reduce inflammation of the skin. [20]. Linoleic acid is key part of ceramide I which is the most important barrier substance in the horny layer of the epidermidis. Linolenic acid improves skin moistening, activates regeneration of damaged lipid barrier of the epidermis, heals inflammation and stabilizes skin metabolism [20-21]. It also stimulates the synthesis of barrier lipids of the skin and proteins precursors of natural moistening factors [20]. Palmitic acid is an important component of both the skin barrier and the acid layer of the skin [21]. It has the most potent skin permeation enhancing effect, which is used mainly as the vehicle for other active ingredients [22-23].

When skin is routinely exposed to stressful factors from the environment, such as UV radiation, smoke, and pollutants, an elevated number of free radicals will be produced, which accelerates skin aging [24]. Free radical activation results in the production of collagenases which degrade collagen by kinase pathways [25]. Based on this theory, an effective approach to delay skin aging is to externally supply antioxidants through skincare products to either suppress production or neutralize excess free radicals [26]. Sacha inchi oil contains antioxidant compounds such as tocopherols, phenolics and flavonoids [6, 12, 17]. Thus, application of sacha inchi lotion is an effective approach to delay the skin aging process.

Additionally, a decrease of melanin in the skin was observed after 28 days of Sacha inchi lotion application. This might be due to the UV absorption property of Sacha inchi oil. Previous studies have found that Sacha inchi oil has UV-absorption properties between 260-320 nm (UVB) and might be used as sunscreen application [8, 12].

Decreases in skin moisture and elasticity are considered to promote wrinkle formation. Exposure to UV radiations also results in skin damage [25]. Thus, Sacha inchi lotion might be effective as anti-aging product. Further investigations are needed for determining the other active chemical components of Sacha inchi. The efficacy of pure Sacha inchi oil on skin also needs to be investigated. In addition, studies about the suitable conditions for Sacha inchi cultivation in Thailand are recommended to determine how to control the quality of the raw material.

In conclusion, Sacha inchi oil contains important quantities of essential fatty acids especially linoleic and linolenic acid. Sacha inchi lotion had significant effects on skin, enhancing skin moisture, increasing skin elasticity and decreasing melanin content. Consequently, Sacha inchi oil is suitable to use as moisturizer and emollient for anti-aging, whitening and as a sun screen product. Finally, this present study indicates that Sacha inchi oil is an active ingredient for cosmetics which can be safely used on the skin.

Acknowledgements

This study was supported by funding from Mae Fah Luang University.

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Bioactivity Screening of *Curcuma bicolor* Extracts for Cosmetic Applications

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Abstract

Curcuma bicolor is one of the plants in the Zingiberaceae family found in northern Thailand. It is used as an ornamental plant and there is no study of their chemical contents or biological activities. Thus, this study aimed to find suitable extraction solvents for this plant, and determine their total phenolic contents and biological activities (free radical scavenging activity, reducing capacity, antimicrobial activity, and anti-inflammation activity). Leaves and stems of *C. bicolor* were extracted with ethanol, water and ethanol/water (the volume ratios of 1:1, 2:1 and 3:1). All extracts were then investigated for their total phenolic content by Folin-Ciocalteu method, free radical scavenging activity by ABTS method, reducing capacity by FRAP assay, anti-microbial activity by broth dilution and anti-inflammation activity by nitric oxide assay. The results showed that the 2:1 ethanol/water extract had the highest extraction yield (39.8%). The ethanol/water extracts showed higher phenolic contents and better bioactivities than those extracted with pure solvents. The 3:1 ethanol/water extract exhibited the highest total phenolic content (12.202 ± 0.37 mg GAE/ g extract) and ABTS radical scavenging activity (43.122 ± 0.724 mg TEAC/ g extract). However, these values were not significantly different from those of the 1:1 ethanol/water extract. For the FRAP assay, the ethanol extract possessed the highest reducing capacity. No extracts inhibited the growth of bacteria, but the 2:1 ethanol/water, 3:1 ethanol/water and ethanol extracts showed inhibition of *C. albicans* (MIC = 0.128 mg/mL). In addition, the extracts did not inhibit NO production in murine macrophage J774.A1 cells. In conclusion, *C. bicolor* extracts may have potential for use as antioxidants and as preservatives against yeast in cosmetics, but the extracts appeared to have no antibacterial and anti-inflammation properties.

Keywords: *Curcuma bicolor*, Total Phenolic Contents, Antioxidant, Anti-Microbial, Anti-Inflammation

Introduction

Humans face many harmful factors, such as sun exposure, stress, poor nutrition, pollutants, and microorganisms, all of which can lead to health degeneration and skin damage. Free radicals are the major cause of skin problems such as wrinkles, dark spots, and aging. Accumulation of free radicals causes damage and oxidative stress which leads to the alteration of biochemical and cellular processes and accumulated aging [1]. Moreover, various microorganisms cause skin problems such as acne and inflammation [2,3].

The role of plant extracts in solving skin problems is gaining more and more attention. They are considered as safer alternatives to synthetic products. Numerous cosmetic products are commercially available for skin aging prevention, UV protection, anti- inflammation, hyperpigmentation, and anti-acne [4-6].

Zingiberaceae is the ginger family of flowering plants made up of more than 1,300 species of aromatic perennial herbs with creeping horizontal or tuberous rhizomes [7]. Many plants in this family have been reported to have bioactive compounds and bioactivities. For example, *Curcuma longa* contains vitamin C and beta-carotene which possess anti-oxidant, anti-inflammatory, anti-viral and anti-fungal activities [8]. Various phytochemicals in *C. sessilis* Gage flower bract extract, such as vitamins, polyphenols, flavonoids and anthocyanins, exhibit high performance anti-oxidant activity [9]. *C. xanthorrhiza* flower bract extract was

found to be a tyrosinase inhibitor, a good anti-oxidant and an antibacterial with activity against *P.acnes* [10].

Curcuma bicolor is one of the plants in the Zingiberaceae family. It is a rhizomatous herb 40-60 cm tall, with terminal inflorescences on pseudostems, 8-20 cm long; bracts orange-red, lanceolate, corolla lobes red; staminodes and labellum orange-yellow with red in lower half (Figure 1a). It grows in deciduous forest and limestone regions in northern of Thailand, such as Doi Tung and Doi Inthanon. It blooms from July to September [11]. This plant is utilized as an ornamental plant. To our knowledge, no research has been reported into the bioactive compounds and bioactivity of *C. bicolor* extract. So, it is interesting to study the bioactivities from this plant, especially in the leaves and stems. Thus, the aims of this study is to find suitable solvents for extraction of *C. bicolor* and to determine total phenolic content, free radical scavenging activity, reducing capacity, antimicrobial activity, and anti-inflammation activity.

(a) (b)

Figure 1 (a) *C. bicolor* appearance, (b) Dried leaves and stems of *C. bicolor*

Materials and Methods

Materials

All chemicals used were analytical grade unless otherwise specified. The solvent for extraction, 95% Ethanol, was purchased from Quality reagent chemical, New Zealand. Amphotericin B, 2, 2'-azinobis (3-ethybenzothiazoline-6-sulfonic acid (ABTS)), Gallic acid, Gentamycin B, Resazurin, Acetic acid, Hepes free acid, Sodium pyruvate, Penicillin G, Streptomycin, Dimethyl sulfoxide (DMSO) Cell grade, Naphthylethylenediamine dihydrochloride (NED), Sulfanilamide, and 3-[4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium] bromide (MTT) were purchased from Sigma-Aldrich corporation, USA. Sodium Carbonate (Na_2CO_3), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), 2,4,6-Tripyridyl-s-Triazine (TPTZ) $FeCl_3 \cdot 6H_2O$, and Sodium acetate trihydrate were purchased from Merck, Germany. Folin-Ciocalteu reagent was purchased from Loba Chemie Pvt Ltd., India. Potassium persulfate ($K_2S_2O_8$) was purchased from Fujian Zhan Hua Chemical Co., Ltd, China. Dulbecco's modified Eagle's medium (DMEM), Fetal bovine serum (FBS), and Lipoprotein Solution (LPS) was purchased from HyClone Laboratories, Inc., USA.

Preparation of the plant extract

Leaves and stems of *C. bicolor* were collected from Doi Tung, Chiang Rai, Thailand in September, 2015. The leaves and stem were washed and air-dried (Figure 1b) before being crushed into small pieces**.** The dried leaves and stem (30 g) were then macerated with water, ethanol and ethanol/water (ethanol/water were mixed at the volume ratios of 1:1, 2:1 and 3:1) at room temperature for 72 hours. After triplicated macerating, the mixtures were filtrated. The mixtures were then concentrated by evaporation under reduced pressure and lyophilized using a freeze dryer to obtain crude extracts. The dried extracts were stored at -20°C until use. The percentage yields were calculated on the dry weight basis as follows.

$$\text{Percentage yield} = \frac{\text{Weight of crude extract (g)}}{\text{Weight of dry plant used (g)}} \times 100$$

Total phenolic content

The total phenolic content of the extracts was determined using the Folin-Ciocalteu method [12] with slight modification. Briefly, 20 µL of the sample in DI water (3 mg/mL) and 100 µL of Folin-Ciocalteu reagent (10 %v/v) were pipetted into a 96-well microplate. Then 80 µL of 7.5 %w/v sodium carbonate solution was added. The mixture was made up to 200 µL with water and incubated for 1 hour at ambient temperature. The absorbance was measured at 765 nm using a microplate reader (Spectrostar Nano, Germany). Gallic acid solution (0.0125 - 0.1 mg/mL) was prepared as a standard. All experiments were carried out in

triplicate. The total phenolic contents of the extracts were expressed as gallic acid equivalents (GAE).

Free radical scavenging activity

The ABTS assay was used to determine free radical scavenging activity of the extracts [12]. ABTS solution (7 mM) and $K_2S_2O_8$ (2.45 mM) were mixed in a ratio of 1:1 and allowed to stand at ambient temperature in the dark for 12-16 hours. Then the mixture was diluted 24 times with DI water to obtain the absorbance in range 0.700 ± 0.02. Twenty µL of sample (1.5 mg/mL) and 180 µL of ABTS solution were pipetted into 96-well microplates. Distilled water was used as a control. The mixture was incubated in dark at ambient temperature for 5 minutes. The absorbance was measured at 734 nm using microplate reader. All of assessments were carried out in triplicate. The results were expressed in mg Trolox equivalent antioxidant capacity per gram of extract.

Reducing capacity

The reducing capacity of the extracts was determined by Ferric reducing antioxidant power (FRAP) assay according to Butsat and Siriamornpun [13] with slight modifications. The FRAP solution was prepared by mixing 300 mM acetate buffer, pH 3.6 (25 mL), 10 mM TPTZ solution in 40 mM HCl (2.5 mL) and 20 mM $FeCl_3$·6H2O solution (2.5 mL). The sample solutions (10 µL) were allowed to react with the FRAP reagent (190 µL) and then incubated at room temperature for 15 minutes in dark. The absorbance of the mixture was measure at 593 nm. The standard curve was linear between 10 and 150 µg/ml Trolox. The reducing capacity was expressed in mg Trolox equivalent antioxidant capacity (TEAC) per gram of extract.

Anti-microbial activity

Antimicrobial activities of all *C. bicolor* extracts were assessed by determining the minimum inhibition concentration (MIC). According to Priya *et al*. [14], MIC determinations were performed by using serial 2-fold broth dilution method. There were seven bacterial strains and one fungal strain: four gram positive bacteria (*Bacillus cereus* TISTR 687; *Staphylococcus aureus* TISTR 1466; *Staphylococcus epidermidis* DMST 15505; *Micrococcus luteus* TISTR 884), three gram negative bacteria (*Escherichia coli* TISTR 780; *Salmonella typhimurium* TISTR 292; *Pseudomonas aeruginosa* TISTR 781) and one yeast; *Candida albicans* TISTR 5779). All bacterial and fungal strains were obtained from the Department of Biology and Biotechnology Laboratory, Mae Fah Luang University. The inoculum was standardized to 0.5 McFarland (approximately 1.5 x 10^8 CFU/mL) and diluted with media (1:200). The standards and extracts were tested in the concentration range of 0.001 - 0.128 mg/mL and 0.01 – 1.28 mg/mL, respectively. Gentamicin and amphotericin B were used as standard drugs for bacteria and fungi, respectively. After incubation at 37 ºC for 18 hours, resazurin was added as an indicator of cell viability, and plates were left for 3 hours. Wells were assessed visually for the colorimetric change from blue to pink, with the highest dilution remaining blue indicating the MIC. The visual assessment was performed in duplicate.

Nitric oxide assay

The nitric oxide (NO) assay was performed as described previously [15]. The murine macrophage J774.A1 cell line was kept in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 100 mM sodium pyruvate, Hepes free acid, penicillin G, and streptomycin. The J774.A1 cells (5×10^4) were incubated for 1 h and pretreated with compounds or vehicle (DMSO) for another 2 h. After pre-incubation of the cells with LPS (10 µg/mL) for 18 h, the quantity of NO production in the culture medium was measured using Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride in distilled water). Briefly, the reagent was mixed with equal volumes of cultured medium and subsequently incubated at room temperature for 10 min. The absorbance at 540 nm was measured in a microplate reader. Percentage inhibition of NO production was calculated as follows.

% inhibition of NO production = 100 - % NO production in the culture medium

MTT assay for cell viability

The measurement of cell viability was performed by using the MTT (4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay with some modification. Briefly, after cultivation and treatment procedure, 10 µL of the MTT (5 mg/mL in saline) solution was added to each well and incubated under the same conditions for another 3 h. MTT was converted into visible formazan crystals during incubation by mitochondrial succinate dehydrogenase in live cells. The crystals of formazan were dissolved in DMSO, and the absorbance was measured at 540nm. Relative cell viability was calculated by comparing the absorbance of the treated group to the untreated control group. All experiments were performed in triplicate. [24]

Statistical analysis

The results were represented as the mean ± SD of

three independent experiments (n = 3). ANOVA was used for the analysis of the test results (LSD test) and Duncan's multiple-range test at the significance level of $P < 0.05$. The correlation between total phenolic contents and ABTS free radical scavenging activity were determined by R-square (R^2) from the linear regression equation.

Results and Discussion

C. bicolor extracts

Five extracts of *C. bicolor* were obtained; ethanol extract, 3:1 ethanol/water extract, 2:1 ethanol/water extract, 1:1 ethanol/water extract, and water extract. The ethanol extract was a viscous semi-solid while the other extracts were a powder. The extracts were green to deep green when the solvent contained higher amounts of ethanol and brown to deep brown when the solvent contained higher amounts of water. This is because of the different polarities of the solvents, which could extract different polarity compounds [16]. The yields of all extracts are shown in Table 1. The 2:1 ethanol/water extract had the highest extraction yield with 39.8%, followed by the 1:1 ethanol/water, 3:1 ethanol/water, ethanol and water extracts, respectively. These results indicate that most of the phytochemicals in *C. bicolor* could be extracted by using the 2:1 ethanol/water mixture, which is the most appropriate solvent based on the highest extraction yield and also for economic considerations.

Table 1 Percentage yield of *C. bicolor* extracts

Extracts	Yield (%)
Ethanol	1.33
3:1 ethanol/water	12.50
2:1 ethanol/water	39.80
1:1 ethanol/water	18.30
Water	1.11

Total phenolic contents and antioxidant activities

Phenolic compounds are phytochemicals which have been reported to have a range of bioactivities, such as antioxidant [8, 9] and anti-bacteria [8, 10] activity. These properties can be used for cosmetics and determination of the total phenolic content in a plant is often the first screening method for cosmetic ingredients. *C. bicolor* extracts had phenolic content in the range 5.433 ± 0.513 to 12.917 ± 0.336 mg GAE/g extract, as shown in Table 2. The total phenolic content in the extracts was higher when mixed ethanol-aqueous solutions were used as the extraction solvent. This is related to Shi *et al*. [17], who reported that the concentration of ethanol in water influenced the yield of extracted phenolic compounds [18]. It was confirmed that the mixture of ethanol and water could extract significantly more potential bioactive compounds from plants than water and absolute ethanol. The most abundant group of phenolic compounds in this genus is curcuminoids (diphenylheptanoids), which are natural hydrophobic polyphenols [21]. They have been shown to exhibit antioxidant, anti-inflammatory, and anti-bacterial activities [22]. Curcuminoid extraction is supported by a mixed ethanol and water solvent that helps to improve their solubility and reduce the dielectric constant of the water [23].

Table 2 Total phenolic contents and antioxidant activities of *C. bicolor* extract (n=3)

Extracts	Total phenolic contents (mg GAE/ g extract)	ABTS scavenging activities (mg TEAC/ g extract)	Reducing capacities (mg TEAC/g extract)
Ethanol	5.433 ± 0.51[a]	30.949 ± 0.079[a]	645.05 ± 8.26[a]
3:1 ethanol/water	12.917 ± 0.34[b]	43.122 ± 0.724[c]	264.86 ± 9.36[b]
2:1 ethanol/water	12.202 ± 0.37[b]	35.895 ± 1.291[b]	261.26 ± 13.60[b]
1:1 ethanol/water	12.337 ± 0.20[b]	42.497 ± 1.085[c]	252.25 ± 11.25[b]
Water	8.281 ± 0.07[c]	42.580 ± 1.032[c]	45.05 ± 8.26[c]

* Data followed by different letters with each column are significantly different according to Duncan's multiple range tests at $P < 0.05$.

Reactive oxygen species (ROS) are an important factor for generating skin problems such as wrinkles, atypical pigmentation and inflammation. The human body has several endogenous oxidative stress-eliminating systems. Antioxidants help to prevent oxidative stress by eliminating ROS [21]. Evaluating the antioxidant activity of plant extracts is a way to find extracts that can be used as natural cosmetic ingredients.

Extraction with water and ethanol/water solutions showed better ABTS scavenging activities than that with pure ethanol. The result was in according to Boonpisuttinant *et al*. [16], who reported that using different solvents for extraction could lead to the different phytochemical contents obtained. Comparing with other plants in Zingiberaceae family, the total phenolic content of *C. bicolor* in 3:1 ethanol/water is higher than that of *C. parviflora* and *C. sessilis* extracts [9] but lower than that of *C. amada* [19]. For reducing activities, the ethanol extract posed the highest value suggesting it to be richer in antioxidant reductants, followed by 3:1 ethanol/water, 2:1 ethanol/water, 1:1 ethanol/water and water extracts. Comparing with other plants, the ethanol extract of *C. bicolor* exhibited reducing capacity higher than methanol extracts of *C. aeroginosa* rhizomes, *Z. officinale* rhizomes, and *C. longa* rhizomes [20].

The difference in ethanol/water ratio did not show significant difference in total phenolic content and reducing capacities ($P < 0.05$). Therefore, it had no effect on the phytochemical contents and bioactivity of *C. bicolor* extract. The uncorrelated results of total phenolic contents, ABTS scavenging activities and reducing capacities indicated that antioxidant activity of *C. bicolor* extract may not be only due to their phenolic compounds. However, the extracts seem to be possible to be used as a natural antioxidant.

Anti-microbial activity

Cosmetics can be spoiled or become harmful to consumers if they're contaminated with microorganisms, such as certain bacteria and fungi. In the search for a safe preservative, plant extracts offer an interesting alternative. The anti-microbial activities of *C. bicolor* extracts are shown in Table 3. The ethanol, 3:1 ethanol/water, and 2:1 ethanol/water extracts inhibited *C. albicans* growth at an MIC of 0.128 mg/mL. This antimicrobial activity was specific for *C. albicans* and can be compared to Amphotericin B which inhibited *C. albicans* at an MIC of 0.016 mg/ml. No extracts showed antibacterial activity against any of the seven Gram positive and Gram negative bacteria, all extracts had an MIC greater than 1.28 mg/mL.

Table 3 Antimicrobial activities of *C. bicolor* extract

Samples	MIC (mg/mL)							
	Gram positive bacteria				**Gram negative bacteria**			**Yeast**
	BC	**SA**	**SE**	**ML**	**EC**	**ST**	**PA**	**CA**
Ethanol	>1.28	>1.28	>1.28	>1.28	>1.28	>1.28	>1.28	0.128
3:1 ethanol/water	>1.28	>1.28	>1.28	>1.28	>1.28	>1.28	>1.28	0.128
2:1 ethanol/water	>1.28	>1.28	>1.28	>1.28	>1.28	>1.28	>1.28	0.128
1:1 ethanol/water	>1.28	>1.28	>1.28	>1.28	>1.28	>1.28	>1.28	>0.128
Water	>1.28	>1.28	>1.28	>1.28	>1.28	>1.28	>1.28	>0.128
Gentamycin	>0.128	0.016	0.008	0.016	0.008	0.008	0.128	>0.128
Amphotericin B	-	-	-	-	-	-	-	0.016

BC = *Bacillus cereus* TISTR 687; SA = *Staphylococcus aureus* TISTR 1466; SE = *Staphylococcus epidermidis* DMST 15505; ML= *Micrococcus luteus* TISTR 884; EC = *Escherichia coli* TISTR 780; ST = *Salmonella typhimurium* TISTR 292; PA = *Pseudomonas aeruginosa* TISTR 781; CA = *Candida albicans* TISTR5779

Anti-inflammatory activity

In murine macrophage J774.A1 cells, LPS has been demonstrated to induce NO production while DMSO does not induce NO production. Therefore, this cell system can be used to screen extracts for potential inhibitors of NO production. The inhibitory activities of extracts toward NO production by LPS-activated macrophages are shown in Figure 2.. The 1:1 ethanol/water, 2:1 ethanol/water and 3:1 ethanol/water extracts showed 36.65 – 49.03 % inhibition of NO production at a concentration of 10 µg/mg. The ethanol and water extracts showed less than 15% inhibition of NO production. As determined by MTT assays (Figure 3), the numbers of viable macrophages revealed that any inhibitory effect of the extracts was not due to cytotoxicity (cell viability > 90%). The result indicated that all extracts had poor potential to use as anti-inflammation ingredients.

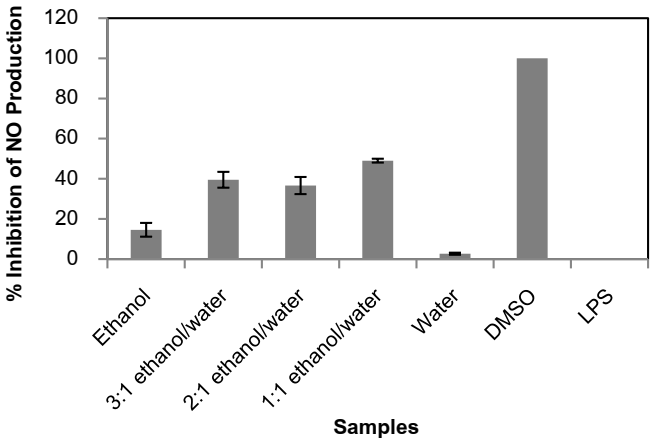

Figure 2 Percentage inhibition of NO production by the extracts and controls at the concentration of 10 µg/mg

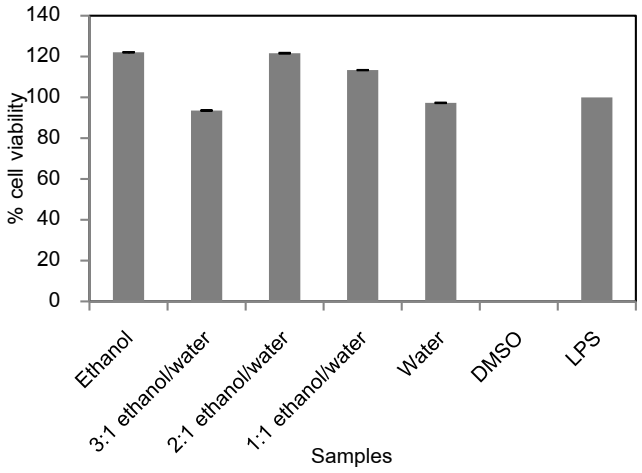

Figure 3 Cytotoxicity of the extracts and controls at the concentration of 10 µg/mg

Conclusions

The present study has shown that the use of mixed ethanol/water solvents produced *C. bicolor* extracts with higher overall phytochemical content and increased bioactivity compared to water and ethanol used alone. The ratio of ethanol/water did not significantly affect the total phenolic content and antioxidant activity of extracts. All extracts did not inhibit bacterial growth or inflammation. The extracts, especially the ethanol, 3:1 ethanol/water and 2:1 ethanol/water extracts, may have potential to be a moderate antioxidant and yeast (*C. albicans*) inhibitior for healthcare and cosmetic applications.

Acknowledgements

This study was partly financial supports by Mae Fah Luang University, Thailand for fiscal year 2016.

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Phytochemical, Composition and Antioxidant Activity of *Cucumis melo* L. By-Products

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Abstract

By-products or the wastes from many agricultural processing industries have value due to richness of various compounds. Phytochemical, cosmetic composition and free radical scavenging activity of *Cucumis melo* L. var. *green sweet* peel and seed were investigated. The extraction was done with *n*-hexane, absolute ethanol, propylene glycol and deionized water using maceration. Phytochemical screening of extracts revealed the presence of tannins, saponins, flavonoids, cardiac glycosides, phenols, and quinones. Quantitative determination of total phenolic (TPC) and total tannin content (TTC) were determined using Folin-Ciocalteu method and total flavonoid (TFC) content was determined using aluminium chloride complex forming assay. The result showed that absolute ethanol had the maximum value of TPC with 5.00 ± 0.00 µgGAE/g for peel extract, and the maximum value of TTC with 4.27 ± 0.10 µgGAE/g for seed extract, while deionized water showed the maximum value of TFC with 6.38 ± 0.07 mg QE/g for peel extract. The antioxidant activity (ABTS assay) was carried out using colorimetric method and expressed in term of IC_{50} (inhibitory concentration of extract in µg/mL that reduces ABTS absorbance to 50% as compared to negative control). The results showed that deionized water extract from peel exhibited the highest antioxidant potential (8.87 ± 0.03 µgAAE/g extract). Therefore, a correlation between flavonoid content and antioxidant activity by ABTS was observed. The present study indicated that the peel and seed of *C. melo* L. var. *green sweet* has antioxidant property for further cosmetic application.

Keywords: *Cucumis melo* L. var. *green sweet*, Phytochemical Screening, Total Phenolic Content, Total Flavonoids Content, Antioxidant Activity.

Introduction

Many agricultural processing industries generate 10-60% of raw materials as waste and some of these were proven to be more valuable than the main products. The waste products that could be recycled as valuable products for industry are called “by-products”. These by-products are mainly from skins, seeds, stems, leaves, waste waters, and unusable pulps which are normally discarded [1]. It is well-known that by-products are rich in sugars, minerals, organic acids, dietary fiber, bioactive compounds such as polyphenols and carotenoids [2]. Utilization of by-products in biotechnological fields including cosmetics, pharmaceutics or foods will increase them into high value products because they are inexpensive, effective, and bio-sustainable.

Cucumis melo L. , commonly called melon, cantaloupe, or honeydew, belongs to the family of Cucurbitaceae. It is popular worldwide due to its sweet-scent and flavor. In Thailand, melon is becoming more popular. It mostly cultivated in the rainy to winter season. Harvesting at 80-90 days will yield the best flavor. Its chemical composition depends on the cultivar, environmental conditions, and also on the stage of fruit maturity [3].

Melon is a significant source of phytochemicals, mainly polyphenols and other antioxidants which provide potential health benefits, especially aiding the cardiovascular system [4]. The extracts from different parts are also valuable for cosmetic ingredients due to containing various phytochemical constituents such as tannins, flavonoids, glycosides, minerals and vitamins that exhibit antioxidant, anti-inflammatory, antimicrobial, antiphlogistic, antiallergic and UV protective activities [5-10].

In addition, previous research indicated that melon pulp extract provides high antioxidant and anti-inflammatory properties. Antioxidants play an important role in defending the body against free radical damage. Antioxidants refer to a group of compounds that are able to delay or inhibit the oxidation of lipids or other biomolecules and thus, prevent or repair the damage to the body cells that is caused by oxygen [11-12] They work by preventing the formation of new free radical species, converting

existing free radicals into less harmful molecules and preventing radical-chained reactions [13].

Therefore, the objectives of this study were to determine the phytochemical constituents by screening test, the phenolic content, the flavonoid content, the tannin content, and antioxidant activity from two parts of melon; peels and seeds extracted with different solvents for application in the cosmetic field in the future.

Methodology

Plant material

(A) Peels (B) Seeds

Figure 1 Peels (A) and seeds (B) of *C.melo* L.

The five fresh fruits of *C. melo* L. var. *greensweet* (melon) were cultivated and harvested during September to November 2016 by Ozone Farm, Mae Chan Sub-district at Chiang Rai Province, Thailand. The five melon fruits were separated into 3 parts; pulp, peel and seed, and calculation for percentage content is shown in Table 1. This study was particularly interested in melon peels (A) and seeds (B) (Fig 1). The peels and seeds were dried in an oven at 40°C for 24 h, then ground to small pieces by using a mechanical blender for extraction.

Chemicals

Absolute ethanol, aluminium chloride, ferric chloride, glacial acetic acid, n-hexane, olive oil, potassium persulfate, propylene glycol, sodium carbonate, sodium hydroxide, sodium nitrite, and 98% sulfuric acid were used from Cosmetic laboratory, Mae Fah Luang University, Thailand. 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), gallic acid, and quercetin were purchased from Sigma-Aldrich Corporation, United States. L-Ascorbic acid was purchased from Chem-Supply Pty. Ltd., Australia. Folin-Ciocalteu was purchased from Loba Chemie Pvt. Ltd., India.

Preparation of sample

The dried and small pieces of peels and seeds (10 g) were macerated with 50 mL of the solvents; *n*-hexane, absolute ethanol, propylene glycol, and deionized water, within an incubator shaker at 30 °C, 150 rpm for 24 h. The extracts were filtrated through Whatman No.1 filter paper, and stored at 4°C in the tight-sealed dark containers for experimental use later.

Percentage yield calculation

Calculation of the percentage yield of extracts was carried out by using the equation [%yield = (gram of crude extract/gram of sample) ×100]. The crude extract was obtained from the difference in weight between the plant extract and its solvent and the plant samples were adjusted equally (50 g).

Phytochemical screening

The preliminary phytochemical analysis was carried out for all the crude extracts to identify various bioactive chemical constituents using the standard procedures [14-16].

Test for tannins

About 1 mL of the extract was boiled in 5 mL of deionized water. A few drops of 0.1% ferric chloride solution were added. A brownish-green or blue-black coloration indicated the presence of tannins [16].

Test for saponins

About 1 mL of the extract was mixed with 5 mL of deionized water and shaken vigorously for a fairly stable froth. Three drops of olive oil were added into the frothing and shaken vigorously for a few minutes. The formation of

a stable emulsion indicated the presence of saponins [15].

Test for flavonoids

About 1 mL of the extract was mixed with 5 mL of deionized water and shaken vigorously. A few drops of 10% aqueous sodium hydroxide were added to produce a yellow coloration. A subsequent change in color from yellow to colorless indicated the presence of flavonoids [15].

Test for cardiac glycosides (Keller-Killiani test)

About 1 mL of the extract was mixed with 5 mL of deionized water and shaken vigorously. Two mL of glacial acetic acid and one drop of ferric chloride solution were added. This was underlaid with 1 mL of concentrated sulphuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while a greenish ring may form above the brown ring and gradually spread through the acetic acid layer [14].

Test for phenols

About 2 mL of the extract was mixed with 5 mL of deionized water and shaken vigorously. A few drops of ferric chloride solution were added. A bluish-green or red coloration indicated the presence of phenols[16].

Test for quinones

About 2 mL of the extract was mixed with 5 mL of distilled water and shaken vigorously. A few drops of concentrated sulphuric acid or aqueous sodium hydroxide solution were added. A color formation indicated the presence of quinones [16].

Total Phenolic Content

Determination of the total phenolic content (TPC) of extracts was carried out with the Folin-Ciocalteu method as described by [17] with some modifications. Gallic acid was used as standard (0.15 mg/mL) and stock standard solutions of gallic acid (0.015, 0.03, 0.06, 0.12, 0.24, 0.75, 1.05 and 1.47 mg/mL) were prepared. Then 0.02 mL of extract was added and mixed with 0.1 mL of Folin-Ciocalteu reagent. The mixture was allowed to stand at room temperature for 5 min. Then, 0.08 mL of sodium carbonate (75 mg/mL) was gently added to the mixture. After standing at room temperature for 1 h, the absorbance was estimated at 765 nm using a microplate reader (BMG LABTECH/SPECTROstar Nano). The TPC was expressed as gallic acid equivalents (GAE) in mg/g of extracts. The concentration of polyphenols in samples was derived from a standard calibration curve of gallic acid ranging from 0.015-1.47 mg/mL.

Total Flavonoid Content

The total flavonoid content of extracts was determined with the aluminium chloride complex forming assay as described by [18] with slight modifications. Quercetin was used as standard (10 mg/mL) and diluted for the concentration from 2 to 6 µg/ml with absolute ethanol. Twenty µl of each of the quercetin dilutions was mixed with 100 µl of distilled water then 20 µl of 5% sodium nitrate was added, incubated at room temperature for 6 minutes. After that, 30 µl of 10% aluminium chloride solution was added and incubated at room temperature for 5 minutes. Then, 40 µl of 1M sodium hydroxide solution was added. The absorbance of the reaction was measured at 510 nm using a microplate reader(BMG LABTECH/SPECTROstar Nano). Total flavonoid content was expressed as quercetin equivalents (mgQE/g).

Total Tannin Content

The tannins were determined by the Folin-Ciocalteu method as described by [19] with slight modifications. Gallic acid was used as standard (1 mg/mL). A set of reference standard solutions of gallic acid (0.2-2 mg/mL) were prepared. A 0.02 mL aliquot of sample extract was added and mixed with 0.1 mL of Folin-Ciocalteu reagent. The mixture was allowed to stand at room temperature for 5 min. Then, 0.08 mL of sodium carbonate (3.5 g/L) was gently added to the mixture. After standing at room temperature for 30 min, the absorbance was measured at 725 nm using a microplate reader(BMG LABTECH/SPECTROstar Nano). The TTC was expressed in terms of mg of gallic acid equivalents (GAE) /g of extracts.

Antioxidant activity by ABTS assay

2, 2′-Azinodi-3-ethylbenzthiazoline sulphonate (ABTS) assay followed the method of [20] with some modifications. The stock solutions of 7 mM ABTS and 2.45 mM potassium persulfate in deionized water were prepared and kept at 4°C until use. Fresh working solution was prepared for each assay by mixing the two stock solutions in equal quantities and incubating for 16 h in the dark under ambient temperature. Then, 1 mL of the working solution was mixed with 29 mL deionized water, to obtain an absorbance of 0.7±0.02 units at 734 nm by using a microplate reader(BMG LABTECH/SPECTROstar Nano). The extracts and standard solution (100 µL) were allowed

to react with 100 µL of the ABTS working solution and incubated for 5 min in the dark under ambient temperature before the absorption was measured at 734 nm. Ascorbic acid was used as a standard and antioxidant activity was expressed as µg of ascorbic acid equivalents (AAE) per g of extract weight (µg AAE/g).

Statistical analysis

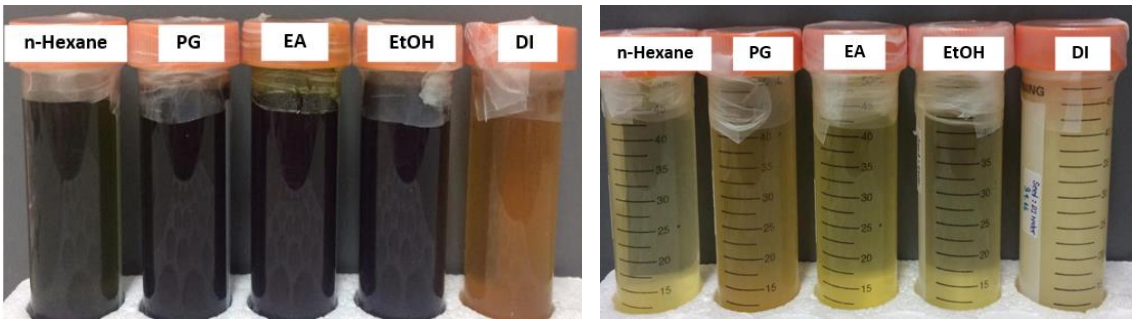
The analyses were carried out in triplicate and regarded to have statistic significant with $P<0.05$. The results are presented as mean ± standard deviation (n=3). The significance of difference was defined by one way analysis of variance (ANOVA) in which the difference between means was determined by Duncan's multiple range test using the SPSS program (Version 24).

Results and discussion

Percentage content of five *C. melo* fruits

The quantity of three parts (pulps, peels and seeds) of five melon fruits were calculated in term of percentage content. Melon pulp has the highest amount (81.49%), followed by seeds (9.71%) and peels (7.3%). The total content of these fruits does not equal to 100%, because of water was loss (1.5%) during the cutting process. Because peel and seed were by-products, or the waste from agricultural processing, both part were selected for further study.

Characteristic of extracts and percent yield



(A) Peel extracts (B) Seed extracts

Figure 2 The physical appearance of *C. melo* L. peel extracts (A) and seed extracts (B) with different solvents

The *C. melo* L. peel extracts (Fig 2A) presented different colors according to the solvents used for extraction; *n*-hexane extract was olive green, propylene glycol and absolute ethanol extracts were a dark green color, while deionized water extract was light brown. For seed extracts (Fig 2B); *n*-hexane and absolute ethanol extracts were clear light yellow, propylene glycol extract was darker yellow, and deionized water had a white turbid appearance. The results indicated that the percentage yield for seeds with propylene glycol was higher than absolute ethanol, followed by deionized water and *n*-hexane respectively. For peel extracts, percentage yield for absolute ethanol was higher than propylene glycol, followed by deionized water, while the percentage yield for *n*-hexane yield was zero, as shown in Table 1. Different yields of extracts might be influenced by the nature of the solvents used for extraction, especially in polarities and total time of extraction [21-22]...

Table 1 Percent yield of *C. melo* L. peel and seed with different solvent for extraction.

Solvent	% yield	
	Peel	Seed
n-Hexane	3.33 ± 0.29	0.00 ± 0.00
Absolute ethanol	13.33 ± 0.76	8.33 ± 0.29
Propylene glycol	11.67 ± 0.76	18.33 ± 1.53
Deionized water	5.00 ± 0.50	5.00 ± 0.50

Phytochemical screening

Phytochemical screening of this plant revealed some differences in constituents of two parts; seeds and peels as shown in Table 2. The result of absolute ethanol extract confirmed the presence of tannins, saponins, cardiac glycosides, flavonoids, phenols and quinones in peel, whereas seed extract exhibited the absence of cardiac glycosides.

Table 2 Phytochemical constituents of peel and seed extracts.

Phytochemical constituents	*n*-Hexane		Absolute Ethanol		Propylene glycol		DI water	
	Peel	Seed	Peel	Seed	Peel	Seed	Peel	Seed
Tannins	-	-	+	+	+	+	+	+
Saponins	-	-	+	+	+	+	-	-
Cardiac glycosides	+	+	+	-	+	-	-	-
Flavonoids	-	+	+	+	+	+	+	+
Phenols	-	-	+	+	+	+	+	+
Quinones	+	+	+	+	+	+	+	-

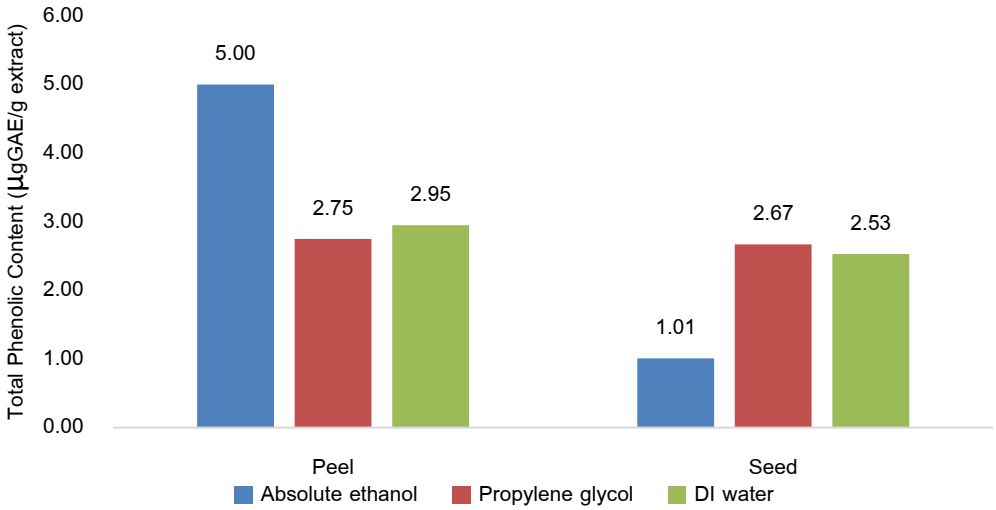
Note: (+); presence, (-); absence.

Seed extract with propylene glycol was also absent for cardiac glycosides, while peel extract contained all phytochemicals. Furthermore, seed extracted by deionized water indicated tannins, flavonoids, and phenols, while peel extract was absent for saponins and cardiac glycosides.

These secondary metabolites have been shown to have therapeutic activities for the treatment of disease [23]. Tannins exhibit the antioxidant, antimicrobial and antiviral effects [24]. Saponins have anti-inflammatory activity [25]. Flavonoids and phenolics play an important role to prevent damage of tissue by activated oxygen species [26]. Cardiac glycosides are known to decrease blood pressure [27]. Due to the low extractive yield and limited phytochemical constituents of *n*-hexane extract, and because of the additional regulatory framework required for hazardous materials, only ethanol, propylene glycol and deionized water extracts were selected for further testing.

Total phenolic content

The result of total content of phenolic compounds in the *C. melo* L. extracts are expressed in term of μgGAE/g extract as shown in Fig 3. The highest total phenolic content was in absolute ethanol extract from peel by 5.00 ± 0.00 μgGAE/g extract, which means that 1 gram of this extract contains phenolic compounds equivalent to 5.00 μg of gallic acid. This was followed by deionized water and propylene glycol extracts. It is harmonized with a previous study [28] which reported that total phenolic content of skin extract was greater than seed extract.



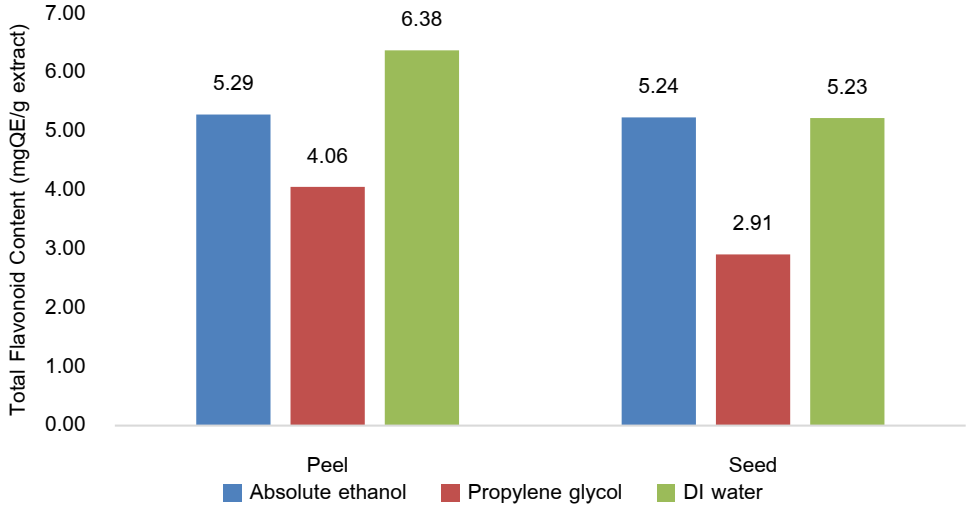
Note: different letters above the columns present significant differences (P<0.05)

Figure 3 Total phenolic content of *C. melo* L. from peel and seed extracts.

Moreover, a previous study identified phenolic compounds in *C. melo* L. peels. The high-performance liquid chromatography analysis indicated that there was a high complexity in the melon peel phenolic composition. They indicated that hydroxybenzoic acid is the major phenolic compound followed by apigenin-7-glycoside. In addition, hydroxybenzoic acids and flavones constitute their main phenolic classes [29]. A previous study used reversed phase high performance liquid chromatography with diode array detection (HPLC-DAD) method to identify phenolic compounds in seed extracts. Gallic acid and its derivative, hydroxybenzoic acid and catechin derivatives and caffeic acid were found in water extract. While nine phenolic compounds were identified in methanol-water extract; caffeic acid, two vanillic acid derivatives, ellagitanins, quercetin-3-rutinoside, derivatives of syringic acid and ellagic acid [30].

Total flavonoid content

The result of total flavonoid content in C. *melo* L. extracts are expressed in term of mgQE/g extract as shown in Fig 4. Deionized water extract from peel was the highest flavonoid content by 6.38 ± 0.07 mgQE/g extract, which means that 1 gram of this extract contains flavonoid content equivalent to 6.38 mg of quercetin. The high value of flavonoid was also found in absolute ethanol extract from both samples and deionized water from seed, but they were not different significantly. The lowest flavonoid content was seed extracted with propylene glycol by 2.91 ± 0.15 mgQE/g extract. As the result, the flavonoid content in this study harmonizes with a previous study [28] that reported the total flavonoid content of skin or peel extract was higher than seed extract.



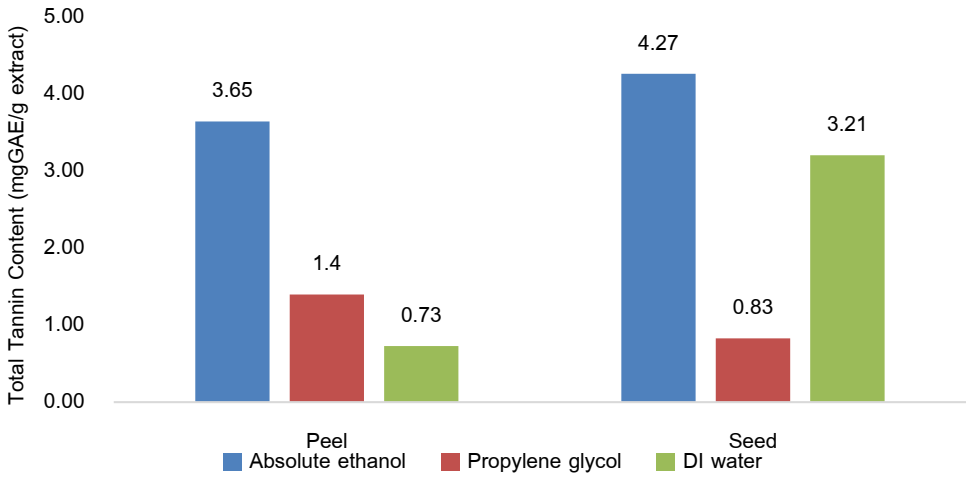
Note: different letters above the columns present significant differences (P<0.05)

Figure 4 Total flavonoid content of *C. melo* L. from peel and seed extracts.

Flavonoid is an antioxidant constituent and possesses a broad spectrum of biological and chemical activity including radical scavenging activity [31]. In addition, flavonoids have been reported to have chelating activity.

Total Tannin Content

The results of total tannin content from peel and seed of *C. melo* L. extract are expressed in term of mgGAE/g extract as shown in Fig 5. The highest total tannin content was seed extracted with absolute ethanol equal to 4.27 mgGAE/g extract, which means that 1 gram of extract contains tannin content equivalent to 4.27 mg of gallic acid, followed with absolute ethanol extract from peel and deionized water extract from seed. The lowest was peel extracted with deionized water 0.73 mgGAE/g extract. One of the numerous flavonoid classes is tannin, which is responsible for astringent flavour of some plant materials and products [32].



Note: different letters above the columns present significant differences (P<0.05)

Figure 5 Total tannin content of *C. melo* L. from peel and seed extracts.

Antioxidant activity

The result of antioxidant activity of samples varies based on the solvent used for extraction and the method of analysis. There is presently no universal method to evaluate antioxidant activity quantitatively [33]. In this study, the analysis of antioxidant activity of peel and seed extract of *C. melo* L. was carried out using ABTS assay. The antioxidant activity of standard solution was measured in term of inhibitory concentration (IC_{50}), the concentration of sample required to scavenge 50% of the free radicals. The range of IC_{50} values was 1.69 to 2.33. The antioxidant activities of peel and seed extracts of *C. melo* L. extracts showed significant radical scavenging activity. From table 7, deionized water seed extract possessed the highest antioxidant activity with 8.87 ± 0.03 μgAAE/g extract, which means 1 gram of extract had antioxidant activity equivalent to 8.87 ± 0.03 μg of ascorbic acid. The lowest antioxidant activity was propylene glycol peel extract with 1.88 ± 0.07 μgAAE/g extract. Since phenols possess the potential to scavenge free radicals due to the presence of a hydroxyl group [34], a correlation between flavonoid content and antioxidant activity by ABTS was observed. These correlations indicated that the scavenging radical activity and antioxidant activity significantly depend on their flavonoid content (R^2 = 0.607) as shown in Table 8. Hence, it was implied that flavonoid which belongs to polyphenol class had effect on antioxidant activity.

Table 7 Antioxidant activity of seed and peel of C. *melo* L. extract from ABTS assays.

Sample	Solvent	Antioxidant activity ABTS (μgAAE/g extract)
Peel	Absolute ethanol	4.28 ± 0.04[a]
	Propylene glycol	1.88 ± 0.07[b]
	Deionized water	7.58 ± 0.24[c]
Seed	Absolute ethanol	6.98 ± 0.32[c]
	Propylene glycol	2.09 ± 0.13[b]
	Deionized water	8.87 ± 0.03[d]

Note: NT; not test, and different letters above the columns present significant differences (P<0.05)

Table 8 The correlation between antioxidant activity and total phenolic content, total flavonoid content, and total phenolic content.

Quantitative analysis	Correlation (R^2) ABTS
Total phenolic content (TPC)	0.102
Total flavonoid content (TFC)	0.607
Total tannin content (TTC)	0.126

Conclusion

Peel and seed extracts from *C. melo* L. var. green sweet contained bioactive compounds and antioxidant (free radical scavenging) activities when compared to ascorbic acid. The results suggested that the antioxidant properties of this plant might contribute as an active ingredient in the cosmetic industry, especially the deionized water extract.

Acknowledgements

The authors would like to thank Ozone Farm for provided melon as materials used in this study, and Mae Fah Luang University for chemical, laboratory facilities, and partial of financial support.

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Total Phenolic Content, Antioxidant and Anti-Tyrosinase Activities of *Zingiber officinale* Root Extracts

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Abstract

Zingiber officinale (ginger) is one of the most famous spices in Thailand and is common in various foods and traditional medicines or therapies, especially its rhizome. This study aimed to compare total phenolic content, antioxidant and tyrosinase inhibitory activities of propylene glycol, 80% ethanol, and distilled water extracts of fresh and dry ginger root. Total phenolic content was determined using Folin-Ciocalteu reagent, antioxidant activity was assessed by DPPH assay and tyrosinase inhibitory activity was evaluated by a modified dopachrome method. The 80% ethanol extract of dry ginger root showed the highest yield (0.3357 ± 0.0003%), antioxidant (11.011 ± 0.071 mg AAE/ 100 g extract) and tyrosinase inhibitory activities (14.642 ± 0.436). It could be concluded that the skin-lightening effect mediated by the tyrosinase inhibitory activity of ginger dry root extract was related to total phenolic content and antioxidant activity. From the above information, 80% ethanol is the best condition for extraction of dry root of *Z. officinale*. Moreover, this condition might be benefit for food, therapeutic and cosmetic applications in the future..

Keywords: *Zingiber officinale*, Total Phenolic Content, Antioxidant Activity, Tyrosinase Inhibitory Activity

Introduction

In vitro bioassay systems are useful for investigating the biological activities of extracts, and can revised to use not only in pharmacology but also in cosmetics. The most famous and important biological activities in cosmetic field are antioxidant and tyrosinase inhibitory activities. Many factors that lead to free radical production, which stimulates aging skin, are increasing nowadays, such as sunlight. Moreover, sunlight or radiation is one factor to stimulate melanocytes of skin [1]. Today, most people are interested in herbal foods, herbal cosmetics, or herbal extracts because they believe these to help them improve their health [2]. Knowledge and application of herbs' is important Thai local wisdom which has grown and been passed down through the generations. In the present, many Thais and foreigners are interested in new alternative medicines, such as herbs, for curing disease or finding ways to protect themselves by changing their diet [3].

Figure 1 *Zingiber officinale* (Ginger)

Zingiber officinale or ginger (Figure 1) is a creeping perennial on a thick tuberous rhizome, which grows underground [4]. *Z. officinale*, the rhizome of ginger, is one of the most famous spices from herbs in Thailand and is commonly used in various foods and beverages. *Z. officinale* has been used as spice and natural ingredient for

over 2000 years [5]. Ginger is now one of the most important and popular spices on the international market and the total global production of ginger is estimated at 100,000 tons a year. All over the world, the ginger rhizome is appreciated for its taste qualities; therefore, it is used as a spice, flavoring agent, and as an additive in the preparation of meals. Ginger is famous as a botanical dietary supplement in North America and Europe because it can be used to treat diverse human aliments like rheumatic disorders, gastrointestinal problems, and inflammatory conditions [6]. The ginger rhizome has been used in many forms as fresh paste, flavoring tea, dried powder and preserved in slices, and has been used as a traditional medicine in Asian and Arabic herbal traditions for many centuries [7]. Active gingerols are the major compounds in ginger, from studies of the lipophilic rhizome extracts, and active gingerols can be converted to shogoals, zingerone, and paradol [8]. The 6-gingerol is responsible for ginger's characteristic taste. Zingerone and shogaols are found in larger amounts in dried or extracted products than in fresh ginger. The researcher studied antioxidant components of *Z. officinale*, including total polyphenols by using Follin-Ciocalteu assays, total flavonoid, total tannin, and another antioxidant activity as DPPH radical scavenging activity [9].

At the present day, ginger has been used in the cosmetic field more than the past. One study proved ginger has antioxidant components following maceration for 3 hours and shaken in electronic shaker at room temperature [8]. Not only antioxidant components but ginger extracts also showed tyrosinase inhibitory activity by investigating sample extracted from methanol absorbance's end point of tyrosinase enzyme [10]. However, to our knowledge there are no reports about the effect of various solvents for extraction on the antioxidant and anti-tyrosinase inhibitory activities of ginger extract.

Therefore, the purpose of this study was comparison of the antioxidant and tyrosinase inhibitory activities of *Z. officinale* in various extracting solvents of both fresh and dry ginger. Furthermore, to study the relationship among total phenolic content, antioxidant and anti-tyrosinase activities in *Z. Officinale* root extracts.

Methodology

Plant materials

Ten month old fresh ginger roots were harvested from Chiang Rai Province in December 2016. They were washed by water and air dried at room temperature. After that, they were chopped and rough blended by electronic blender. This method was used for fresh sample. Whereas dry sample preparation, they were blended by electronic blender before drying by hot air oven at 60°C for 3 hours (until the weight was stable).

Chemical reagents and solvents

Solvents were all analytical grade including gallic acid (Sigma-Aldrich, US), ascorbic acid (Sigma-Aldrich, US), kojic acid (Sigma-Aldrich, US), DPPH or 2,2-diphenyl-1-picrylhydrazyl (Sigma-Aldrich, US), Follin-Ciocalteu reagent (Sigma-Aldrich, US), mushroom tyrosinase enzyme (Sigma-Aldrich, US), phosphate buffer (Univar, US). Sodium carbonate (Fisher, UK) were purchased were used.

Extraction of sample

Forty grams of each sample was macerated in 120 ml of each solvent. They were shaken for 3 hours in an electronic shaker (Shellab, SI4-2) at room temperature. Then they were centrifuged at 4000 rpm for 25 min and filtered by Whatman No.1 filter paper. All crude extracts were kept at 4°C until use.

Total phenolic content analysis

Folin-Ciocalteu reagent was used to estimate total polyphenol content [11-12]). First, mix 20 µl of sample or standard, 100 µl of 10% v/v Follin-Ciocalteu reagent in water and add 80 µl of sodium carbonate solution (75 mg/ml). Gallic acid solution in various concentrations was used for positive control (standard). In the blank or negative control, Folin-Ciocalteu reagent was replaced by 100 µl of distilled water. Each concentration of standard and each sample was assessed in triplicate and plates were incubated at room temperature for 1 hour. All of these processes were carried out in 96 well microplate. After 1 hour incubation, measured absorbance by using microplate reader (BMG LABTECH/ SPECTROstar) at 765 nm. Calibration curve was performed by absorbance of gallic acid (0-25 µg/ml). Total phenolic content of sample was expressed as mg GAE or gallic acid equivalent per g of sample.

DPPH radical scavenging activity

The effect of *Z. officinale* root extracts on DPPH free radical was measured according to [8]. One hundred µl of $6x10^{-5}$ M of DPPH solution was added into 100 µl of sample or standard prepared in ethanol. Ascorbic acid (0.25-10 µg/ml) was used as positive control or standard to plot the calibration curve of absorbance and percentage inhibition. The negative control was prepared by adding 100 µl of

DPPH solution ($6x10^{-5}$ M) to 100 µl of each solvent (propylene glycol, 80% ethanol, and distilled water). This assay was accomplished in 96 well microplate, each concentration of ascorbic acid and each sample was performed in triplicate. After incubating the microplate in the dark for 30 minutes, the absorbance of each mixture was measured by microplate reader at 517 nm wavelength. The DPPH radical scavenging activity of ascorbic acid was calculated by the following equation:

$$\%\text{inhibition} = (A_c-A_s)/A_c*100$$

Where, A_c is absorbance of negative control and A_s is absorbance of sample or positive control. And the DPPH radical scavenging results of sample were expressed by mg of ascorbic acid equivalent (mg AAE) per g of sample.

Tyrosinase inhibitory activity

A modified dopachrome method with L-DOPA as substrate was used to determine the tyrosinase inhibitory activity of the samples [13]. Each sample was prepared to different concentration using distilled water. Forty µl of standard and each sample were added to 20 mM of phosphate buffer (pH 6.8) and 40 µl of 240 unit/ml mushroom tyrosinase enzyme and pre-incubated at room temperature for 10 minutes. Then 40 µl of L-DOPA (0.85 mM) was added and the mixture was incubated for 20 minutes. Kojic acid was used to perform standard curve and the blank contained all components except mushroom tyrosinase enzyme. The absorbance was measured by microplate reader at 490 nm.

$$\%\text{inhibition} = (A_c-A_s)/A_c*100$$

Where, A_c is absorbance of negative control and A_s is absorbance of sample or positive control. Percent of inhibition was used to express the result.

Statistical analysis

All data are presented as the mean ± SD. (n=3). Differences between bioactivities in each extract from different solvents and types of sample were considered statistically significant by one way ANOVA when $p < 0.05$ using IBM SPSS Statistics version 24.

Results and discussion

The sample was divided into two types; fresh and dry samples, and extracted using different solvents; propylene glycol, 80% ethanol, and distilled water.

Table 1. Percent yield of *Z. officinale* root extracts.

Solvent	% Yield (%w/w)	
	Fresh	Dry
Propylene Glycol	0.1144 ± 0.0145*	0.1188 ± 0.0088*
80% Ethanol	0.2708 ± 0.0003	0.3357 ± 0.0003
Distilled water	0.0611 ± 0.0004	0.0843 ± 0.0007

*No Significantly different means ($p < 0.05$)

Data are presented as means ± SD.

The 80% ethanol extract of dry sample showed the highest extractive yield (0.3357 ± 0.0003%) as shown in Table 1. Moreover, all 80% ethanol extracts were significantly higher for the amount of crude extract ($p < 0.05$) than propylene glycol and distilled water extracts, respectively. The previous report by Pilerood and Prakash [8] also indicated that alcoholic extraction exhibited higher activities than other solvents, especially methanol. However, methanol was not used as an extracting solvent in the current study because of concerns about methanol toxicity [14]. Furthermore, extracts from dry *Z. officinale* showed higher amounts of crude extract ($p < 0.05$) than extracts from fresh *Z. officinale,* except for propylene glycol extract ($p > 0.05$).

Table 2. Total phenolic content, antioxidant and tyrosinase activities of *Z. officinale* root extracts.

Solvents	Total phenolic content (mg GAE/ 100 g extract)		Antioxidant by DPPH assay (mg AAE/ 100 g extract)		Tyrosinase inhibitory activity (% inhibition at 200 mg/ml of extract)	
	Fresh	Dry	Fresh	Dry	Fresh	Dry
Propylene Glycol	3.071± 0.159	5.852 ± 0.698	8.135 ± 0.168	8.575 ± 0.275	5.729 ± 0.467	13.068 ± 0.5719
80% Ethanol	3.071 ± 0.058	7.912 ± 0.442	10.061 ± 0.068	11.011 ± 0.071**	14.464 ± 0.861***	14.642 ± 0.436***
Distilled water	2.872 ± 0.499*	2.879 ± 0.330*	11.208 ± 0.006**	11.248 ± 0.018**	11.257 ± 0.155	11.517 ± 0.595

*No Significantly different means ($p < 0.05$)

Data are presented as means ± SD

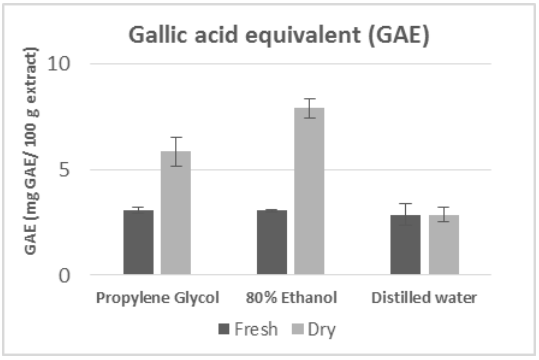

Figure 2 Gallic acid equivalent (mg GAE/ 100 g of extract) for each type sample in different extracting solvent. (n=3)

The total phenolic content of fresh and dry samples of *Z. officinale* was determined as mg Gallic acid equivalent or mg GAE per 100 mg of extract. Pilerood and Prakash [8] reported that 80% ethanol extract of dry sample had higher total phenolic content than other solvents ($p < 0.05$). From Table 2 and Figure 2, the dry sample preparation showed significantly superior quantities ($p < 0.05$) to fresh sample preparation when extraction with propylene glycol and 80% ethanol because of many phenolic contents in the ginger such as gingerol, quercetin, catechin, and epicatechin by HPLC analysis [15]. The polyphenol extraction and antioxidant activity of plant extracts depend highly on solvent polarity [16]. For this reason, the 80% ethanol extracts gave the highest total phenolic contents.

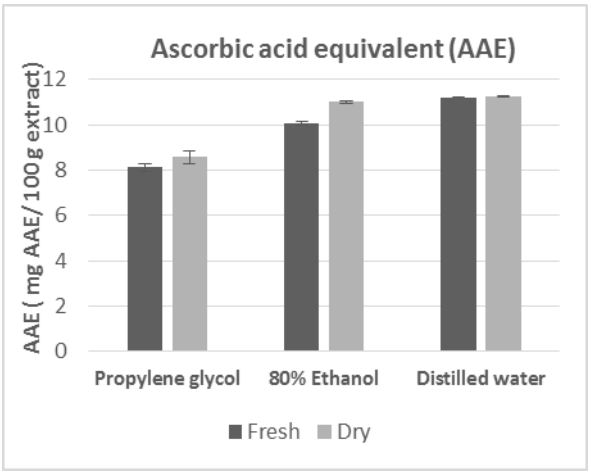

Figure 3 Ascorbic acid equivalent (mg AAE/ 100 g of extract) in each type sample and extracting solvent. (n=3)

From Table 2 and Figure 3, antioxidant activity was estimated using a single-electron transfer with free radical by DPPH assay. The results showed that distilled water extract of fresh sample and 80% ethanol and distilled water extracts of dry sample were found to be highest antioxidant activity with no significant difference ($p > 0.05$). Also, the antioxidant activity of distilled water extracts from fresh and dry samples were not different.

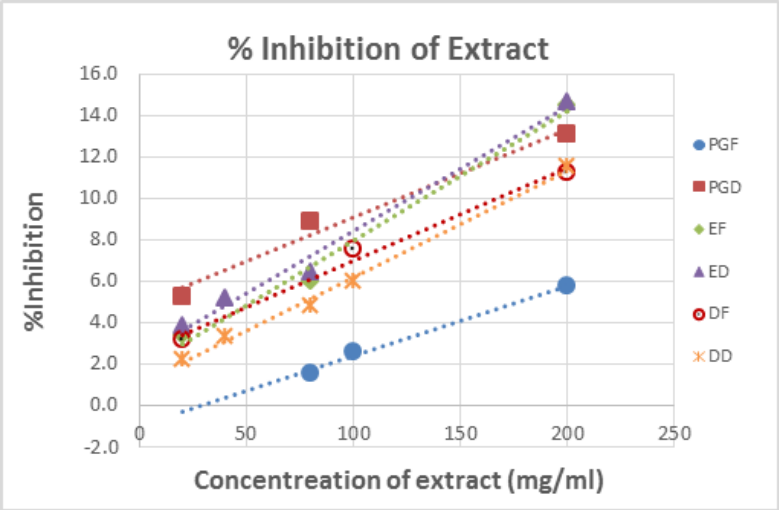

Figure 4 Percent inhibition (% inhibition at 200 mg/ml of extract) for each type of sample in different extracting solvents. Whereas, PGF: Fresh sample in Propylene Glycol solvent, PGD: Dry sample in Propylene Glycol, EF: Fresh sample in 80% Ethanol solvent, ED: Dry sample in 80% Ethanol solvent, DF: Fresh sample in Distilled water, DD: Dry sample in Distilled water

The skin-lightening efficacy of *Z. officinale* extracts were assessed using *in-vitro* anti-tyrosinase activity. At 200 mg/ml of extract, the highest activity was extract from 80% ethanol of fresh and dry samples ($p < 0.05$), followed by propylene glycol and distilled water (Table 2, Figure 4). Therefore, the 80% ethanol extract was able to deactivate the tyrosinase enzyme in melanin production. By combining the data of total phenolic content, antioxidant and tyrosinase inhibitory activities of 80% ethanol extract from dry *Z. Officinale* root, it could be determined that the anti-tyrosinase activity appeared to be related to total phenolic content and antioxidant activity [17]. However, all extracts had low percent inhibition, which it might be from the starting material.

From the above results, the total phenolic, antioxidant activity, and tyrosinase inhibitory activity showed correlation. However, AAE equivalent and GAE equivalent were not dependent; which might be from differences in the solubility of each extract in various polarity of extracting solvents [18].

Conclusion

The most potent biologically active extract determined by *in vitro* bioassays was 80% ethanol extract from dry *Z. officinale* root. It had the highest total phenolic content by Folin-Ciocalteu reagent assay, highest antioxidant activity using DPPH radical scavenging assay and highest skin-lightening efficacy using tyrosinase inhibitory assay. Although it had less capacity to inhibit mushroom tyrosinase enzyme. In addition, the biological activity of ginger root extract was influenced from various factors which are; amount of sample, sample preparation, extracting solvent, and concentration of extract. The correlation among total phenolic content, antioxidant and yrosinase inhibitory effect is offered as an achievable method for standardization of ginger root extract in 80% ethanol. From this study, 80% ethanol is the best condition for extraction of dry root of *Z. officinale*. Moreover, this condition might be benefit for food, therapeutic and cosmetic application in the future.

Acknowledgements

The authors would like to acknowledge Mae Fah Luang University, Chiang Rai, Thailand for facilities and partial of

financial support.

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Stability of Anthocyanin of Hom Nin Rice Extract:Preliminary Study

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Abstract

This preliminary study aimed to investigate the stability of anthocyanin of Hom Nin rice extract (*Oryza sativa*).Hom Nin rice was extracted by different two solvents (deionized water, 95% ethanol) and these acidified by 1% (v/v) 1M HCl by shaking extractionin ratio of 1:4 (sample:solvent w/v) at 150 rpm for 12 hours. All organic solvents were removed and the extracts were freeze dried and then collected them as crude extract for determination of anthocyanin content. The results showed that 95% ethanol (EtOH) acidified extract was given the highest anthocyanin content (40.15±0.77 mg C3G/g extract). The stability of anthocyanin from 95% ethanol acidified was studied in various pH and temperature as well as accelerate condition (heating and cooling cycles) and light exposure. In various pH condition, anthocyanin was higher stability in the acidic condition (pH 3 and 5) then basic condition (pH 9 and 11) that anthocyanin contents were decreased when pH increased. For the stability in various temperature, anthocyanin content was slightly changed. In accelerate stability condition, the amount of anthocyanin and its color was changed after heating and cooling cycles. For light stability, the comparison of Hom Nin extract between light and without light exposure in 14 days were determined. Light exposure condition was shown rather degradation in anthocyanin contents thanwithout light exposure. And they also showed color fading from dark purple red to yellowish. These studies may conclude that anthocyanin from Hom Nin extract was not stable. Therefore, the encapsulation for Hom Nin extract for maintain the stability in cosmetic application should be considered.

Keywords: Anthocyanin, Hom Nin Extraction, Rice Extraction, Stability

Introduction

Black rice is a type of rice in species *Oryza sativa* L. that is also known as purple rice, forbidden rice, heaven rice, imperial rice, king's rice and prized rice because it is rich in nutrients, especially anthocyanin that is located in the pericarp and makes the rice color black [6]. Hom Nin is one species of black rice in Thailand. The characteristic of Hom Nin is tapered and dark-purple to black grain color, which contains high nutrition [5]. Bran contains delphinidin-3- glucoside, cyanidin-3-glucoside and pelargonidin-3-glucoside, which are common anthocyanin that can be found in rice [9]. There are many research reports of the benefits of black rice to decrease cholesterol because of anthocyanin. Moreover, black rice also contains iron, zinc, calcium, copper and manganese higher than red and white rice [13]. Anthocyanin is the pigment responsible for red, blue, and purple color of plants. The different color of anthocyanins is caused by the pattern of B-ring in aglycone, the pattern of glycosylation, and the degree and nature of esterification of the sugars with aliphatic or aromatic acids, and also by the pH, temperature, type of solvent, and the presences of co-pigments [7]. Even though anthocyanin is health beneficial there are many studies that anthocyanin is limited in the stability, although they come from different sources. Therefore, the stability of anthocyanin contents from Hom Nin extract were investigated at various pH along with thermal stability and effect of light exposure.

Methodology

Hom Nin rice extraction

Hom Nin (HN) rice was purchased from Phetchabunprovice; the Central of Thailand. Hom Nin rice was extracted by four solvents (Deionized water, 95%ethanol, and these solvents acidified with 1% 1M HCl) in ratio of 1:4 (w/v) for 12 hours at 150 rpm in shaker. All sample solutions were filtered by Buchner funnel with whatman paper No.4. Organic solvent was removed by evaporation rotary at 45°C. All rice extracts were dehydrated by freeze-dryer and they were referred as Hom Nin crude extract. The extraction was repeated in triplicate.

Determination of total monomeric anthocyanin contents (TAC)

The total monomeric anthocyanin contents in HN extracts was determined according to pH differential method [1]. Briefly, sample solutions were diluted with pH 1.0 buffer (potassium chloride, 0.025 M) and pH 4.5 buffer (sodium acetate, 0.4M) by 1 part test portion, 4 parts buffer and measured for absorbance at 520 and 700 nm. DI water was used as blank. Sample solutions were measured for absorbance within 20 minutes of preparation. Total monomeric anthocyanin content was calculated and expressed as Cyanidin-3-glucoside equivalents (mg G3G/g extract). The measurement was repeated in triplicate.

Stability evaluation of Hom Nin crude extracts

Effect of pH

The stability of Hom Nin extract in different pH was determined according to [10] with some modification. Hom Nin crude extract solutions were prepared by dissolving Hom Nin crude extract 5 mg in propylene glycol 1 ml. The solutions were diluted 5 times with different buffers (pH 3,5,7,9,11) and incubated for 15 minutes. Anthocyanin contents were determined.

Effect of temperature

The stability of Hom Nin extract in different temperature was determined according to [10] with some modification. 1 mg Hom Nin crude extract was dissolved in 1 ml propylene glycol, which referred as Hom Nin crude extract solution. Hom Nin crude extract solutions were stored at 30˚C to 9 0 ˚C. All Hom Nin crude extract solutions were sampled periodically every 15 minutes for testing for anthocyanin content.

Effect of heating and cooling cycles

The stability of Hom Nin extract in different heating and cooling cycles was determined according to [10] with some modification. 10 mg Hom Nin crude extract was dissolved in 1 ml propylene glycol, which referred as Hom Nin extract solutions. Hom Nin extract solutions were stored in heating condition at 45˚C for 24 hours and cooling condition at -4˚C for 24 hours. Anthocyanin contents were determined in each cycle until 3 cycles were completed.

Effect of light

The stability of Hom Nin extract in light was determined according to [10] with some modification.10 mg Hom Nin crude extract was dissolved in 1 ml propylene glycol, which referred as Hom Nin extract solutions. Hom Nin extract solutions were stored for 14 days in different conditions; first condition is all extract solutions were exposed light and the second is stored in dark room. Anthocyanin contents were determined in day 0, 1, 3, 5, 7, and 14.

Results

The optimization of Hom Nin extraction determined the best solvent to extract anthocyanin from Hom Nin rice. The solvent systems based on acidified and non-acidified hydroethanolic solution were used: (1) water; (2) water acidified by 1% of 1M HCl; (3) 95%ethanol; (4) 95%ethanol acidified by 1% of 1M HCl. The evaluation of anthocyanin concentration was performed under pH different method which is shown on Figure 1. The result of anthocyanin contents was presented as follow 95%ethanol acidified (40.15±0.77 mg C3G/g extract) > Water (15.86±0.77 77 mg C3G/g extract) > 95% ethanol (8.77±0.77 77 mg C3G/g extract) > Water acidified (7.59±0.00 77 mg C3G/g extract).

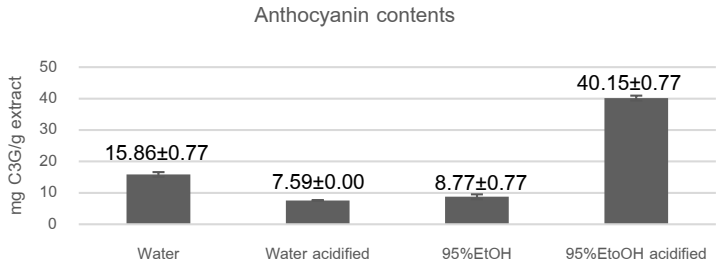

Figure 1 Anthocyanin contents of Hom Nin extract from different solvents; water, water acidified, 95%EtOH and 95%EtOH acidified.

Anthocyanin of Hom Nin extract from 95% ethanol acidified was selected to studied the stability in various pH and temperature and light exposure due to it being the richest in anthocyanin content. For the study of anthocyanin

stability, Hom Nin extract was prepared as solution by dissolving into propylene glycol before further processing.

For result of anthocyanin contents in various pH is shown on table 1. The estimated pH for anthocyanin from Hom Nin extract was pH 3-5, which is in acidic range, because the decreasing percentage was lower than for the basic range. The increasing pH also affected anthocyanin content between pH 7 to pH 11. This was evidence that Hom Nin anthocyanins were not stable in neutral and alkaline buffer solutions. pH was one factor which affected anthocyanin color stability. The color of Hom Nin extract changed by increasing pH, turning from dark red-purple to blue.

The stability in various temperature (room temperature to 90˚C) of anthocyanin from Hom Nin extract is shown in table 2. The temperature did not significantly affect anthocyanin content. Therefore, the color after incubation in various temperature did not change. But, when compared with the stress condition of heating and cooling cycles (at 45˚C and -4˚C), the results showed degradation of anthocyanin contents and the fading color of Hom Nin extract (Table 3).

The result of stability in light exposure over 14 days is shown in Table 4. Anthocyanin contents degraded under both light and without light exposure and under light exposure color also turned fom dark red-purple to yellowish. The comparison in both of light and without light exposure presented that in light exposure increased degradation than without light exposure. The color of Hom Nin extract was directly related to anthocyanin content.

Table 1 Anthocyanin contents of Hom Nin extracts in various pH

pH	Anthocyanin content (mg C3G/g extract)		Color	
	Before	After		
Normal (pH 7)	44.96±1.77	44.01±0.44		Dark red-purple
pH 3	29.59±0.43[a]	12.87±1.13[a]		Light red-purple
PH 5	20.56±0.52[b]	13.87±3.41[a]		Light red-yellow
pH 7	17.22±0.38[c]	11.20±2.44[a]		Blue
pH 9	16.46±1.16[c]	5.18±1.04[b]		Blue
pH 11	15.38±1.26[c]	0.00[c]		Dark blue

N=3, *p*-value < 0.05

Table 2 Anthocyanin contents of Hom Nin extracts in various temperature

Temperature	TAC (mg C3G/g extract)	Color	
Normal (Room temperature)	39.20±0.95		Dark red-purple
30	39.53±0.81		Dark red-purple
40	39.87±0.90		Dark red-purple
50	38.86±0.54		Dark red-purple
60	38.78±1.04		Dark red-purple

Temperature	TAC (mg C3G/g extract)	Color
70	38.53±0.14	Dark red-purple
80	38.28±0.14	Dark red-purple
90	38.28±0.29	Dark red-purple

N=3, p-value < 0.05

Table 3 Anthocyanin content after heating and cooling cycle

Cycle	TAC (mg C3G/g extract)	Color
Before	45.38±1.89[a]	Dark red-purple
Cycle 1	41.20±1.47[a]	Dark red-purple
Cycle 2	42.04±2.37[a]	Dark red-purple
Cycle 3	34.18±1.01[c]	Dark red-purple

N=3, p-value < 0.05

Table 4 Anthocyanin content after expose light

Day	Light (mg C3G/g extract)	Color	Dark (mg C3G/g extract)	Color
Day 0	66.70±3.04[a]	Dark red-purple	66.70±3.04[a]	Dark red-purple
Day 1	48.73±3.39[b]	Dark red-purple	53.91±1.15[b]	Dark red-purple
Day 3	31.09±2.39[c]	Red-purple	42.71±1.18[c]	Dark red-purple
Day 5	29.50±5.39[c,d]	Red-purple	40.87±0.66[c]	Dark red-purple
Day 7	21.40±2.33[d,e]	Red-purple	34.68±0.38[d]	Dark red-purple
Day 14	13.21±2.26[e,f]	Yellow-brown	30.17±1.04[e,f]	Red-purple

N=3, *p*-value < 0.05

Discussion and Conclusion

The polarity and pH of solvent affected anthocyanin extraction because low pH extraction solvents can solubilize and stabilize anthocyanin contents [11]. The best solvent for anthocyanin extraction from Hom Nin rice was 95% ethanol acidified. Even though water acidified was gave the lower pH, the polarity did not suit extraction of anthocyanin from Hom Nin extract. Hom Nin extracts were prepared by dissolving in propylene glycol because the hydroxyl group of propylene glycol covalently bonds with anthocyanin [8]. From the result of the stability in various pH, color turned from dark red-purple to blue when pH increased may because the main anthocyanins in Hom Nin rice are cyanidin-3-glucoside (C3G) and peonidin-3-glucoside (P3G) [11]. The color of anthocyanin depends on the structure of anthocyanin by 3-glucosides of pelargonidin, peonidin, and malvidin were presented blue shades with intensity and stability above pH 8 [12].From previous studies, heat affects anthocyanin stability [4]. The result of this study presented that temperature did not affect the stability of anthocyanin in Hom Nin extract. But, heating and cooling cycles decreased anthocyanin content. We can conclude that anthocyanin from Hom Nin extract cannot resist fast thermal changes. Light was one factor that influenced the decrease in anthocyanin content because light may cause the transformation of flavylium salts to chalcone [2]. In this study, light was the factor that had the most effect on anthocyanin stability. These results showed that the Hom Nin extract was a good source of anthocyanin but it was not stable under stability test in different environments. Thus, the encapsulation of Hom Nin extract should be considered to improve the stability of Hom Nin extract for usage in cosmetic product as active ingredient. Microencapsulation is the process of encapsulating a material containing an active ingredient in a shell of a second material. The shell can be either permanent or temporary. This process can be improve the stability of active ingredient and also improve the delivery and control the release of active ingredients for topical cosmetic application [3]

Acknowledgements

This work was supported from School of Cosmetic Science and Office of the Postgraduate Studies, Mae Fah Luang University. The authors thank the Center of Excellence in Natural Product Innovation and the Scientific and Technological Instrument Center, Mae Fah Luang University (MFU) for supports theequipment.

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Synthesis and Cytotoxic Activity of Azanaphthoquinone Annelated Pyrrole Derivatives

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Abstract

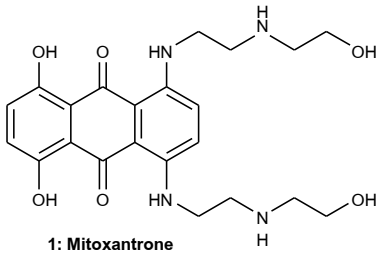
Azanaphthoquinone annelated pyrroles have been developed from the natural anticancer drug, mitoxantrone to reduce cardiotoxic side-effects. This paper reports the synthesis of azanaphthoquinone annelated pyrrole derivatives as cytostatic compounds. The synthetic pathway was started from the commercially-available 5-hydroxyisoquinoline via a three step reaction to obtain 1*H*-pyrrolo[3,2-*g*]isoquinoline-4,9-dione. *N*-Alkylation of nitrogen atom in pyrrole ring was carried out under basic conditions with different side chains to obtain mono-substituted azanaphthoquinone annelated pyrroles (**2a-e**) with 2 to 3-carbon side chains in moderate to good yields. The hydrazone and thiosemicarbazone derivatives were synthesized by a condensation reaction of mono-substituted products **2** with hydrazine and thiosemicarbazide respectively. The reactions occurred regio-selectively at C-4 to give hydrazones (**3a-e**) and thiosemicarbazones (**4a-e**) respectively. The synthesized compounds were purified by column chromatography and characterized by spectroscopic techniques, including ^{1}H NMR, ^{13}C NMR, IR, UV and LC-MS. The antiproliferative activity of the synthesized compounds was evaluated on cervical carcinoma: KB/HeLa by using xCELLigence from Roche. The results showed that the mono-substituted products with 2-carbon side chain (**2b**) exhibited the highest activity with IC_{50} value of 0.008 μM. The mono-substituted product **2** showed higher inhibition in comparison to hydrazone and thiosemicarbazone derivatives. These results lead to the optimization of the target molecules in further works.

Keywords: Anticancer agents, Azanaphthoquinone, Intercalating agents

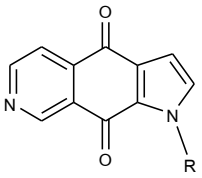
Introduction

Mitoxantrone is an anthracenedione agent which is used to treat certain types of cancer, including metastatic breast cancer, acute myeloid leukemia, and non-Hodgkin's lymphoma [1-7]. Mitoxantrone, as with other drugs in its class, may cause adverse reactions of varying severity, including nausea, vomiting, hair loss, heart damage, and immunosuppression, possibly with delayed onset. Because of the risk of cardiomyopathy, mitoxantrone carries a limit on the cumulative lifetime dose. The development of mitoxantrone analogs has been driven by the requirement for lower cardiotoxicity.

Azanaphthoquinoine annelated pyrrole core structures were synthesized by H. Spreitzer [8]. After that, the mono- and di-substituted derivatives were synthesized [9]. The biological evaluation of the synthesized compounds with human cancer cell lines showed promising cytotoxicity. To avoid the quinone system, the oxime derivatives and carbinol derivatives were studied to enhance the biological activity and lower semiquinone radical generation [10-11]. Thiosemicabazone derivatives have been incorporated into a large number of compounds with potential medicinal values, such as antifungal, antibacterial, antiviral, antimalarial and antitumor activities [12-13]. Drugs containing thiosemicabazone, such as Triapine and Dp44mT, have potent antiproliferative activity and have been evaluated as anticancer agents. The action of Triapine as an agent that slows conversion of RNA and DNA, decreasing cellular proliferation, suggests that it would be an excellent chemotherapeutic agent [14-15]. As a part of our studies on the design and synthesis of pharmaceutical active compounds as potential anticancer compounds, we report on the synthesis of azanaphthoquinone annelated pyrrole derivatives, including mono-substituted products (**2a-e**), hydrazones (**3a-e**), and thiosemicarbazones (**4a-e**) to overcome the disadvantages of mitoxantrone. We believe the nitrogen-nitrogen bond to be more stable and consisting of thiosemicarbazone moiety could interact with DNA via hydrogen bonding, thus leading to potential anticancer compounds. The length of side chain and the end-group could affect the interaction with the DNA strand. Therefore, different side chains were attacthed to the core structure in an attempt to improve the potency of activity.



1: Mitoxantrone



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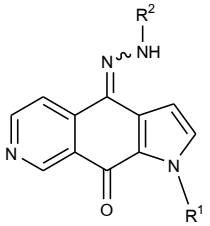

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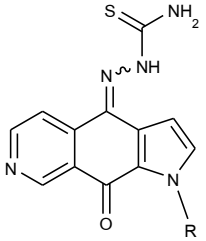


4

Methodology

^{1}H NMR spectra were recorded on a Bruker AVANCE (300 MHz) spectrometer. The residue of the non-deuterated solvent was used as internal standard which was related to tetramethylsilane with δ = 7.26 ppm for $CDCl_3$. ^{13}C NMR spectra were recorded on a Bruker AVANCE (75 MHz) with the residue of the non-deuterated solvent peak as the internal standard, δ = 77.0 ppm for $CDCl_3$. The IR spectra were recorded on Perkin-Elmer FT-IR spectroscopy Spectrum RXI. Mass spectrometric analyses were recorded on a LC-MS Bruker Daltonics DataAnalysis 3.3. All reactions and atmospheric pressure distillations were performed under a positive pressure of dry nitrogen. Reaction flasks were dried at 120°C for 2 h and connected with N_2-line when they were still warm. Extracts were dried over anhydrous magnesium sulfate ($MgSO_4$). Solvents were removed by rotary evaporator at water aspirator pressure. A trace amount of solvent was further removed under vacuum (Ca. 0.01 mmHg).

Thin layer chromatography (TLC) was performed with Merck silica gel 60 PF_{254} plate (Merck-Nr 1.05554: 0.2 mm, 20 x 20 cm) or Merck aluminium oxide plate (Merck-Nr 1.05550: 0.2 mm, 20 x 20 cm). Chromatography was performed using Merck silica gel 60, 70-230 mesh ASTM, Nr 1.07734 or aluminium oxide activated basic, 50-200 Micron (Acros Organics Nr 189990010).

*Preparation of isoquinoline-5,8-dione (**6**)*

[Bis(trifluoroacetoxy)iodo]benzene (9.4670 g, 22 mmol) was dissolved in CH_3CN/H_2O; 2/1 (20 ml), then the solution was cooled down to 0°C in an ice bath. The suspension of 5-hydroxyisoquinoline (**5**, 1.4670 g, 10 mmol) in CH_3CN/H_2O; 2/1 (40 ml) was added dropwise. The reaction mixture was stirred at 0°C for 2.5 h. Afterwards it was diluted with water (100 ml) and the mixture was extracted with EtOAc (8 x 100 ml). The organic layers were dried over anh.$MgSO_4$. The solvent was removed in a rotary evaporator. The crude product of **6** was obtained in quantitative yields and used in the next reaction step without further purification.

*Preparation of 7-aminoisoquinoline-5,8-dione (**7**)*

The crude product of compound **6** (1.4670 g, 10 mmol) was dissolved in glacial acetic acid (10 ml) and THF (25 ml). The mixture was warmed up to 40°C before a solution of sodium azide (0.6500 g, 10 mmol) in water (3.5 ml) was added dropwise. Stirring was continued for addition 2.5 h at 40°C. After complete conversion starting material as indicated by TLC, the solvents were removed in rotary evaporator. The crude product was purified by column chromatography (silica gel) eluting with EtOAc to furnish compound **7** (0.8332 g, 47 %) as yellow solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 9.29 (s, 1H), 9.03 (d, *J* = 5.1 Hz, 1H), 7.95 (d, *J* = 5.1 Hz, 1H), 6.12 (s, 1H), 5.32 (br s, 2H) ppm.

*Preparation of 1H-pyrrolo[3,2-g]isoquinoline-4,9-dione (**8**)*

The solution of **7** (0.2719 g, 1.56 mmol) in glacial acetic acid (35 ml) was added by manganese (III) acetate dihydrate (1.6723 g, 2.73 mmol) and acetaldehyde diethyl acetal (0.4 ml, 2.73 mmol). The reaction mixture was stirred at 60°C overnight (18 h). Afterwards, EtOAc (100 ml) and the aqueous solution of sodium pyrosulfite (50 ml) were added to the reaction mixture respectively. The residue was exhaustively extracted with EtOAc (2 x 100 ml). The combined organic phase was washed with water (2 x 50 ml) and saturated in an aqueous solution of $NaHCO_3$ (50 ml). A small amount of product was extracted back from the aqueous phase with EtOAc. The combined organic layers were dried (anh. MgSO4) and concentrated in a rotary evaporator. The crude product of **8** (0.1763 g, 57%) as a yellow solid was used in the next reaction without further purification.^{1}H NMR (300 MHz, $CDCl_3$): δ = 9.69 (br s, 1H), 9.39 (s, 1H), 9.04 (d, *J* = 5.0 Hz, 1H), 8.01 (d, *J* = 5.0 Hz, 1H), 7.21(d, *J* = 2.6 Hz, 1H), 6.89 (d, *J* = 2.6 Hz, 1H) ppm.

Synthesis of mono-substituted derivatives of azanaphthoquinone annelated pyrrole

General procedure

NaH (60% suspension in mineral oil, 2.65 equiv.) was dissolved in DMF. The mixture was cooled to 0°C, then

compound **8** (1 equiv.) in DMF was added dropwise. The reaction mixture was stirred at 0°C for additional 30 min. The appropriate side chain (1.5 equiv) in DMF was added dropwise. The reaction mixture was stirred at 67-70°C until the completion of the reaction. Afterward, water was added and the solvent was removed in a rotary evaporator.

*1-[2-N,N-(dimethylamino)ethyl]-1Hpyrrolo[3,2-g]isoquinoline-4,9-dione (**2a**)*

The mixture of NaH (0.0590 g, 1.47 mmol) in DMF (2 ml), compound **8** (0.0905 g, 0.45 mmol) in DMF (3 ml) and 2-*N,N*-dimethylamino-1-ethly chloride hydrochloride (0.1990 g, 1.38 mmol) in DMF (3 ml) was refluxed at 67-70°C for 4 h. The crude product was purified by column chromatography (aluminium oxide) eluting with gradient EtOAc to EtOAc/MeOH; 8/2 to give product **2a** (0.0853 g, 70 %) as an orange solid [16]. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 9.37 (s, 1H), 8.98 (d, *J* = 4.5 Hz, 1H), 7.95 (d, *J* = 4.5 Hz, 1H), 7.14 (s, 1H), 6.78 (s, 1H), 4.64 (t, *J* = 6.6 Hz, 2H), 2.87 (t, *J* = 6.6 Hz, 2H), 2.38 (s, 6H) ppm; ^{13}C-NMR (75 Hz, $CDCl_3$): δ = 179.7, 175.4, 154.9, 148.0, 139.2, 133.1, 129.8, 128.7, 126.8, 119.0, 107.2, 59.3, 47.2, 45.5 ppm; IR (KBr Disc): ν_{max} = 2924, 1639, 1616, 1018, 618, 467 cm^{-1} [10].

*Synthesis of 1-(2-(pyrrolidin-1-yl)ethyl)-1Hpyrrolo[3,2-g]isoquinoline-4,9-dione (**2b**)*

The mixture of NaH (0.1083 g, 2.67 mmol) in DMF (4 ml), compound **8** (0.1763 g, 0.89 mmol) in DMF (4 ml) and *N*-(2-chloroethyl)pyrrolidine hydrochloride (0.2270 g, 1.34 mmol) in DMF (4 ml) was stirred at 67°C for 4 h. The crude product was purified by column chromatography (aluminium oxide) eluting with gradient to EtOAc/MeOH; 8/2 to give product **2b** (0.1257 g, 48 %) as orange solid [16]. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 9.38 (s, 1H), 9.01 (d, *J* = 5.1 Hz, 1H), 7.96 (d, *J* = 5.1 Hz, 1H), 7.25 (d, *J* = 2.7 Hz, 1H), 6.80 (d, *J* = 2.7 Hz, 1H), 4.76 (t, *J* = 6.8 Hz, 2H), 3.14 (t, *J* = 6.8 Hz, 2H), 2.38 (br s, 4H), 1.90 (br s, 4H) ppm.

*1-(2-(piperidin-1-yl)ethyl)-1Hpyrrolo[3,2-g]isoquinoline-4,9-dione (**2c**)*

The mixture of NaH (0.6598 g, 16.29 mmol) in DMF (6 ml), compound **8** (1.2026 g, 6.10 mmol) in DMF (6 ml) and 1-(2-chloroethyl)piperidine hydrochloride (1.6794 g, 9.10 mmol) in DMF (6 ml) was stirred at 67°C for 18 h. The residue was purified by column chromatography (aluminium oxide) eluting with gradient EtOAc to EtOAc/MeOH; 8/2 to give product **2c** (0.3563 g, 19 %) as yellow solid. ^{1}H NMR (300 MHz, Acetone D_6): δ = 9.25 (s, 1H,), 9.02 (d, *J* = 5.1 Hz, 1H), 7.90 (d, *J* = 4.8 Hz, 1H), 7.44 (d, *J* = 2.7 Hz, 1H), 6.72 (d, *J* = 2.7 Hz, 1H), 4.65 (t, *J* = 6.3 Hz, 2H), 2.74 (t, *J* = 6.3 Hz, 2H), 2.60-2.30 (m, 4H), 1.55-1.25 (m, 6H) ppm.

*1-[3-N,N-(dimethylamino)propyl]-1Hpyrrolo[3,2-g]isoquinoline-4,9-dione (**2d**)*

The mixture of NaH (0.2502 g, 6.25 mmol) in DMF (8 ml), compound **8** (0.4129 g, 2.08 mmol) in DMF (8 ml) and 3-*N,N*-dimethylamino-1-propyl chloride hydrochloride (0.5070 g, 3.21 mmol) in DMF (8 ml) was refluxed at 67°C for 4 h. The residue was purified by column chromatography (aluminium oxide) eluting with gradient EtOAc to EtOAc/MeOH; 8/2 to give product **2d** (0.3295 g, 56 %) as orange solid [16]. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 9.34 (s, 1H), 8.97 (d, *J* = 4.5 Hz, 1H), 7.92 (d, *J* = 4.5 Hz, 1H), 7.08 (s, 1H), 6.75 (s, 1H), 4.51 (t, *J* = 6.8 Hz, 2H), 2.45 (t, *J* = 6.8 Hz, 2H) 2.27 (s, 6H), 2.04 (p, *J* = 6.6 Hz, 2H) ppm.

*1-(3-(piperidin-1-yl)propyl)-1Hpyrrolo[3,2-g]isoquinoline-4,9-dione (**2e**)*

The mixture of NaH (0.6324 g, 15.72 mmol) in DMF (6 ml), compound **8** (1.1661 g, 5.89 mmol) in DMF (6 ml) and 1-(3-chloropropyl)piperidine hydrochloride (1.8078 g, 8.84 mmol) in DMF (6 ml) was stirred at 67°C for 24 h. The crude product was purified by column chromatography (aluminium oxide) eluting with gradient EtOAc to EtOAc/MeOH; 8/2 to give product **2e** (0.2525 g, 13 %) as green-brown solid. ^{1}H NMR (300 MHz, $CDCl_3$): δ = 9.45 (s, 1H), 8.80 (d, *J* = 5.4 Hz, 1H), 8.07 (d, *J* = 5.4 Hz, 1H), 7.54 (br s, 1H), 7.32 (d, *J* = 2.9 Hz, 1H), 6.91 (d, *J* = 2.9 Hz, 1H), 4.63 (t, *J* = 6.8 Hz, 2H), 2.53-2.38 (m, 4H), 2.33 (t, *J* = 6.8 Hz, 2H), 2.09 (t, *J* = 6.8 Hz, 2H), 1.70-1.30 (m, 6H) ppm.

Synthesis of azanaphthoquinone annelated pyrrolo hydrazones

4-(2-(2-(dimethylamino)ethyl)hydrazono)-1-(2-(dimethylamino)ethyl)-1H-pyrrolo[3,2-g] isoquinoline-4,9-dione **(3a)**

The solution of 2-(*N,N*-dimethylamino)ethylhydrazine (0.1560 g, 1.2 mmol) in EtOH (0.7 ml) was slowly added to the mixture of compound **2a** (0.2762 g, 1.0 mmol) and triethylamine (0.5 ml, 3.6 mmol) in THF (2 ml) at room temperature. The reaction mixture was stirred at room temperature for 2 h. Then the second portion of the hydrazine (0.1560 g, 1.2 mmol) in EtOH (0.7 ml) was added. The reaction mixture was stirred for an additional 18 h. After that, solvent was removed in *vaccuo*. The crude product was purified by column chromatography (Aluminium oxide) eluting with EtOAc to give product **3a** (0.1042 g, 29%) as an orange liquid. ^{1}H-NMR (300

MHz,$CDCl_3$): δ = 9.33 (s, 1H), 8.73 (d, *J* = 5.4 Hz, 1H), 7.74 (d, *J* = 5.1 Hz, 1H), 7.05 (d, *J* = 2.7 Hz, 1H), 6.90 (d, *J* = 2.4 Hz, 1H), 5.65 (br s, 1H), 4.40-4.60 (m, 2H), 2.91 (t, *J* = 6.5 Hz, 2H), 2.66 (t, *J* = 6.5 Hz, 2H), 2.38-2.48 (m, 2H), 2.35 (s, 6H), 2.27 (s, 6H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$): δ= 174.0, 157.5, 152.5, 149.5, 142.5, 131.8, 128.5, 126.5, 120.9, 118.5, 107.5, 60.0, 55.8, 47.1, 45.6, 45.4, 42.0 ppm; IR (in MeOH) ν_{max} = 3434, 2911, 2733, 2367, 1651, 1591, 1460, 1376, 1258, 1209, 1040, 919, 723 cm^{-1}; MS: Calcd. for $C_{19}H_{31}N_6O$ (M^+ + 5H): 359.2559; found 359.1944.

4-(2-(2-(dimethylamino)ethyl)hydrazono)-1-(2-(pyrrolidin-1-yl)ethyl)-1H-pyrrolo[3,2-g] isoquinoline-4,9-dione **(3b)**

The solution of 2-(*N,N*-dimethylamino)ethylhydrazine (0.4042 g, 3.9 mmol) in EtOH (0.7 ml) was slowly added to the mixture of compound **2b** (0.1905 g, 0.6 mmol) and triethylamine (0.2 ml, 1.5 mmol) in THF (3 ml) at room temperature. The reaction mixture was stirred at room temperature for 2 h. Then the second portion of the hydrazine (0.4102 g, 3.9 mmol) in EtOH (0.7 ml) was added. The reaction mixture was stirred for an additional 2 h. After that solvent was removed in *vaccuo*. The crude product was purified by column chromatography (aluminium oxide) eluting with EtOAc to give product **3b** (0.0713 g, 29%) as an orange liquid. ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 9.34 (s, 1H), 8.76 (d, *J* = 5.1 Hz, 1H), 7.77 (d, *J* = 5.1 Hz, 1H), 7.11 (d, *J* = 2.4 Hz, 1H), 6.42 (d, *J* = 2.4 Hz, 1H), 4.75-4.45 (m, 2H), 3.17 (q, *J* = 5.3 Hz, 2H), 2.96 (t, *J* = 6.9 Hz, 2H), 2.70 (br s, 4H), 2.49 (t, *J* = 5.7 Hz, 2H), 2.40 (s, 6H), 1.90 (br s, 4H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 174.0, 157.5, 152.5, 149.5, 142.5, 131.8, 128.5, 126.5, 120.6, 118.5, 107.5, 56.0, 55.9, 54.1, 47.5, 44.7, 42.0, 23.5 ppm**;** IR (in CH_2Cl_2) ν_{max} = 3376, 2926, 2853, 1651, 1593, 1427, 1399, 913, 762 cm^{-1}; MS: Calcd. for $C_{21}H_{33}N_6O$ (M^+ + 5H): 385.5263; found 385.2137.

4-(2-(2-(piperidinyl)ethyl)hydrazono)-1-(2-(piperidin-1-yl)ethyl)-1H-pyrrolo[3,2-g] isoquinoline-4,9-dione **(3c)**

The solution of 2-(piperidinyl)ethylhydrazine (0.2649 g, 2.4 mmol) in EtOH (1 ml) was slowly added to the mixture of compound **2c** (0.1586 g, 0.5 mmol) and triethylamine (0.2 ml, 1.5 mmol) in THF (4 ml) at room temperature. The reaction mixture was stirred at room temperature for 6 h. Then the second portion of the hydrazine (0.2649 g, 2.4 mmol) in EtOH (1 ml) was added. The reaction mixture was stirred for an additional 6 h. After that, the solvent was removed in *vaccuo*. The crude product was purified by column chromatography (aluminium oxide) eluting with EtOAc to give product **3c** (0.1205 g, 56%) as an orange liquid. ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 9.34 (s, 1H), 8.75 (d, *J* = 5.1 Hz, 1H), 7.75 (d, *J* = 5.1 Hz, 1H), 7.06 (d, *J* = 2.4 Hz, 1H), 6.38 (d, *J* = 2.4 Hz, 1H), 4.70-4.40 (m, 2H), 3.13 (t, *J* = 5.7 Hz, 2H), 3.04 (dd, *J* = 18.3, 5.7 Hz, 2H), 2.70 (t, *J* = 6.8 Hz, 2H), 2.55-2.30 (m, 8H), 1.80-1.30 (m, 12H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 174.5, 157.5, 152.0, 148.5, 141.0, 138.0, 131.5, 127.0, 124.0, 120.0, 107.0, 61.5, 60.0, 54.0 (4 x C), 46.5, 42.5, 26.0, 25.5, 24.5 (2 x C), 24.0 (2 x C) ppm**;** IR (in CH_2Cl_2) ν_{max} = 2936,1656, 1583, 1384, 1247 cm^{-1}; MS for $C_{25}H_{34}N_6O$: m/z (%) = 436 (M^++ 2H, 0.22), 404 (5), 306 (60), 223 (10), 98 (100).

4-(2-(3-(N,N-dimethylamino)propyl)hydrazono)-1-(3-(N,N-dimethylamino)propyl)-1H-pyrrolo[3,2-g] isoquinoline-4,9-dione **(3d)**

The solution of 3-(*N,N*-dimethylamino)propylhydrazine (0.5105 g, 5.0 mmol) in EtOH (2 ml) was slowly added to the mixture of compound **2d** (0.2803 g, 0.99 mmol) and triethylamine (0.2 ml, 1.5 mmol) in THF (6 ml) at room temperature. The reaction mixture was stirred at room temperature for 2 h. Then the second portion of the hydrazine (0.5105 g, 5.0 mmol) in EtOH (2 ml) was added. The reaction mixture was stirred for an additional 120 h. After that solvent was removed in *vaccuo*. The crude product was purified by column chromatography (aluminium oxide) eluting with EtOAc to give product **3d** (0.0937 g, 24%) as an orange liquid. ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 9.27 (s, 1H), 8.68 (d, *J* = 5.1 Hz, 1H), 7.70 (d, *J* = 5.4 Hz, 1H), 6.99 (d, *J* = 2.4 Hz, 1H), 6.35 (d, *J* = 2.7 Hz, 1H), 4.60-4.30 (m, 2H), 3.15 (t, *J* = 7.5 Hz, 2H), 2.90 (t, *J* = 4.5 Hz, 2H), 2.30-2.05 (m, 14H), 1.67 (p, *J* = 7.5 Hz, 4H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 173.5, 157.5, 152.0, 148.7, 141.3, 131.3, 126.2, 124.0, 122.5, 120.5, 106.5, 59.8, 57.3, 56.0, 50.6, 47.1 (4 x C), 29.0, 22.3 ppm**;** IR (in CH_2Cl_2) ν_{max} = 3406, 2947,1648, 1561, 1463, 1404, 1256 cm^{-1}; MS for $C_{21}H_{31}N_6O$: m/z (%) = 383 (M^++ H, 0.22), 404 (0.18), 352 (15), 284 (25), 239 (25), 84 (66), 54 (100).

4-(2-(3-(piperidinyl)propyl)hydrazono)-1-(3-(piperidinyl)propyl)-1H-pyrrolo[3,2-g] isoquinoline-4,9-dione **(3e)**

The solution of 3-(piperidinyl)propylhydrazine (0.3650 g, 2.3 mmol) in EtOH (1 ml) was slowly added to the mixture of compound **2e** (0.1530 g, 0.46 mmol) and triethylamine (0.2 ml, 1.5 mmol) in THF (4 ml) at room temperature. The reaction mixture was stirred at room

temperature for 6 h. Then the second portion of the hydrazine (0.3650 g, 2.3 mmol) in EtOH (1 ml) was added. The reaction mixture was stirred for an additional 120 h. After that, the solvent was removed in *vaccuo*. The crude product was purified by column chromatography (Aluminium oxide) eluting with EtOAc to give product **3e** (0.0529 g, 24%) as an orange liquid. ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 9.29 (s, 1H), 8.69 (d, *J* = 4.1 Hz, 1H), 7.70 (d, *J* = 4.1 Hz, 1H), 6.99 (d, *J* = 2.4 Hz, 1H), 6.31 (d, *J* = 2.7 Hz, 1H), 4.60-4.30 (m, 2H), 2.94 (t, *J* = 7.5 Hz, 2H), 2.56 (t, *J* = 7.5 Hz, 2H), 2.50-2.30 (m, 4H), 2.24 (t, *J* = 7.4 Hz, 2H),1.70-1.35 (m, 16H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 174.0, 157.0, 153.0, 149.0, 142.5, 136.5, 132.0, 128.0, 123.0, 120.5, 105.6, 59.0, 56.9, 55.0 (2 x C), 54.5 (2 x C), 48.5, 47.5, 34.5, 28.6, 27.5, 25.0 (2 x C), 24.5 (2 x C), 22.30 ppm**;** IR (in CH_2Cl_2) ν_{max} = 3420, 2935,1738, 1658, 1499, 1228 cm^{-1}; MS for $C_{27}H_{38}N_6O$: m/z (%) = 383 (M^+, 0.28),433 (23), 432(72), 322 (54), 320 (45), 124 (100).

Synthesis of azanaphthoquinone annelated pyrrolo thiosemicarbazones

1-[2-N,N-(dimethylamino)ethyl]-1H-pyrrolo[3,2-g]isoquinoline-4,9-dione thiosemicarbazone (**4a**)

The suspension mixture of **2a** (0.0853 g, 0.32 mmol) and thiosemicarbazide (0.0337 g, 0.37 mmol) in ethanol (4 ml) and conc. HCl was stirred at 80°C for 6 h. The crude product was purified by column chromatography (aluminium oxide) eluting with gradient EtOAc/MeOH; 9:1 to give product **4a** (0.0223 g, 26%) as an orange solid. ^{1}H-NMR (300 MHz, CD_3OD): δ = 9.24 (s, 1H), 8.69 (d, *J* = 5.1 Hz, 1H), 8.46 (d, *J* = 5.1 Hz, 1H), 7.54 (s, 1H), 7.06 (s, 1H), 4.73 (t, *J* = 6.8 Hz, 2H), 2.91 (t, *J* = 6.8 Hz, 2H,), 2.44 (s, 6H) ppm; ^{13}C-NMR (75 MHz, CD_3OD): δ = 181.0, 174.0, 151.0, 150.0, 147.5, 142.5, 133.0, 127.0, 126.0, 122.0, 118.5, 107.5, 59.0, 46.5, 44.0 (2 x C) ppm. IR (KBr): ν_{max} = 3426, 2925, 1623, 1442, 1219,11136, 917 cm^{-1}; MS for $C_{16}H_{18}N_6OS$: m/z (%) = 342 (M+, 16.2), 252 (19), 196 (9), 91 (100).

1-(2-(pyrrolidin-1-yl)ethyl)-1H-pyrrolo[3,2-g]isoquinoline-4,9-dione thiosemicarbazone (**4b**)

The suspension mixture of **2b** (0.1257 g, 0.34 mmol) and thiosemicarbazide (0.0328 g, 0.36 mmol) in ethanol (7 ml) and conc. HCl was stirred at 80°C for 20 h. The crude product was purified by column chromatography (aluminium oxide) eluting with gradient EtOAc/MeOH; 9:1 to give product **4b** (0.1064 g, 68%) as orange solid. ^{1}H-NMR (300 MHz, Acetone D_6): δ = 9.33 (s, 1H), 8.75 (d, *J* = 5.7 Hz, 1H), 8.50 (d, *J* = 5.7 Hz, 1H), 8.20 (br s, 1H), 7.66 (d, *J* = 3.0 Hz, 1H), 6.99 (d, *J* = 2.7 Hz, 1H), 4.76 (t, *J* = 6.3 Hz, 2H), 3.60 (br s, 2H), 2.94 (t, *J* = 6.3 Hz, 2H), 2.58 (br s, 4H), 1.72 (br s, 4H) ppm; ^{13}C-NMR (75 MHz, Acetone D_6): δ = 181.0, 174.0, 171.0, 152.0 (C-6), 148.0, 143.0, 134.0, 133.0, 125.0, 121.0, 118.0, 107.0, 57.0, 49.0, 43.0, 23.0 (2 x C) ppm; IR (KBr): ν_{max} = 3417, 2926, 1625, 1572, 1425, 1217, 1141, 923 cm^{-1}; MS for $C_{18}H_{20}N_6OS$: m/z (%) = 368 (M^+, 10), 281 (12), 198 (13), 91 (57), 54 (100).

1-(2-(piperidin-1-yl)ethyl)-1H-pyrrolo[3,2-g]isoquinoline-4,9-dione thiosemicarbazone (**4c**)

The suspension mixture of **2c** (0.0591 g, 0.19 mmol) and thiosemicarbazide (0.0183 g, 0.19 mmol) in ethanol (4 ml) and conc. HCl was stirred for 45 h at 80°C. The crude product was purified by column chromatography (aluminium oxide) eluting with gradient EtOAc/MeOH; 9:1 to give product **4c** (0.0702 g, 96%) as an orange solid. ^{1}H-NMR (300 MHz, Acetone D_6): δ = 9.35 (s, 1H), 8.78 (d, *J* = 5.4 Hz, 1H), 8.54 (d, *J* = 5.4 Hz, 1H), 8.30 (s, 1H), 7.72 (d, *J* = 3.0 Hz, 1H), 7.02 (d, *J* = 2.7 Hz, 1H), 4.78 (t, *J* = 6.3 Hz, 2H), 2.95 (s, 2H), 2.79 (t, *J* = 6.3 Hz, 2H), 2.60-2.40 (m, 4H), 1.70-1.40 (m, 6H) ppm; ^{13}C-NMR (75 MHz, Acetone D_6): δ = 181.0, 175.5, 157.5, 152.0, 150.0, 145.0, 133.0, 128.0, 127.0, 122.0, 119.5, 108.5, 60.0, 55.0 (2 x C), 49.0, 28.0 (2 x C), 25.0 ppm; IR (KBr): ν_{max} = 3355, 2924, 1616, 1560, 1420, 1267, 1217, 937 cm^{-1}; MS for $C_{19}H_{22}N_6OS$: m/z (%) = 384 (M^+ + 2H, 1.8), 295 (2), 198 (2), 91 (26).

1-[3-N,N-(dimethylamino)propyl]-1H-pyrrolo[3,2-g]isoquinoline-4,9-dione thiosemicarbazone (**4d**)

The suspension mixture of **2d** (0.3295 g, 1.16 mmol) and thiosemicarbazide (0.1195 g, 1.31 mmol) in ethanol (20 ml) and conc. HCl was stirred at 80°C for 5 h. The crude product was purified by column chromatography (aluminium oxide) eluting with gradient EtOAc/MeOH; 9:1 to give product **4d** (0.0771 g, 19%) as an orange solid. ^{1}H-NMR (300 MHz, CD_3OD): δ = 9.28 (s, 1H), 8.75 (d, *J* = 4.5 Hz, 1H), 8.62-8.49 (m, 1H), 7.59 (s, 1H), 7.41 (s, 1H), 4.83-4.60 (m, 2H), 3.30-3.10 (m, 2H) 2.90 (s, 6H), 2.50-2.25 (m, 2H) ppm; ^{13}C-NMR (75 MHz, CD_3OD): δ = 180.0, 176.0, 172.0, 152.5, 149.0, 142.0,133.0, 132.0,125.0, 121.0, 117.0, 107.0, 55.0, 54.0 (2 x C), 48.0 ppm; IR (KBr): ν_{max} = 1590, 1428, 1212, 1142, 1967 cm^{-1}; MS for $C_{17}H_{20}N_6OS$: m/z (%) = 355 (M+, 3.2), 267 (9), 198 (8), 91 (40), 67 (100).

1-(3-(piperidin-1-yl)propyl)-1H-pyrrolo[3,2-g]isoquinoline-4,9-dione thiosemicarbazone (**4e**)

The suspension mixture of **2e** (0.0350 g, 0.11 mmol) and thiosemicarbazide (0.0119 g, 0.13 mmol) in ethanol (3 ml) and conc. HCl was stirred for 45 h at 80°C. The crude product was purified by column chromatography (aluminium oxide) eluting with gradient EtOAc/MeOH; 8:2 to give product **4e** (0.0410 g, 99%) as an orange solid. ^{1}H-NMR (300 MHz, $CDCl_3$): δ = 9.45 (s, 1H), 8.80 (d, *J* = 5.4 Hz, 1H), 8.07 (d, *J* = 5.4 Hz, 1H), 7.54 (br s, 1H), 7.32 (d, *J* = 2.9 Hz, 1H), 6.91 (d, *J* = 2.9 Hz, 1H), 4.63, (t, *J* = 6.8 Hz, 2H), 2.53-2.38 (m, 4H), 2.33 (t, *J* = 6.8 Hz, 2H), 2.09 (t, *J* = 6.8 Hz, 2H), 1.70-1.30 (m, 6H) ppm; ^{13}C-NMR (75 MHz, $CDCl_3$): δ = 181.0, 174.0, 171, 152.0, 148.0, 143.0, 134.0, 134.0, 133.0, 125.0, 121.0, 118.0, 107.0, 57.0, 49.0 (2 x C), 43.0, 23.0 (2 x C) ppm; IR (KBr): ν_{max} = 3355, 2924, 1616, 1560, 1420, 1267, 1217, 937 cm^{-1}; MS for $C_{20}H_{24}N_6OS$: m/z (%) = 395 (M+, 7.4), 309 (8), 197 (7), 148 (100), 91 (40).

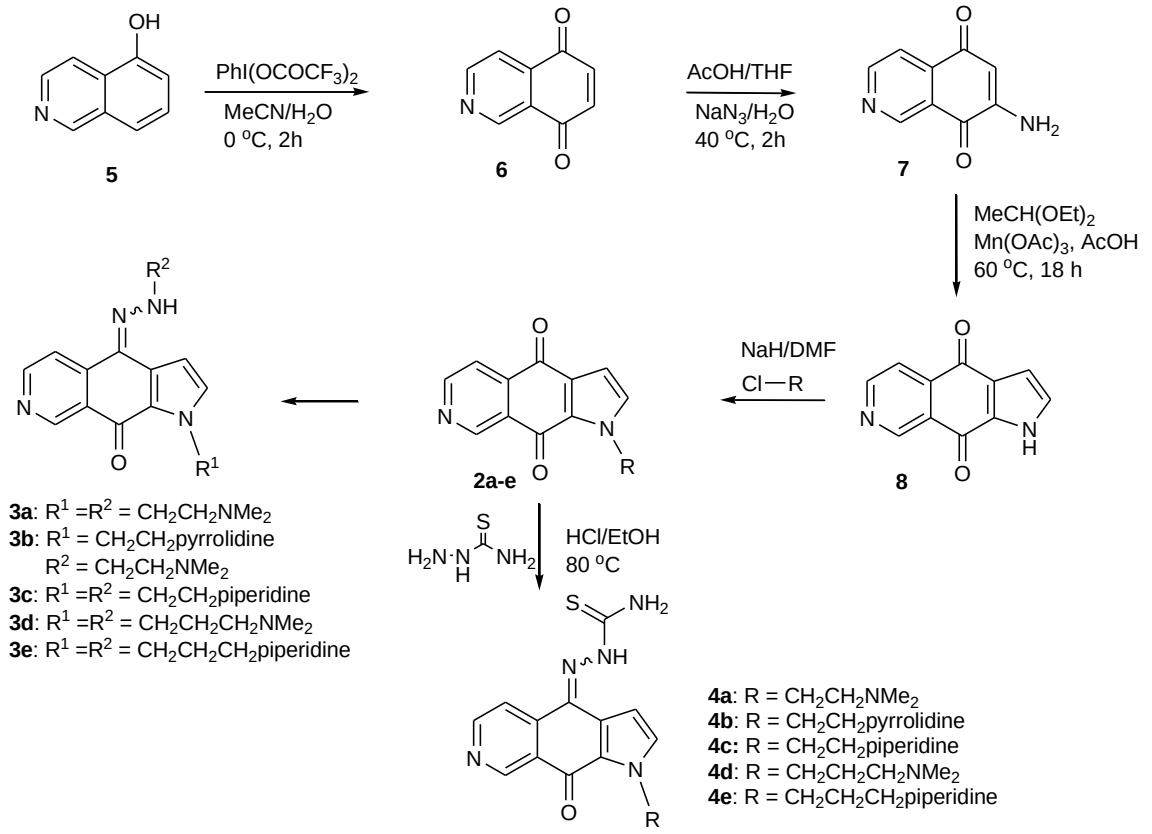
Biological evaluation

Antiproliferative acitivity of the synthesized compounds was evaluated with human cancer cell line: KB/Hela (cervical carcinoma). The concentration of compounds that inhibited 50% (IC_{50}) of cell proliferation after 48 h was detected by automatic xCELLigence System (Roche) using the data from at least two independent MTT cytotoxicity assays and reported in μM.

Results and discussion

The core structure azanaphthoquinone annelated pyrrole skeletion; 1*H*-pyrro[3,2-*g*]isoquinoline-4,9-dione (**8**), was synthesized by a three step reaction starting with the commercially available 5-hydroxyisoquinonline (**5**) [10]. The side chains were introduced by alkylation reaction of compound **8** with 2 to 3-carbon side chains under a basic condition at 67ºC (4 h) to give mono-substituted azanaphthoquinone annelated pyrroles 2a-**e** in moderate to good yield (13-70%). We found that the steric effect played an important role in yield of alkylation and condensation reaction. The yield of alkylation decreased from 70% for a compound with 2-carbon (**2a**) to 13% for a compound with 3-carbon side chain (**2e**).The condensation reaction of **2** with hydrazine derivative in THF/EtOH using triethylamine as a base at room temperature (24 h) occurred regio-selectively at C-4 position to furnish the target molecules **3** as shown in scheme 1. The shifting of the ^{13}C-NMR signal of C-4 in compound **2a** from 179.7 ppm to 157.5 ppm in product **3a** confirmed that the hydrazine moiety was condensed into the mono-substituted compound **2a** at C-4. The structure elucidation of **2a** was accomplished by 2D-NMR and NOE experiment. Irradiation of methylene group in hydrazone moiety showed a significant nuclear Overhauser enhancement of hydrogen atom at C-5 thus indicating the condensation at C-4. We suggested that the steric effect of the first side chain probably prevents the condensation at C-9 [10]. Hydrazones **3a-e** were obtained in moderate yields in the range of 24-56% yield.

The condensation of compound **2a-e** with thiosemicarbazide was performed in EtOH /conc.HCl at 80°C (4-45 h) occurred regio-selectively at C-4 position to furnish the target molecules **4a-e** in a moderate to good yield (19-99%). Structure elucidation of thiosemicarbazone **4a** was confirmed by ^{1}H-NMR, ^{13}C-NMR, IR and MS techniques. The ^{1}H-NMR showed shifting of the signal of H-6 from δ = 8.98 ppm in starting material **2a** to 8.69 ppm in the product **4a**, H-5 from δ 7.95 ppm in starting material **2a** to 8.46 ppm in the product **4a**. ^{13}C-NMR signal of C=S at 181 ppm and the shifting of signal of C-4 from 179.7 ppm to 172.0 ppm were the evidence to confirm that condensation reaction was occurred regio-selectively at C-4.



Scheme 1. Synthetic pathway of azanaphthoquinone annelated pyrrolo hydrazones (**3**) and thiosemicarbazones (**4**)

The biological evaluation of compound **3** and **4** were screened for antiproliferative activity against human cancer cell line: HeLa (cervical carcinoma). The concentration of compounds that inhibited 50% (IC_{50}) of cell proliferation after 48 h were detected by automatic xCELLigence System (Roche) using the data from at least two independent MTT cytotoxic assays. The results of the cytotoxic assays are shown in Table 1. Interestingly, mono-substituted products **2** showed significantly higher inhibition across Hela cell line compared to hydrazones **3** and thiosemicarbazones **4**. Compound **2b** with two-carbon side chain ending with pyrrolidinyl group was the most active compound with IC_{50} value of 0.008 μM. Hydrazone series showed low to moderate activity with IC_{50} in the range of 0.282-8.650 x 10^3 micro molar. Compound **3a** was the most active compound in this series. Thiosemicarbazones **4** showed slightly higher activity in comparison with hydrazone series with IC_{50} value of 10.850-5.500 x 10^3 μM. Compound **4b** was the most active in this series with IC_{50} value of 10.850 μM.

In those three series of synthesized compounds, we found that the derivative with 2-carbon side chains exhibited higher activity than those of 3-carbon side chains. This can suggest that the steric effect could be the major factor in antiproliferative activity of these potential intercalating agents.

Table 1. The antiproliferative activity of synthesized compounds on cervical carcinoma: HeLa expressed in IC_{50} (μM)

Compounds	IC_{50} [μM]		
	2	**3**	**4**
a	11.040	0.282	14.600
b	0.008	26.282	10.850
c	42.000	2.110×10^3	52.289
d	0.141	8.650×10^3	2.090×10^3
e	inactive	inactive	5.500×10^3

Conclusion

A series of thiosemicarbazone derivatives of azanaphthoquinone annelated pyrrole was successfully synthesized by a five step reaction. We found that the steric effect played an important role in the yields of alkylation and condensation reaction. The yield of alkylation decreased from 70% for compound with 2-carbon (**2a**) to 13% for compound with 3-carbon side chain (**2d**). A similar effect was informed for the condensation with thiosemicarbazide at C-4. Thiosemicarbazone of the pyrrolo[3,2-g]isoquinoline series exhibited moderate IC_{50} values compared with the mono-substituted precursors, but slightly higher than a series of hydrazones. In those three series of synthesized compounds, we found that the derivative with 2-carbon side chains exhibited higher activity than those of 3-carbon side chains. This can suggest that the steric effect could be the major factor in antiproliferative activity of these potential intercalating agents. These results provide useful information for further studies and for the development of an extended compound library.

Acknowledgements

We would like to thank NRCT for financial support in this research work, Dr. Pawana Phanomket at the College of Medicine and Public Health, Ubon Ratchathani University for biological testing, and the Faculty of Science, Ubon Ratchathani University for use of the facilities.

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Synthesis of Fluorescently-Labeled Huwentoxin-X (HWTX-X) Microtoxin

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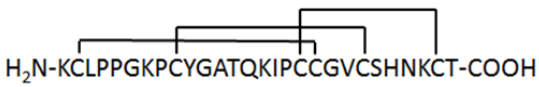
Abstract

Huwentoxin-X microtoxin (HWTX-X) is classified as a neurotoxic peptide isolated from the venom of the Chinese Bird spider *Ornithoctonus huwena.* It is comprised of 28 amino acids with three disulfide bridges and well-known as an important cystine knot (ICK) motif inhibitor. HWTX-X demonstrated a promising biological activity in blocking the Ca^{2+} channel, which is extensively known as a great candidate for therapeutic applications, especially for pain treatment. This research aimed to chemically synthesize native HWTX-X microtoxin *via* the solid phase peptide synthesis (SPPS) strategy. It also focused on the synthesis of fluorescently-labeled HWTX-X, which demonstrated a promising applicability for bio-imaging purposes. The purity of HWTX-X was confirmed by high performance liquid chromatography (HPLC) and the molecular mass was then characterized by electrospray ionization (ESI) mass spectrometry.

Keywords: Huwentoxin-X, Ion Channel, Fluorescently Labeled Toxin, Solid Phase Peptide Synthesis

Introduction

Huwentoxin-X microtoxin (HWTX-X) is recognized as a neurotoxic peptide comprised of 28 residues with three disulfide bridges arranged in the pattern of C_1-C_4, C_2-C_5, and C_3-C_6 respectively, as shown in Figure 1. HWTX-X microtoxin was firstly isolated from the venom of the Chinese Bird spider *Ornithoctonus huwena*, and demonstrated a prominent biological activity for blocking N-type Ca^{2+} channels [1]. Interestingly, HWTX-X is known as the smallest microtoxin in the Huwentoxin family. This Huwentoxin family spans from HWTX-I which contains 33 amino acids and known as the N-type Ca^{2+} channel inhibitor [2]. HWTX-II, HWTX-VII, and HWTX-VIII are comprised of 37, 36, and 35 amino acid residues respectively. Importantly, they have been regarded as insecticidal neurotoxins [3-4]. HWTX-IV is composed of 35 amino acids residues with a dominant activity on tetrodotoxin-sensitive Na^+ channel blocker [5].



a)

b)

Figure 1 (a) The sequence alignment of HWTX-X microtoxin comprised of 28 amino acids. Disulfide bridges are depicted with black lines. (b) A picture of the Chinese Bird spider adopted from https://www.pinterest.com/pin/375839531376450249.

Voltage-gated ion channels (VGICs) are transmembrane proteins that play a critical role in the electrical signaling of cells by allowing the movement of ions passed through a cellular membrane [6]. The structure of the ion channel is depicted in Figure 2. These VGICs can be classified as voltage-gated sodium, potassium, calcium, and chloride channels. These ion channels can also be divided into sub-types, for example voltage-gated sodium channel consisting of nine sub-types (Nav1.1-Nav1.9) [7], voltage-gated calcium channel containing five types (T, L,

N, P/Q, and R) [8]. These sub-types show very high specificity with tissue and perform the function differently within the cell. Therefore, each sub-type is an important target of exploring new therapeutic agents for treating diseases. The development of new therapeutic agents for pain treatment is crucial, especially, N-type of Ca^{2+} channels, well-known as a key factor in cell signaling in dorsal root ganglion (DRG) cells. Thus, this sub-type of Ca^{2+} channels is the subject of great interest for the development of analgesic drugs [9]. This research developed a synthetic approach of HWTX-X, which is a specific N-type Ca^{2+} channel blocker. This study further included the fabrication of fluorescently-labeled HWTX-X which could show the great potential for bio-imaging applications, for instance, the mechanistic investigation of voltage-gated ion channels in living cells [10].

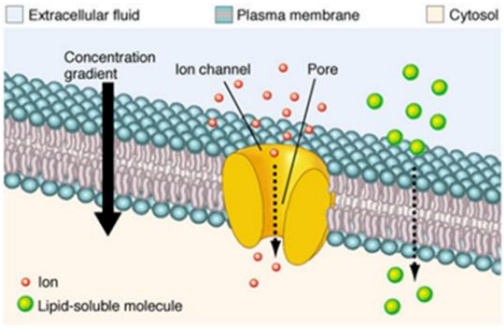


Figure 2 The general function of voltage-gated ion channels in cells. The picture is adopted from http://classroom.sdmesa.edu/eschmid/Chapter 3-Zoo145.htm.

The fluorescent label technique is recognized as an important tool for monitoring some biological processes in living cells [11]. Also, this method can be applied in a number of physical studies, ranging from the synthesis of fluorescently-labeled Hongotoxin1 ($HgTx_1$) which was applied for the study of voltage-gated K^+ channel [12], including the study of protein-protein interaction [13]. This work reported the synthesis of native HWTX-X and fluorescently-labeled HWTX-X microtoxin, which could be applied as an important tool for investigating the mechanism of actions between peptides and specific targeting membrane proteins, such as voltage-gate ion channels in living cells.

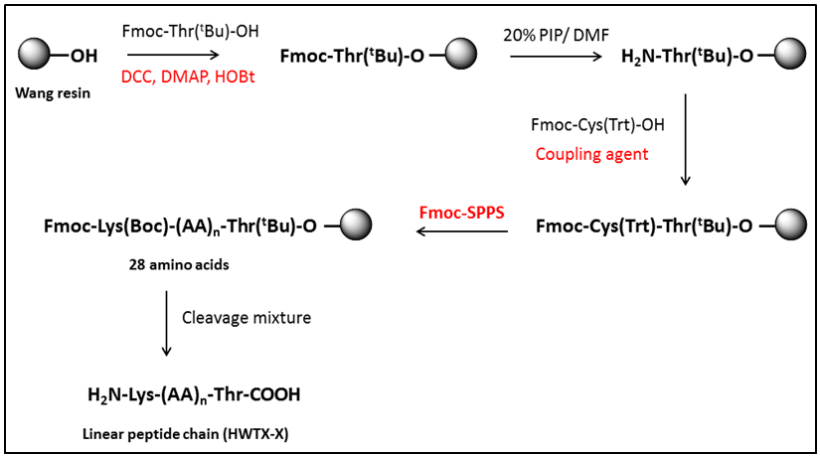
Materials and Methods

1.1 Chemicals and Equipment

Fmoc (N-(9-fluorenyl)methoxycarbonyl)-amino acids and wang resin were purchased from Aapptec, USA. Reagents used for peptide synthesis were purchased from Merck, Germany. 5-Fluorescein isothiocyanate was purchased from Carbosynth, UK. Trifluoroacetic acid was purchased from Fisher chemical, UK. All other reagents were analytical grade. Peptides were purified by high performance liquid chromatography (HPLC), Shimadzu (Japan). Mass spectra were measured by Thermo Finigan mass spectrometer (UK).

1.2 Linear peptide synthesis

Huwentoxin-X microtoxin was manually synthesized by using the solid phase peptide synthesis strategy as displayed in scheme 1 [14]. In the research, wang resin (0.06 mmol, 150 mg) was employed as a resin for peptide syntheses. Next, amino acids were assembled stepwise on a 0.2 mmol scale. Before each coupling step, the Fmoc protecting groups were removed by using 20% piperidine in DMF. The coupling steps were followed by using Dicyclohexylcarbodiimide (DCC), 4-Dimethylaminopyridine (DMAP), and 1-Hydroxybenzotriazole hydrate (HOBt) as coupling agents dissolved in DMF. After linear peptide chains were successfully synthesized, the fully protected linear peptides were treated with the deprotection cocktail (TFA/H_2O/EDT/TIS: 94:2.5:2.5:1) for 3 h at room temperature. Then, fully unprotected peptides were precipitated with cold methyl-*t*-butylether, and then resulting peptides were washed by methyl-*t*-butylether three times. White powder peptides were obtained by lyophilization.



Scheme 1 Solid phase peptide synthesis (SPPS) strategy of HWTX-X microtoxin

Attachment of the first amino acid on Wang resin

Fmoc-Thr(^{t}Bu)-OH (0.2 mmol, 3 eq.), DCC (0.2 mmol, 3 eq.), DMAP (0.2 mmol, 3 eq.), and HOBt (0.2 mmol, 3 eq.) were dissolved in DMF (5 mL). This solution was then added to wang resin (0.06 mmol, 150 mg) and was stirred continuously at an ambient temperature for 24 h. The resin was washed with DMF (3 × 1 mL), DCM (3 × 1 mL) and diethyl ether (3 × 1 mL) respectively. The resin was dried *in vacuo* and the loading of resin was estimated by Fmoc test.

Amino acids assembly *via* SPPS

After the first amino acid attachment, the resin swelled in DMF for 1 h and then drained off. Propanoic anhydride, DIPEA, and DMF were added to the resin and stirred at room temperature for 1 h. The capped resin was washed with DMF (4 × 1 mL). Next, the Fmoc deprotection was carried out by using 20% piperidine in DMF for 30 min and washed with DMF (3 × 1 mL).Then, a resin was coupled by Fmoc-Cys(Trt)-OH by using DCC, DMAP ,and HOBt as coupling agents. The reaction was allowed to proceed for 1 h and washed with DMF (3 × 1 mL). The resin was de-protected by using 20% piperidine in DMF (de-protection step) and coupled (coupling step) with the next amino acid in the presence of DCC, DMAP, and HOBt. The syntheses was continuously performed until the last amino acid (Fmoc-Lys(Boc)-OH) was coupled. The resin was de-protected and washed with DMF (3 × 1 mL), DCM (3 × 1 mL), and diethyl ether (3 × 1 mL) respectively. The resin was dried *in vacuo* and was then cleaved by treating with a de-protection cocktail (TFA/H_2O/EDT/TIS: 94:2.5:2.5:1) for 3 h. Then, the filtrate was dried under nitrogen gas and then precipitated by cold methyl-*t*-butylether. Finally, the white solid was obtained by lyophilization. The solid phase peptide syntheses (SPPS) were assembled by following this procedure (above).

1.3 Peptide purification and peptide folding experiment

Crude linear peptides were purified by reverse phase HPLC (C18 column) by using the gradient system (15-60% acetonitrile in the presence of 0.1 % trifluoroacetic acid), flow rate 3 mL/min. The peptide fraction was collected and obtained as a white solid by lyophilization. Then, peptides were folded by using an oxidizing condition (5 mM GSH/0.5 mM GSSG in 0.1 M Tris-HCl solution at pH 8.0) and the reaction was allowed to proceed at room temperature for 24 h. The folding experiment was then confirmed by RP-HPLC.

1.4 Synthesis of fluorescently-labeled HWTX-X microtoxin

The folded peptide was labeled with fluorescent dye with a ratio of 2:1 by following these procedures. Pipetted peptide (1 mg/mL) 10 µL to eppendorf tube and then added 1M $NaHCO_3$ (pH 9) 2 µL, fluorescent dye 2.6 µL, and deionized water 5.4 µL respectively. The reaction was allowed to proceed at room temperature for 2 h. The reaction was then quenched by adding 10% TFA. The reaction was confirmed by RP-HPLC at wavelength 215 and 280 nm.

Results and discussion

Huwentoxin- X (HWTX- X) microtoxin was successfully synthesized. The synthetic strategy was carried out by using solid phase peptide syntheses (SPPS). In this research, wang resin was utilized as a resin for a linear HWTX- X assembly. The first amino acid (Fmoc- Thr(^{t}Bu) - OH) was firstly attached to wang resin by using DCC, DMAP, and HOBt as coupling agents. The loading capacity was investigated by using Fmoc test, and it was found to be approximately 64%. The resulting resin was further coupled with Fmoc- Cys(Trt) - OH by using DCC, DMAP, and HOBt as coupling agents. The SPPS was continuously coupled to other amino acids (the order of amino acid is depicted in Figure 1). Fmoc-Lys(Boc)-OH was lastly coupled to wang resin. In this step, Fmoc protecting group was then removed by 20% piperidine in DMF, and the linear HWTX-X was then cleaved from the resin by using TFA/ H_2O/ EDT/ TIS: 94: 2. 5: 2. 5: 1. The resulting HWTX- X microtoxin was purified by reverse phase HPLC (on ACE C18 column) with the gradient (15- 60% acetonitrile gradient). The HPLC chromatogram is shown in Figure 3. The expected linear HWTX- X was observed at a retention time of 35 min (as shown in Figure 3).

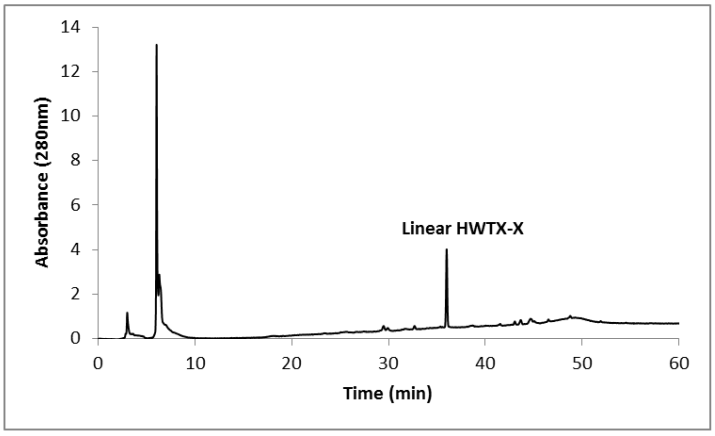


Figure 3 HPLC chromatogram of a linear HWTX-X microtoxin on ACE C18 column (4.6 mm x 250 mm). The peptides were eluted by using low pressure acetonitrile gradient (15-60% acetonitrile) for 60 min with the flow rate of 1.0 mL/min. The linear HWTX-X was monitored at 280 nm.

The molecular mass was confirmed by electrospray ionization- mass spectrometry (ESI- MS) , indicating the molecular mass of 1470. 11 (M+ 2H) $^{2+}$ and 980.5 $(M+3H)^{3+}$ respectively, as depicted in Figure 4. Both found molecular masses corresponded to that of linear HWTX- X (M. W. = 2936. 54 Da). The yield of chemical syntheses of linear HWTX- X peptide was found to be approximately 12%. Generally, the yield of peptide was low mainly due to the steric effect and peptide aggregation involved at the coupling step, directly affecting the obtained yield.

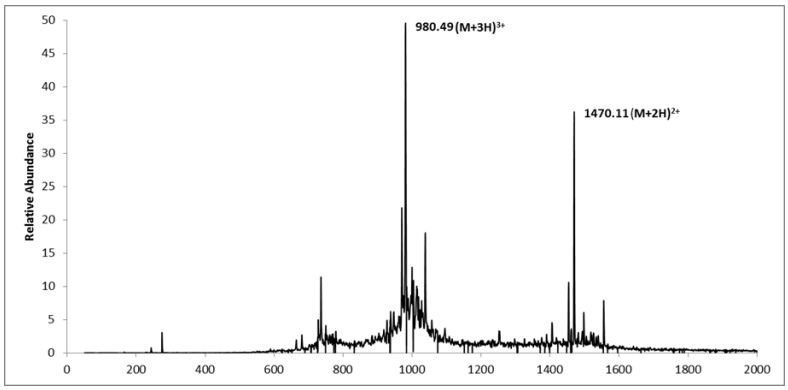


Figure 4 Electrospray ionization-mass spectrometry (ESI) analysis of linear HWTX-X microtoxin and the labeled peaks indicated all m/z ratios observed as 1470.11 $(M+2H)^{2+}$ and 980.49 $(M+3H)^{3+,}$ respectively

Then, a linear HWTX-X peptide was oxidized by using 5 mM GSH and 0.5 mM GSSG in 0.1 M Tris-HCl solution at pH 8.0. The folding experiment was carried out at an ambient temperature overnight. The formation of disulfide bonds was monitored by RP-HPLC, as shown in Figure 5. The HPLC chromatogram clearly showed that a folded HWTX-X (native HWTX-X) was eluted at 32 min relative to that of linear HWTX-X (35 min), meaning a folded HWTX-X was slightly higher polar when compared to that of a linear HWTX-X.

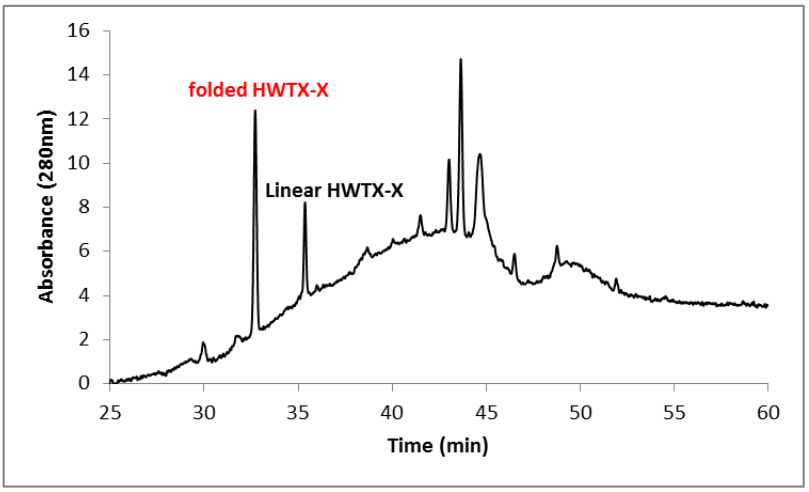


Figure 5 HPLC chromatogram of folded HWTX-X microtoxin on ACE C18 column (4.6 mm x 250 mm). The folded peptides were eluted by using low pressure acetonitrile gradient (15-60% acetonitrile) in 60 min at flow rate of 1.0 mL/min. The folding reaction was checked after 24 h at 280 nm.

Furthermore, the fluorescently-labeled HWTX-X for bio-imaging applications was also of great interest. The synthesis of fluorescently-labeled HWTX-X was successively carried out by using 5-Fluorescein isothiocyanate (FITC) as a fluorescent reporter mainly because of its intrinsic properties, namely high fluorescence intensity and quantum yield. The labeling reaction condition was carried out under an optimized condition (FITC: HWTX-X= 2:1). The reaction was allowed to proceed at an ambient temperature for 2 h, and quenched by adding 10% TFA. The fluorescent label of HWTX-X was confirmed by RP-HPLC. According to HPLC chromatogram, the fluorescently labeled HWTX-X was found at 38 min as displayed in Figure 6. We expect that this fluorescently-labeled HWTX-X can be used as a crucial tool to monitor the binding interaction mechanism between HWTX-X and Ca^{2+} channels.

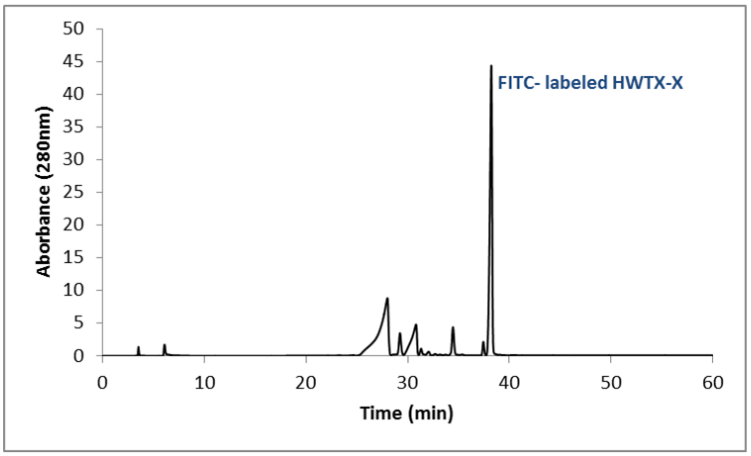


Figure 6 HPLC Chromatogram of Fluorescently labeled HWTX-X microtoxin on ACE C18 column (4.6 mm x 250 mm).The FITC-labeled HWTX-X was eluted by using acetonitrile gradient (15-60% acetonitrile) for 60 min with the flow rate of 1.0 mL/min. The labeling reaction was monitored at 280 nm.

Conclusion

This study reported the chemical synthesis of Huwentoxin-X (HWTX-X) and fluorescently-labeled HWTX-X protein. The Huwentoxin-X (HWTX-X) was successively synthesized *via* the solid phase peptide synthesis strategy (SPPS) and the molecular mass was confirmed by ESI-mass spectrometry. This HWTX-X will be further investigated for their biological activities against the Ca^{2+} channel, including the mutation of HWTX-X. The fluorescently-labeled HWTX-X will be further studied for bio-imaging applications, which is useful for monitoring the HWTX-X protein-Ca^{2+} channel binding interaction for medical purposes, such as pain treatment.

Acknowledgements

The authors are grateful to the Department of Chemistry, Faculty of Science and Technology, Thammasat University for MS analyses and Center of Scientific Equipment for Advanced Research (TUCSEAR), Thammasat University. The authors also acknowledge the Science Achievement Scholarship of Thailand (SAST).

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Effect of Mahanine in Combination with MG132 and Cisplatin on Growth Inhibition of Multidrug Resistant Human Oral Squamous Cell Carcinoma Cells

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Abstract

Drug resistance is a major problem in cancer therapy resulting in a failure of successful cancer treatment. To overcome drug resistance, anticancer drug combination has been used as a therapeutic strategy to enhance anticancer activity against many types of drug-resistant cancer cells. The present study demonstrated anti-cancer activity of carbazole alkaloid mahanine and its combinations with anticancer drug cisplatin and proteasome inhibitor MG132 against multidrug resistant human oral squamous cell carcinoma (CLS-354/DX) cells. CLS-354/DX cells were treated with mahanine, cisplatin, MG132, and their combinations at various concentrations (0–60 μM) for 24 h. Cell viability was determined by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. CompuSyn software was used to calculate combination index (CI) values and dosage reduction index (DRI) values. The results showed that a combination of mahanine with MG132 exhibited a synergistic effect against cell growth with CI value of 0.69±0.07. CI value of mahanine in combination with cisplatin was 1.49±0.13, suggesting an antagonistic effect. In addition, dosage reduction index (DRI) values were calculated. The combination of mahanine and MG132 reduced IC_{50} concentrations of mahanine and MG132 by 7.94 and 2.26 folds respectively in comparison to single treatments. Thus, the present study suggests that mahanine combined with MG132 might be a potential strategy for overcoming drug-resistant oral cancer.

Keywords: Carbazole Alkaloid, Multidrug Resistant Human Oral Squamous Cell Carcinoma, Drug Combination

Introduction

Resistance to targeted drug therapy has been an issue of concern as it constitutes a major barrier to progress in managing cancer treatment [1]. Chemotherapeutic drug treatment of oral squamous cell carcinoma can cause drug resistance and long-term side-effects as well as toxicity to normal cells [2]. Chemotherapy kills drug-sensitive cells but leaves behind a higher proportion of drug-resistant cells, resulting in therapeutic failure and cancer recurrence [2]. Discovery of novel effective combinations of anticancer agents is therefore an important step in the development of a therapeutic strategy to overcome drug-resistant cancers [3].

Cisplatin ($Pt(NH_3)_2Cl_2$) or cis-diamine-dichloroplatinum (II) is the most common chemotherapeutic drug used to treat many types of solid tumors, including oral cancers [4]. The mode of action of cisplatin is related to apoptosis mechanism by crosslinking with adenine and guanine based on the DNA, resulting in DNA damage and inducing apoptosis in cancer cells [4,5]. However, prolonged cisplatin treatment causes many side-effects, such as kidney problems, allergic reactions, decreased immunity, gastrointestinal disorders, hemorrhage, and hearing loss [4]. Prolonged treatment with cisplatin does not only exert deleterious effects but also facilitates the acquisition of multidrug resistance. Evidence showed that many cancer cell types have been developed to be cisplatin-resistant via diverse mechanisms. Resistance mechanisms include overexpression of ABC transporter gene of drug efflux pumps, activation of the PI3-K/Akt pathway, loss of p53 function, overexpression of anti-apoptotic bcl-2, loss of damage recognition, and interference in caspase activation [6]. In advanced-stage oral cancer, the most frequently reported cisplatin-resistant mechanisms are related to

increased ABC transporter proteins to reduce intracellular drug accumulation [7,8]. Thus, novel drug combination appears as an alternative strategy to overcome the limitations of cisplatin resistance in cancer.

Proteasome is an important proteolytic regulator responsible for degradation of intracellular proteins involved in cell death and cell survival, such as an inhibitor of apoptosis proteins, and it also plays a role in inducing drug resistance in cancer [9]. It has been demonstrated that treatment of the proteasome inhibitor could potentially promote apoptosis in drug-resistant cancer [10]. MG132 (carbobenzoxy-Leu-Leu-leucinal), an inhibitor of proteasome, has been reported as a novel cancer therapeutic agent. The inhibitor can induce cell cycle arrest and apoptosis through reactive oxygen species (ROS) generation [11]. In addition, MG132 is able to enhance sensitivity to apoptosis of cisplatin in various cancer cells [12,13].

Mahanine, a carbazole alkaloid, has been found abundantly in leaves of *Murraya koenigii* and related species which has been consumed in some parts of Southeast Asia. Mahanine has been reported to display interesting biological activities, including cytotoxic activity against oral squamous cell carcinoma (OSCC) cells [14]. Mahanine has been reported to induce apoptosis via various mechanisms, for example, disrupting mitochondrial membrane potential [15], inducing cytochrome c release [15], activating caspases (caspase-3, -6, -8, and -9) [14-16], stimulating reactive oxygen species production [16], cell cycle arrest [17], epigenetic regulation [18], and autophagic flux inhibition [14]. Moreover, mahanine also exerted synergistic effects with the anticancer drugs cisplatin and 5-fluorouracil in promoting cervical and colon cancer cell death [19,20]. Therefore, it is interesting to explore mahanine as a promising herbal compound for combination with other anticancer agents.

Our recent data reported that oral cancer cell line CLS-354/DX became drug-resistant via overexpression of ABC transporter protein and increased drug efflux pump activity [21]. This cell line also showed a good response to the cytotoxic effect of carbazole alkaloid [21]. Hence, this present study was designed to analyze whether drug combination of mahanine and cisplatin or MG132 would exert synergistic effects against CLS-354/DX cell growth. Our findings suggested that mahanine could enhance MG132 cytotoxicity, which indicated the assumption that mahanine might be used as a potent anticancer agent to overcome drug-resistant OSCC cells.

Methodology

Materials

Cisplatin, proteosome inhibitor MG132, and dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich Corp., St. Louis, MO, USA. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reagent were obtained from Invitrogen, Grand Island, NY.

Plant material

Mahanine was extracted using dichloromethane as a solvent from *Murraya koenigii* leaves as previously described [14]. This plant was verified and authenticated by Professor Thawatchai Santisuk. A voucher specimen (Apichart Suksamrarn, number 064) is deposited at the Faculty of Science, Ramkhamhaeng University, Bangkok, Thailand. Mahanine was dissolved in 100% dimethyl sulfoxide to 5 mM stock solution before conducting the experiments.

Cell lines and culture conditions

Multidrug resistant human oral squamous cell carcinoma (CLS-354/DX) at passage number 50–60 was cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (PAA Laboratories GmbH, Pasching, Austria) supplemented with 10% fetal bovine serum (Biochrom GmbH, Berlin, Germany), 1% penicillin/streptomycin (PAA Laboratories GmbH), and 2 mM stable L-glutamine (Gibco, Life Technologies, Carlsbad, CA, USA). The CLS-354/DX cells were maintained in an atmosphere of 95% humidity and 5% CO_2 at 37°C.

Cytotoxicity test

The cytotoxicity was determined by using MTT assay. Briefly, CLS-354/DX cells were seeded into the 96-well plates at a density of 1.2×10^4 cells/cm^2 and allowed to grow for 48 h. Cells were then treated with various concentrations of mahanine, cisplatin, and MG132 for 24 h at 37°C in 5% CO_2 atmosphere. After treatment, MTT solution (0.5 mg/ml) was added into each well following the incubation for 3 h. Formazan crystals were dissolved with DMSO and absorbance was detected at 560 nm with subtraction of background at 670 nm by using a microplate reader. Cell viability was calculated using the following equation:

% Cell viability = [(Abs treated sample/ Abs untreated sample) x100].

The drug concentration causing 50% inhibition (IC_{50}) value was calculated by plotting from the viability graph.

Analysis of cytotoxic synergy

The combination experiment was conducted using a constant ratio as previously described by Chou (2006) [22]. Mahanine in a range of 0–75 μM was combined with cisplatin (0–25 μM) or MG132 (0–25 μM) based on a constant molar ratio 3:1. In this case, the optimal concentrations of synergistic interactions were observed *in vitro* at a certain mahanine/drug molar ratio 3:1 whereas other ratios were additive or antagonistic. These combinations were tested in CLS-354/DX cells for 24 h followed by MTT assay. Drug combination index was derived from median-effect equation correlated to the interaction of the combination of two drugs and the cytotoxicity effect in the following equation:

$$CI = D_{(1)}/D_{50(1)} + D_{(2)}/D_{50(2)}$$

where D_{50} is the doses of drug 1 and 2 alone that inhibits 50 percent of cell growth. $D_{(1)}$ and $D_{(2)}$ are the concentrations of drug 1 and 2 used in combination treatment which inhibit 50 percent of cytotoxicity. The CI values reflect the ways of interaction between two drugs; CI < 1 indicates synergism; CI = 1 indicates an additive effect; and CI > 1 indicates antagonism. Description and symbols of synergism or antagonism in drug combination studies analyzed with the combination index method are defined in Table 1.

Table 1 Interpretation of drug interaction value is defined as follows (Chou, 2006) [22]:

Range of Combination Index	Interpretation	Graded Symbols
< 0.10	Very strong synergism	+++++
0.10 – 0.29	Strong synergism	++++
0.30 – 0.69	Synergism	+++
0.70 – 0.84	Moderate synergism	++
0.85 – 0.89	Slightly synergism	+
0.90 – 1.09	Nearly additive	±
1.10 – 1.19	Slightly antagonism	-
1.20 - 1.44	Moderate antagonism	- -
1.45 – 3.29	Antagonism	- - -
3.30 – 9.99	Strong antagonism	- - - -
≥ 10.00	Very strong antagonism	- - - - -

Dose reduction index (DRI) were used to determine when the drug combination achieved synergy interaction as how many fold number or ratio of the concentration of drug alone can be reduced to achieve the effect levels in combination treatment. If the drug combination achieved the synergy, it could lead to reduced toxicity and maintain or increase therapeutic efficacy [22]. DRI was calculated using the following formula:

$$DRI = IC_{50} \text{ cytotoxic drug alone}/IC_{50} \text{ cytotoxic drug in combination treatment}$$

DRI = 1 indicates no dose reduction, whereas DRI > 1 and DRI < 1 indicate favorable and unfavorable dose-reduction respectively [22].

Results

Cytotoxic effects of individual treatment

Cisplatin, MG132 and mahanine were treated in CLS-354/DX cells and cell viability was evaluated by MTT assay. The individual treatment of cisplatin, MG132, and mahanine showed IC_{50} values of 21.06±2.25 (Figure 1a), 3.96±0.72 (Figure 1b), and 50.5±4.27 (Figure 1c) μM respectively. The results indicated that CLS-354/DX cells were sensitive to proteasome inhibitor MG132 than cisplatin and mahanine (Figure 1).

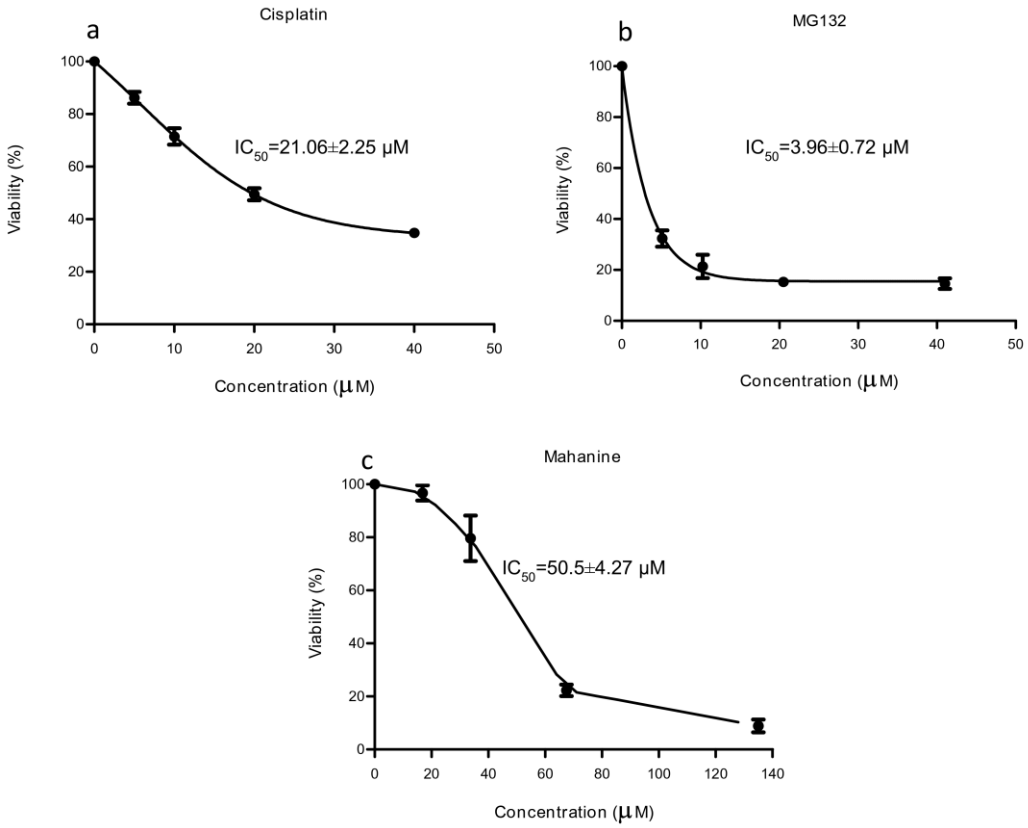

Figure 1 IC_{50} values of CLS-354/DX cells. Cells were treated with cisplatin (a), MG132 (b) and mahanine (c), followed by MTT assay. Data were expressed as mean ± SD from three independent experiments in triplicate.

Cytotoxic effects of drug combination treatment

Mahanine was combined with cisplatin and MG132 in a constant molar ratio of 3:1. Mahanine in combination with cisplatin and MG132 revealed the IC_{50} values of 13.09±1.11 and 1.75±0.05 μM respectively (Table 2). The combination index (CI) was calculated to determine the drug interaction. The combination of mahanine with cisplatin and MG132 showed CI values of 1.49±0.13 and 0.69±0.07 respectively (Figure 2). Mahanine combined with MG132 gave CI value of less than 1, interpreting the synergistic effect of this combination compared with the treatment of mahanine and cisplatin (CI > 1).

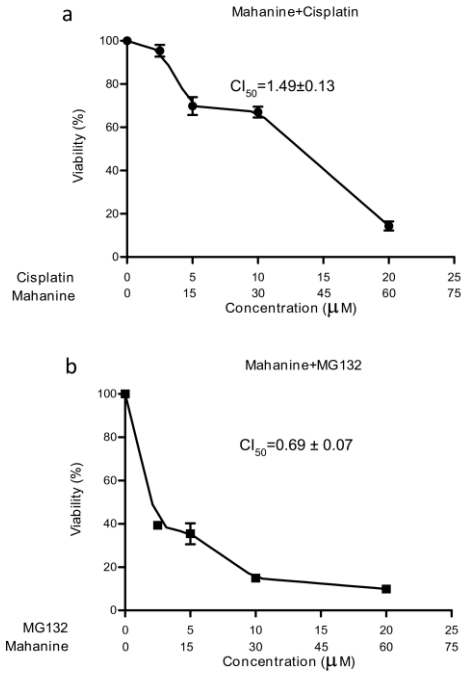

Figure 2 Drug combination treatments in CLS-354/DX cells. Cells were treated with mahanine combined with cisplatin (a), MG132 (b), and MG132 combined with cisplatin (c) for 24 h, followed by MTT assay.
Data were expressed as mean ± SD from three independent experiments in triplicate.

Dosage reduction index of the combination treatment

The dosage reduction index (DRI) of MG132 was calculated in the combination treatments. The results showed that the DRI value of MG132 in combination with mahanine was 2.26 fold. Moreover, the concentration of mahanine in combination with MG132 was approximately an 8 fold dose reduction compared with mahanine treatment alone (Table 2).

Table 2 CI and DRI values of the combination treatment

IC_{50} (μM)								
Mahanine	**Drugs**	**Mahanine+Drug Molar ratio 3:1**		**CI**	**Grade Symbol**	**Interpretation**	**$DRI_{Mahanine}$**	**DRI_{Drug}**
		Mahanine	**Drugs**					
50.5±4.27	Cisplatin 21.06±2.25	39.27±1.81	Cisplatin 13.09±1.11	1.49±0.13	- - -	Antagonism	N/A	N/A
	MG132 3.96±0.72	6.36±1.32	MG132 1.75±0.05	0.69 ± 0.07	+++	Synergism	7.94	2.26

N/A = not applicable

Discussion and conclusions

Mahanine has been shown to have a potent anticancer activity against various cancer cell lines. It is a DNA minor groove binding agent exerting cellular cytotoxicity on cancer cells with minimal toxicity to normal cells [23]. Mahanine was reported to synergistically enhance cytotoxicity of 5-fluorouracil through ROS-mediated activation of phosphatase and tensin homolog and p53/p73 in colon carcinoma [20]. Furthermore, it was reported that mahanine sensitizes cisplatin action on cervical cancer through STAT3 inhibition [19]. Mahanine had a cytotoxic effect against the parental CLS-354 cells with IC_{50} value of 15 μM [14]. We found that the drug-resistant CLS-354/DX cells had IC_{50} value greater than the parental CLS-354 by ~ 3-fold, suggesting the low responsiveness to mahanine. We revealed that mahanine in combination with cisplatin might not enhance cytotoxicity of cisplatin resistance CLS-354/DX cell line. CLS-354/DX was reported to overexpress multidrug resistance-

associated protein 1 (MRP1) and drug efflux pump activity which contributed to resistance to cisplatin and camptothecin [21]. It may be concluded that mahanine combined with cisplatin has no synergy against CLS-354/DX cytotoxicity. Mahanine treatment alone was reported to induce apoptosis in various cancer cells via caspase activation by intrinsic apoptosis pathway [15,16]. MG132 was reported to sensitize apoptosis of standard anticancer drugs in multidrug-resistant gastric cancer cells by downregulating the expression of anti-apoptotic Bcl-2 and MDR1 (P-gp), leading to apoptosis [24]. Furthermore, MG132 combined with verapamil was reported to reduce ABCB1 overexpression and decrease drug efflux pump activity in multidrug-resistant human uterine sarcoma cells [25]. This present study showed that mahanine in combination with MG132 had a INCREASED OR REDUCED? synergistic effect on cytotoxicity of CLS-354/DX cells rather than cisplatin. The possible mechanism of synergism may involve apoptosis induction as it was previously shown that mahanine could induce apoptosis and autophagic flux inhibition in CLS-354 cells [14]. However, mechanism of this combination will be further elucidated.

In summary, the present study showed that mahanine enhances the cytostatic and cytotoxic activities of the proteasome inhibitor MG132 in multidrug resistant OSCC cells. Mahanine in combination with MG132 may result in apoptosis and autophagic flux inhibition. The mechanism of the synergistic effects of mahanine combined with MG132 will be investigated in further studies to develop a novel effective drug combination for oral cancer treatment.

Acknowledgements

This research was granted by Walailak University Fund [WU58601] and [12/2559] for graduate studentship to Sirinya Thungtong and The Thailand Research Fund [DBG5980003].

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Evaluation of *in vitro* Bioactivity of Ethanolic Extract of *Cladogynos orientalis* Leaves: Antioxidation, Antimelanogenesis, and Anti-inflammation

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Abstract

Cladogynos orientalis (Euphorbiaceae) is a native medicinal plant in tropical regions of Asia. It has traditionally been used for treatments of stomach ache and abdominal pain. The aim of this study was to investigate the pharmacological activity of *C. orientalis* leaves. Alcohol-based extract of *C. orientalis* leaves was prepared in 50% ethanol by maceration (CLE). CLE contained high total phenolic content and strong DPPH scavenging activity (IC_{50} of 9.24±0.47 µg/ml). After 48 hr of incubation, CLE 250 µg/ml inhibited melanin production in B16-F10 murine melanoma cells (37.80±1.95%). Moreover, CLE significantly inhibited the mRNA expression of tyrosinase, tyrosinase-related protein-1 (TRP-1), and TRP-2 in B16-F10 cells. In addition, CLE suppressed the expression of LPS-induced pro-inflammatory genes (COX-2, IL-1β and TNF-α) in RAW264.7 murine macrophage cells in a dose-dependent manner. The findings revealed the antioxidant, antimelanogenesis, and anti-inflammation properties of *C. orientalis* leaves extract. This study recommends that this plant should be further developed for the utilization as an ingredient in traditional medicine and cosmetics.

Keywords: *Cladogynos orientalis*, Antioxidation, Antimelanogenesis, Anti-inflammation

Introduction

Aerobic organisms normally produce reactive oxygen species (ROS) via metabolic pathways. However, excessive ROS production may cause harmful effects on cells, tissues, and organs. Antioxidants may prevent the detrimental effects of ROS and treat oxidative stress-related diseases, such as atherosclerosis, stroke, diabetes, and cancer. Vitamin C, E, and phenolic compounds are well-known natural antioxidants and widely used to protect the human body from free radicals and delay the progress of several diseases [1]. Antioxidants are found in many natural plants and various natural phytochemicals are extremely influential agents in health promotion and disease prevention. They catch oxidants effectively or terminate the free radical chain-reaction and reduce the oxidative stresses from external and intrinsic resources in human physical conditions [2]. The exposure to oxidative stress induces inflammation of healthy organs and tissues [3]. During the inflammatory process, the inflammatory biomarkers are highly produced, such as ROS, reactive nitrogen species (RNS), tumor necrosis factor-α (TNF-α), interleukin (IL)-1, IL-6, and cyclooxygenase (COX)-2. The TNF-α, a pro-inflammatory cytokine, is a key cytokine that plays a role as a master regulator of inflammatory cytokine production involved in inflammation, immunity, and tumor progression. In addition to TNF-α, IL-1 and IL-6 also play roles as endogenous pyrogens by stimulating the release of prostaglandins catalyzed from COX [4]. In addition, free radicals can induce the melanin production process [5]. Melanin is synthesized from a unique organelle, called melanosomes [6]. Several steps of melanogenesis can be controlled, such as inhibition of tyrosinase gene expression or tyrosinase enzyme activity [5, 7]. Although there are three types of melanogenic enzymes, tyrosinase, and tyrosinase-related proteins (TRP)-1 and TRP-2, it is well known that tyrosinase plays the most critical role in pigmentation [6, 8].

Cladogynos orientalis Zipp. Ex Span (Thai name: Chet-ta-phang-khi), a member of the family Euphorbiaceae, is a shrub up to 2 cm high native in tropical regions of Asia. It is used in Thai traditional medicine for the relief of stomach ache and abdominal pain. The chemical constituents of this plant are diterpene, guainane sesquiterpene, flavones, aromatic glycosises, and megastigmane glucoside [9]. The bioactivity evaluation of this plant had not been previously reported. Therefore, in the present work, a semi-quantitative RT-PCR technique

was used to determine antimelanogenesis via the expression of tyrosinase, TRP-1, and TRP-2 in murine B16-F10 melanocytes, and anti-inflammation via the expression of COX-2, IL-1β, and TNF-α in murine RAW264.7 macrophages. Moreover, antioxidant activities were evaluated *in vitro* using DPPH and FRAP assays. The total phenol contents of the extract were measured by using the Folin-Ciocalteau method. The biological activity study of *C. orientalis* is very useful for further development of this plant in drug or cosmetic products.

Methodology

Reagents

Molecular biology grade agarose was obtained from Bio-Rad (Spain). 1kb DNA ladder and Blue/Orange 6X loading dye were sourced from Promega (USA). Primer β-actin, tyrosinase, TRP-1, TRP-2, COX-2, IL-1β, and TNF-α were obtained from Eurofins MWG Operon (Germany). Tris base and ethylenediaminetetraacetic acid are products of Ajax (Australia). 3-[4, 5-dimethylthiazol-2-yl]-2,5-dyphenyl tetrazolium bromide, Dulbecco's modified eagle medium (DMEM), Roswell Park Memorial Institute medium (RPMI), and fetal bovine serum (FBS) are products of Invitrogen (UK). Melanin, Kojic acid, 2,2-diphenyl-1-picrylhydrazyl and folin phenol are products of Sigma (USA). DNase Set, Omiscript RT Kit, TopTaq Master Mix kits were obtained from QIAGEN (Germany). All other chemicals including Novel Juice and RNA extraction kit were obtained from GeneDirex and GE Healthcare (UK) respectively.

Cell lines

B16-F10 murine melanoma cells were cultured in DMEM containing 10%-heat inactivated fetal bovine serum (FBS), 100 units/ml penicillin, and 100 µg/ml streptomycin, whereas RAW264.7 murine macrophage cells were carefully grown and maintained in complete media containing RPMI-1640, 10% FBS 100 units/ml penicillin, and 100 µg/ml streptomycin. The cells were cultured at 37°C in a humidified atmosphere with 5% CO_2.

Preparation of the plant extract

The mature leaves of *Cladogynos orientalis* were collected from Chulabhorn Dam, Chaiyaphum Province, Thailand. The plant was identified and authenticated by Thitimetharoch, T (taxonomist). A voucher specimen (TT-OC-SK-912) was deposited at the Faculty of Pharmaceutical Sciences, Khon Kaen University. The samples were pulverized and macerated in 50% ethanol for 5 days, then filtered through thin cloth and centrifuged at 3000 g, 25°C for 10 min using laboratory centrifuge (Kubota, Japan). The clear supernatant was concentrated using a rotary evaporator (ETERA, Japan) at 45-50°C, then freeze-dried (Christ, Germany). The ethanolic extract of *C. orientalis* was obtained and called CLE.

Determination of total phenolic content, DPPH radical scavenging activity, and ferric reducing ability

The total phenolic content was determined by the Folin-Ciocalteu method and expressed as µg gallic acid equivalent (GAE)/mg dried extract [10]. The DPPH radical scavenging activity was determined by the method described by Shimada et al. (1992) [11]. The negative (methanol) and positive (vitamin C and vitamin E) controls were parallel run. The ferric reducing ability of plasma (FRAP) was determined by a modified micro-assay method [12]. The extract was dissolved in 50% ethanol (1–10 mg/ml concentrations), then 10 µl of the extract solution was added into a 96-well microplate containing 200 µl of FRAP regent (freshly prepared by mixing 300 mM acetate buffer (pH 3.6) with 10 mM 2, 4, 6-tripyridyl-s-triazine in 40 mM HCl and 20 mM ferric chloride in distilled water at a ratio of 1:1:0.1). After mixing well and incubating at room temperature for 4 min, the absorbance was measured at 600 nm by a microplate reader (Anthos, Austria). The standard ferrous sulfate solution dissolved in 40 mM HCl was tested in a parallel process. The FRAP values were calculated from the standard curve of ferrous sulfate.

Cell viability assay

The viability assay of plant extract on RAW264.7 cells and B16-F10 cells was determined by using MTT assay [13]. Cells were seeded in a 96-well plate at a density of 1 x 10^5 cells per well and allowed to attach for 24 hr. Various concentrations of the samples were then added and incubated for 24 and 48 hr for RAW264.7 cells and B16-F10 cells respectively. After incubation, the MTT solution was added to each well at a final concentration of 0.1 mg/mL, and further incubated for 3 hr. The solution was removed and dimethyl sulfoxide was added. Finally, the cell viability was estimated in terms of absorbance at 570 nm.

Determination of melanin content

The determination of melanin content was slightly modified from a previously described method [14]. B16-F10 cells were seeded in a 12-well plate at a density of 1x10^5 cells per well and incubated overnight, followed by addition of the plant extract. After 48 hr of incubation, the cells were trypsinized with 0.25% trypsin and washed with DMEM

media. After centrifugation, the cell pellets were solubilized in 100 µl of 1M NaOH at 60°C for 1 h. The melanin content was determined at the absorbance of 405 nm and compared with the standard curve of melanin.

Determination of gene expression

For melanogenetic related genes, B16-F10 cells were plated at a density of $1x10^5$ cells/well in a 12-well tissue culture plate in a humidified atmosphere with 5% CO_2 overnight. The cells were treated with the samples and then incubated at 37°C for 48 h. For pro-inflammatory related genes, RAW264.7 cells were plated at a density of $1x10^6$ cells/well in a 12-well tissue culture plate in a humidified atmosphere with 5% CO_2 overnight. The cells were treated with the samples for 22 hr. After adding *E. coli* LPS to a final concentration of 1 µg/ml, the cells were further incubated at 37°C for 2 hr. The total RNA was isolated from the treated cells using a RNA extraction kit. The first strand cDNA was synthesized from 40 ng of total RNA with Omniscript reverse transcriptase. The primers used for amplifying the respective fragments were as shown in previous reports [14-16]. Specific primer for β-actin was used as a control. PCR was carried out by incubation of each cDNA sample with the primers (10 pmol each), Taq polymerase (1.25 U), and deoxynucleotide mix (0.2 mM each). Amplification was completed for 30 cycles. The conditions were denaturation at 95°C for 2 min (tyrosinase, TRP-1, TRP-2, COX-2, β-actin) and 94°C for 2 min (TNF-α, IL-1β); annealing at the annealing temperature (TA) for 1 min (COX-2, TNF-α, β-actin), 45 s (IL-1β), 30 s (tyrosinse, TRP-1, TRP-2), and primer extension at 72°C for 1 min (all other genes). The reactions were finally extended at 72°C for 10 min. The PCR products were then analyzed on 1.5% agarose gel, visualized by Novel Juices staining, and RT-PCR product densities were measured by a Gel Documentation and System Analysis machine. The expression of melanogenetic and pro-inflammatory related genes were calculated for the relative mRNA expression level with β-actin. All experiments were performed in triplicate.

Statistic analysis

All experiments were performed in triplicate and the results expressed as means ±S.D. One-Way ANOVA and multiple comparisons were used to analyze the significant differences ($P<0.05$) by using SPSS version 19.0 software.

Results

Phenolic content and anti-oxidative activity of CLE

The CLE with 7.40% yield exhibited strong antioxidant activity (IC_{50} of DPPH at 9.24±0.47 µg/ml and FRAP values at 0.78±0.64 mg $FeSO_4$/g extract), and high phenolic contents as shown in Table 1.

Table 1 Phenolic content, antioxidant activity by DPPH and FRAP determinations of CLE

Test sample	Yield (%)	Total phenolic content* GAE (µg/mg extract)	DPPH IC_{50}* (µg/ml)	FRAP* (mg $FeSO_4$/g extract)
CLE	7.40	307.95±12.20	9.24±0.47	0.78±0.64
Ascorbic acid	-	-	3.21±0.10	-
Tocopherol	-	-	6.99±0.12	-

* Values are expressed as means ±SD (n=3)

Toxicity of CLE on B16-F10 and RAW264.7 cells

The effect of CLE on the viability of B16-F10 and RAW264.7 cells was determined using MTT assay. The B16-F10 and RAW264.7 cells were treated with various concentrations of CLE at 0.00-1000 µg/ml for 48 and 24 hr respectively. The effect of CLE on the viability of B16-F10 and RAW264.7 cells was calculated as half inhibitory concentration (IC_{50}) values which were 600.74±55.16 and 961.51±35.26 µg/ml respectively (Figure 1). Based on these results, we evaluated the effect of CLE on melanogenesis and inflammation activity tests at doses lower than its IC_{50} value.

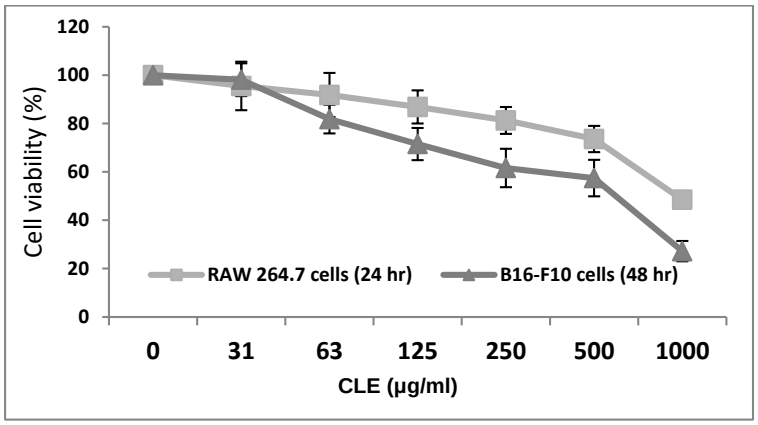

Figure 1 Effects of CLE on viability of RAW264.7 and B16-F10 cells.

Anti-melanogenesis effect of CLE

The results showed that CLE had the ability to inhibit melanin production in B16-F10 cells in a dose-dependent manner. CLE at a dose of 250 µg/ml inhibited melanin production by 33.55±0.66% and 37.80±1.95% after incubation with the cells for 24 and 48 hr respectively (Figure 2). The IC_{50} values of CLE and Kojic acid after incubation with the cells for 48 hr were 290.09±21.44 and 135.22±9.96 µg/ml respectively. In addition, as shown in Figure 3, the tyrosinase mRNA expression of CLE-treated cells decreased in a dose-dependent manner. CLE treatment at 65-250 µg/ml concentrations significantly decreased tyrosinase level. The expressions of TRP-1 and TRP-2 mRNA similarly decreased after treatment with CLE.

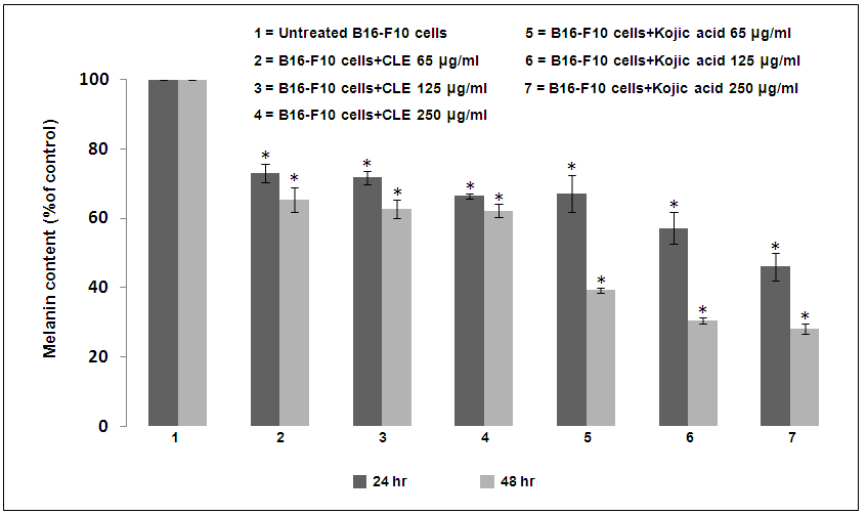

Figure 2 Effect of CLE and Kojic acid on melanin production in B16-F10 cells. (*P < 0.05 vs. untreated cells)

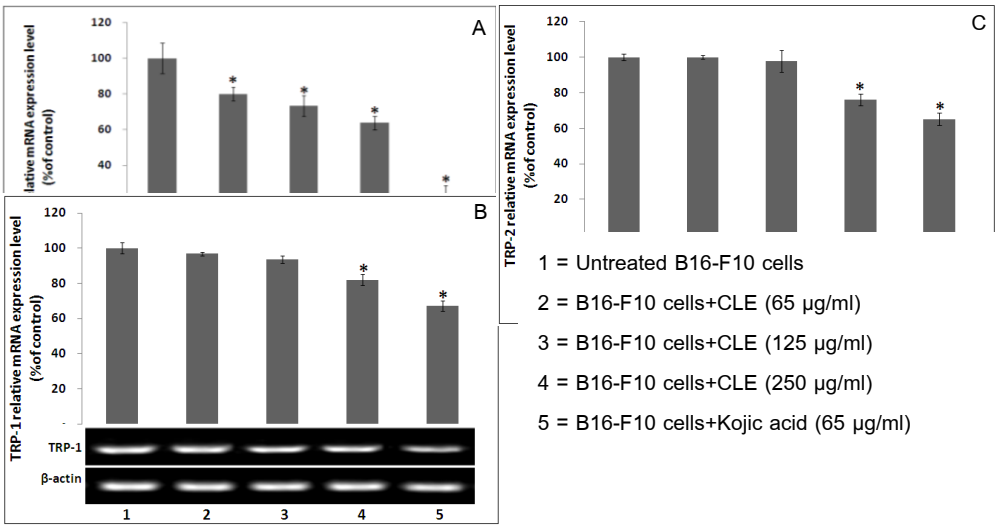

Figure 3 Effects of CLE and Kojic acid on mRNA expression of tyrosinase (A), TRP-1 (B) and TRP-2 (C). (Results are expressed as % of control, **P* < 0.05 vs. untreated cells)

Anti-inflammatory effect of CLE

In vitro RT- PCR results demonstrated the significant dose- dependence on several pro- inflammatory gene expressions, including COX- 2, IL- 1β, and TNF- α in LPS- induced RAW264. 7 cells. CLE inhibited the up-regulation of these pro-inflammatory genes in LPS-induced cells and did not change the expressions in the untreated. However, the suppressive effect of CLE on the expression of these genes was still less than those of Indomethacin-treated group (Figure 4).

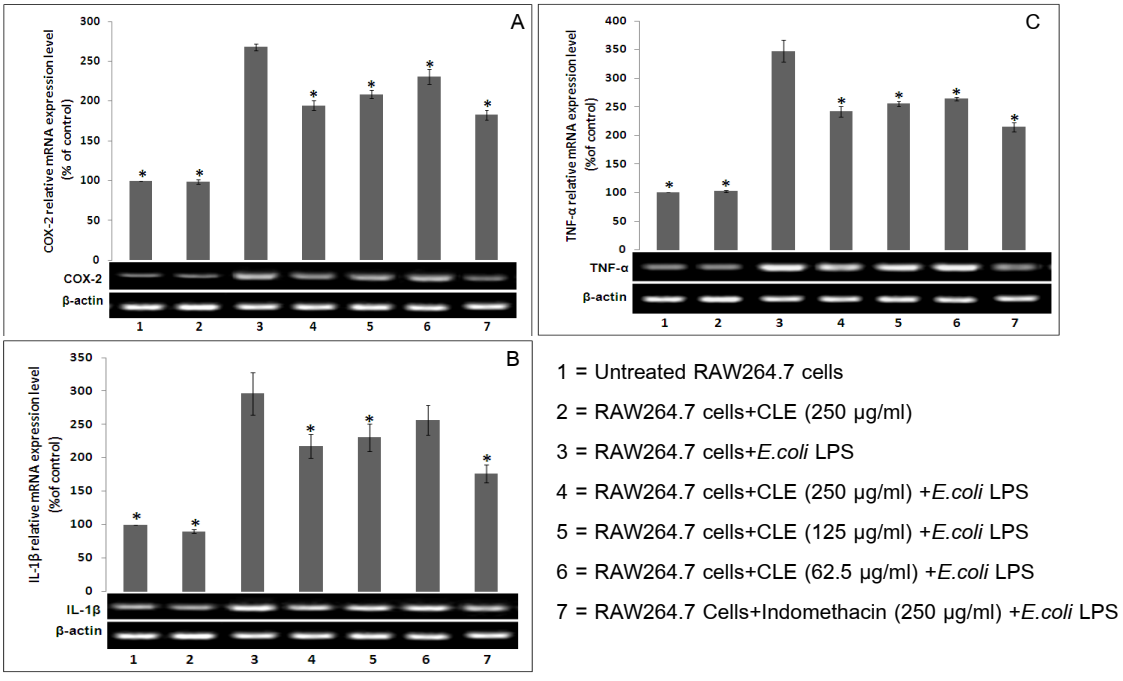

Figure 4 Inhibitory effects of CLE on mRNA expression of pro-inflammatory mediators. Relative mRNA expression of COX-2 (A), IL-1β (B) and TNF-α (C) compared with β-actin mRNA expression. *Significant difference from LPS treatment alone ($p<0.05$).

Discussion and conclusion

As shown in Table 1, CLE exhibited high phenolic contents and strong antioxidant activity. The antioxidant activity of CLE from the DPPH assay was close to vitamins C and E with IC_{50} of 9.24±0.47, 3.21±0.10, and 6.99±0.12 μg/ml respectively. CLE also showed phosphomolibdenum-

reducing antioxidant power (FRAP) (0.78±0.64 mg $FeSO_4$/g extract). The phenolic compound is strongly associated with antioxidant activity [16-18], and occurs as a defense mechanism against UV-damage [19]. CLE also showed antimelanogenesis in the cell culture model used in this study. After 48 hr of incubation, CLE at concentrations of 31-500 µg/ml showed low cytotoxicity on B16-F10 melanoma cells (IC_{50}= 600.74±55.16 µg/ml) (Figure 1). Moreover, at non-toxic concentrations (65-250 µg/mL), CLE exhibited significant melanin inhibition in B16-F10 cells, especially when the incubation time increased from 24 to 48 hr and the melanin pigmentation was reduced. However, antimelanogenesis of CLE was still less than that of Kojic acid. The results from melanin production and cell viability tests suggested that the ability to inhibit melanin formation came from a direct effect on melanogenesis, not cell death (Figure 2). Furthermore, RT-PCR analyses showed that the mechanism to inhibit melanin of CLE was via the down regulation of tyrosinase gene expression, TRP-1, and TRP-2. CLE at doses of 65-250 µg/mL significantly suppressed the level of these genes in B16-F10 cells at dose-dependent manners. In addition, the results revealed that CLE was able to inhibit the expression of tyrosinase better than that of TRP-1 and TRP-2 genes (Figure 3). CLE inhibited tyrosinase which plays the most critical role in melanogenesis.[5] Furthermore, to expand the usage of CLE for anti-inflammation, *in vitro* RT-PCR exhibited the significant dose-dependent inhibitory effect of CLE on several pro-inflammatory gene expressions including COX-2, IL-1β, and TNF-α in LPS-induced RAW264.7 murine macrophage cells (Figure 4). The results were consistent with the ethnopharmacological uses of *C. orientalis*.[9] Moreover, its chemical compounds, such as diterpene, guainane sesquiterpene, flavones, aromatic glycosides, and megastigmane glucoside, had been shown to have pharmacological benefits related to antioxidant, antimelanogenesis, and anti-inflammatory activities [20-22].

In conclusion, the present study demonstrated the high phenolic content and strong antioxidant activity of ethanolic extract of *C. orientalis* leaves. Research on selected pharmacological activities revealed the potential effects of the *C. orientalis* leaves extract on antimelanogenesis and anti-inflammation activities. The findings suggested that *C. orientalis* may be further used as an ingredient in traditional medicine and cosmetics.

Acknowledgments

This research was financially supported by the Plant Genetic Conservation Project under The Royal initiative of Her Royal Highness Princess Maha Chakri Sirindhorn and the Center for Research and Development of Herbal Health Products, Khon Kaen University, Thailand.

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Antibacterial Activity of *Centella asiatica* Extract-loaded Gelatin Nanoparticles Against Foodborne Pathogens

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Abstract

Centella asiatica is used as a traditional drug. Crude extracts of it showed excellent potential *In-vitro* but poor *In-vivo* antibacterial activity resulting from its poor lipid solubility and undesired molecular size. The most economical and readily-used material for generation of the nanoparticles is gelatin. It can act as the carrier and primary protection for crude extracts to increase the antibacterial activity. Therefore, this study was aimed to develop *C. asiatica* crude extract-loaded gelatin nanoparticles (CGNP) to improve the antibacterial activity. CGNP was prepared by two methods, gelatin one-step desolvation and gelatin two-step desolvation, at three different ratios of 95% ethanolic *C. asiatica* crude extracts:Gelatin (1:2, 1:3, and 1:4). The well agar diffusion method was used for the evaluation of antibacterial activity of CGNP with different concentrations (100, 200, and 300 µg/ml) against seven foodborne pathogens (*Escherichia coli* ATCC25822, *Bacillus cereus*, *B. subtilis*, *Staphylococcus aureus*, *Salmonella enterica* Typhimurium U302 (DT104b), *S. enterica* Enteritidis (human), and *S. enterica* 4,5,12:i:- (human) US clone). The results showed that the highest inhibition zone of CGNP was 1.65±0.57 cm against *S. aureus* using the gelatin one-step desolvation method at the ratio 1:4, 200 µg/ml. There were no significant differences in CGNP's antibacterial activity using different preparation methods and ratios. The antibacterial activity of CGNP was almost three times higher than 95% ethanolic *C. asiatica* crude extracts. It was found that the antibacterial activity of CGNP was not concentration-dependent against all tested foodborne pathogens. The results indicated that CGNP showed a promising increase in antibacterial activity.

Keywords: *Centella asiatica*, Gelatin, Nanoparticles, Antibacterial

Introduction

One challenging mission that bio-medicists must deal with is the solution to treatment of antibiotic-resistant bacterial species. Resistance to antibiotics appears when people misuse them for a long period, thus affecting people's lives either from environment or food. Food sources have been widely treated with antibiotics for the last few decades. Nevertheless, people try to alter the use of antibiotics with the source of natural antibiotics [1].

Centella asiatica was known as a medicinal plant in ancient times. It grows in tropical and sub-tropical areas mostly in South East Asia and spread through the Western world. It generally consists of various chemical compositions which have the ability to cure many symptoms and diseases related to bacterial infection, and these compositions are found in the roots and leaves [2]. As shown in much research, *C. asiatica* crude extract has the ability to inhibit the growth of *B. cereus* and *L. monocytogenes* under normal, osmotic stress, and high acidic conditions, growth of pathogenic bacteria in intestines, and growth of both Gram-positive and Gram-negative bacteria [3]-[6]. Specifically, triterpene can help to break down cell walls of microorganisms as a result of weakening tissue membranes [5].

Even though bioactive compounds in the triterpene group in *C. asiatica* have the ability to inhibit the growth of pathogenic bacteria, they are likely destroyed by complex enzymes and gastric juice. One research study stated that only 50% or less can pass through the gastrointestinal tract, indicating that it has a limitation on the delivery of bioactive compounds from the crude extract of *C. asiatica* when tested under *in vivo* and acidic conditions [5], [7].

The world of technology is changing rapidly so there are many available techniques, such as capsules, solid dispersion, dry emulsion, pellets and tablets, microspheres, nanoparticles, suppositories, and implants, which can be applied to existing drugs or herbs to increase their efficiency [8].

The nanoparticle technique has been used with various types of medicinal components and it is considered one of the most effective techniques to help in the enhancement of bioavailability, especially antibacterial activity [9]-[11]. The definition of nanoparticles is the solid particles with a size in the range of 1–1000 nm [12]. As shown in various studies, the nanoparticle technique is applicable to improve bioavailability. Examples of nanoparticles are PGLA-nanoparticles, BSA-nanoparticles, and Gelatin-nanoparticles. Gelatin is defined as the hydrophilic proteins derived from collagen extracted from bones, ligaments, skin, and tendons of animals. It is the most economical and readily-available material used for generating nanoparticles. It helps to entrap and primarily protect crude extracts from being damaged [13]-[15]. One research work investigated antibiotic delivery by using gelatin nanoparticles as carriers for the drug. The result showed that a small dose of antibiotics could treat the bacterial infection with a controllable releasing drug [16].

This research aimed to study the antibacterial activity of *C. asiatica* crude extract-loaded gelatin nanoparticles against foodborne pathogens by the application of one-step and two-step desolvation gelatin nanoparticles compared to *C. asiatica* crude extract. The ratio concentrations of *C. asiatica* of 1:2, 1:3, and 1:4 were loaded into gelatin desolvation nanoparticles.

Materials and Methods

Preparation of *C. asiatica* crude extract

C. asiatica was purchased from a local market in Bangkok, Thailand. The mixture of the aerial part of *C. asiatica* dried powder with 95% ethanol at a ratio of 1:10 (g/ml) was soaked at 30ºC, 120 rpm, for 48 hours. The mixture was filtered through Whatmann filter paper no. 4 after 48 hours. The crude extract was evaporated at 45ºC by rotary evaporators (BUCHI Rotavapor R-205) and was stored at -20 ºC prior to use in preparation of *C. asiatica*-gelatin nanoparticles [6].

Preparation of gelatin one-step desolvation *C. asiatica* nanoparticles

Gelatin was prepared under constant heat and pH at 40 ± 1ºC, pH 3 (adjusted by 0.1 M HCl) by dissolving 600 mg of gelatin in 30 ml of sterile distilled water. The gelatin nanoparticles were formed after adding *C. asiatica* crude extract at the ratios of 1:2, 1:3, and 1:4 and adding 30 ml acetone dropwise. 100 µL 8% v/v glutaraldehyde solution was added to stabilize the *C. asiatica* crude extract-loaded gelatin nanoparticles (CGNP) and the solution was stirred gently for 2 hours. CGNP solution was centrifuged at a speed of 4000 rpm for 5 minutes. CGNP residue was purified by centrifuging in sterile distilled water three times. After purification, CGNP particles were added with 3% w/w mannitol and freeze-dried to obtain the free-flowed powder of CGNP [13].

Preparation of gelatin two-step desolvation *C. asiatica* nanoparticles

The encapsulation of *C. asiatica* in gelatin nanoparticles was adapted from Azimi [13]. 600 mg gelatin was added in 30 ml of sterile distilled water under a constant temperature at 40 ± 1ºC. The precipitation of high molecular weight gelatin (HMWG) was obtained by adding 30 ml of acetone to the gelatin solution. After 15 minutes, the HMWG was re-dissolved with 30 ml of sterile distilled water at 40± 1ºC, pH 3, and stirred gently. The CGNP was formed by adding *C. asiatica* at different ratios (1:2, 1:3, and 1:4) and 30 ml of acetone to the gelatin solution. The stabilization of CGNP was generated by adding 100 µL glutaraldehyde solution (8% v/v) and stirring gently for 2 hours. The CGNP solution was centrifuged at 4000 rpm for 5 minutes. The purification of CGNP was performed by centrifuging with sterile distilled water three times. The freeze-dried was applied to transform the CGNP liquid into CGNP powder.

Antibacterial activity

C. asiatica crude extract and CGNP were dissolved in sterile distilled water at concentrations of 100, 200, and 300 µg/ml. These solutions were added on Mueller Hinton agar plates which were swabbed with 100 µL of bacterial cultures (approx. $1.5x10^8$ CFU/ml). This was referred to as the modified agar well diffusion method adapted from Rattanakom [6]. Penicillin G (300 µg/ml) was used as a positive control. After 24 hours of incubation at 37ºC, the inhibition zone were measured to determine how effective *C. asiatica* crude extract and CGNP could inhibit the microorganisms *Escherichia coli* ATCC25822, *Bacillus cereus, B. subtilis, Staphylococcus aureus, Salmonella enterica* Typhimurium U302 (DT104b), *S. enterica* Enteritidis (human), and *S. enterica* 4,5,12:i:- (human) US clone.

Statistical analysis

All experiments were conducted in three replications and statistical analysis was accomplished using ANOVA with

Duncan's multiple range tests ($p < 0.05$) by SAS software version 9.3.

Results and discussion

The application of nanoparticles with herbal crude extracts could enhance their efficacy on small size, hydrophilicity, and drug-regulated release [17], [18]. One-step and two-step gelatin desolvation methods were used to generate CGNPs from ethanolic *C. asiatica* crude extract and gelatin with the ratio concentrations of 1:2, 1:3, and 1:4 [13]. The determination of antibacterial activity on CGNPs and crude extract was done by the well agar diffusion method [6]. 100, 200, and 300 µg/ml of CGNPs and crude extract were chosen to test against seven foodborne pathogens (*Escherichia coli* ATCC25822, *Bacillus cereus*, *B. subtilis*, *Staphylococcus aureus*, *Salmonella enterica* Typhimurium U302 (DT104b), *S. enterica* Enteritidis (human), and *S. enterica* 4,5,12:i:- (human) US clone).

The results showed that a larger inhibition zone was discovered when six pathogenic bacteria, except *S. enterica* Enteritidis (human), were treated with at least 200 µg/ml of CGNPs compared with ethanolic crude extract. However, S. aureus was remarkably inhibited by all types of CGNPs and concentrations. Moreover, the inhibition zone significantly increased by almost three times compared with ethanolic crude extract, as shown in table 1 ($p<0.05$). Research found that triterpenes in *C. asiatica* is effective to inhibit the growth of bacteria due to bacterial membrane disturbance [19]. Nevertheless, gelatin, an economical, readily-available, and harmless material, can entrap bioactive compounds to form nano-sized particles and beneficially help those compounds to attach to bacterial cells. The slow release rate of a compound trapped in gelatin desolvation nanoparticles was proved [13]. From the above basis, gelatin nanoparticles can increase the absorption rate into bacterial cells compared to *C. asiatica* crude extract. The CGNP likely affected both gram-negative (*E. coli* ATCC25822, *S. enterica* Typhimurium U302 (DT104b), *S. enterica* Enteritidis (human), and *S. enterica* 4, 5, 12: i: - (human) US clone) and gram-positive bacteria (*B. cereus, B. subtilis*, and *S. aureus*). The structures of gram-negative and gram-positive bacteria are different in the way that gram-negative bacteria is constructed from an inner membrane, a thin layer made of peptidoglycan, and an outer membrane, whereas gram-positive has an inner membrane covered with a thick layer of peptidoglycan [20], [21]. From the result, *S. aureus* and *B. subtilis* were highly inhibited due to their ability to generate the enzyme gelatinase, which helps *C. asiatica* to regularly release during CGNP adherence to bacterial cells [22]-[24]. Therefore, the types of gelatin desolvation nanoparticles, concentrations, and ratios between ethanolic *C. asiatica* crude extracts were independent of the antibacterial activity of CGNP against all pathogenic bacteria. The highest inhibition zone of CGNP was 1.65±0.57 cm against *S. aureus* using the gelatin one-step desolvation method at a ratio of 1:4, 200 µg//ml. The improvement of antibacterial activity of ethanolic *C. asiatica* crude extracts against foodborne pathogens can be achieved by the application of one-step and two-step gelatin desolvation nanoparticles.

Table 1: The inhibition zone of CGNP and crude extract against 7 different microorganisms in the unit of centimeter

Samples	Ratio (Crude:Gelatin	Concentration (Ug/ml)	Inhibition Zone of 7 Microorganisms (cm.)						
			E. coli	*B. cereus*	*B. subtilis*	*S. aureus*	*ST*	*SE*	*SUS*
Gelatin One-Step Desolvation Nanoparticles	***1:2***	***100***	0.66±0.04^{B,abcd}	0.59±0.01^{B,bcd}	0.73±0.15^{AB,cdefg}	0.96±0.14^{A,bcdef}	0.75±0.32^{AB,bcde}	0.70±0.22^{AB,bcd}	0.67±0.05^{B,bc}
		200	0.81±0.27^{A,a}	0.62±0.06^{A,abcd}	0.67±0.31^{A,cdefg}	0.84±0.26^{A,bcdef}	0.72±0.34^{A,bcde}	0.70±0.33^{A,bcd}	0.73±0.11^{A,b}
		300	0.72±0.09^{AB,abc}	0.59±0.03^{B,bcd}	1.10±0.52^{A,bc}	0.85±0.49^{AB,bcdef}	0.59±0.08^{B,cde}	0.88±0.09^{AB,abc}	0.74±0.09^{AB,b}
	1:3	***100***	0.73±0.07^{B,abc}	0.63±0.06^{B,abcd}	0.68±0.14^{B,cdefg}	1.33±0.41^{A,bcde}	0.65±0.24^{B,cde}	0.87±0.45^{B,abc}	0.72±0.07^{B,b}
		200	0.70±0.22^{B,abc}	0.64±0.10^{B,abc}	0.64±0.09^{B,defg}	1.40±0.39^{A,bcde}	0.61±0.07^{B,cde}	0.77±0.24^{B,bc}	0.73±0.03^{B,b}
		300	0.72±0.15^{AB,abc}	0.62±0.05^{B,abcd}	0.75±0.33^{AB,cdefg}	1.46±0.92^{A,bcd}	0.65±0.10^{B,cde}	1.17±0.90^{AB,abc}	0.69±0.08^{AB,b}
	1:4	***100***	0.64±0.15^{A,abcd}	0.57±0.01^{A,bcd}	0.61±0.09^{A,efg}	0.76±0.25^{A,bcdef}	0.56±0.05^{A,de}	0.59±0.16^{A,bcd}	0.61±0.23^{A,bc}
		200	0.72±0.15^{B,abc}	0.59±0.05^{B,bcd}	0.72±0.31^{B,cdefg}	1.65±0.57^{A,b}	0.69±0.25^{B,cde}	0.84±0.54^{B,abc}	0.63±0.07^{B,bc}
		300	0.69±0.12^{AB,abc}	0.57±0.08^{B,bcd}	0.57±0.17^{B,fg}	0.82±0.27^{A,bcdef}	0.61±0.18^{AB,cde}	0.62±0.17^{AB,bcd}	0.59±0.09^{B,bc}
Gelatin Two-Step Desolvation Nanoparticles	***1:2***	***100***	0.63±0.09^{B,abcde}	0.54±0.08^{B,bcde}	1.03±0.43^{AB,bcde}	1.50±0.85^{A,bc}	0.70±0.15^{B,cde}	0.69±0.23^{B,bcd}	0.70±0.18^{B,b}
		200	0.65±0.20^{BC,abcd}	0.60±0.12^{C,bcd}	1.07±0.33^{AB,bcd}	1.30±0.60^{A,bcde}	0.73±0.11^{BC,bcde}	0.71±0.12^{BC,bcd}	0.77±0.18^{BC,b}
		300	0.59±0.10^{A,abcde}	0.62±0.13^{A,abcd}	1.22±0.31^{A,b}	1.48±1.10^{A,bc}	1.12±0.44^{A,ab}	0.93±0.61^{A,abc}	0.69±0.11^{A,b}
	1:3	***100***	0.73±0.13^{AB,abc}	0.68±0.10^{B,ab}	0.97±0.16^{AB,bcdef}	1.08±0.44^{AB,bcde}	1.01±0.41^{AB,abc}	1.24±0.50^{A,ab}	0.70±0.07^{B,b}
		200	0.84±0.16^{A,a}	0.66±0.04^{A,ab}	0.65±0.03^{A,defg}	1.33±0.66^{A,bcde}	0.72±0.12^{A,bcde}	0.98±0.67^{A,abc}	0.65±0.18^{A,bc}
		300	0.75±0.15^{AB,ab}	0.61±0.06^{B,abcd}	0.81±0.22^{AB,bcdefg}	1.25±0.60^{A,bcde}	0.90±0.37^{AB,bcd}	1.15±0.62^{AB,abc}	0.68±0.04^{AB,b}
	1:4	***100***	0.64±0.17^{A,abcd}	0.57±0.09^{A,bcd}	0.62±0.09^{A,efg}	0.63±0.08^{A,cdef}	0.56±0.06^{A,de}	0.60±0.10^{A,bcd}	0.60±0.03^{A,bc}
		200	0.66±0.13^{AB,abcd}	0.66±0.03^{AB,ab}	0.58±0.13^{B,efg}	0.88±0.45^{A,bcdef}	0.56±0.09^{B,de}	0.71±0.34^{AB,bcd}	0.68±0.17^{AB,b}
		300	0.53±0.08^{BC,bcde}	0.66±0.10^{AB,ab}	0.60±0.16^{ABC,efg}	0.58±0.05^{ABC,cdef}	0.54±0.05^{ABC,de}	0.50±0.08^{C,bcd}	0.69±0.13^{A,b}
Crude		***100***	0.48±0.16^{A,cde}	0.48±0.09^{A,de}	0.53±0.05^{A,fg}	0.48±0.14^{A,ef}	0.46±0.12^{A,e}	0.56±0.02^{A,bcd}	0.57±0.04^{A,bc}
		200	0.40±0.02^{AB,e}	0.43±0.09^{AB,e}	0.43±0.03^{AB,g}	0.49±0.11^{A,def}	0.35±0.05^{AB,ef}	0.46±0.09^{AB,cd}	0.33±0.06^{B,d}
		300	0.43±0.00^{A,de}	0.49±0.09^{A,cde}	0.53±0.04^{A,fg}	0.46±0.14^{A,ef}	0.52±0.26^{A,de}	0.62±0.27^{A,bcd}	0.47±0.05^{A,cd}
Penicillin G (Positive control)		***300***	0.72±0.10^{D,abc}	0.76±0.07^{D,a}	1.61±0.00^{B,a}	3.30±0.17^{A,a}	1.37±0.16^{C,a}	1.52±0.06^{BC,a}	1.64±0.03^{B,a}
DMSO (Negative control)		***300***	0.00±0.00^{A,f}	0.00±0.00^{A,f}	0.00±0.00^{A,h}	0.00±0.00^{A,f}	0.00±0.00^{A,f}	0.00±0.00^{A,d}	0.00±0.00^{A,e}

Note: Superscript in capital letters (A, B, C) and small letters (a, b, c) represented significantly different value in a row and a column at $p<0.05$, respectively.

ST stands for *S. enterica* Typhimurium, SE stands for *S. enterica* Enteritidis, and SUS stands for *S. enterica* 4,5,12:i:- (human) US clone.

Conclusion

The antimicrobial efficacy of these nanoparticles was evaluated against gram-negative bacterium and gram-positive bacterium and showed concentration-independence for both gelatin desolvation nanoparticles methods. The results showed that a larger inhibition zone was discovered when six pathogenic bacteria, except *S. enterica* Enteritidis (human), were treated with at least 200 µg/ml of CGNPs compared with ethanolic crude extract. However, S. aureus was remarkably inhibited by all types of CGNPs and concentrations. Moreover, the highest inhibition zone of CGNP was 1.65±0.57 cm against *S. aureus* using the gelatin one-step desolvation method at a ratio of 1:4, 200 µg/ml. The antibacterial activity of CGNP was almost three times higher than 95% ethanolic *C. asiatica* crude extracts. The higher inhibition zone of CGNPs over *C. asiatica* crude extract was beneficial from gelatin nanoparticles to slowly release triterpenes into the cell membranes of pathogenic bacteria. This experiment showed a further possibility to investigate different solvents for extracting *C. asiatica* and testing under a simulated digestive system.

Acknowledgements

This work was supported by STEM Workforce, National Science and Technology Development Agency (SCA-CO-2560-3545-TH).

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Effect of Nanoparticle Preparation Methods on Antioxidant Activity of Bua-Bok (*Centella asiatica*) Chloroform Extracts-loaded Nanoparticles

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Abstract

Bua-bok (*Centella asiatica*) is rated as one of the top five Thailand Champion Herbal Products by the Department for Development of Thai Transitional and Alternative Medicine, Ministry of Public Health. Bua-Bok has been investigated for its bioavailability activities, antimicrobial activity, antioxidant activity, anti-inflammatory activity, wound healing activity, and anticancer activity. Bua-Bok crude extracts showed excellent potential *in-vitro* but poor activity *in-vivo* due to their poor lipid solubility or improper molecular size, resulting in poor bioavailability. Nanotechnology has been used to improve drug bioavailability and drug delivery systems. The purpose of this study was to study the antioxidant activities of Bua-Bok chloroform extracts-loaded nanoparticles prepared by three different preparation methods, the Bovine Serum Albumin (BSA) desolvation method, gelatin one-step desolvation method, and gelatin two-step desolvation method. Three different ratios between Bua-Bok crude chloroform extracts:nanoparticle carrier (1:2, 1:3, and 1:4) were used in nanoparticle preparation. The antioxidant activities were evaluated by using DPPH radical scavenging activity and Ferric reducing antioxidant power (FRAP) antioxidant power. The results showed that the Bua-Bok chloroform extracts-loaded two-step gelatin nanoparticles using a ratio of 1:3 showed the highest ferric reducing (FRAP) antioxidant power (1.42±1.20 mmol Fe^{2+}/mg dried weight). Meanwhile, Bua-Bok chloroform extracts-loaded one-step gelatin nanoparticles using a ratio of 1:3 showed the highest DPPH radical scavenging activity (17.47±2.34 % DPPH radical scavenging). From DPPH radical scavenging activity, there were no significant difference of antioxidant activity between Bua-Bok chloroform crude extracts and all Bua-Bok chloroform extracts-loaded nanoparticles ($p>0.05$). As reported previously, it was found that Bua-Bok extract-loaded BSA nanoparticles showed the slow release rate up to 6 hrs. The antioxidant reaction time was only 30 min so active compound in all Bua-Bok chloroform extracts-loaded nanoparticles might need longer release time to show antioxidant activity. The stimulus digestive system is needed for further study to investigate the antioxidant activity of Bua-Bok chloroform extracts-loaded nanoparticles.

Keywords: *Centella asiatica*, Bua-Bok, Gelatin, Nanoparticles, Antioxidant

Introduction

Bua-Bok *(Centella asiatica)* is rated as one of the top five of Thailand Champion Herbal Products (TCHP) by the Department for Development of Thai Transitional and Alternative Medicine, Ministry of Public Health. Ithas been investigated for its bioavailability activities, antimicrobial activity, antioxidant activity, anti- inflammatory activity, wound healing activity and anticancer activity [1]. The major bioactive compounds of *Centella* extract are triterpene glycosides (saponins) , such as asiaticoside and madecassoside, and their respective aglycones (sapogenins), including asiatic acid and madecassic acid [2].

Although Bua-Bok has many beneficial activities for humans, it is not easy for water soluble biological active compounds to enter through the cell membranes of both humans and pathogenic microorganisms which have hydrophobic characteristics [3].

Nanoparticlse (NP) have a relatively large (functional) surface which is able to bind, adsorb, and carry other compounds, such as drugs, probes, and proteins [4]. These particles can be prepared from a variety of natural and synthetic materials, such as proteins, polysaccharides, and synthetic polymers. Gelatin is one of the low cost compounds, biodegradable, biocompatible, non- toxic, and easy to crosslink and to modify chemically, and has therefore an immense potential to be used for the preparation of drug delivery systems, such as protein-containing nanoparticles [5]. Used as bio- degradable as drug carriers, gelatin nanoparticles (GNP) represent a

promising carrier system for controlled drug delivery technology [6]. Antioxidants may be of great benefit in improving the quality of life by preventing or postponing the onset of degenerative diseases. In addition, they have a potential for substantial savings in the cost of health care delivery. [7]

The aim of this study was to prepare the three methods of making the Bua-Bok chloroform extract loaded nanopatices to see the antioxidant activities of the *Centella asiatica* (Bua-Bok).

Methodology

1. Preparation of Bua-Bok crude extract

The mixture of Bua-Bok with chloroform in a ratio of 1:10 (g/ml) was soaked at 30°C, 120 rpm, for 48 hours. The mixture was filtered through Whatmann filter paper no. 4 after 48 hours. The crude extract was evaporated at 45°C by rotary evaporators (BUCHI Rotavapor R-205) and stored at -20°C prior to use in preparation of Bua-Bok chloroform extracts- loaded Bovine Serum Albumin (BSA) nanoparticles, Bua-Bok Chloroform extracts- loaded one step nanoparticles, and Bua-Bok Chloroform extracts-loaded two step nanoparticles. [8]

2. Preparation of Bovine Serum Albumin (BSA) desolvation Bua-Bok nanoparticles

Bua-Bok Bovine Serum Albumin Nanoparticles were prepared by the desolvation method. 100 mg of BSA was dissolved in 1 ml of sodium chloride solution (10 mM sodium chloride solution). Then, 8.0 ml of ethanol was added dropwise into the BSA solution under magnetic stirring (400 rpm) at room temperature. Subsequently, the prepared BSA nanoparticles were cross-linked with 0.2% glutaraldehyde (GA). Then, Bua-Bok crude chloroform extract was added into the solution for 24 hours at different ratios of Bua-Bok to BSA (1:2, 1:3, and 1:4) in the preparation of the Bua-Bok Bovine Serum Albumin Nanoparticles. The particles were centrifuged and washed with distilled water. The centrifuged particles were re-suspended and dispersed in 2% mannitol, then freeze-dried for 24 hours. The dried nanopowder was kept at room temperature before use. [9]

3. Preparation of gelatin one-step desolvation Bua-Bok nanoparticles

Gelatin was prepared under constant heat and pH at 40 ± 1ºC, pH 3 (adjusted by 0.1 M HCl) by dissolving 200 mg of gelatin in 10 ml of sterile distilled water. The gelatin nanoparticles were formed after adding Bua-Bok crude extract at the ratios of 1:2, 1:3, and 1:4 and adding 30 ml of acetone dropwise. 100 µL 25% v/v glutaraldehyde solution was added to stabilize the Bua-Bok Gelatin Nanoparticles and the solution was stirred gently for 30 minutes. Bua-Bok Gelatin Nanoparticles solution was centrifuged at a speed of 12000 rpm for 30 minutes. Bua-Bok Gelatin Nanoparticles residue was purified by twice centrifuging in sterile distilled water. After purification, Bua-Bok Gelatin Nanoparticles particles were freeze-dried to obtain the free-flowed powder of Bua-Bok extract-loaded Gelatin Nanoparticles. [10]

4. Preparation of gelatin two-step desolvation Bua-Bok nanoparticles

The encapsulation of Bua-Bok in gelatin nanoparticles was adapted from Azimi et al. [11] : 200 mg gelatin was added in 10 ml of sterile distilled water under a constant temperature at 40 ± 1ºC. The precipitation of high molecular weight gelatin was obtained by adding 10 ml acetone into gelatin solution. The high molecular weight gelatin (HMWG) was dissolved with 10 ml of sterile distilled water at a constant temperature and pH of 40ºC, pH 3, and stirred gently. The Bua-Bok extract- loaded gelatin nanoparticles was formed by adding Bua-Bok at different ratios (1:2, 1:3, and 1:4) and 10 ml of acetone into a gelatin solution. The stabilization of Bua-Bok extract-loaded Gelatin Nanoparticles was generated by adding 100 µL glutaraldehyde solution (25 % v/v) and stirring gently at 40 ± 1ºC for 30 minutes. The Bua-Bok extract- loaded Gelatin Nanoparticles solution was centrifuged at 12000 rpm for 30 minutes. The purification of Bua-Bok extract-loaded Gelatin Nanoparticles was performed by two centrifugations with sterile distilled water. The freeze drying technique was applied to transform the liquid Bua-Bok extract- loaded Gelatin Nanoparticles into the form of Bua-Bok extract-loaded Gelatin Nanoparticles powder.

5. Antioxidant efficiency

5.1 DPPH radical scavenging activity

Brand-William et al. [12] reported: DPPH method to measure antioxidant activities. This method was modified to determine the antioxidant activities of Bua-Bok crude extract, Bua-Bok Chloroform extracts-loaded Bovine Serum Albumin (BSA) nanoparticles, Bua-Bok Chloroform extracts-loaded one step nanoparticles, and Bua-Bok Chloroform extracts-loaded two step nanoparticles. The mixture of 100 µL Bua-Bok crude extract or Bua-Bok nanoparticles and

3.9 mL of methanol DPPH solution (6 x 10^{-5} mol/L) was shaken thoroughly and kept in the dark room for 3 hours. The mixture was measured by UV-vis spectrophotometer at 515 nm. The unit of µL/mL of gallic acid equivalent (GAE) per 100 g sample was used to express results as percentage reduction of

$$\text{Percentage reduction} = 100\left(\frac{Ao}{Ao} - \frac{Ac}{Ao}\right) \quad \ldots (1)$$

where A_0 is the initial absorbance and A_c is the value of added sample concentration. All measurements were done in triplicate.

5.2 Ferric reducing (FRAP) antioxidant power

The FRAP method was used to measure the antioxidant activities of *Bua-Bok* crude extract and *Bua-Bok* nanoparticles adapted from Benzie and Strain [13]. The mixture (30 µL *Boa- Bog* crude extract or *Bua-Bok* nanoparticles with 270 µL FRAP reagent) was incubated in the dark room for 30 minutes. The absorbance of solution was measured by a UV-vis spectrophotometer at 595 nm. The unit of µL/mL of trolox equivalent (TE) per 100 g sample was used to express results. The mixtures were incubated in the dark room for 30 minutes. The absorbance of solution was measured by UV-vis spectrophotometer at 595 nm. The unit of µL/mL of trolox equivalent (TE) per 100 g sample was used to express results.

6. Statistical analysis

All experiments were conducted in three replications and statistical analysis was accomplished using ANOVA with Duncan's multiple range tests ($p < 0.05$) by SAS software version 9.3.

Results

Table 1: The antioxidant efficiency in DPPH radical scavenging activity and Ferric reducing antioxidant power of Bua-Bok BSA nanoparticles, Bua-Bok gelatin one step nanoparticles, Bua-Bok gelatin two step nanoparticles, and Bua-Bok crude extract

Sample (ratio)	**DPPH** **% reduction**	**FRAP** **mmol Fe^{2+}/mg dried weight**
BSA nanoparticle (1:2)	11.55±1.68^{A}	0.53±0.18^{C}
BSA nanoparticle (1:3)	13.57±7.01^{A}	0.49±0.04^{C}
BSA nanoparticle (1:4)	5.76±9.88^{A}	0.50±0.01^{C}
Gelatin One Step (1:2)	16.64±2.70^{A}	0.65±0.25^{BC}
Gelatin One Step (1:3)	17.47±2.34^{A}	0.74±0.38^{B}
Gelatin One Step (1:4)	14.72±23.08^{A}	0.84±0.81^{BC}
Gelatin Two Step (1:2)	11.97±1.79^{A}	0.77±0.03^{BC}
Gelatin Two Step (1:3)	4.96±3.59^{A}	1.42±1.20^{BC}
Gelatin Two Step (1:4)	12.60±2.79^{A}	0.93±0.31^{BC}
Crude	1.05±5.15^{A}	2.29±0.22^{A}

Table1 shows the antioxidant efficiency in DPPH radical scavenging activity and Ferric Reducing Antioxidant Power (FRAP). For DPPH radical scavenging activity, the result showed that there were no significant differences between DPPH% reduction of Bua-Bok BSA nanoparticles, Bua-Bok gelatin one step nanoparticles, Bua-Bok gelatin two step nanoparticles, and Bua-Bok crude extract ($p>0.5$). For FRAP, the highest value was Bua-Bok crude extract (2.29 ± 0.22 mmol Fe^{2+}/mg) and for the nanoparticles the highest value was Gelatin Two Step (1:3) 1.42 ± 1.20 mmol Fe^{2+}/mg. On the other hand, statistical analysis found that there were no significant differences between Bog BSA nanoparticles, Bua-Bok gelatin one step nanoparticles, and Bua-Bok gelatin two step nanoparticles. ($p>0.5$)

Discussion and conclusion

Antioxidant activity represents the ability to inhibit the process of oxidation [14] or the ability to catch the free radical compounds or chelates metal ions. However, it cannot be measured directly but rather by the effects of the antioxidants in controlling the extent of oxidation [15], so, it can be measured by the scavenging or trapping methods. Bua-Bok Chloroform crude extract, Bua-Bok Bovine Serum Albumin nanoparticles, and Bua-Bok Gelatin nanoparticles were used for evaluating antioxidant activities by DPPH and FRAP methods which were interpreted by using Randomized Complete Block Design (RCBD) with Duncan's multiple rage tests in SAS ver. 9.3 program, as shown in table1. The antioxidant activity for DPPH radical scavenging activity percentage reduction showed no significant differences between Bua-Bok crude extract and Bua-Bok chloroform extracts loaded nanoparticles in both Bovine Serum Albumin, Gelatin one step, and two step method ($p>0.5$). For FRAP ferric reducing antioxidant power, Bua-Bok crude extract showed the significantly highest antioxidant activity (2.29 ± 0.22^{A}), followed by Bua-Bok Gelatin one step nanoparticles using a ratio of 1:3 (0.74 ± 0.38^{B}). Nanoparticles did not show significant differences between each other from table 1.

The results showed that there was no significant differences between the antioxidant activity from Bua-Bok Chloroform crude extract, Bua-Bok Chloroform loaded Bovine Serum Albumin nanoparticles, and Bua-Bok Chloroform loaded Gelatin nanoparticles in both one and two steps from the DPPH method in all ratios ($p>0.5$), but for the FRAP method, Bua-Bok chloroform crude extract showed the highest antioxidant activity ($p>0.5$). For the Bua-Bok Chloroform extract loaded nanoparticles, Bua-Bok chloroform extracts-loaded two-step gelatin nanoparticles using a ratio of 1:3 showed the highest ferric reducing (FRAP) antioxidant power (1.42 ± 1.20 mmol Fe^{2+}/mg dried weight. It can be said that the active compound in Bua-Bok may be in an inactive form when testing with the FRAP and DPPH methods because the active compound might react or bind with proteins (the BSA and the gelatin). The proteins protect the active compound to not release in that condition or at the time of testing. Future studies may investigate the antioxidant activities of Bua-Bok chloroform extract loaded nanoparticles with a digestive system.

Acknowledgements

We extend our sincere thanks to all who contributed to preparing the instructions.

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Antioxidant Activity of Bua-Bok (*Centella asiatica*) Ethanolic Extract-Loaded Gelatin Nanoparticles

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Abstract

Bua-Bok or *Centella asiatica* has been used widely in Asia as a traditional medicine. *C. asiatica* crude extracts showed excellent potential *in vitro* but less or none *in vivo* activity due to their poor lipid solubility and/or improper molecular size, resulting in poor absorption and bioavailability. Nanotechnology has been used to improve drug bioavailability and drug delivery systems. Gelatin is one of several readily available and economical sources for generating nanoparticles to increase drug bioavailability. It acts as a carrier and primary protection during drug delivery. The aim of this study was to compare the antioxidant activity of *C. asiatica* crude ethanolic extract, *C. asiatica* extract-loaded gelatin nanoparticle (CGNP) prepared by gelatin one-step and two-step desolvation methods, using three different ratios between *C. asiatica* crude ethanolic extract and gelatin (1:2, 1:3, and 1:4 w/w). The antioxidant activity of CGNP was evaluated by using DPPH radical scavenging assay and ferric reducing antioxidant power (FRAP) assay. *C. asiatica* crude ethanolic extract showed significantly greater FRAP (1.33±0.31 mmol Fe^{2+}/mg dried weight) than CGNP prepared by both one-step and two-step methods at all ratios. However, there was no significant difference in DPPH radical scavenging activity between *C. asiatica* crude ethanolic extracts and the CGNP generated by any methods. Previous study of *C. asiatica* extract-loaded Bovine Serum Albumin nanoparticles showed that it has a slow release rate of up to 6 hours, but the antioxidant reaction time only lasts for 30 minutes. The study concludes that the active compound in CGNP may need a longer release time to show higher antioxidant activity.

Keywords : *Centella asiatica*, Bua-Bok, Gelatin, Nanoparticles, Antioxidant

Introduction

Centella asiatica (Bua-bok) is used as a traditional medicine widely in Asia. In Ayurvedic, an Indian system of medicine, *C. asiatica* is used for the treatment of leprosy, insanity, asthma, ulcers, eczema, skin and gastrointestinal disorders, arthritis, varicose veins, and high blood pressure [1]. It is known that herbs are rich in phenolic compounds [2] which have antioxidant properties as protective agents against free radical compounds by scavenging or trapping methods [3], [4]. The reduction of hydroperoxides, inactivation of free radicals, chelation of metal ions, or combinations may lead to the antioxidant activity of *C. asiatica* [5]. *C. asiatica* may have different antioxidant functional properties, such as reactive oxygen species scavenging (quercetin and catechins) [6], inhibition of the generation of free radicals, and chain-breaking activity, such as p-coumaric acids [7] and metalchelation [8]. The major biologically active compounds of *C. asiatica* extract are monoterpenes [9], sesquiterpene [9], triterpenoids [9], polyphenols [5], and triterpenes [10]. Asiaticoside and asiatica acid, the bioactive compounds of *C. asiatica*, can transfer only or less than 50% to humans. There is a limitation on the absorption of bioactive compounds from the crude extracts of *C. asiatica* which showed significantly high efficiency on the drug absorption and drug delivery system *in-vitro* but less *in-vivo* due to their poor lipid solubility and/or improper molecular size [11]. Especially, it is also difficult for water-soluble biological active compounds to enter through cell membranes of both humans and pathogenic microorganisms which have hydrophobic characteristics.

There are many available techniques, such as capsules, solid dispersion, dry emulsion, pellets and tablets, microsphere, nanoparticles, suppositories, and implants, which can be applied to existing drugs or herbs to increase their efficiency [12]. Nanotechnology has improved drug

bioavailability and drug delivery systems. Previous studies of nanoparticles, including PGLA- nanoparticles, BSA-nanoparticles, and Gelatin- nanoparticles, indicated that nanoparticle technique is applicable to improve bioavailability [13]-[16]. *C. asiatica* extract-loaded Bovine Serum Albumin nanoparticles (CBNP) was developed and the results indicated that CBNP showed promise to increase bioavailability of *C. asiatica* [15]. Therefore, gelatin nanoparticles are another way of processing *C. asiatica* extract- loaded nanoparticles. Gelatin is defined as the hydrophilic proteins derived from collagen extracted from bones, ligaments, skin, and tendons of animals. Gelatin, known as a readily and economical source, is one of several available materials used for generating the nanoparticles, acting as the carrier and primary protection for the medicine to be able to increase bioavailability activity.

This research aimed to compare the antioxidant activity of *C. asiatica* crude ethanolic extracts, *C. asiatica* ethanolic extract- loaded gelatin nanoparticle (CGNP) prepared by the gelatin one- step desolvation method and two- step desolvation methods on three different ratios between *C. asiatica* crude ethanolic extracts and gelatin (1:2, 1:3, and 1:4 w/w). As the antioxidant activity of *C. asiatica* come from the complex of phytochemicals and involvement of many reactions, the measurement of the activity cannot be done accurately with only one assay. The combination of DPPH radical scavenging activity and ferric reducing antioxidant power (FRAP) were used to evaluate the antioxidant activity.

Methodology

1. Preparation of sample

C. asiatica was purchased from Bangkapi markets in Bangkok, Thailand. The aerial part of *C. asiatica* was used. Fresh *C. asiatica* was washed with tap water, cut into small pieces, and air-dried in an oven (Memmert UM500) at 45°C. The dried samples were finely ground into a powder. The powder was kept at 4°C before use [9].

2. Preparation of *C. asiatica* crude ethanolic extract

C. asiatica was extracted with 95% ethanol using 1:10 ratio (g/ml). The mixtures were macerated at room temperature, 120 rpm, for 48 hours and then filtered using whatman filter paper no. 4. The *C. asiatica* crude ethanolic extracts were evaporated at 45°C by rotary evaporators (BUCHI Rotavapor R-205). The crude was stored at -20°C prior to use in preparation of *C. asiatica*-gelatin nanoparticles [9].

3. Preparation of Gelatin one- step desolvation *C. asiatica* nanoparticles

Gelatin was prepared under constant heat and pH at 40 ± 1ºC, pH 3 (adjusting by 0.1 M HCl) by dissolving 600 mg of gelatin in 30 mL sterile distilled water. The gelatin nanoparticles were formed after adding *C. asiatica* crude extract at different ratios. The ratios between *C. asiatica* crude ethanolic extracts and gelatin were 1:2, 1:3, and 1:4 w/w. Then, 30 ml acetone was added dropwise. 100 µL 8% v/v glutaraldehyde solution was added to stabilize the CGNP and the solution was stirred gently for 2 hours. CGNP were centrifuged and washed with distilled water. The centrifuged particles were re-suspended and dispersed in 3% mannitol, then freeze- dried for 48 hours. The dried CGNP were kept at 4°C prior to use in the antioxidant assay [16].

4. Preparation of Gelatin two- step desolvation *C. asiatica* nanoparticles

The encapsulation of *C. asiatica* in gelatin nanoparticles was modified from Azimi [16]. 600 mg gelatin was added in 30 mL sterile distilled water under a constant temperature of 40 ± 1ºC. The precipitation of high molecular weight gelatin was obtained by adding 30 mL acetone into gelatin solution. After 15 minutes, the high molecular weight gelatin was re- dissolved with 30 mL sterile distilled water at 40± 1ºC, pH 3 and stirred gently. The *C. asiatica* extract-loaded gelatin nanoparticles was formed by adding *C. asiatica* at different ratios. The ratios between *C. asiatica* crude ethanolic extracts and gelatin were 1:2, 1:3, and 1:4 w/w. Then, 30 mL acetone was added dropwise. The stabilization of CGNP was generated by adding 100 µL glutaraldehyde solution (8% v/v) and stirring gently for 2 hours. CGNP were centrifuged and washed with distilled water. The centrifuged particles were re-suspended and dispersed in 3% mannitol, then freeze-dried for 48 hours. The dried CGNP were kept at 4°C prior to use in the antioxidant assay

5. Antioxidant activity by ferric reducing antioxidant power (FRAP) assay

The modified ferric reducing antioxidant potential assay [17] was used to determine the FRAP value of *C. asiatica* crude ethanolic extract and CGNP. The FRAP reagent was prepared using 300 mmol sodium acetate buffer at pH 3.6, 20 mmol iron chloride and 10 mmol 2,4,6-tripyridyl-s-triazine dissolved in 40 mmol hydrochloric acid at a ratio of 10:1:1 (v:v:v). The reagent was incubated at 37°C for 10 minutes before use. The 20 µL of 1 mg/mL the extract and CGNP was added, followed by adding 1000 µL of FRAP reagent

vigorously and kept in the dark for 30 minutes. The optical density (OD) of this mixture was measured at 593 nm. FRAP values were expressed as mmol Fe^{2+}/mg of sample. All measurements were done in triplicate with three replications independently.

6. Antioxidant activity by 2,2-Diphenyl-1-picrylhydrazil (DPPH) radical scavenging activity

1 mg/mL sample was pipetted between 0-200 µL. The 50 µM DPPH solution in methanol was added into the following volume of sample to get the total volume of 2 mL. The mixture was mixed gently and left in a dark room for 30 minutes. The optical density (OD) of this mixture was measured at 517 nm. The results were expressed as IC_{50} ug/mL. All measurements were done in triplicate with three replications independently.

7. Statistical analysis and experimental design

All experiments were conducted in three replications and statistical analysis was accomplished using Randomized Complete Block Design (RCBD) with Duncan's multiple comparison ($p< 0.05$) by SAS software version 9.3.

The strategy of applying nanotechnology with plant extracts has been widely used, because nanostructure systems could increase the effect of action of plant extracts, promote sustained release of active constituents, reduce required doses, decrease side-effects, and improve activity [18-19]. Nanoparticles could be used as effective transport and delivery systems. There are six differences in the nanoparticles preparative in this study. CGNP were prepared by gelatin one-step and two-step desolvation methods [16] at three different ratios between *C. asiatica* crude ethanolic extracts and gelatin (1:2, 1:3, and 1:4 w/w). Antioxidant activity of all CGNPs and crude extract were evaluated by using ferric reducing antioxidant power (FRAP) assay [17] and DPPH radical scavenging. The antioxidant activity of all CGNPs and crude extract were statistically analyzed to see the differences between each sample that were prepared from different preparation methods and ratios. The results were interpreted by using Randomized Complete Block Design (RCBD) with Duncan's multiple range tests in SAS program version 9.3, as shown in table1.

Results and discussion

Table 1 Antioxidant activity of CGNP and crude extract

Sample	FRAP	DPPH
	mmol Fe^{2+}/mg dried weight	IC_{50} (ug dried weight/ml)
OS1:2	0.97 ± 0.10^{B}	50.52 ± 0.33^{A}
OS1:3	0.79 ± 0.09^{B}	50.53 ± 0.56^{A}
OS1:4	0.70 ± 0.06^{B}	50.30 ± 0.77^{A}
TS1:2	0.92 ± 0.15^{B}	50.01 ± 1.27^{A}
TS1:3	0.90 ± 0.19^{B}	51.58 ± 6.93^{A}
TS1:4	0.70 ± 0.10^{B}	50.98 ± 1.20^{A}
Crude	1.33 ± 0.31^{A}	50.74 ± 1.09^{A}

Note: OS and TS represented *C. asiatica* extract-loaded gelatin nanoparticle one-step and two-step *C. asiatica* extract-loaded gelatin nanoparticle respectively. A, B represented significantly different values in a column at $p<0.05$.

DPPH radical scavenging assay measures the reducing ability of antioxidants toward DPPH. The antioxidant effect is proportional to the disappearance of DPPH in a methanolic solution when added with CGNPs or *C. asiatica* crude ethannolic extracts. The FRAP assay is different from the DPPH assay as there are no free radicals involved. The assays determine the ability of CGNPs or *C. asiatica* crude ethanolic extracts to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). [20]

C. asiatica crude ethanolic extracts showed significantly greater ferric reducing antioxidant power, FRAP (1.33±0.31 mmol Fe^{2+}/mg dried weight) than both one-step and two-step CGNP at all ratios ($p<0.05$). CGNP prepared with one-step desolvation method at ratio 1:2 showed the highest FRAP (0.97±0.10 mmol Fe^{2+}/mg dried weight) among CGNP. However, there were no significant differences between *C. asiatica* crude ethanolic extracts and both one-step and two-step CGNP in DPPH radical scavenging activity ($p<0.05$). CGNP prepared with two-step

desolvation method at ratio 1:3 showed the highest DPPH radical scavenging activity (IC_{50}: 51.58±6.93 ug/mL) among CGNP and crude extract. In addition, there were no significant differences between CGNP preparation method, gelatin one-step and two-step desolvation methods ($p<0.05$). So, CGNP prepared by using the one-step desolvation method at ratio 1:4 was most effective in an economical way of the CGNP production focusing on antioxidant activity because it consumed the least times and material and showed no significant differences in antioxidant activity compared with the others.

According to a previous study on *C. asiatica* Extract-Loaded BSA nanoparticles, the nanoparticles has a slow release rate of up to 6 hours and its antioxidant activity showed less or equal compared to crude extract [15]. The result of this study showed that CGNP also have less or equal antioxidant activity compared to crude extract. Thus, the antioxidant activity may need longer reaction time to release more active compounds of CGNP to get higher antioxidant activity.

Conclusion

The antioxidant activity of CGNP prepared by gelatin one-step desolvation method and two-step desolvation methods on three different ratios between *C. asiatica* crude ethanolic extracts and gelatin (1:2, 1:3, and 1:4 w/w) were evaluated by using DPPH radical scavenging activity and ferric reducing antioxidant power (FRAP). The resulta of this study showed that CGNP are significantly lower in FRAP and have no significantly difference in DPPH radical scavenging activity compared to *C. asiatica* crude ethanolic extracts ($p<0.05$). A previous study of *C. asiatica* Extract-Loaded BSA nanoparticles showed releasing of compounds up to 6 hours [15]. To get higher antioxidant activity, CGNP may need a longer reaction time to release more active compounds. The stimulus digestive system is needed for further study to investigate the antioxidant activity of CGNP.

Acknowledgments

This work was supported by the research grant of Assumption university (P-58-388).

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Immuno-stimulatory Effect of Water Extract of *Streblus asper* Seeds on Macrophage Cells

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Abstract

The seeds of *Streblus asper* have been traditionally used as anti-flatulents, appetizers, element tonics, or longevity agents. They are also a part of various Thai traditional medicinal recipes. This study aimed to investigate the immunomodulating effects of the water extract of *S. asper* seeds on macrophage J774A.1 cells. The water extract at 100 µg/ml significantly stimulated nitric oxide (NO) production determined by Griess reaction assay. The stimulatory effect of the extract was further evaluated by reverse transcription-polymerase chain reaction (RT-PCR) to determine the mRNA expression of markers of the activated macrophages which were inducible nitric oxide synthase (iNOS), TNF-α, and IL-1β. At 25-100 µg/ml, the extract increased the mRNA expression of iNOS which is responsible for NO production during phagocytosis and inflammation. At the same concentrations, the extract also increased the expression of TNF-α and IL-1 which are the pro-inflammatory cytokines and commonly used as the markers of activated macrophages. The extract did not have any effect on the viability of J774A.1 cells. The results from this study suggest that the water extract of *Streblus asper* seeds may have immune-enhancing activity. This activity may be a part of pharmacological activities of the seeds when they are used in traditional medicinal recipes, such as longevity recipes.

Keywords: *Streblus asper*, nitric oxide, iNOS, pro-inflammatory cytokines, J774A.1 cells

Introduction

Macrophages are multifunction immune cells originating from pluripotent hematopoietic stem cells in bone marrow [1]. They play roles in both innate and adaptive immune responses in the defense of the body from invading pathogens. They are major tissue phagocytes of the innate immunity by recognizing pathogen-associated molecule patterns (PAMPs) of pathogens through pattern recognition receptor (PRRs). For example, lipopolysaccharide (LPS) from Gram negative bacteria is recognized by toll like receptor 4. Once they recognize pathogens, the cells become activated macrophages for eliminating and limiting pathogen invasion by phagocytosis and generating several enzymes and molecules, such as proinflammatory cytokines, iNOS, COX-2, NO, and PGE2 [2-6]. Activated macrophages can act as antigen-presenting cells to initiate adaptive immune response [7]. They are also one of the major effector cells of adaptive immune response against pathogens [7]. Stimulation macrophages can cause immune enhancement.

Streblus asper is a plant in the Moraceae family. It is named Siamese rough bush or tooth brush tree because the leaves and stem bark of this plant were used as toothbrushes and for oral cleansing in ancient times. The seeds of this plant have been used as anti-flatulents, appetizers, element tonics, or longevity agents in Thai traditional medicine [8]. It is also a component in a variety of Thai traditional medicinal recipes for body restoration or nourishment. The reported pharmacological activities of this plant were anti-bacterial, cardiotonic, anti-oxidant, anti-cancer, antifilarial, and anti-allergic activities. The water extract of *S. asper* seeds had anti-allergic activity by inhibiting β-hexosaminidase of mast cells [9]. There is no report of the effect of *S. asper* seeds on macrophage function. This study purposed to investigate the immunomodulatory effect of the water extract of *S. asper* seeds on macrophage J774A.1 cells.

Materials and Methods

Preparation of the water extract of *S. asper* seeds

S. asper grounded seeds were obtained from Chao Krom Poe herbal dispensary (Bangkok). The seeds were cleaned and decocted thrice with distilled water at 75 °C for 15 min. The decocted solution was filtered,

lyophilized to powder, and kept at - 20ºC until used. The extract was diluted in Dulbeco's Modified Eagle's Medium (DMEM) to required concentrations for all experiments in this study.

Cell culture

Macrophage J774A.1 cells were obtained from American Type Culture Collection (ATCC, USA). The cells were cultured in DMEM containing 10% fetal bovine serum, 1% penicillin-streptomycin at 37ºC in 5% CO_2, and 95% humidity. The cells at $4x10^5$ cells/ml were used in all experiments. DMEM and 100 ng/ml LPS were used as negative and positive controls respectively.

Measurement of NO production

J774A.1 cells were treated with 12.5-100 µg/ml of the water extract of *S. asper* seeds for 24 h. The supernatants of the treated cells were collected and determined for NO production by Griess reagent system (Pomega, USA). The concentrations of NO in the supernatant were calculated from a nitrite standard curve.

Measurement of cell viability

J774A.1 cells were treated with the water extract of *S. asper* seeds at 12.5-100 µg/ml for 24 h. The viability of the treated cells was evaluated by resazurin reduction assay. Viable cells change resazurin to rezasurin product. The viability of the treated cells was compared to DMEM control.

Measurement of iNOS, IL-1β, and TNF-α mRNA expression

J774A.1 cells were treated with the water extract of *S. asper* seeds at 25, 50, and 100 µg/ml for 4 or 24 h. The total RNA of the treated cells was isolated by using TRIzol® reagent (Invitrogen, UK), and converted to cDNA by ImProm-II™ reverse transcription kit (Promega, USA). The PCR products were synthesized from cDNA with specific primers by reverse transcription-polymerase chain reaction (RT-PCR). Primer sequences used in this study were as follows: iNOS; 5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3' (forward) and 5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3' (backward); IL-1β, 5'-CAGGATGAGGACATGAGCACC-3' (forward) and 5'-CTCTGCAGACTCAAACTCCAC-3' (backward); TNF-α, 5'-TTGACCTCAGCGCTGAGTTG-3' (forward) and 5'-CCTGTAGCCCACGTCGTAGC-3' (backward). They were run on 1.5% agarose gel electrophoresis and measured densities by Molecular Imager® Gel Doc™XR+ System (Bio-Rad, USA). Lastly, they were calculated as a ratio by comparing with the β-actin PCR product. The expression of these studied genes in the treated cells was compared to the DMEM control.

Statistical analysis

Data are presented as means ± standard deviation (S.D.) values from three independent experiments. Statistical evaluation was determined by one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. $P < 0.05$ was considered as statistical significance.

Results and Discussion

Effects of the water extract of *S. asper* seeds on NO production and iNOS mRNA expression

The water extract of *S. asper* seeds at 100 µg/ml significantly increased NO production without any effect on cell viability [Figure 1A]. In order to confirm the stimulatory effect of the extract on this mediator, its effect on iNOS expression was also evaluated. The extract at all concentrations used in this study (25, 50, and 100 µg/ml) significantly increased iNOS mRNA expression when compared to the media control, as shown in Figure 1B. iNOS is usually expressed in activated macrophages and involved in the production of NO which is one of the mediators in phagocytosis and inflammatory process during immune responses against microorganisms. A previous study demonstrated that the ethanol extract of *S. asper* leaves inhibited iNOS mRNA expression in LPS-induced macrophages. Flavonoid named quercetin-3-O rutinoside was found as a major compound in the ethanol extract from *S. asper* leaves [10]. The active compound in the water extract of *S. asper* seeds was carbohydrate consisting of D-mannose and D- glucose [11]. The contrasting results between this study and the previous one suggest that each solvent extract contains different active compounds from the plants. Different parts of the plant used in the studies may also be another reason for these different results.

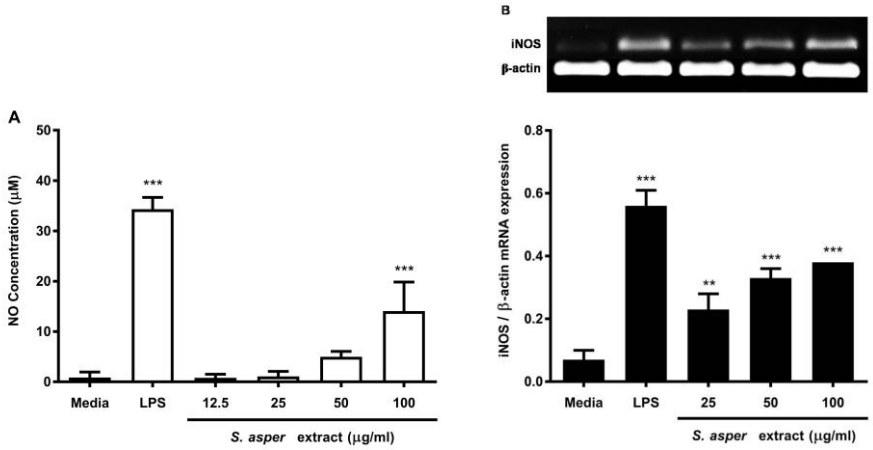

Figure 1 Effects of the water extract of *S. asper* seeds on (A) NO production and (B) iNOS mRNA expression in J774A.1 cells after 24 h of treatment. Data are presented as means ± S.D. from three independent experiments. ***p*<0.01, ****p*<0.001 compared to the media control.

Effects of the water extract of *S. asper* seeds on the mRNA expression of IL-1β and TNF-α

The water extract of *S. asper* seeds at 25, 50, and 100 μg/ml significantly up-regulated mRNA expression of TNF-α and IL-1β at 4 h [Figure 2A and 2B]. These cytokines are key cytokines of activated macrophages during generation of both innate and adaptive immune responses. They are commonly used as the markers of macrophage activation [5, 12]. These results imply that the water extract of *S. asper* seeds can activate macrophages. The effect of *S. asper* on these cytokine expressions has not been reported before.

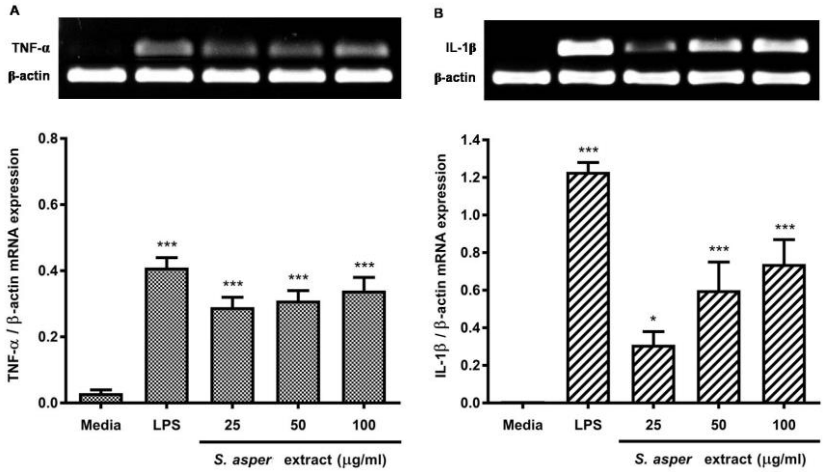

Figure 2: Effects of the water extract of *S. asper* seeds on the mRNA expression of (A) TNF-α and (B) IL-1β in J774A.1 cells after 4 h of treatment. Data are presented as means ± S.D. from three independent experiments. **p*<0.05, ****p*<0.001 compared to the media control.

Conclusion

The results from this study demonstrated that the water extract of *S. asper* seeds stimulates macrophage functions. It is possible that the active compound(s) in the water extract may enhance immunity.

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Efficiency Testing of Herbal Spray as Mouse Repellent

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Abstract

Rodent infestation is considered a major problem as it is a health hazard and also causes damages to households and agricultural fields. The use of rodenticides is a common approach to this problem but most are poisonous to humans and the environment. The use of natural extracts as repellents may be a better alternative. This research studied the efficiency of herbal sprays as mice repellents. The radial eight arms maze and dark/light test were used to assess the acute and long-term effects of mice repellant sprays. In relation to the acute effects, formula spray showed significantly long latency time (compared with water), followed by ginger oil and plai oil respectively in the radial arms maze. Formula spray showed a significantly short time spent followed by lemongrass oil and orange oil respectively. In the dark/light test, ginger oil showed significantly long latency time followed by formula spray and lemongrass oil respectively. Peppermint oil showed a significantly long time spent in the light compartment followed by lemongrass and formula spray respectively. Peppermint oil showed a significantly short time spent in the target (dark) compartment followed by lemongrass oil and formula spray respectively. After one week, only formula spray and lemongrass oil still retained their repellant effects in both tests. These results suggested that herbs are very effective but it due to the duration of use. It is recommended that spraying is repeated every week. The repellant effect may come from the major compound in lemongrass oil, citral, a mixture of terpenoids. The E-isomer is known as geranial and the Z-isomer is known as neral. Citral has been reported to be toxic to some insects. The formula herb spray contains many essential oils which possess the repellant activity, such as orange oil, lemongrass oil, and ginger oil.

Keywords: Mice Repellent, Herbal Spray, Lemongrass Oil, Orange Oil, Ginger Oil, Peppermint Oil, Menthol, Plai Oil

Introduction

Rodent infestation is considered a major problem as it is a health hazard and also causes damages to households and agricultural fields. Worldwide consumption of synthetic repellents has increased to minimize losses in stored grains, fruits, and other cellulosic materials infested by various pests. The use of rodenticides is a common approach to the problem but most are poisonous to humans and the environment, and cause economic problems because of damage to agricultural systems, the environment, social problems associated with their close proximity to human habitation, and health problems as carriers of zoonoses [1]. Therefore, the use of natural extracts as mice repellents may be a better alternative. Essential oils from plants belonging to several species have been extensively tested to assess their repellent properties.

Several studies reported the efficiency of herbal mice repellents after investigations of the efficacy of pure natural repellents on rat responses using a circular open field (Figure 1). The studies used wintergreen oil+ chilli (F1), wintergreen oil + peppermint oil (F2), bergamot oil (F3), wintergreen oil+ peppermint oil + bergamot oil (F4), and bergamot oil + geranium oil (F5). The effect of various repellents on rat behavior in the circular open field revealed that different ones had significant effect on reducing the numbers of visits that rats made to the testing rod compared to control on both day 0 and day 7. The studies also found that repellents had a significant effect on the time the rats spent in the inner zone, middle zone, and the outer zone. The time spent in the middle zone was significant only on day 0 in that the rats were exposed to F2, F3, and F4 spent less time than the control and F1 and F5 spent less time than F1, but not different from the control but more than F2, F3 and F4. This effect was not seen on day 7. For the time spent in the outer zone, the rats exposed to all repellents spent more time in the outer zone than the control on day 0. On day 7, the rats exposed to repellents spent more time in the outer zone than the control [2].

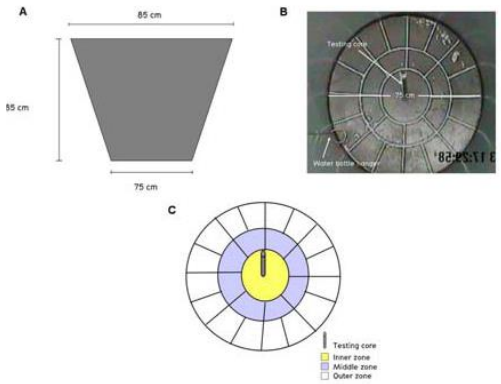

Figure 1 The circular open field, an aluminum tub-shaped apparatus with a based-diameter of 75 cm, an opening-diameter of 85 cm with a height of 85 cm (A). The water bottle hanger and a metal testing core are installed for water dispensing during 24-hour test and for cardboard paper placer, respectively (B). The base of the apparatus was divided into 3 zones; inner zone, middle zone and outer zones as shown in C.

In addition, the studies investigated the naturally-occurring bioactive compounds from four repellent essential oils against *Bemisia tabaci* whiteflies. The repellent essential oils of lemongrass (*Cymbopogon citratus*), cinnamon (*Cinnamomum zeylanicum*), cumin (*Cuminum cyminum*), and citronella (*Cymbopogon winternarius*) were identified. It was found that the pure compound geraniol and citronellol from lemongrass were the most promising net coatings owing to their repellent effect. The repellency, irritancy, or toxicity varied with the product and concentration, and these features were independent, indicating that the repellent and the irritant/ toxic mechanisms were not the same. The combined effects of these different compounds accounted for the bioactivity of the mixture, suggesting interactions between the compounds [3]. Moreover, they investigated the rat repellent from eucalyptus oil. Mature and healthy house rat, *Rattus rattus* of both sexes, was exposed to 5, 10, and 20% eucalyptus oil applied as a spray in laboratory pens in bichoice tests. Each concentration was applied through three different modes of application, daily, once, and alternatively in a week. The repellent effect of the oil was assessed based on food consumption from treated and untreated sides for four days. Overall, food consumption was significantly lower from the treatment side compared to the untreated side, indicating significant repellent effect of the oil at all the three concentrations tested. The repellent effect of the oil was, however, not found to differ significantly between the two sexes. The percent repellency in both male and female rats was apparently more with daily application of 5 and 10% eucalyptus oil. Present studies revealed the potential of eucalyptus oil in repelling *R. rattus*. However, further studies may be conducted to enhance the persistence of the repellent effect for longer periods of time [4].

The objectives of this current study were to observe the herbal sprays, orange oil, peppermint oil, lemongrass oil, menthol, ginger oil, and plai oil, as mice repellents in the radial 8-arm maze and dark/light test for acute and long-term effects.

Methodology

***Repellents*:**

The repellent substances included orange oil, peppermint oil, lemongrass oil, menthol, ginger oil, and plai oil and were supplied by ARK@RICH, Co. Ltd, and the formula spray was supplied by Forlife (Thailand) Co. Ltd. The repellents were freshly sprayed on cloth (size 5x10 cm) before being placed into the radial 8-arm maze and dark/light apparatus.

***Animals*:**

Male ICR mice (20-30 g, 7 weeks old) were obtained from the Nomura Siam International. Mice were housed on wood chip bedding in stainless steel cages with free access to food and water in the Laboratory Animal Unit of the Faculty of Pharmaceutical Sciences, Khon Kaen University, Thailand. Housing was thermostatically maintained at 22 ± 2ºC with constant humidity (45% ± 2%) and a 12-hlight–dark cycle (lights on: 06:00–18:00). The experimental procedures used in the present study were in accordance with the Guiding Principles for the Care and Use of Animals (NIH Publications No. 80-23, revised in 1996). The experimental protocols for the present study were approved by Animal Ethics Committee for Use and Care of Khon Kaen University, Khon Kaen, Thailand (Approval No. AEKKU 14/2560).

***Behavioral test*:**

1. Radial 8-arm maze

A radial 8-arm maze consists of an array of eight arms that radiate from a central starting point (Figure 2). The apparatus was an acrylic with enclosed arm 15x50x20 cm. The end of each arm has a cup that contains a food reward. Mice were placed in the center of the radial 8-arm maze. The cloth (size 5x10 cm) was placed at the start of each arm and orange oil, peppermint oil, lemongrass oil, menthol, ginger oil, plai oil, and formula spray were sprayed on the cloth. Investigators measured the latency time it took for animals to reach the arm leading to the food and time spent in each arm over 5 minutes [5, 6].

Figure 2 Radial 8-arm maze apparatus

2. Dark/Light test

The dark/light exploration test (Figure 3) has two chambers, one bigger and brighter and the other smaller and darker. Mice were placed in the lighted chamber. Two milliliter of formula spray and tested oil were dropped on the cloth (size 5x10 cm). Then each cloth was placed in the dark compartment. The latency time and time spent in each division were recorded for 5 minutes [7].

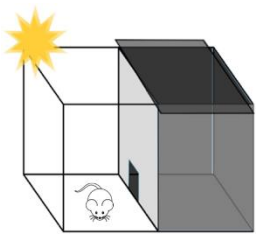

Figure 3 Dark/Light apparatus

The radial eight arms maze and dark/light test were used to test the acute and long-term effects. The acute effect were assessed immediately after spraying the herbals or formula repellent on the cloth and the long-term effects were assessed after herbal sprays or formula repellent for one week.

***Statistic analyses*:**

The results were expressed as the mean ± S.E.M. and analyzed by t-test (compared with water). Differences of $p < 0.05$ were considered to be statistically significant. The analysis was conducted using SigmaStat® ver. 3.5 (SYSTAT Software Inc., Richmond, CA, USA).

Results

1. The effects of herbal and formula sprays in radial 8-arm maze

Figure 4A shows the acute effects. Formula spray showed significantly long latency time when compared with water followed by ginger oil and plai oil respectively in the radial arms maze. After one week (long-term effect), only the formula spray showed significantly long latency time when compared with water (Figure 4B).

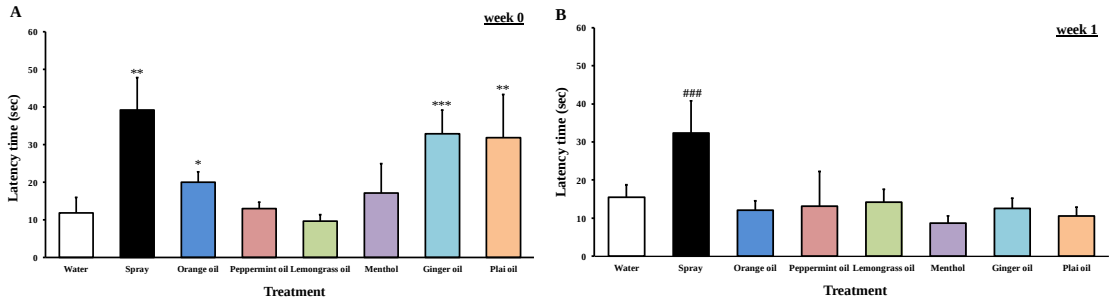

Figure 4 The latency to each arm of radial 8-arm maze. Value giver are the mean ± SEM (n = 20). Panel A is week 0 and panel B is week 1. $^{***}p < 0.001$, $^{**}p < 0.01$ and $^{*}p < 0.05$ compared with water. $^{###}p < 0.001$ compared with water.

We also recorded the time spent by each mouse in each arm (each herbal). Figure 5A shows the acute effects. The formula spray showed significantly short time spent when compared with water followed by lemongrass oil and orange oil respectively. After one week (Figure 5B), the formula spray trended to short time spent in radial 8-arm maze but not different from water, followed by ginger oil and menthol (not significant) when compared with water.

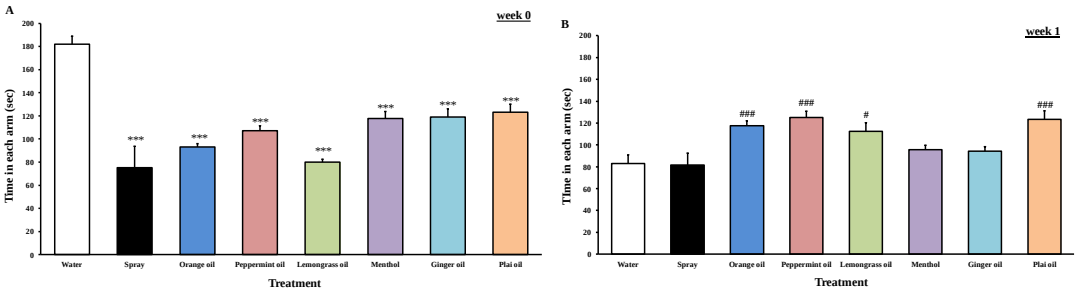

Figure 5 The time spending in each arm of radial 8-arm maze. Value giver are the mean ± SEM (n = 20). Panel A is week 0 and panel B is week 1. *** $p < 0.001$, ** $p < 0.01$ and * $p < 0.05$ compared with water. $^{###}$ $p < 0.001$ compared with water.

2. The effects of herbal and formula sprays on dark/light test

Figure 6A shows the acute effects in the dark/ light test. Ginger oil showed a significantly long latency time when compared with water, followed by formula spray and lemongrass oil respectively. After one week (long- term effect) , only lemongrass oil showed significantly long latency time when compared with water, followed by spray (not significant) (Figure 6B).

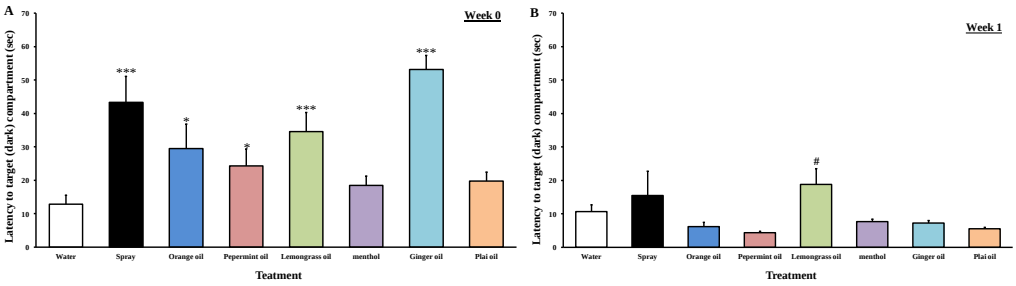

Figure 6 The latency to first entry into the dark area of dark/light test. Value giver are the mean ± SEM (n = 20). Panel A is week 0 and panel B is week 1. *** $p < 0.001$ and * $p < 0.05$ compared with water. $^{\#}$ $p < 0.05$ compared with water.

We also measured the time spent in the light compartment in the dark/ light test. Figure 7A shows the acute effects. Peppermint oil showed significantly long time spent in the light compartment when compared with water, followed by lemongrass and formula spray respectively. Figure 7B shows the long- term effects. Lemongrass oil showed a significantly long time spent in the light compartment when compared with water, followed by orange oil and ginger oil respectively.

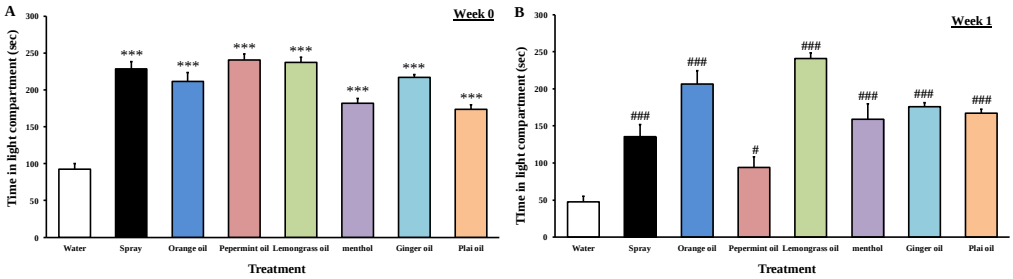

Figure 7 The time spending in light compartment of dark/light test. Value giver are the mean ± SEM (n = 20). Panel A is week 0 and panel B is week 1. *** $p < 0.001$ compared with water. $^{###}$ $p < 0.001$ and $^{\#}$ $p < 0.05$ compared with water.

Moreover, we recorded the time spent in the target (dark) compartment. Figure 8A shows the acute effects. Peppermint oil showed a significantly short time spent in the target (dark) compartment when compared with water, followed by lemongrass oil and formula spray respectively. After one week, lemongrass oil showed a significantly short time spent in the target (dark) compartment when compared

with water, followed by orange oil and ginger oil respectively (Figure 8B).

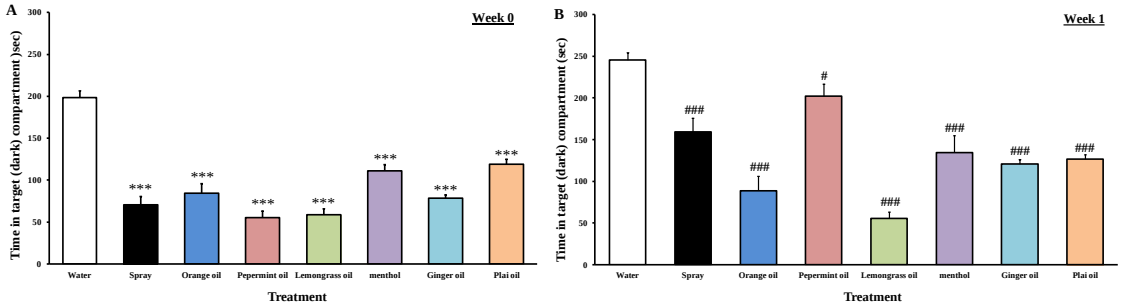

Figure 8 The time spending in target (dark) compartment of dark/light test. Value giver are the mean ± SEM (n = 20). Panel A is week 0 and panel B is week 1. *** $p < 0.001$ compared with water. ### $p < 0.001$ and # $p < 0.05$ compared with water.

Discussion and conclusion

The effects of herbal and formula sprays used as mice repellents were investigated by a radial 8-arm maze and dark/light test. Twenty male mice were food-deprived for 18 hours before the test with the radial 8-arm apparatus. The repellents were freshly sprayed on the fabric strip (5x10 cm) before being placed mice into the radial 8-arm maze and dark/light apparatuses with food in the ends of the arms. Mice were placed in the center of the maze and recorded for 5 minutes. In the dark/light (D/L) exploration test, a fabric strip was placed in the target (dark) compartment. Mice were placed in the light compartment and the latency time and time spent in light and target (dark) compartment were measured during 5 minutes. Both the radial 8-arm maze and D/L test tested acute and long-term effects.

The results showed the latency time that mice spent in the radial 8-arm maze. Long latency time indicated the best efficacy of the repellent. In the radial 8-arm maze, the acute effects of formula spray showed a significantly long latency time when compared with water, followed by ginger oil and plai oil respectively. After one week, the formula also showed a significantly long latency time, followed by lemongrass oil and peppermint oil respectively. We also measured the time spent in each arm. A short time spent indicated the best efficacy of the mice repellent. The results related to the acute effects of formula spray showed a significantly short time spent when compared with water, followed by lemongrass oil and orange oil respectively. In relation to the long-term effects (after one week), the formula spray also showed a significantly short time spent followed by ginger oil and menthol respectively. In the dark/light test, we measured latency time and time spent in the light and target (dark) compartments. The results related to the acute effects of the dark/light of ginger oil showed a significantly long latency time when compared with water, followed by formula spray and lemongrass oil respectively. Peppermint oil showed a significantly long time spent in the light compartment, followed by lemongrass and formula sprays respectively. Peppermint oil showed a significantly short time spent in the target (dark) compartment, followed by lemongrass oil and formula sprays respectively. After one week, lemongrass oil showed a significantly long latency time, followed by spray (not significant). Lemongrass oil showed a significantly long time spent in the light compartment, followed by orange oil and ginger oil respectively. Lemongrass oil showed a significantly short time spent in the target (dark) compartment, followed by orange oil and ginger oil respectively. A short time spent in the target (dark) compartment indicated the best efficacy of the mice repellent.

In the comparison of mice repellents between week 0 to week 1, only formula spray and lemongrass oil still retained their repellant effects in both tests because lemongrass contains a mixture of terpenoids such as limonene. Limonene can be used as cat, dog, and insect repellent [8].

These results suggested that herbs are very effective but it depends on the duration of use. The recommendation is repeated spraying every week. The repellant effect may come from the major compound in lemongrass oil, citral, a mixture of terpenoids. The E-isomer

is known as geranial and the Z- isomer is known as neral. Citral has been reported to be toxic to some insects. The formula herb spray contains many essential oils which possess the repellant activity, such as orange oil, lemongrass oil, and ginger oil.

Acknowledgement

The authors would like to thank Khon Kaen University, Khon Kaen, Thailand for financial support for the presentation of this work.

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In vitro Studies on α-Glucosidase Inhibition and Antioxidant Properties of *Boletus colossus* Heim.

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Abstract

Diabetes is a metabolic disease characterized by a high blood glucose level that can cause serious damage to the body system. Postponement of the absorption of glucose in humans through inhibition of alpha-glucosidase enzyme is used for type 2 diabetes treatment. Alpha-glucosidase is considered to be involved in a metabolism because the enzyme plays a key role in the digestion of carbohydrates. Mushrooms are one of the diet supplementations that have been reported as organisms with antioxidant, anti-inflammatory, antitumor, and anti-α-glucosidase activities. *Boletus colossus* Heim. is one of the higher fungi reported as an important source of bioactive compounds. The aim of this study was to investigate the anti-alpha-glucosidase activity and compare the antioxidant properties of different solvent extracts from *Boletus colossus* Heim. in the forms of mycelium and filtrate (cultural medium). Dried mycelium and filtrate of *Boletus Colossus* Heim. were extracted at room temperature for 48 hours three times with ethyl acetate, n-butanol, and water respectively. All extracts were tested for their alpha-glucosidase inhibition, DPPH radical scavenging and reducing power activities, and total phenolic content by the Folin-Ciocalteu method. All of the investigated extracts exhibited potent anti-alpha-glucosidase activities with the IC_{50} in range 0.04-1.32 mg/mL. BECM, WECM, EAECM and WEM showed better activities than the acarbose that was used as a standard (IC_{50} = 1.32 mg/mL). The crude ethyl acetate fractions from mycelium and filtrate provided better results of DPPH radical scavenging (IC_{50} = 0.31, 0.11 mg/mL respectively) and reducing power activities than others. In addition, both crude fractions showed higher total phenolic content as 100 and 289.30 mg GAE/g of crude extracts respectively than others. ***Conclusion:*** These results indicate that all extracts from mycelium and filtrate of *B. colossus* have the potential to be natural anti-α-glucosidase and antioxidant sources.

Keywords: Antioxidant, Alpha-Glucosidase, Mycelum, Cultural Medium, *Boletus Colossus* Heim.

Introduction

Diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia resulting from defects in insulin secretion, insulin action, or both. Along with hyperglycemia and abnormalities in serum lipids, diabetes is associated with micro- and macro-vascular complications, which are the major causes of morbidity and death in diabetes subjects [1, 2]. Diabetes is divided into two main types. Type 1 diabetes, insulin-dependent diabetes, is a chronic condition in which the pancreas produces little or no insulin. Type 2 diabetes is insulin-independent caused by the defect of insulin to carry the glucose into the body's cells to use for energy, or the failure of the pancreas to make enough insulin when blood sugar levels increase after meals. To postpone absorption of glucose in humans through inhibition of the carbohydrate-hydrolyzing enzymes: α-amylase and α-glucosidase in the digestive tract, Alpha-glucosidase enzyme, considered to be involved in a metabolism because the enzyme plays a key role in digesting carbohydrates, is used for type 2 diabetes treatment [3]. Oxidative stress, including free radicals, is believed to cause a variety of diseases such as carcinogenesis, diabetes, heart disease, cancer, inflammation, and Parkinson's and Alzheimer's diseases [4]. These results in the damage of biopolymers including nucleic acids, proteins, polyunsaturated fatty acids, and carbohydrates. Antioxidants help organisms to deal with oxidative stress caused by free radical damage. Free radicals are chemical species which contain one or more unpaired electrons which are highly unstable and cause damage to other molecules by extracting electrons from them to attain stability. In recent years, alternative health

care using diet and dietary components has become increasingly popular due to its discovered beneficial effects [5-7]. Mushrooms are one of dietary supplements reported as organisms with antioxidant, anti-inflammatory, antitumor, and anti- α- glucosidase activities [8-14]. They have powerful biological activities derived from different compounds such as selenium, ergothioneine, phenolics, and polysaccharides. Therefore, mushrooms are considered to be a good source of phenolic antioxidants.

Boletus colossus Heim. (Bolete mushroom), in the Boletaceae family, is an edible ectomycorrhizal mushroom group. It was recently popular as a source of bioactive compounds used in agriculture for controlling insects or weeds. Furthermore, other bioactive metabolites in this mushroom were reported. Kitwicha *et al.* found that crude polysaccharide extracted from the mycelium of *B. colossus* Heim. showed antioxidant activities [15]. However, the inhibitions on alpha-glucosidase activity of extracts from mycelium and filtrate of *B. colossus* has rarely been studied as the growth rate of *B. colossus* is very slow and it takes a long time to cultivate in the green house. Thus, it is expensive to obtain fruiting bodies. Therefore, using a submerged culture to produce effective substances might be an alternative way to overcome this problem. Mycelium and its byproduct fermentation filtrate, mainly prepared from submerged culture, are alternative or substitute products of mushrooms. The objectives of this study were to investigate the inhibitory effects of crude extracted from *B. colossus* mycelium and filtrate on anti-α-glucosidase and antioxidant activities.

Methodology

Materials and chemicals

Boletus colossus Heim. in submerged culture was obtained from the Department of Horticulture, Faculty of Agriculture at Kamphaeng Saen, Kasetsart University. 1,1-diphenyl-2-picrylhydrazyl (DPPH), α-glucosidase and gallic acid were purchased from Sigma-Aldrich whereas Folin-Ciocalteu reagent was from Fluka. All other chemicals and solvents used were analytical or laboratory grades (used with further purification).

Preparation of samples

The strain of *B. colossus* Heim. in PDB (potato dextrose broth) medium 5 L was incubated at room temperature for 60-70 days. The mycelium and filtrate were separated by suction. Thereafter, the mycelium was cleaned with water, air-dried, and further freeze-dried. Meanwhile the filtrate was stored at 4°C until used.

Extraction of crude extracts

Extraction of mycelia by maceration: The extraction method used was a modified method of Tuzz-Ying *et al* [16]. The freeze-dried mycelium of *B. colossus* was ground to a powder form, and then extracted with hexane to remove fat. The residue was separated by suction filtration and further extracted three times with ethyl acetate, n-butanol, and water respectively in ratio 1:10 at room temperature for 48 h. The extracted solutions were filtered by suction and evaporated to dryness under reduced pressure. After evaporation, each extract was further freeze-dried to provide three fractions of extracts, ethyl acetate (EAEM), butanol (BEM), and water (WEM) extracts of mycelium.

Extraction of filtrate by liquid-liquid extraction: 5 L of filtrate (cultural medium) was partitioned three times with the equal volume of ethyl acetat and, n-butanol. The organic layers were combined and evaporated to dryness. All extracts were freeze-dried to give three fractions as ethyl acetate (EAECM), butanol (BECM), and water (WECM) extracts of cultural medium.

α-Glucosidase inhibitory activity assay

The enzymatic activity of α-glucosidase was determined colorimetrically by monitoring the release of *p*-nitrophenol from the *p*-nitrophenol glycoside substrate. The assay was carried out according to the modified Kim *et al.* Method [17]. Briefly, 50 µl of 0.2 M phosphate buffer (pH 6.8) was mixed with 50 µl of α-glucosidase (0.5 U/ml) and 50 µl of each extract at various concentrations. The solution was pre-incubated at 37°C for 15 min. Then, 100 µl of 0.3 mM 4-nitrophenyl-α-D-glucopyranoside (pNPG) was added. The enzymatic reaction was allowed to proceed at 37°C for 20 min. The reaction was stopped by adding 750 µl of 0.1 M Na_2CO_3 solution. Then, the mixture was measured at 405 nm by UV-Vis spectrophotometer. An acarbose was used as a positive control. A solution without sample was used as a control and a solution without substrate was used as a blank. The percentage of α-glucosidase inhibition was calculated as follows:

$$(\%)\ \text{inhibition of } \alpha\text{-glucosidase} = \left[1 - \frac{(A_{sample} - A_{blank})}{A_{control}}\right] \times 100$$

DPPH radical scavenging capacity assay

DPPH assay was measured by the modified method of Chu *et al.* [18]. One milliliter of 0.3 mM DPPH in methanol was added into 3.0 mL of each extract solution at various concentrations. The mixture was shaken vigorously and left to stand for 30 min in the dark at room temperature. The absorbance was measured at 514 nm. Butylated hydroxytoluene (BHT) was used as a positive control. The percentage of DPPH inhibition was calculated as follows:

$$(\%)\ \text{DPPH inhibition} = \left[1 - \left(\frac{A_{sample} - A_{blank}}{A_{control}}\right)\right] \times 100$$

Reducing power assay

Reducing power assay was measured by the modified method of Isabel *et al.* [8]. 2.5 mL of each extract at various concentrations was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% (w/v) $K_2Fe(CN)_6$ solution. The reaction was incubated at 50°C for 20 min and then 2.5 mL of 10% (w/v) of trichloroacetic acid was added and mixed. The mixture solution was centrifuged at 650 rpm for 10 min. Five milliliter of upper solution was mixed into 5.0 mL of DI water and 1.0 mL of 0.1% (w/v) $FeCl_3$ solution. The absorbance was measured at 700 nm against blank. BHT was used as positive control.

Determination of total phenolic content

The total phenolic content was determined by the modified Folin-Ciocalteu method of Thetsrimuang *et al.*[10]. Briefly, 0.5 mL of each extract in methanol was added into 10% (v/v) Folin-Ciocalteu reagent 0.5 mL. The reaction was left at room temperature for 3 min and then 35% (w/v) Na_2CO_3 0.5 mL was added. The mixture solution was adjusted to 5.0 mL with DI water. The absorbance of the mixture was read at 725 nm after left to stand in the dark for 90 min. The quantification of phenolic compound was determined based on a standard curve of gallic acid with the concentrations as 5, 10, 25, 50, 100, 150, and 200 mg/L. The amount of total phenolic content was expressed as mgGAE/g of crude extract.

Statistical analysis

All determinations were performed at least in triplicate and the results expressed as mean ± standard deviation (SD). The IC_{50} values were calculated from linear regression analysis. Analysis of the variance and significance of differences among samples were analyzed using One way analysis of variance (ANOVA) and the Duncan Test.

Results and discussion

Extraction of mycelium and filtrate

The extraction of *B. colossus* mycelium (44.1476 g) by maceration at room temperature produced three fractions of extracts. The yields of ethyl acetate (EAEM), butanol (BEM), and water (WEM) extracts of mycelium were 0.5220, 0.4836, and 16.8644 g respectively. The extraction of filtrate with different solvents gave another three fractions as EAECM (0.3112 g), BECM (4.9972 g) and WECM (1.4165 g), respectively.

α-Glucosidase inhibitory activity

α-glucosidase inhibitors are effective in delaying glucose absorption and preventing elevation of the post-prandial blood glucose level and therefore, they play a significant role as chemotherapeutic agents for type 2 diabetes [19]. To investigate the inhibitory activity of extracts against α-glucosidase, an acarbose was used as a positive control in the assay. As acarbose is an oral α-glucosidase inhibitor for use in control of type 2 diabetes. The results are presented in Table 1. All extracts showed the potential of α-glucosidase inhibition.

Table 1 IC_{50} of α-glucosidase and DPPH inhibitions and total phenolic content of extracts from *B. colossus* mycelium and filtrate

Extracts	IC_{50} (mg/mL)		Total phenolic content (mg GAE/g of crude extract)
	α-glucosidase inhibition	DPPH inhibition	
EAEM	1.32±0.88^{c}	0.31±0.32^{b}	100.00±3.44^{b}
BEM	1.27±0.84^{c}	1.26±0.25^{d}	34.63±0.74^{a}
WEM	0.99±0.78^{b}	1.70±0.41^{e}	52.82±1.50^{a}
EAECM	0.09±0.79^{a}	0.11±0.17^{a}	289.30±2.63^{d}
BECM	0.04±0.85^{a}	0.33±0.19^{b}	238.90±1.31^{c}

Extracts	IC_{50} (mg/mL)		Total phenolic content (mg GAE/g of crude extract)
	α-glucosidase inhibition	DPPH inhibition	
WECM	0.05±0.77[a]	0.75±0.21[c]	228.00±2.70[c]
Acarbose	1.32±0.75[c]		
BHT		0.04±0.43[a]	

Values in each column with different letters are significantly different at $p<0.05$.

It was found that the α-glucosidase inhibition of extracts from *B. colossus* mycelium and filtrate depended on extract concentrations. Increases of extract concentrations were accompanied by increases in the α-glucosidase inhibitory activities. The inhibition order of mycelium extracts was WEM > BEM ≈ EAEM with IC_{50} of 0.99, 1.27 and 1.32 mg/mL respectively. The filtrate extracts showed the α-glucosidase inhibition in order BECM ≈ WECM > EAECM. In addition the results showed that the filtrate extracts exhibited better activities than the mycelium extracts. Moreover, BECM, WECM, EAECM, and WEM had better activities than an acarbose (IC_{50} 1.32 mg/mL) which was used as a standard. Fatmawati *et al.* [11] extracted the bioactive compounds from the fruiting body of *Ganoderma lucidum* with chloroform and hot water. They found that the chloroform extract showed inhibitory activity on α-glucosidase (IC_{50} of 88.7 μg/mL) better that the hot water extract. Additionally, the chloroform extract had stronger activity than the positive control, acarbose with an IC_{50} of 336.7 μg/mL. Hsu *et al.* [20] separated and purified the intramolecular polysaccharides from *Coriolus versicolor* LH1 mycelia for their α-glucosidase inhibitory properties. Three fractions of polysaccharide (iPL-F5-2-1, iPL-F5-4-1, and iPL-F5-5-1) demonstrated strong α-glucosidase inhibition with IC_{50} of 1.7, 1.8 and 0.8 mg/mL respectively.

Antioxidant activity of extracts

The antioxidant activities of all extracts from *B. colossus* were evaluated by DPPH radical scavenging capacity and reducing power activities and determination of total phenolic content. DPPH is a useful reagent for investigating the free radical- scavenging activities of materials. In the DPPH test, the antioxidants were able to reduce the stable DPPH to the yellow- colored diphenylpricrylhydrazine. By DPPH assay, the inhibitory activity depended on extract concentrations. The inhibition of DPPH radical of mycelium extracts was found in the order of EAEM > BEM > WEM while the extracts from filtrate were found in order of EAECM > BECM > WECM. The crude ethyl acetate fractions from mycelium and filtrate showed better DPPH inhibition than others with IC_{50} 0.31 and 0.11mg/mL respectively, as shown in Table 1, according to Ajith *et al*. [21] who studied the *in vitro* antioxidant activity and antihepatotoxic activity of the extracts from *Phellinus rimosus*. From preliminary screening, they found that the ethyl acetate extract showed significantly higher activity than methanol and water extracts. Therefore, dried fruiting bodies of *Phellinus rimosus* were extracted with petroleum ether and then with ethyl acetate using soxhlet apparatus for 8-10 h. They found that ethyl acetate extract of *Phellinus rimosus* shows potent scavenging activity of free radical such as superoxide, hydroxyl, and nitric oxide and inhibition of lipid peroxidation activity. Their results indicated that ethyl acetate extract of *Phellinus rimosus* exhibited significance *in vitro* antioxidant activity.

Fe^{3+} reduction is often used as an indicator of electron-donating activity. In the reducing power assay, the presence of antioxidant in the extracts resulted in the reduction of Fe^{3+} to Fe^{2+} by donating an electron. This reducing capacity of compounds could serve as an indicator of potential antioxidant properties and the increasing absorbance suggests an increase in reducing power [8]. The reducing power of all extracts from *B. colossuss* mycelium and filtrate increased with an increase of concentration as shown in Figure 1 **(A)** and **(B)**. The highest activity was observed from EAECM and EAEM. However, the extracts from filtrate seemed to provide higher activity than mycelium extracts at all concentrations. In addition all extracts showed higher reducing power activities than BHT.

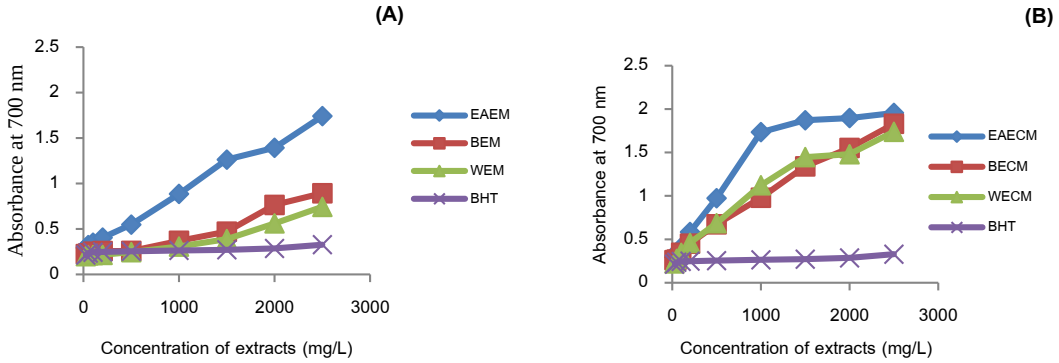

Figure 1 Comparison of reducing power of extracts from *B. colossus* **(A)** mycelium **(B)** filtrate

The total phenolic content of extracts that determined by the Folin-Ciocalteu method were in the range of 34.63-289.30 mgGAE/g crude extract, as shown in Table 1 and Fig 2.

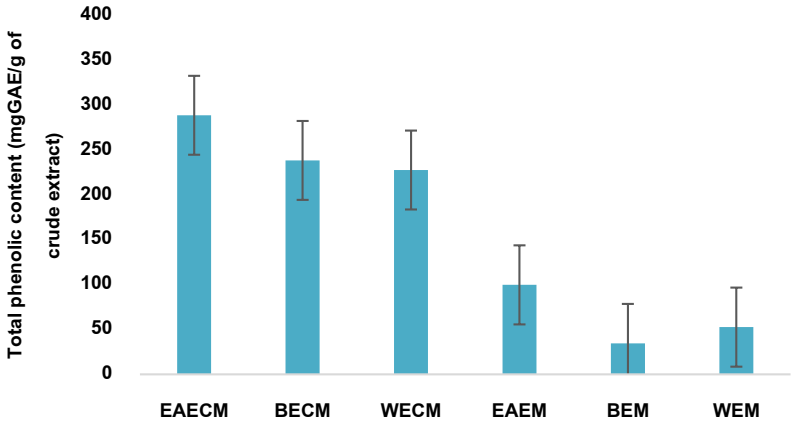

Figure 2 Total phenolic content of extracts from *B. colossus* mycelium and filtrate

Figure 2 shows EAECM had a higher amount of phenolic compounds (289. 3±2. 63 mgGAE/ g of crude extract) than other extracts from filtrate and also from mycelium. Comparison of extracts from mycelium show EAEM had the highest phenolic content (100. 0±3. 44 mgGAE/g of crude extract). The results were in agreement with the findings that the higher a total phenolic content, the stronger the DPPH radical scavenging and reducing power. The correlation coefficient between total phenolic contents and antioxidant activity was 0. 859. Thetsrimung *et al.* reported the relationship between the total phenolic content and antioxidant activity.[10] Therefore, phenolic compounds might be the main active compounds responsible for antioxidant activity.

Conclusion

The alpha-glucosidase inhibitory and antioxidant activities of extracts from *B. colossus* mycelium and filtrate were investigated. The inhibitory activities on alpha-glucosidase depended on the extract concentrations. All extracts had the anti-α-glucosidase activity. However, the extracts from filtrate exhibited stronger activities than the extracts from mycelium. The best potent of antioxidant activities by the DPPH method and reducing power assay was EAECM. In addition, the highest value of total phenolic content was also observed from EAECM. There was correlation between DPPH inhibition and total phenolic content at a high level. These suggested that all extracts from *B. colossus* mycelium and filtrate were helpful to control the rapid increase in blood glucose level and had the potential to be natural antioxidant.

Acknowledgements

This work was supported by the Foundation of Student Development and the Department of Chemistry, Faculty of Liberal Arts and Science, Kasetsart University Kamphaeng Saen Campus.

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Flavonol Glucosides Contents of *Allium ascalonicum* L. Bulbs and Their Anti-Oxidant Activity

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Abstract

Shallot (*Allium ascalonicum* L.) is an edible plant in a family Amaryllidaceae. It is commonly used as spice in oriental food and traditionally used to relieve nasal congestion from common cold. The chemical compositions of shallots contain volatile and non-volatile sulfur-containing organic compounds, flavonoids, saponins and tannins. Flanovol gulcosides are main compounds found in shallot bulbs. This study aimed to determine flavonol glucosides in these shallots and evaluate their anti-oxidant activity. Shallot samples were obtained from 15 sources in Thailand. Shallot from Srisaket exhibited the highest anti-oxidant activity (IC_{50}=1.50±0.05 mg/mL). In addition, HPLC analysis of the relative area percent of flanovol gulcosides showed that high quercetin contents (87.72%±0.08) were in water shallot extract from Srisaket. The highest quercetin-4′-glucoside content (63.59%±0.04) was found in 80%ethanolic shallot extract from Nakornratchasima. Moreover, Pearson's correlation showed IC_{50} values of ethanolic shallot extract were significantly correlated with their quercetin contents (p-value < 0.05). This study indicated some sources of high quality shallot bulbs. However, it needs further study to improve the extract to obtain high potency and search for other potential pharmacological activities.

Keywords: *Allium ascalonicum* L, Anti-oxidant, Flavonol glucosides, Quercetin

Introduction

It is known that shallot, *Allium ascalonicum* L. is a plant of the largest *Allium* genus of the Amaryllidaceae family. A lot of research has been studied about shallots. Shallot bulbs exhibited antioxidant [1], antimicrobial activities, [2], [3], [4] and antifungal activities [5]. In addition, shallot was used for both prevention and therapy and prescribed as hypoglycemic, hypotensive, hypocholesterolemic, antiartherosclerotic and antithrombotic agents. Aqueous extracts exhibited anti-inflammatory and anticancer activities in Jurkat and K562 cell lines [6]. To search for the sources of good quality shallots, fifteen shallot samples were collected and determined the chemical compositions.

Research studies reported volatile organosulfur compounds or thiosulfinates, mainly disulfides and trisulfides, were the characteristics of pungent taste of shallots, onions and garlics [7]. The other polar compounds were flavonoids, saponins and phenolic compounds [8], [9], [10] which were previously found in shallots. Flavonol glucosides; for example, quercetin-3,4′-diglucosides, quercetin-4′-glucosides and isorhamnetin-3,4′-diglucosides were identified in methanolic shallot extracts [9], [11]. Traces of isorhamnetin 3′-diglucosides, free quercetin and ascalonicosides were also detected [12]. These compounds were studied some biological activities i.e. antioxidant, antibacterial, antifungal activities [13], [14], [15], [16]. In this study shallot samples were evaluated for their antioxidant activity.

Methodology

Quality of shallot bulbs

The shallot bulbs were evaluated for foreign matter, total ash, acid insoluble ash, alcohol-soluble extractive, water-soluble extractive and loss on drying according to the procedures in Thai Herbal Pharmacopoeia [17].

Sample preparation

Shallot bulbs were bought from 15 sources; 6 from north eastern, 4 from north and 5 from the central part of Thailand. The fresh bulbs were removed outer shell and washed thoroughly in water. Shallot bulbs were mashed properly in a kitchen mixer and extracted with distilled water and 80%ethanol using the ultrasonic bath for 1 hour. The

suspension was then filtered through Whatman No. 1 filter paper. Ethanolic extract were removed solvent in a rotary evaporator (Buchi-Germany) at 50ºC and water extracts were dried using a lyophilizer. Percent yield was calculated as % of dried extract in the fresh weight.

DPPH radical scavenging assay

DPPH solution was prepared at a concentration of 200 µM in ethanol. The shallot extracts were solubilized in 80% ethanol and diluted to final concentrations of 0 - 1.0 mg/mL, (N=3). Standard quercetin solutions were prepared in ethanol (0.5-200 µg/mL). Both 100 µL of the sample solutions and 100 µL of the DPPH solution were added into a 96-well plate and mixed well. After leaving it at a room temperature for 30 minutes, absorbance was measured at the 517 nm wavelength using a Biorad® microplate reader. The assessment of the antioxidant activities was expressed in IC_{50} value and compared with quercetin hydrate. Each measurement was conducted in triplicate.

HPLC analysis

HPLC analysis was performed on the Agilent 1260 HPLC system with EZchrome software. Poroshell C18 column (2.1 x 150 mm, 4 µm) at 30 ^{o}C was used as a stationary phase, and the mobile phase composition was 0.1% formic acid in water (solvent A) and acetonitrile (solvent B) in step gradient elution. The mobile phase was eluted from 5–20 %B (0-5 min), 20–30 %B (5-10 min), 30–35 %B (10-20 min), 35–50 %B (20-35 min), 50–95 %B (35-38 min) and remained at 95%B, then back to 5%B and equilibrated for 4 minutes, with a total run time of 45 minutes modified from Bonaccorsi *et al.* [9]. The flow rate was 0.3 mL/min, and the injection volume was 10 µL. Chromatogram was detected using a diode array at the wavelengths 260 and 370 nm. A peak area of each flavonol glucosides was recorded. The relative quercetin-4′-glucoside and quercetin area percent were calculated from total area of flavonol glucosides.

Statistical analysis

Data were obtained from independent experiments and presented through means ± standard deviation (SD). Statistical analysis was performed using independent t-test, one-way ANOVA, and post hoc test at a significance level of *p*-values < 0.05. IBM SPSS software version 21.0 was used for all statistical analyses.

Results

Shallot bulbs were obtained from 15 different sources and categorized to 3 regions; north: Chiangrai (CR), Petchaboon (PB), Udtaradit (UD), Nan (NN), north eastern: Srisaket (SK), Nakornratchasima (NR), Nakornpanom (NP), Burirum (BR), Roi-Et (RE), Yasothorn (YT), and central part: Nakorn Pathom (NT), Ratchaburi (RB), Kanchanaburi (KB), Pathumthani 1 (PT1), Pathumthani 2 (PT2) of Thailand. Percent yield of shallot water extract and 80% ethanolic extract were in the range of 5.16-7.02%w/w and 6.01-6.93%w/w, respectively. As the quality assurance, these shallot samples were examined for pharmacognostic characters and physicochemical identities according to Thai Herbal Pharmacopoeia [17] and WHO Pharmacopoeia [18]. The foreign matter (0.68±0.08 %w/w), acid insoluble ash (0.45±0.11 %w/w), water (82.35±4.48 %w/w), and ethanol (7.93±1.28 %w/w), soluble extractives and loss on drying (7.01±0.99 %w/w) were with the limit of pharmacopoeia except that acid insoluble ash (7.77±0.89 %w/w) was higher than 1.0 % w/w [19]. The physicochemical identities of shallot bulbs from 15 sources are summarized in Table 1.

Table 1 The physicohemical identities of shallot bulbs

Samples	Foreign matter	Total ash	Acid-insoluble ash	Alcohol-soluble extractive	Water-soluble extractive	Loss on drying
North						
PB	0.73±0.14	8.07±0.09	0.28±0.18	6.27±1.33	81.17±1.36	6.80±0.04
CR	0.80±0.07	6.47±0.34	0.55±0.10	8.83±0.06	85.67±5.26	7.50±0.47
NN	0.72±0.05	6.67±0.52	0.42±0.19	9.20±2.07	81.10±2.05	5.67±0.38
UD	0.68±0.06	7.94±0.85	0.65±0.13	8.63±0.40	73.60±4.99	8.61±0.14
North eastern						
SK	0.60±0.04	8.84±0.37	0.47±0.28	9.07±0.45	84.40±0.29	6.42±0.47
NR	0.75±0.05	9.15±0.35	0.48±0.06	6.47±2.05	83.67±1.34	8.60±0.32
NP	0.61±0.07	6.59±0.50	0.45±0.09	6.57±1.95	81.97±1.89	5.57±0.27
BR	0.73±0.01	7.54±1.71	0.57±0.10	6.63±1.46	84.07±3.23	8.45±0.42

Samples	Foreign matter	Total ash	Acid-insoluble ash	Alcohol-soluble extractive	Water-soluble extractive	Loss on drying
RE	0.78±0.04	8.94±0.94	0.32±0.06	8.60±1.06	86.60±3.15	6.48±0.30
YT	0.76±0.10	6.55±0.68	0.50±0.10	8.10±0.46	75.00±11.35	6.65±0.31
Central						
NT	0.70±0.03	7.06±0.72	0.60±0.38	7.70±0.87	87.40±2.86	6.59±1.73
RB	0.50±0.07	7.67±1.45	0.30±0.10	7.87±0.93	90.17±1.37	6.37±0.42
KB	0.54±0.06	8.13±0.35	0.50±0.00	7.90±0.66	83.90±1.85	6.57±0.30
PT 1	0.74±0.05	8.92±0.92	0.43±0.21	9.47±2.45	85.93±1.86	8.29±0.22
PT 2	0.66±0.11	8.10±0.65	0.48±0.08	10.50±0.26	79.40±27.33	6.50±0.46

These shallot extracts were analyzed for chemical compositions and they were mainly flavonol glucosides. The sulfur-containing organic compounds were not detected in all samples. Flavonol glucosides were identified using ESI-MS/MS in our previous study [20] mostly were quercetin-4′-glucoside, quercetin-3,4′-diglucoside and quercetin. The identified flavonol glucosides were quercetin-3,4′-diglucosides, isorhamnetin-3,4′-diglucosides, quercetin-3-glucoside, quercetin-4′-glucoside, isorhamnetin-4′-glucoside, quercetin and isorhamnetin according to their elution order from HPLC analysis. All shallot extracts showed similar HPLC elution pattern, a representative of HPLC chromatogram is shown in Figure 1. All shallot extracts were found high quercetin content in water extract while quercetin-4′-glucoside contents were high in 80%ethanolic extracts (Table 2). Water shallot extract from Srisaket showed the highest quercetin content while that from Petchaboon showed the highest quercetin-4′-glucoside content. In similar, 80%ethanolic shallot extract from Srisaket showed the highest quercetin content although it was lower than that of water extract. 80%Ethanolic shallot extract from Nakornratchasima showed the highest quercetin-4′-glucoside content.

Anti-oxidant activity of these shallot samples were evaluated using DPPH radical scavenging assay. Shallot extracts in both water and 80% ethanol from Srisaket showed the highest anti-oxidant activity (IC_{50} values = 1.92 and 1.50 mg/mL, respectively); however, they were much lower potency compared with standard quercetin (Table 3). At the concentration of 1 mg/mL percent scavenging of shallot water extracts was in the range of 17.14 - 30.62% while that of 80%ethanolic extracts was 11.24 - 36.60% compared with quercetin (85%, 0.1 mg/mL). The anti-oxidant activity of NP water extract (IC_{50} values = 1.95 mg/mL) was also in similar potency to that of SK water extract. Between groups the anti-oxidant activity of SK and PT1 water extracts were statistically different and so were SK and PT2 ethanolic extracts (p-value < 0.05). Moreover, these results showed that among different locations shallots from Srisaket contained relatively high flavonol glucosides and this may contribute to its anti-oxidant activity.

Table 2 Percent yield and area percent of quercetin-4′-glucoside and quercetin of water and 80% ethanolic shallot extracts

Samples	Water extracts			80% Ethanolic extracts		
	%yield (%w/w)	quercetin-4-glucoside[1]	quercetin[1]	%yield (%w/w)	quercetin-4-glucoside[1]	quercetin[1]
PB	5.16±0.22	55.25±0.05*	35.76±0.03	6.47±0.10	57.87±0.09	3.96±0.00
CR	5.63±0.19	13.86±1.23	53.31±1.02	6.35±0.11	59.55±0.17	3.86±0.20
NN	5.63±0.17	24.40±1.02	48.58±0.87	6.13±0.14	57.67±0.31	2.78±0.15
UD	5.60±0.17	30.48±1.13	41.17±0.90	6.18±0.10	60.49±0.08	9.91±0.05*
SK	7.02±1.64	3.20±0.05*	87.72±0.08*	6.93±0.13	57.73±0.07	12.14±0.09*
NR	5.74±0.11	46.39±0.12*	45.30±0.03	6.18±0.17	63.59±0.04	4.16±0.08
NP	5.29±0.20	29.15±0.06	62.26±0.05*	6.09±0.10	61.59±0.18	8.07±0.03
BR	5.33±0.15	27.70±0.07	59.59±0.05	6.15±0.15	59.22±0.09	5.51±0.07
RE	5.60±0.19	28.20±0.84	48.46±0.74	6.82±0.18	54.65±0.08	5.40±0.07

Samples	Water extracts			80% Ethanolic extracts		
	%yield (%w/w)	quercetin-4-glucoside[1]	quercetin[1]	%yield (%w/w)	quercetin-4-glucoside[1]	quercetin[1]
YT	5.28±0.18	16.94±0.02	55.44±0.33	6.60±0.16	47.80±0.24	3.05±0.10
NT	5.77±0.19	25.12±0.94	51.09±0.51	6.01±0.13	57.13±3.42	3.25±0.31
RB	5.64±0.12	12.92±4.43	50.85±7.34	6.60±0.10	55.29±2.48	3.88±0.30
KB	5.69±0.14	24.44±5.67	50.61±2.29	6.30±0.12	55.17±0.77	3.81±0.23
PT1	5.74±0.17	14.26±2.42	48.38±11.25	6.60±0.18	54.22±0.69	4.45±0.40
PT2	5.78±0.16	21.64±0.02	52.77±0.05	6.80±0.13	49.48±0.14	5.24±0.00

[1]Relative area percent (N=1, duplicate)

*p-value < 0.05 between groups

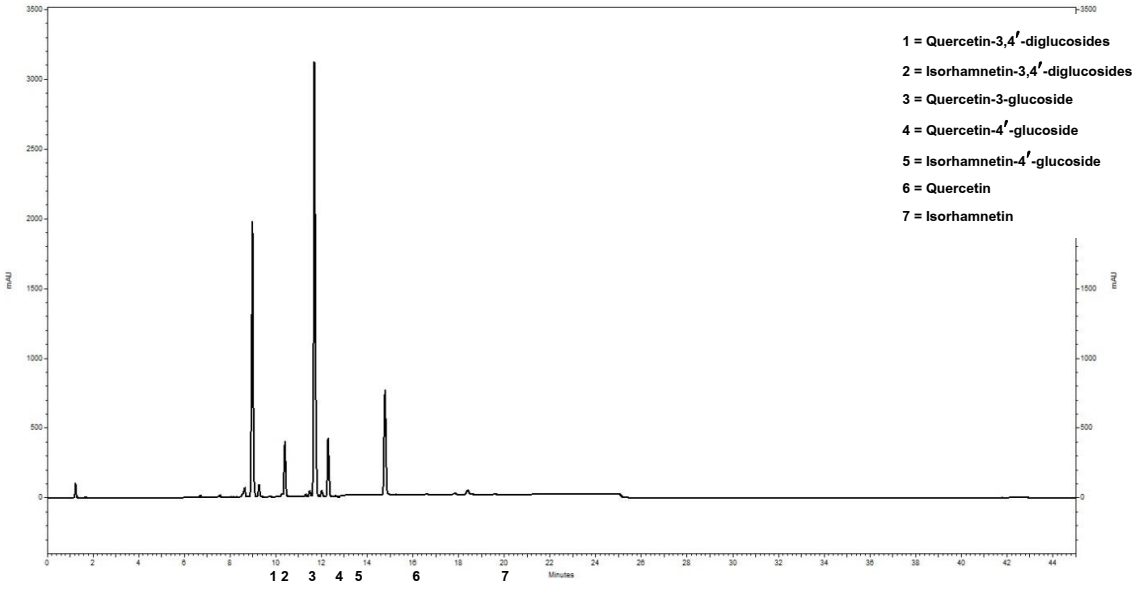

Figure 1 HPLC chromatograms of SK ethanolic extract at 370 nm

Discussion and Conclusions

Although the percent yields of these shallot extracts were not significantly different between water and 80% ethanol, they showed varied relative quercetin and quercetin-4′-glucoside contents depending on geographical diversity. HPLC chromatograms showed similar patterns and elution orders among these samples. The flavonol glucosides in these shallot extracts were similar in compositions compared with the methanolic Italian shallot extracts [9]. Quercetin contents form Srisaket water and 80% ethanolic extracts were significantly different between groups (p-value < 0.05). Quercetin-4′-glucoside contents in water extract from Srisaket, Petchaboon and Nakornratchasima were significantly different between groups (p-value < 0.05). Relative quercetin contents in 80%ethanolic extracts from Srisaket and Udonthani were significantly different between groups (p-value < 0.05) while quercetin-4′-glucoside contents were not significantly different in these extracts.

Table 3 Anti-oxidant activity of shallot extracts

Samples	DPPH, IC_{50} (mg/mL)		%Scavenging (1 mg/mL)	
	Water extract	80% Ethanolic extract	Water extract	80% Ethanolic extract
PB	2.31 ± 0.31	3.29 ± 0.09	28.47 ± 7.98	19.82 ± 5.26
CR	2.65 ± 0.10	3.70 ± 0.21	21.43 ± 4.41	12.44 ± 6.06
NN	3.05 ± 0.27	5.04 ± 0.21	19.99 ± 5.57	11.24 ± 4.52

Samples	DPPH, IC_{50} (mg/mL)		%Scavenging (1 mg/mL)	
	Water extract	80% Ethanolic extract	Water extract	80% Ethanolic extract
UD	3.29 ± 0.30	3.46 ± 0.30	19.12 ± 5.33	18.93 ± 4.11
SK	1.92 ± 0.24*	1.50 ± 0.05*	29.00 ± 10.37	36.60 ± 4.72
NR	2.40 ± 0.16	3.16 ± 0.35	24.97 ± 6.67	31.04 ± 1.38
NP	1.95 ± 0.13	3.25 ± 0.27	30.62 ± 3.70	18.38 ± 4.52
BR	2.15 ± 0.17	3.47 ± 0.20	28.15 ± 5.29	16.28 ± 7.41
RE	2.46 ± 0.10	3.79 ± 0.19	27.59 ± 6.45	15.33 ± 6.64
YT	3.16 ± 0.18	3.65 ± 0.18	19.97 ± 4.50	15.28 ± 4.03
NT	2.57 ± 0.36	4.10 ± 1.34	27.11 ± 7.24	20.95 ± 5.83
RB	2.75 ± 0.11	3.47 ± 0.66	21.54 ± 1.16	15.05 ± 3.61
KB	3.03 ± 0.19	3.74 ± 1.00	23.84 ± 0.63	21.20 ± 6.46
PT1	4.48 ± 0.28*	3.27 ± 0.32	18.90 ± 1.52	16.93 ± 6.18
PT2	3.08 ± 0.10	5.93 ± 0.20*	17.14 ± 1.19	13.28 ± 2.94
Quercetin	3.99 ± 0.09 µg/mL		85% (0.1 mg/mL)	

*p-value < 0.05 between groups

Quercetin and it glucosides are one of polyphenolic compounds which affect antioxidant capacity to a greater extent [21]. The structure features and nature substitutions on rings of quercetin glucosides determined the active anti-oxidant capacity of these compounds. In this study only quercetin content in 80%ethanolic extract showed significantly correlated with the anti-oxidant activity (IC_{50}, DPPH) by Pearsons' correlation (p-value < 0.05). These results were consistent with a previous study of flavonoid aglycone and glycosides that the glycosylation of blocking 3-OH in C-ring of flavonoids could reduce anti-oxidative activity of this compound [22]. The similar findings were also found in *Allium cepa* that treatment with low temperature and high pressure could increase the extractability of quercetin-3,4′-diglucosides, quercetin and quercetin-4′-glucoside in onion and maintained antioxidant activity compared with untreated onions [15]. Another study reported that fermented yellow onion (*Allium cepa*) increased quercetin content which showed strong anti-oxidative effect in the DPPH assay [23]. In conclusions, the quality of shallots in Thailand was examined and the results were within the criteria of pharmacognostic specification except for total ash as summarized in Table 4. Flavonol glucosides were identified as one of the chemical markers in shallot extracts especially quercetin and quercetin-4′-glucoside. In this study quercetin content of 80%ethanolic extracts showed significant relationship with anti-oxidant activity in the DPPH assay.

Table 4 Quality of shallot bulbs

Areas	Foreign matter	Total ash	Acid insoluble ash	Alcohol soluble extractive	Water soluble extractive	Loss on drying
North	0.68-0.80	6.47-8.07	0.28-0.65	6.27-9.20	73.60-85.67	5.67-8.61
North eastern	0.60-0.78	6.55-9.15	0.32-0.57	6.47-9.07	75.00-86.60	5.57-8.60
Central	0.50-0.74	7.06-8.92	0.30-0.60	7.70-10.50	79.40-90.17	6.37-8.29
Average values	0.68 ± 0.08	7.77 ± 0.89	0.45 ± 0.11	7.93 ± 1.28	82.35 ± 4.48	7.01 ± 0.99
Criteria[1]	NMT 2.0%	NMT 1.0%	-	-	-	NMT 87.0% (Azeotropic)

[1] Thai Herbal Pharmacopoeia 2016

Acknowledgements

The authors would like to express their sincere thanks to Agricultural Research Development Agency (Public Organization) funding this project (Grant number: CRP5905020030) as well as Faculty of Pharmacy, Rangsit University for provision of laboratory facilities and equipment. The authors appreciate Assoc. Prof. Thatree Padungcharoen for her advice in this study.

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Nutritional Effects of Long-Lub-Lae Durian (*Durio zibethinus*) on Improvement of Learning and Memory in Aged Male Rats

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Abstract

Long-Lub-Lae durian (LD) is one of the most famous fruit varieties of Uttaradit province. Since several studies indicates that durian polyphenols have neuroprotective effects, thus, this study aimed to investigate a) nutrients and antioxidant activity of LD and b) the cognitive improving effects of LD in aged male rats. The rats were divided into 5 groups comprising: 1) vehicle group treated with reverse osmosis (RO) water, 2) group fed with 0.5 g/kg LD, 3) group fed with 1 g/kg LD, 4) group fed with 2.0 g/kg LD and 5) group fed with vitamin E 80 IU/kg bodyweight. The cognitive function tests consisted of 1) radial arm maze (RAM) test, 2) Morris's water maze (MWM) test, and 3) novel object recognition test (NOR) for assessing the working memory, spatial memory and the object recognition, respectively. For analysis of fresh ripe durian (100 g), there were 23.0±0.46 g sugar, 3.37±0.0 g protein, and 5.51±0.01 g fat. For the analysis of antioxidants, there were 10.15 ± 0.35 µg alpha-carotene, 182.55 ± 5.16 µg beta-carotene, 129.1 ± 5.54 mg (eq GA) total polyphenol, and 28.8 ± 0.11 mgAA total antioxidant activity. The behavioral results showed that aged rats treated with all doses of LD once a day for 21 consecutive days exhibited significant improvements of their cognition functions as indicated by higher spatial and recognition indexes when compared with control aged group. Therefore, it is possible that improvement of cognitive functions observed in this study may result from antioxidant activities of LD.

Keywords: Long-Lub-Lae durian, nutrients, learning and memory, aging

Introduction

Durian (*Durio zibethinus* Murray) is one of the exotic tropical fruits in South East Asia countries, particularly Malaysia, Indonesia, The Philippines and Thailand. It has been reported that durian has nutritional and health value especially antioxidant and anti-inflammatory properties [1]. Bioactive compounds such as beta carotene, lutein, and total phenolic content have been reported in four varieties of durian [2]. Besides high antioxidant activity, durian fruit consumption has also been reported to decrease the incidence of several diseases such as metabolic syndromes, hyperlipidemia, hyperglycemia, inflammation and oxidative stress [3-5]. For cognitive function, natural polyphenolic compounds including flavonoids has been shown to exert neuroprotective effects in the reduction of neuronal death as a result, enhancing cognitive ability [6-8]. So far, most of studies have focused on nutrients and bioactive compounds in the two popular cultivars; Mon-thong and Cha-nee and neuroprotective effects of durian in general has not yet been investigated. Long-Lub-Lae durian (LD) is one of the most famous traditional native fruits of Uttaradit province that its demand increase dramatically due to its unique taste. Hence, it would be valuable to explore the nutrient values and the health benefits of this Long-lub-lae especially in improvement of cognitive functions. Therefore, this study was aimed to investigate nutrient values and the health effect of LD on improvement of learning and memory abilities in naturally aging male rats through the Morris water maze (MWM), the Radial arm maze (RAM), and the Novel object recognition (NOR) tests.

Methodology

Preparation of durian solution for administration to the rats

The ripened Long-Lub-Lae durians were collected from the durian orchard in Tambon Huadong, Lublae district of Uttaradit province (post-harvesting period was June – August, 2016). The edible part was cut into pieces by plastic knife, and then divided into small portions and kept frozen at -20 ^{o}C until use for administration to the animals. Durian 100% w/v, was prepared freshly each day by weighting the frozen ripe durian 30 g and blended with distilled water 30 ml. This durian solution was then

administered orally to all animals in different groups daily for 21 consecutive days.

Nutrients analysis of ripe durian

Durian flesh (10 g) was blended with 60% methanol (50 ml) and centrifuged at 10000 rpm, 4 ^{O}C for 10 min. Supernatant was then collected and filtered through 0.45 µm nylon membrane before injecting into the high-performance liquid chromatography (HPLC) system with UV detector for ascorbic acid, gallic acid, caffeic acid, quercetin and catechin analyses. In addition, HPLC with fluorescence detector was used to analyze amino acids content such as aspartate, glutamate, serine, glutamine, glycine and gamma aminobutyric acid (GABA).

The frozen durian was also sent to the Institute of Nutrition Laboratory, Mahidol University which conformed to ISO 17025:2005, the international standard for laboratory quality systems to analyze its nutrients and antioxidant activity. Content of protein, total fat and total sugar as well as analyses of total antioxidant activity (DPPH), total polyphenol and carotenoid profile were analyses by using standard official method of analysis (AOAC 2012).

Animal experimentation

The experiment was carried out at the Naresuan University Center for Animal Research (NUCAR) which has received full accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. Total of 50 retired Sprague Dawley rats (aged 8 months) were purchased from National Laboratory Animal Center, Salaya, Nakorn Pathom. They were housed 2-3 per cage, maintained in a 12:12 (light:dark) cycle, light on at 23.00 – 11.00 and off at 11.00 – 23.00 (reverse dark-light cycle) and given a standard pellet diet and water ad libitum. All experimental procedures conformed to the guidelines established by the National Research Council of Thailand and were approved by the Naresuan University Animal Care and Use Committee (NUACUC). Upon arrival, all rats were further raised until they reached 15 months of age. They were then randomly divided into five groups of 8 animals each including vehicle, D 0.5 g, D 1.0 g and D 2.0 g and vitamin E groups. Aged rats in the vehicle control group were administered orally with reverse osmosis (RO) water 1 ml and aged rats in D 0.5 g, D 1.0 g and D 2.0 g groups were administered with the ripened Long-Lub-Lae durian solution (100% w/v) 0.5, 1.0 and 2.0 mg/kg bodyweight (BW) respectively. Aged rats in the vitamin E group were administered with Vitamin E 80 IU/kgBW. All rats were subjected to behavioral testing (RAM and NOR) two times: before substance administration (Day 0) and at the end of substance administration (Day 21).

Measurement of fasting blood glucose (FGB) level

To check whether repeated LD feeding could cause a rise in blood sugar level, measurement of FGB were conducted two times: before start LD feeding (Day 0) and at the end of LD feeding (Day 21). Each rat was subjected to food deprivation for 8-12 hours prior to blood sampling and a blood glucose meter was used to analyze the FBG.

Learning and memory tests

- Radial arm maze (RAM) test

Before and after substance administration, rats were subjected to measure their spatial working memories by using RAM test. The RAM apparatus is made of dark surface plastic and consisted of 8 arms. Each arm has dimensions of 60 cm long X 10 cm wide X 20 cm high and has a food cup at the end. The central platform is the regular octagon and its size defined by each of the eight arms widths. One day before starting the test, rats were reduced to 85% of their ad lib feeding weights to obtain the “drive” to execute this task. Rats were given one habituation session in which they were allowed to explore the maze freely. The test consisted of three phases: the training phase, the delay phase and the test phase. In training phase, only 4 arms were open (such as arms 2, 4, 7 and 8) and the food cup in these 4 arms were baited. This phase started when placing the rat in the central platform of RAM to find food in the open arm and finished when entries into all open arms or 5 minutes. After that the rat was returned to its home cage for 5 minutes (the delay phase) and the RAM apparatus was cleaned by wiping with 70% alcohol in this period. In the testing phase, the baited were switched to the closed arm (such as arms 1, 3, 5 and 6) and all eight arms were open. This phase was finished when rat entered all baited arms or 5 minutes. Data recorded was which arms the rat visited once versus those visited more than once up to the limit that once all baits are obtained the trial ends. Two parameters: working memory errors (WME) and reference memory errors (RME) were recorded from this test. WME is defined as re-entry into the arms and RME are entries into never-baited arms.

- Novel object recognition (NOR) test

Before and after substance administration, rats were subjected to measure their abilities to recognize a novel object in the environment by using NOR test. This test consisted of three phases: the familiarization phase, the delay phase and the test phase. In the familiarization phase, a rat was allowed to freely be exploring the open-field area containing two sample objects (object A and B) for 5 minutes, before returning to their home cage for 5 minutes (the delay phase). In the test phase, the same rat was returned to the open-field arena containing two objects, one was identical to the previous sample and the other (object C) was novel. The time of exploration of the objects in the familiarization phase and the test phase were recorded. The recognition index was calculated by using the formula: recognition index = (time of spent exploring object C / time of spent exploring objects A and C) × 100

- Morris's water maze (MWM) test

The spatial memory was conducted by employing the MWM test, in which the rats learned to swim in the water tank to find a submerged platform guided by external visual cues. The water maze used in this study was a circular pool of 100 cm diameter, 50 cm high, filled with water to a depth of 30 cm. The only escape from the water was a platform, diameter of 20 cm, submerged 1 cm below the surface and located in the only one of the quadrants. The starch was added into the water to make the platform in the pool invisible. There were 4 visual cues (star, circle, triangle and square) on the curtain walls visible from inside the pool. This test had two phases: training and testing phases. In the training phase, rats were trained to escape by swimming to the hidden platform for 3 trials per day for 7 days. In each trial, a rat was released from 3 different quadrants except a target (star) quadrant and allowed to swim freely in the water until the platform was found. The latency (the time taken to climb onto the platform) of each rat was recorded and the cut-off time was 90 sec. After completing each trial, rat was lifted out of the water, dried, and returned to its home cage. In testing phase (probe trial), the platform was removed and rats were allowed to swim to search for it for 90 sec. All movements (including the path course) were recorded by a CCTV camera above the pool that connected directly to the computer. Live images from a CCTV camera were tracked and analyzed by ANY-maze video tracking software. The latency in testing phase was measured in all quadrants and spatial memory index was calculated as followed: spatial memory index = (time of spent in target quadrant / time of spent in non-target quadrant) × 100.

Statistical analysis

The results were processed for statistical analysis using Graphpad prism (Version 7.0). The results are presented as the mean ± SEM. Data of FGB, numbers if errors (WME, RME) from RAM test and, recognition index from NOR test were analyzed using paired *t* tests. Comparisons of spatial memory index of the MWM test were made among the groups with one-way analysis of variance, followed by Dunnett's test. A value of $p < 0.05$ was used to indicate significant differences.

Results

Long-Lub-Lae durian analysis

To our knowledge, this is the first report showing nutrients and bioactive compounds in Long-Lub-Lae durian. The present study showed 100 g of ripe Long-Lub-Lae durian had 10.15 ± 0.35 µg alpha-carotene, 182.55 ± 5.16 µg beta-carotene, 129.1 ± 5.54 mg (eq GA) total polyphenol, and 28.8 ± 0.11 mgAA total antioxidant activity. In 100 g of LD also had 5.5 ± 0.01 g fat, 23.0 ± 0.46 g sugar, and 3.37 ± 0.0 g protein. Furthermore, ascorbic acid, gallic acid, and catechin concentrations in 200 mg LD were 83.35 ± 0.16 µg, 7.77 ± 0.66 µg, 18.02 ± 0.74 µg, respectively.

For amino acids profile (Fig. 1), there were 418.95 ± 8.83 mg aspartate, 1242.34 ± 40.38 mg glutamate, 156.17 ± 4.11 mg serine, 794.84 ± 20.66 mg glutamine, 85.13 ± 1.74 mg glycine, and 508.98 ± 40.02 mg GABA in ripe Long-Lub-Lae durian (100 g).

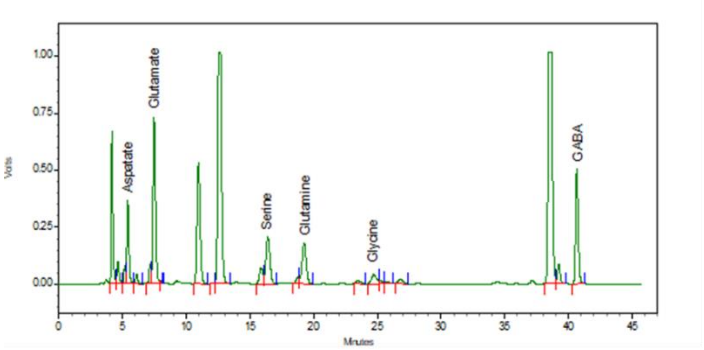

Figure 1 HPLC chromatogram showing amino acids profile of the edible part of Long-Lub-Lae durian.

Level of fasting blood glucose (FGB)

Basal levels of FGB in 3 groups of aged rats were 99.60 ± 3.64, 96.00 ± 9.00 and 90.50 ± 2.59 mg/dl, respectively. Following 21 days of LD oral administration, FGB in the same rats measured 94.67 ± 2.14, 98 ± 4.00 and 93.00 ± 1.97 mg/dl, respectively. No significant difference was found in FBG levels of rats fed with LD (D 0.5 g, D 1.0 g and D 2.0 g) when compared to the basal levels before LD administration (paired *t* test $p > 0.05$) (Fig. 2).

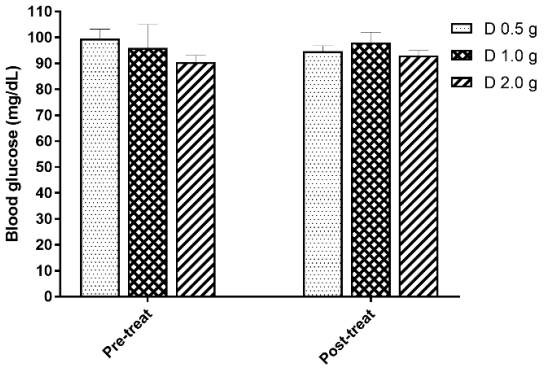

Figure 2 shows the average level of glucose in serum of aged rats during pre- and post- 21 days of durian administration.

Learning and memory tests

- Radial arm maze (RAM) test

The RAM is one of the standard apparatuses used to assess spatial memory. Fig. 3 shows the set up and the use of software in analyzing rat's foraging behavior in this study. The total number of working memory errors (WME), reference memory errors (RME) before administration (pre-treat) and after 3 weeks period of LD administration (post-treat) in aged rats are shown in Fig. 4 (left) and Fig. 4 (right), respectively. The WME of pre-treatment showed no significant difference among groups; vehicle (4.31 ± 0.22), D 0.5 g (4.06 ± 0.35), D 1.0 g (4.11 ± 0.44), D 2.0 g (4.86 ± 0.50) and Vit E (4.22 ± 0.44). For post-treatment, WME trended to be lower and significant differences were found in D 0.5 g (1.86 ± 0.37), D 1.0 g (2.48 ± 0.25), D 2.0 g (2.53 ± 0.43) and Vit E (2.57 ± 0.32).

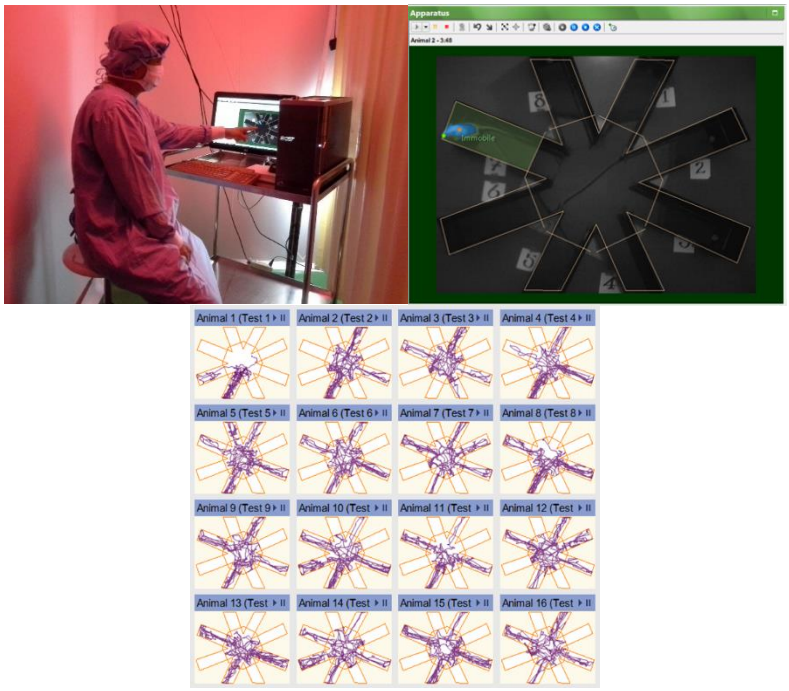

Figure 3 A RAM set up with an experimenter (left), a computer display of a rat searching for food in the RAM apparatus (middle), example of RAM traces of rats in the test phase (right).

Similarly, the RME of pre-treatment in all groups were not different among groups; vehicle (5.15 ± 0.21), D 0.5 g (5.01 ± 0.35), D 1.0 g (4.68 ± 0.39), D 2.0 g (5.47 ± 0.47) and Vit E (5.50 ± 0.44). Following 21 days of administration, all aged rats received LD at all doses as well as vitamin E demonstrated significant decreases in WME (D 0.5 g = 3.46 ± 0.40, D 1.0 g = 3.57 ± 0.27, D 2.0 g = 3.90 ± 0.31 and Vit E = 3.14 ± 0.29) when compared to theirs pre-treat values. The results demonstrated that no significant difference was detected between pre-treat and post-treat of vehicle control group and lesser mean numbers of error were observed for the LD-treated and vit E-treated groups in both parameters as compared to the vehicle-treated group. This data indicates that LD and vitamin E could enhance the spatial working memory performance of aged rats on a RAM which was indicated by the declining trend of the mean numbers of error in RAM tests.

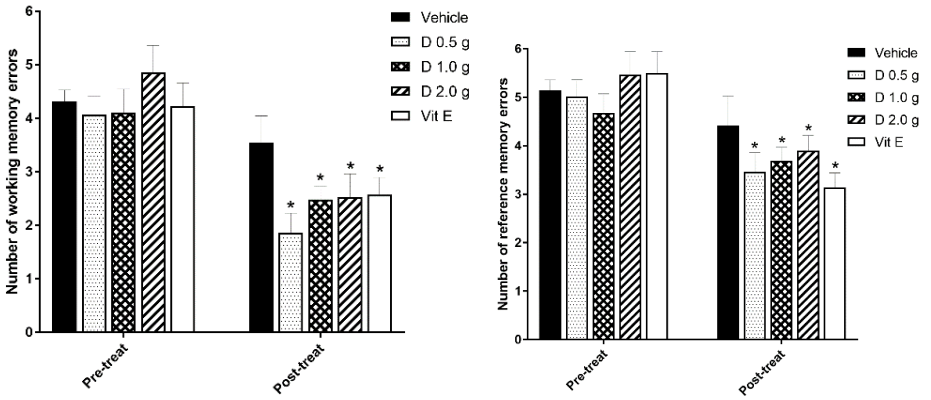

Figure 4 Numbers of WME (left) and RME (right) of the RAM test from 5 groups of aged rats before and after received 21 days of substance administration. (* P< 0.05, paired *t*-tests).

- Novel object recognition test

Memory recognition of each group was determined by using NOR test. Recognition index of aged rats before administration (pre-treat) and after 21 days of substance administration (post-treat) were compared. As shown in Fig. 5, the recognition indexes of pre-treat period were similar among 5 groups (Vehicle = 52.08 ± 3.04, D 0.5 g = 47.65 ± 10.69, D 1.0 g = 52.95 ± 5.07, D 2.0 g = 47.10 ± 8.97 and Vit E = 55.12 ± 3.00). Following 21 days of LD administration, recognition index of post-treat period in vehicle control group (56.74 ± 4.47) showed no significant difference. Nevertheless, aged rats treated with LD at all doses (0.5 g, 1.0 g, and 2.0 g) and vitamin E showed significantly increase in their recognition indexes (D 0.5 g = 76.41 ± 4.32, D 1.0 g = 68.31 ± 4.28, D 2.0 g = 72.89 ± 7.03 and Vit E = 78.29 ± 3.35) (P< 0.05, paired *t*-tests).

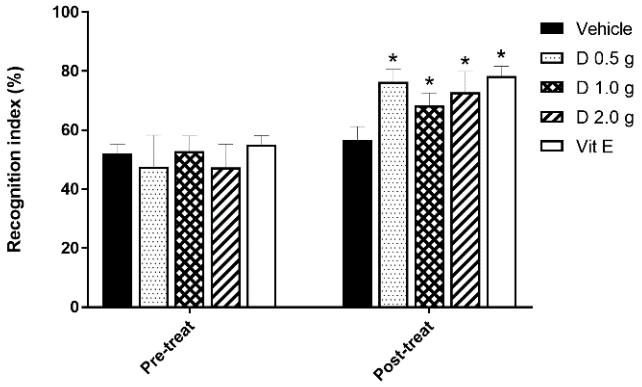

Figure 5 Recognition index from NOR test from 5 groups of aged rats before and after received 21 days of substance administration (* P< 0.05, paired *t*-tests).

- Morris's water maze (MWM) test

The Morris Water Maze (MWM) equipped with a video tracking system (Fig. 6) was utilized to investigate spatial learning and memory in aged rats. After 2-week periods of LD administration, rats were trained to swim to find a hidden platform and their escape latencies were recorded. As shown in Fig. 7 (left), swimming time of all aged rats during the probe trial were measured in each quadrant. On day 1 of training phase, there was no difference between the aged treated and control groups in latency and path length (data not shown) indicating that all the groups had similar motor and visual capabilities. The test phase (probe trail) results on the last day showed that the number of times the rats traveled into the star quadrant, where the hidden platform was previously placed, was significantly greater with LD administration compared to control (Fig. 7 left).

The spatial memory index (Fig. 7 right) of all doses of LD (0.5 g, 1.0 g and 2.0 g) were 42.76 ± 3.09, 43.6 ± 5.20 and 52.99 ± 6.93, which were significant higher compared to vehicle group (23.15 ± 1.75) (P < 0.05, one-way ANOVA with Dunnett's post-hoc test). For aged rats received vitamin E, there was only a slight increase in spatial memory index (34.17 ± 1.35). These data indicate that LD administration significantly improves the memory deficits seen in normal aged rats.

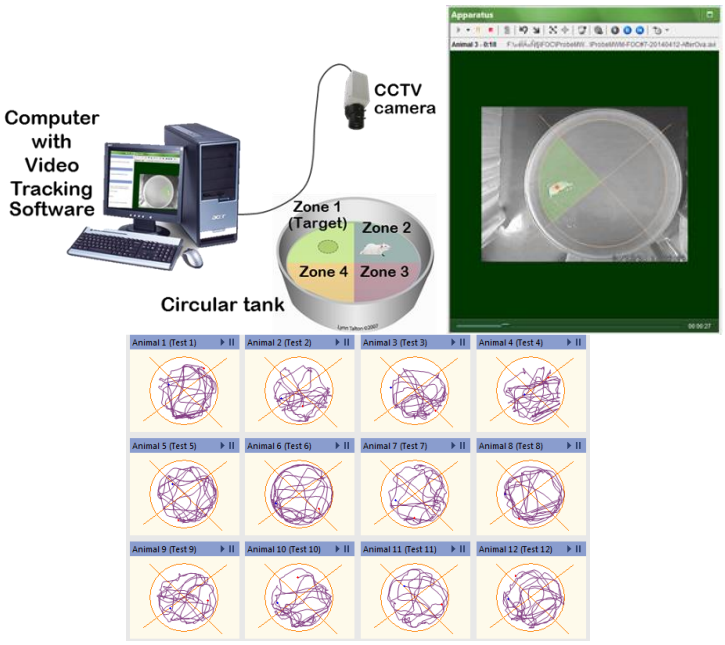

Figure 6 A diagram of MWM set up (left), a computer display of a rat swimming in the pool to find submerged platform (middle), examples of swimming traces of rats in the training phase (right).

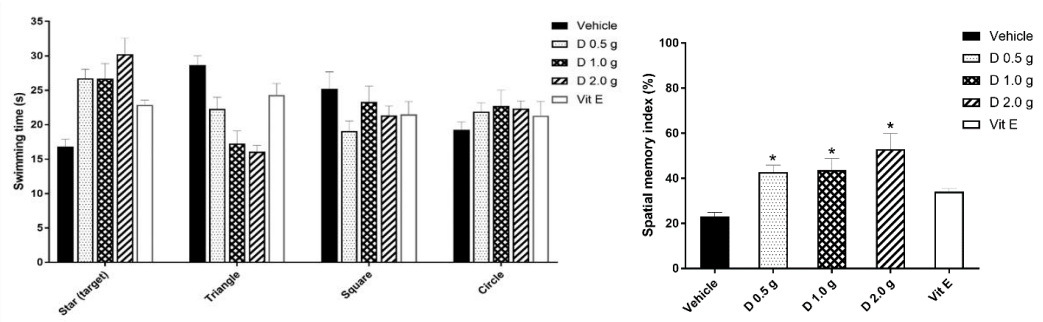

Figure 7 Spatial memory index from MWM test from 5 groups of aged rats before and after received substance administration (* P< 0.05, one-way ANOVA with Dunnett's post-hoc test).

Discussion

Previous study reported the antioxidant activity of Mon- thong cultivar was 260. 8 ± 20. 2 μM trolox equivalent/ 100 g fresh weight determined by ferric-reducing/ antioxidant power (FRAP), 1,075. 6 ± 81. 4 μM trolox equivalent/ 100 g fresh weight determined by cupric reducing antioxidant capacity (CUPRAC) and 2,352. 7 ± 124. 2 μM trolox equivalent/ 100 g fresh weight [10], however, there was no values of antioxidant activity obtained from DPPH assay as employed in this study. According to recent study investigated in other durian varieties, Charoenkiatkul et al, (2016) [11] reported fat and total sugars in Mon-thong, Cha-nee and Kra-dum were 10.1 ± 1.6 g & 48.3 ± 11.0 g, 14.0 ± 2.5 g & 51.7 ± 3.3 g, and 11.2 ± 0.4 g & 47.9 ± 2.9 g, respectively. Accordingly, LD trends to have lower amounts of fat and total sugars. When compared to those found in Mon- Thong, beta-carotene and antioxidant activity (DPPH) in LD were relatively higher (117 ± 30 μg and 8 ± 1 μg, respectively) but amount of alpha- carotene in LD was almost the same average as found in Mon-thong (13±5 μg). However, lutein in Monthong, Chanee, and Kra- dum was markedly higher (136±48 μg, 129±33 μg, and 130±66 μg, respectively) than LD [11]. The amount of beta carotene observed in the present study also higher than reported in both ripe Cha-nee (95.8 μg) and ripe Mon-thong (41.4 μg) [12]. The data in this study is also supported by the report of Arancibia-Avila showing that in ripe stage had the most amount of total polyphenol, flavonoids, anthocyanins and flavonols as compared to other stages [1]. Since amino acid composition has been previously reported only in durian seed gum [13], this is the first study showing that edible part of LD also contains high level of amino acids such as glutamate and GABA which may benefit a wide range of brain functions.

The FBG level in normal rats was around 90 mg/dl (range of 70 to 110 mg/dl) [14], the values obtained from this study both pre and post LD administrations were considered to be in the normal range. This result indicate that repeated consumption of LD did not affect blood glucose level. And the results from learning and memory tests showed that consuming Long-Lub-Lae durian at the doses of 0.5, 1.0 and 2.0 g/kg BW led to improve cognition, specifically working memory, object recognition and spatial memory in aged rats by using RAM, NOR and MWM test, respectively.

Conclusion

Overall, the results from this present study showed that Long-Lub-Lae durian had several important bioactive compounds and consuming Long-Lub-Lae durian improve learning and memory functions. However, the mechanism of Long-Lub-Lae durian in this effect is still unknown. The beneficial effects of LD on memory improvements are most likely due to the ability of its constituents through antioxidant activity mechanisms since natural polyphenolic compounds including flavonoids has been shown to exert neuroprotective effects in the reduction of neuronal death as a result, enhancing cognitive ability [6-9]. Thus, it is likely that the cognitive enhancing effect of Long-Lub-Lae durian may cause by antioxidant property of its bioactive compounds such as polyphenols, flavonoid and beta-carotene as well as amino acids component.

Acknowledgements

We would like to thank Ms. Supapan Eak-u-ranpun for her technical assistance in HPLC analysis. This study was financially supported by Thailand Research Fund *(*TRF*)*.

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Antioxidant Activity of Anthocyanin Extracts from Six Varieties of Local Colour Rice in Loei Province

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Abstract

The aim of the present study was to investigate the antioxidant activity of anthocyanin extracts from six varieties of local colour rice in Loei province by spectrophotometric technique. Khao Kam, Khao Kam Jaow, Khao Kam Poon, Khao Neaw Kam, Khao Jaow Dum and Khao Dum Leum Pua were used in experiment. 1 % of hydrochloric acid diluted in methanol is generally used to extract anthocyanins. The total anthocyanin in six varieties of local colour rice was determined by spectrophotometric method. We found that, Khao Kam Poon, Khao Jaow Dum and Khao Dum Leum Pua show the higher total anthocyanin content than other local Loei colour rice. The anthocyanin extracts from Khao Dum Leum Pua and Khao Kam Poon displayed high antioxidant activity with IC_{50} values of 48.16±1.62 µg/ml and 49.80±1.50 µg/ml, respectively, determine by DPPH assay. The extracts of Khao Kam Jaow showed moderate antioxidant activity whereas Khao Jaow Dum, Khao Neaw Kam and Khao Kam gave low antioxidant activity. In ABTS assay, the highest antioxidant activity from anthocyanin extract found in Khao Kam Poon with IC_{50} values of 6.05±0.36 µg/ml. In addition, other varieties of colour rice showed moderate antioxidant activity when determine by ABTS assay. The advantage of ABTS assay was that samples reacted rapidly with ABTS in the aqueous solution reaching a steady state within 30 min.

Keywords: Antioxidant activity, Anthocyanin, Local colour rice

Introduction

In normal condition, human body has a redox system trying to keep human life to be at a healthy balance. Free radicals are necessary for the living state of the cells and organisms. Some free radicals such as nitric oxide, superoxide radical anion and related reactive oxygen species (ROS) and or reactive nitrogen species (RNS) mediate cells in signalling processes [1]. However the redox homeostasis could be off balance. And an oxidative stress occurs. Oxidative stress is an imbalanced state where excessive quantities of ROS/RNS overcome endogenous antioxidant capacity, leading to oxidation of enzymes, proteins, DNA and lipids. Oxidative stress is important in the development of chronic degenerative diseases including coronary heart disease, cancer and aging [2]. Antioxidants may be defined as being compounds that can delay, inhibit or prevent the oxidation of oxidizable materials by scavenging free radicals and diminishing oxidative stress. The antioxidant properties of different plant extracts, food and beverages can be evaluated using various in vitro assays [3].

Anthocyanins are generally accepted as the largest and most important group of water soluble pigments in nature. They are responsible for the blue, purple, red and orange colors of many fruits and vegetables. The diversity of anthocyanins are due to the number and position of hydroxyl and methoxy groups on the basic anthocyanidin skeleton, the identity, number, and positions at which sugars are attached, and the extent of sugar acylation and the identity of the acylating agent [4]. Anthocyanin-rich foods are becoming more popular [5]. Epidemiologic studies suggest that the consumption of anthocyanins lowers the risk of cardiovascular disease, diabetes, arthritis and cancer, due to their anti-inflammatory and antioxidant properties [5].

Colour rice is a type of the rice species Oryza sativa L. which is glutinous, packed with high level of nutrients and mainly cultivated in Northeast Thailand. The pericarp (outer part) of kernel of this rice colour is black, purple and red due to a pigment known as anthocyanin. A commonly found anthocyanin in colored rice is acelylated procyanidins, which is reported to possess an antioxidant properties [6]. Six varieties of local colour rice, Khao Kam, Khao Kam Jaow, Khao Kam Poon, Khao Neaw Kam, Khao Jaow Dum and Khao Dum Leum Pua were cultiveted in Loei province of Northeast Thailand. Many reports have been written about the antioxidant activity and anthocyanin content of other various colour rice. But not included these rice.

Therefore, the aim of this article was to examine the anthocyanin contents present in six varieties of local colour rice, their antioxidant activity and their potential beneficial effects on health.

Methodology

Materials

Six varieties of local colour rice exhibit in Table 1. Khao Kam, Khao Kam Jaow, Khao Kam Poon, Khao Neaw Kam, Khao Jaow Dum and Khao Dum Leum Pua were collected during November-December 2015, from Loei Province, Thailand. Chemicals and most of the pure standards of anthocynanin were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA)

Table 1: Six varieties of local colour rice in Loei province

Rice Sample	Colour of Kernel
Khao Kam	Purple-Red
Khao Kam Jaow	Black-Red
Khao Kam Poon	Purple-Black
Khao Neaw Kam	Purple
Khao Jaow Dum	Black
Khao Dum Leum Pua	Black-Red

Sample Preparation

Ten grams of air-dried ground six varieties of local colour rice were macerated and periodically stirred in 100 mL of 1 % hydrochloric acid in methanol for 6 hours. The suspension was filtered through Whatman No. 4 filter paper and centrifuged at 5,000 rpm for 15 minutes. The supernatant was air-dried to yield an anthocyanin crude extract. The residue was reconstituted in 1 % hydrochloric acid in methanol before testing and the solvent was used as a negative control.

Total Anthocyanin Content

The assessment of total anthocyanin content (TAC) was carried out by the pH differential method according to AOAC as described by [7]. Absorbance was measured at 510 and 700 nm in buffers at pH 1.0 and 4.5. Anthocyanin concentration is expressed as mg cyanidin equivalents/100 g dry sample and calculated using the formula (1) below:

$$\text{TAC (mg/100 g)} = \frac{A \times M.W. \times DF \times 10^3}{\varepsilon \times 1} \quad (1)$$

A = (A510 nm - A700 nm) pH 1.0 - (A510 nm - A700 nm) pH 4.5; M. W. (molecular weight) = 322.70 g/mol; DF = dilution factor; 1 = cuvette pathlength in cm; ε = 35,000 L/mol.cm, molar extinction coefficient for cyanidin. 10^3: factor to convert g to mg. All spectrometric measurements were performed using a UV-160A spectrophotometer.

Liquid Chromatography Analysis

A portion of anthocyanin crude extract obtained as explained above was dissolved in 5 mL 1 % HCl in MeOH, filtered through a 0.45 μm micropore membrane (PTFE, Waters, Milford, MA, USA) before use and was injected into the HPLC-UV-VIS equipment. Qualitative HPLC- UV-VIS analysis of the anthocyanin crude extract was performed using a SHIMADZU system equipped with LC-10AD separation module unit and SCL-10A UV-VIS detector and a 250 × 4.6 mm, 5 μm, 100 Å, ODS C-18 column (GL Sciences, USA), with a linear gradient solvent system of 4 % aqueous formic acid (solvent A) and 100 % acetonitrile (solvent B) as follows: 90 % - 80 % solvent A until 5 min, followed by 80%–70% solvent A over 15 min, then going back to 90% solvent A until 30 min. and finally reconditioning the column with 90% solvent A isocratic for 15 min. The flow rate and the injection volume were 1.5 mL/min and 20 μL, respectively. The compounds were monitored using a wavelength range of 520 nm.

Validation of the HPLC Method

Quantification was done by external standardization, using the respective standard anthocyanins

(cyanidin), at the wavelengths of maximum absorption of the anthocyanins. For the validation of the analytical method based on HPLC factors, linearity, precision, detection limits and accuracy were evaluated following [8]. Stock solution of cyanidin standard was prepared by dissolving one milligram of cyanidin in 1 % hydrochloric acid in methanol (1,000 µg/mL). Several calibration levels were prepared by diluting the stock solutions with 1 % hydrochloric acid in methanol yielding concentrations of 5, 10, 20, 30, 40 and 50 µg/mL. The calibration curves (R^2 > 0.9998) were obtained by plotting peak areas versus concentrations. Limits of detection (LOD) and quantitation (LOQ) were measured for cyanidin and are reported as the concentrations that gave signal-to-noise ratios of cyanidin, from five replicate injections. Accuracy was determined by spiking cyanidin standard 100 µg/mL in ten gram of sample, which was then extracted and assayed as described before. Mean percentage recovery in relation to the theoretically present amounts (% recovery = amount detected × 100/theoretical amount) were used as a measure of accuracy. The relative standard deviation (RSD%) within the measurements was considered as a measure of precision and repeatability. The samples were prepared and analyzed for anthocyanin concentration on the same day (n = 3).

Free Radical Scavenging Capacity by DPPH Assay

The free radical scavenging activity was measured *in vitro* by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay according to the method described earlier [9]. One hundred micro molar of DPPH solution was prepared by dissolving 4 mg of DPPH with 100 ml ethanol. A 200 µL of DPPH solution was mixed with 5 µL of anthocyanin crude extract at various concentrations in 96-well plate. The solution was mixture and incubated in the dark for 10 min at room temperature. Then, the absorbance was taken by a microplate reader at 515 nm. The control was prepared as above without any sample. Cyanidin, gallic acid, quercetin, α-tocopherol and ascorbic acid were used as a positive control. The scavenging activity was estimated based on the percentage of DPPH radical scavenged as the followed equation (2):

$$\text{DPPH radical scavenging activity (\%)} = \frac{(A_c - A_s) \times 100}{A_c} \quad (2)$$

A_c as refer to control absorbance; A_s as refer to sample absorbance

Free Radical Scavenging Capacity by ABTS assay

The 2, 2-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay was based on the reduction of blue/green ABTS radical cations chromophore ($ABTS^{\bullet\cdot}$) generated from the reaction between ABTS and potassium persulfate (K_2S_2O8) by an electron-donating antioxidant. The ABTS assay according to the method described earlier [10]. Briefly, 7.4 mM of ABTS solution was reacted with 2.6 mM of potassium persulfate solution and kept for overnight in the dark. Prior to use in the assay, the ABTS radical cation was diluted with 50 % methanol for an initial absorbance of about 0.70±0.02 at 745 nm. Free radical scavenging activity was assessed by mixing 5 µL of anthocyanin crude extract at various concentrations with 195 µL of ABTS working solution. The solution was mixture and incubated in the dark for 30 min at room temperature. Then the absorbance was taken by microplate reader at 745 nm. Cyanidin, gallic acid, quercetin, α-tocopherol and ascorbic acid were used as a positive control. The control was prepared as above without any sample. The scavenging activity was estimated based on the percentage of ABTS radical scavenged as the followed equation (3):

$$\text{ABTS radical scavenging activity (\%)} = \frac{(A_c - A_s) \times 100}{A_c} \quad (3)$$

A_c as refer to control absorbance; A_s as refer to sample absorbance

Statistical Analysis

Data are expressed as means±standard deviation (SD) from five independent experiments. Tests for differences between vehicle control and sample were carried out using one-way ANOVA with Duncan's post hoc test. The criterion for statistical significance was set at $p < 0.05$.

Results and Discussion

Anthocyanin Content in Six Varieties of Local Colour Rice

The total anthocyanin of six varieties of local colour rice is shown in Table 2. The total anthocyanin content ranged from 12.53 to 31.60 mg /100g DW. For Khao Dum Leum Pua have a highest of extraction yield (7.43±0.07 g/100 g DW) and total anthocyanin content (31.60±0.18 mg/100 g DW) (Table 2). Many studies have reported that black rice is more abundant in anthocyanin and other phenolic compounds compared to that of white rice [11]. These phytochemical compounds usually accumulated in pericarp or bran of rice kernels. These compounds are pigmented-containing related to unique colors such as purple, red or black.

Table 2: Extracted yields, total anthocyanin and cyanidin content in six varieties of local colour rice.

Rice Samples	Extraction Yields	Total Anthocyanin	Cyanidin
	(g/100 g Dry Sample)	(mg/100g Dry Sample)	(mg/100 g Dry Sample)
Khao Kam	5.36 ± 0.11 ^{c}	12.53 ± 0.23^{d}	2.42 ± 0.17^{d}
Khao Kam Jaow	6.79 ± 0.04 ^{b}	18.52 ± 0.02^{c}	1.14 ± 0.07^{d}
Khao Kam Poon	3.96 ± 0.04 ^{d}	22.03 ± 0.03^{b}	6.35 ± 0.23^{b}
Khao Neaw Kam	7.28 ± 0.15 ^{a}	12.65 ± 0.02^{d}	4.09 ± 0.36^{c}
Khao Jaow Dum	5.20 ± 0.07 ^{c}	29.08 ± 0.07^{a}	4.50 ± 0.43^{c}
Khao Dum Leum Pua	7.43 ± 0.07^{a}	31.60 ± 0.18^{a}	14.34 ± 0.93^{a}

Values in the same column followed by a different latter ($^{a-d}$) are significantly different ($P < 0.05$). Each value in table is represented as mean±SD (n = 5).

Anthocyanins in six varieties of local colour rice detected and identified using HPLC with UV-visible detection (Figure 1, Table 2). The three anthocyanins identified in the six varieties of local colour rice (Figure 1) were consited of cyanidin and their derivatives. For Khao Dum Leum Pua have a highest of cyanidin content was 14.34±0.93 mg/100 g DW (Table 2). In previous reported, black and red rice contain 2 main compounds of anthocyanin; cyaniding 3-glucoside (C3G) and peonidin 3-glucoside (P3G), in which C3G comprises approximately 93% of the quantified anthocyanin [12].

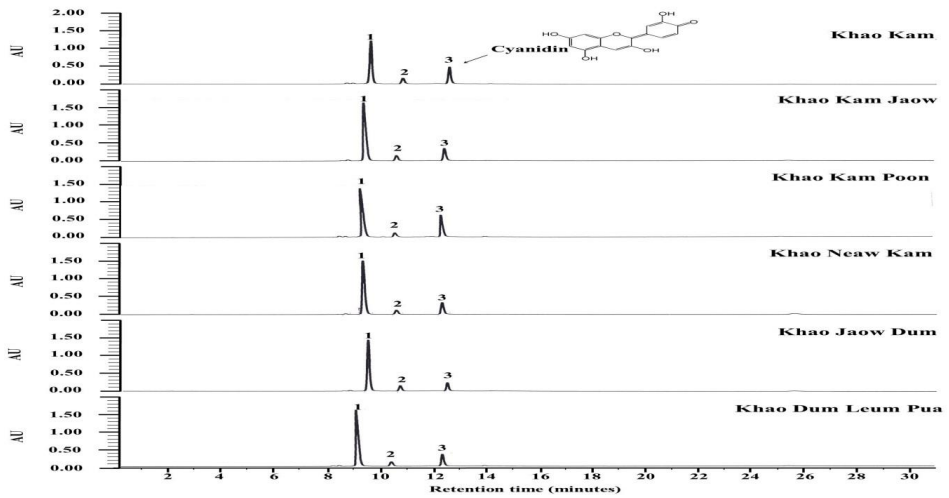

Figure 1: HPLC-UV-VIS chromatograms of six local colour rice in Loei province. (a) Khao Kam, (b) Khao Kam Jaow, (c) Khao Kam Poon, (d) Khao Neaw Kam, (e) Khao Jaow Dum and (f) Khao Dum Leum Pua, monitored at 520 nm. A number 1, 2 as refer to unknown anthocyanin; 3 as refer to cyanidin compound.

The HPLC quantification method showed good performance, baseline was good (Figure 1), and the correlation coefficients for the standard curves of the cyanidin standard anthocyanins was 0.9998 (data not show). The limits of detection for cyanidin were 0.15 μg/mL and the limits of quantification was 0.46 μg/mL (data not show). As seen in Table 3 all recovery results varied from 96.87±0.47 to 99.03±0.76 (data not show) and were within the usually required recovery range of 100% ± 5% [13].

Antioxidant Activity of Six Varieties of Local Colour Rice

Concerning the study of antioxidant effectiveness, the use of different in vitro models has recently been recommended, due to the differences between the various free radical scavenging assay systems [14]. Thus, the determination of the antioxidant activity of the anthocyanin extracts was carried out using the DPPH and ABTS methods. The order of the antioxidant activity measured by the bleaching of the radical DPPH showed in Table 3. The 50 % inhibition concentration or IC50 of anthocyanin extracts from six varieties of local colour rice was Khao Neaw Kam > Khao Kam > Khao Jaow Dum > Khao Kam Jaow > Khao Kam Poon > Khao Dum Leum Pua (Table 3). This result indicate that, the anthocyanin extracts from Khao Dum Leum Pua displayed high antioxidant activity which is also the order found for the sum of the individual major anthocyanins measured by HPLC (Table 2). For DPPH assay, Khao Kam Poon and Khao Dum Leum Pua did not significantly. In ABTS assay, Khao Kam Poon has the lower of IC_{50} (Table 3). This result indicate that, the anthocyanin extracts from Khao Kam Poon displayed a highest antioxidant activity when compared the other rice.

To describe the relationship between anthocyanin content and antioxidant activity, the correlation study was performed and reported as correlation coefficient (r). It showed that positive correlations between total anthocyanin content and IC_{50} from both assay (r=0.53 and r=0.64 for DPPH and ABTS assay, respectively), This result indicate that, anthocyanins play an important role as they are responsible for the antioxidant activity in the six varieties of local colour rice.

Table 3: Antioxidant activity of six varieties of local colour rice.

Samples	Antioxidant activity; IC_{50} (μg/mL)	
	DPPH assay	ABTS assay
Cyanidin	2.57 ± 0.17^{a}	1.67 ± 0.08^{c}
Quercetin	10.09 ± 1.03^{c}	2.31 ± 0.03^{c}
α-Tocopherol	11.96 ± 0.48^{c}	5.76 ± 0.14^{d}
Khao Kam	133.57 ± 6.89^{g}	16.54 ± 0.91^{e}
Khao Kam Jaow	65.27 ± 2.27^{e}	19.44 ± 0.58^{e}
Khao Kam Poon	49.80 ± 1.50^{d}	6.05 ± 0.36^{d}
Khao Neaw Kam	210.30 ± 6.70^{h}	18.86 ± 0.71^{e}
Khao Jaow Dum	111.35 ± 6.63^{f}	42.10 + 2.55^{g}
Khao Dum Leum Pua	48.16 ± 1.62^{d}	23.45 ± 1.06^{f}

Values in the same column followed by a different latter (a-h) are significantly different (P < 0.05). Each value in table is represented as mean ± SD (n = 5).

Conclusions

Six varieties of local colour rice in Loei province containing pigments is one of good sources of antioxidant compounds, including anthocyanin. Cyanidin and Cyanidin 3-glucoside is major anthocyanin compounds in six varieties of local colour rice. As far as antioxidant and their activity are concerned, it was found that colored rice contains more anthocyanin and antioxidant activity. Especially, Khao Dum Leum Pua and Khao Kam Poon displayed a high antioxidant activity in both assay.

Acknowledgement

The author would like to thank the National Research Council of Thailand for supporting the study.

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Amount of Protein, Crude Fiber and β-glucan, and Anti-Oxidative Activity in Edible Wild Mushrooms Used as Food in Ubon Ratchathani Province

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Abstract

This research aim to determine amount of protein, crude fiber, and β-glucan from 20 species of edible wild mushrooms compared with *Lentinus edodes* (Berk.) Sing (Shitake mushroom or Hed Hom), a famous commercial mushroom. In addition, anti-oxidative activity was conducted to determine in 3 species edible wild mushrooms. These mushroom samples were collected during July to August in 2014 from a roadside market in Ubon Ratchathani province. The results of this research found that, the edible wild mushrooms which had the highest amount of protein, crude fiber, and β-glucan was *Amanita vaginata* var. vaginata (Bull. & Fr.) Vitt. (Hed Sai Deun) (25.02±0.08 %w/w), *Russula aeruginea* Lindbl. (Hed Din) (48.00±15.01 %w/w) and *Russula foetens* (Pers.) Fr. (Hed Poong Moo) (43.83±0.28 %w/w), respectively. While, amount of protein, crude fiber and β-glucan in *Lentinus edodes* (Berk.) Sing were 14.77±0.37, 38.00±7.21 and 26.68±1.01, respectively. For anti-oxidative research, DPPH, ABTS, FRAP and FTC assays were conducted to determine this activity and indicated that, *Amauroderma subresinosum* (Murrill) Corner. (Hed Zin) is good free electron scavenger, while *Mycoamaranthus cambodgensis* (Pat.) Trap. (Hed Hum Fran) is good electron donator, and *M. cambodgensis extract* has high anti-lipid peroxidation activity. The results of this research will be information to support consuming of edible wild mushroom and yield the source of β-glucan for processing as nutraceutical products.

Keywords: edible wild mushroom, protein, crude fiber, β-glucan, anti-oxidative activity

Introduction

Mushrooms are organisms which are belonging to Kingdom of fungi. Mushrooms are the reproductive structures of fungi which have a large size and enough to be seen by the naked eye. There are many reports demonstrated that Thai population is consuming mushrooms more and more. Resulted from mushrooms have been considered healthy food because mushrooms contain high amount of protein, vitamins, minerals and fiber but fat is low [1]. In addition, high amount of β-glucan is also found in mushrooms. β-glucan is the substance which had various of biological activities. [2-3]

β-glucan is a polysaccharide compound which composed of glucose sugar linked by glycosidic bond. From a number of researches, found 1-3-β-glucan as the backbone structure and 1-4,1-6-β-glucan as the branch of structure. The type structure of β-glucan was classified into two groups. First group is 1-3/1-6-β-glucan which founded in cell wall of yeasts and mushrooms, and the other is 1-3/1-4-β-glucan founded in oats and barleys [4].

Up to date, there have many biological activities of β-glucan, for example immunomodulating [5-7], anti-tumor and cancer cell [8], anti-lipidemia and anti-diabetes [9-11], anti-inflammation [12] and anti- bacterial [13-14], fungi [15-16], viral [17] and parasitic activities [18]. In addition, anti-oxidative activity of β-glucan was found from mushroom extract [19]. From the biological activities mentioned above, β-glucan has been used as dietary and nutraceutical [20].

Nowadays, the consuming rate of an edible mushroom in Thai population is increased, especially in Northeast area [21]. Most of mushrooms obtained from two sources: cultivated and wild mushrooms. From literature review, less of evidences reported the amount of protein, crude fiber and β-glucan in edible wild mushroom in Ubon Ratchathani province. Therefore, this research aim to determine the amount of these compounds and anti-oxidative activity of edible wild mushroom extracts. The results obtained from this study will be information to support consuming of edible wild mushroom and yield the source of β-glucan as nutraceutical product.

Methodology

1. Materials

- **Sample mushrooms**

The edible wild and commercial mushrooms used in this study were purchased from roadside market and supermarket in Ubon Ratchathani province during July to August in 2014. The species of all mushrooms obtained were identified by comparing with voucher specimen of this mushroom deposited in Pharmaceutical and Pharmacognosy Laboratory, Faculty of Pharmaceutical Sciences, Ubon Ratchathani University.

- **Chemicals**

The mushroom and yeast β-glucan assay kit were supplied by Megazyme Int., Ireland. 2-Thiobarbituric acid (TBA), and 1,1-diphenyl-2-picrylhydrazyl radical (DPPH) were obtained from Sigma-Aldrich (Germany) and linoleic acid from Sigma Chemical Company. Trolox, and TPTZ (2,4,6-tripyridyl-s-triazine) were supplied by Acros (Belgium). Ferric chloride hexahydrate ($FeCl_3.6H_2O$) were purchased from Fluka (Switzerland). 2,2-Azino-bis (3-ethylbenzothialine-6-sulfonic acid) or ABTS was supplied by Wako (Japan). All chemicals and analytical grade solvents were purchased from Merck (Germany).

2. Methods

- **Mushroom preparation and extraction**

The sample mushrooms were rinsed and cut into small pieces and dried by freeze drying. The dried powder mushrooms were kept in an airtight container until use. For extraction, the dried powder mushrooms were mixed with deionized water in 1:1 ratio. The mixture were boiled and stirred at 100 °C for 3 hr then left to cool at room temperature. Filter the mixture and filtrate was collected and dried by freeze drying. The extracts were calculated the % yield and kept in a desiccator until use. [22]

- **Determination amount of protein**

The total nitrogen contents were determined according to Chang, 2010. Briefly, 0.5 g of dried powder mushrooms were digested in a Kjeldahl digestion flask by boiling with 15 mL of concentrated H_2SO_4 and a catalyst until the mixture was clear. The digest was filtered into a 250 mL flask and connected for distillation. Ammonia was distilled from the digest by adding 90 mL of 40% sodium hydroxide and 75 mL of 4% boric acid, 150 mL of the distillate was collected in the flask. The sample solution was then titrated against 0.1 N hydrochloric acid solutions. Bromocresol green and methyl red was used as indicator. Titration was carried out until color changes from green to pink. Results were obtained in triplicate. % Nitrogen is calculated as follows: (14.01 x (Vs-Vb) x N HCL x 100) / (Weight of sample in g x 100). When Vs is volume of hydrochloric acid of sample and Vb is volume of hydrochloric acid of blank.

- **Determination amount of crude fiber**

Weighted out 1 gram of the sample and placed in beaker. Added 1.25% sulfuric acid 25 mL and boiled for 30 min. Filtrated samples with Buchner funnel washed with distilled water and tested acidity by pH paper. Then, placed the samples in beaker again and added 1.25% sodium hydroxide 25 mL and boiled for 30 min. After that, filtrated, washed and tested pH similar to above step. Placed the samples in crucibles and washed with 95 % ethanol 15 mL, removed and evaporated ethanol at 25°C for 15 min. After the final washing, transferred the sample to crucibles and incubated at 110 °C for 2 hr or until weight constant. The weight of sample plus crucible were recorded. The samples were ashed at 500 °C in the oven for 3-4 hr and weight were recorded again. The % crude fiber was calculated as follows: {(B - C) x 100} / A; A = Weight of sample in g, B = weight of crucible and sample before ash in g and C = weight of crucible and sample after ash in g. [23]

- **Determination amount of β-glucan**

The content of total glucans and α-glucan were determined in the polysaccharide extracts using the mushroom and yeast β-glucan assay procedure (Megazyme Int.). The enzyme kit contains exo 1,3-β-glucanase, β-glucosidase, amyloglucosidase, invertase, glucose determination reagent (GOPOD-glucose oxidase, peroxidase, 4-ami-noantipyrine) and glucose standard solution. Briefly, the total glucan content samples were hydrolyzed with ice-cold 60% sulfuric acid (v/v) for 1 hr and for 2 hr at 100°C. After neutralization, polysaccharide hydrolysis was carried out using a mixture of exo-β-(1-3)-D-glucanase plus β-glucosidase in sodium acetate buffer at pH 4.5 for 1 hr at 40°C. Total glucan content was measured after GOPOD reagent was added and absorbance of all solutions was analyzed spectrophotometrically at 510 nm. α-glucan content was measured upon enzymatic hydrolysis with amyloglucosidase plus invertase. The β-glucan content was calculated by subtracting the α-glucan from the total glucan content. All values of β-glucan contents were expressed as g/100g of dried powder mushroom weight.

- Determination anti-oxidative activity of mushroom extracts

Anti-oxidative activities of mushroom extracts were focused on *R. foetens*, *M. cambodgensis*, *A. subresinosum* because they have a high amount of β-glucan compared with other edible wild mushrooms in this study. The DPPH, ABTS, FRAP and FTC assays were used for measuring the anti-oxidative activity.

- DPPH (1,1-diphenyl-2-picrylhydrazyl) free radical scavenging activity assay

DPPH radical scavenging activity was determined. Briefly, the 0.075 mM DPPH radical solution was mixed with various concentrations of trolox (the water-soluble α-tocopherol analogue) and other test solutions to produce the final concentration at 1-100 µg/mL. The mixture was then vortexed vigorously and left for 30 min at 25 °C under protection from light. The absorbance at 517 nm of ethanolic solution of DPPH radical was recorded at different concentrations of the extract. Triplicates were made for each test sample. The lower absorbance of the reaction mixture showed the presence of a higher free radical scavenging activity. The inhibition ratio (%) of the DPPH radical by the test compounds were calculated as follows: % inhibition = [(absorbance of control - absorbance of test compound)/absorbance of control] x 100. The anti-oxidative activity was expressed as the concentration of test compounds inhibiting the formation of DPPH radicals by 50% or IC_{50}. The IC_{50} values were derived from the inhibition curves. [24]

- ABTS (2,2'-azinobis-[3-ethylbenzthiazoline-6-sulfonic acid]) scavenging activity assay

The scavenging activity of ABTS was measured according to the method described by Lo and Cheung (2004) [25] with some modifications. The ABTS (Wako, Japan) assay was employed to measure the anti-oxidative activity of the extracts. ABTS was dissolved in deionized water to 7 mM concentration and potassium persulphate was added to a concentration of 2.45 mM. The reaction mixture remained in the dark and at 25 °C overnight (12 to 16 hr) before used. The resultant intensely colored ABTS radical cation was diluted with deionized water to give an absorbance value of ~0.70±0.02 at 734 nm. The test compounds were diluted 100 times with the $ABTS^{\bullet+}$ solution to a total volume of 2 mL. They were then incubated for 6 min and an absorbance was measured at 734 nm. The absorbance of 1 mg/mL extracts in methanol was prepared and calculated absorbance in the range of the standard curve. The assay was performed at least in triplicates. Controls without $ABTS^{\bullet+}$ were used to allow for any absorbance of the extracts themselves and deionized water was added to these control samples instead. Fresh stocks of $ABTS^{\bullet+}$ solution were prepared every two days due to self-degradation of the radical. The assay was first carried out on trolox which served as the standard. The results of the assay were expressed relative to trolox in terms of TEAC (Trolox Equivalent Antioxidant Capacity).

- Ferric reducing ability power (FRAP) assay

According to previously published methods [26], FRAP was conducted with some modifications. In this assay, three reagents were used: 1) sodium acetate and acetic acid buffer (pH 3.6); 2)10 mM solution of 2,4,6-tripyridyl-s-triazine in a 40 mM solution of hydrochloric acid; and 3) 20 mM solution of ferric chloride hexahydrate, prepared in deionized water. The FRAP reagent was prepared daily with 25 mL of reagent one and 2.5 mL of reagent two and three, which were warmed to 37 °C before used. The various concentrations (50-1000 µg/mL) of standard $FeSO_4$ or test compounds were added to the FRAP reagent. The absorbance was determined at 593 nm for 4 min through the use of a spectrophotometer. The FRAP values were determined from a five point curve using the $FeSO_4$ standard and also represent the concentrations of antioxidants which having a ferric ability equivalent to the standard $FeSO_4$ solution.

- Anti lipid peroxidation by ferric thiocyanate (FTC) assay.

Lipid peroxidation produces a peroxide free radical. The peroxide formed reacts with ferrous chloride, resulting in ferric ions. After that, a ferric ion merges with ammonium thiocyanate to form a ferric thiocyanate that absorbs light at a wavelength of 500 nm. The absorbance values vary with the amount of fee radical generated, so the substance that reduces the amount of peroxide shows the ability of antioxidants. The measured absorbance was calculated as a percentage of lipid peroxidation increased from the first day. The FTC assay was conducted according to the method described by Yassa et al. (2008) [27] with some modifications. Briefly, a mixture of sample was prepared in a screw cupped tube from mushroom extract in ethanol (0.01 µg/µL), 2.5% linoleic acid in absolute ethanol and 0.02 M phosphate buffer pH 7. Incubated in oven at 40 °C for 7 days, then transferred 50 µL of mixture into test tube every 24 hr, then, added 4.85 mL of 75% ethanol and 100 µL of 30% ammonium thiocyanate. Incubated for 3 min

after addition of 100 µL of 0.02 M ferrous chloride in 3.5% HCl and absorbance was measured at 500 nm. The % of increased peroxide was calculated using the following formula: % increased peroxide = [(absorbance at Di-absorbance at D0)/ absorbance at D0] x 100. The same reaction mixture without extract was used as negative control. Trolox was added and used as standard antioxidant for the comparison.

Results and Discussions

1. Amount of protein

Protein contents were determined by Kjeldahl crude protein method based on the measurement the amount of total nitrogen. *L. edodes* had a protein content of 14.77±0.37 %w/w. *A. vaginata* had the highest protein content, while the lowest content of protein was found in *R. foetens* as shown in Table 1. The results of this study showed that the soft texture mushrooms had higher protein content. Srikram and Supapvanich in 2016 [28] reported the protein contents of some edible mushrooms from Sakon Nakhon province were about 18.97±1.29 to 49.20±23.61 %w/w. The overall of protein content obtained from current study is in the same range compared with those report. However, the protein content of the same mushroom is different might have been dependent on climate differences and geographic area.

Table 1: Protein contents in *L. edodes* and some edible wild mushrooms.

Mushrooms (Scientific name)	Local Thai name	Amount of protein (%w/w; Mean±SEM, n=3)
Lentinus edodes (Berk.) Sing.	Hed Hom	14.77±0.37
Amanita vaginata var. vaginata (Bull. & Fr.) Vitt.	Hed Sai Deun	25.02±0.08
Russula virescens Fr.	Hed Klai	20.87±0.07
Russula aeruginea Lindbl.	Hed Din	20.25±0.08
Russula lepida Fr.	Hed Kho	19.53±0.04
Termitomyces globules Heim & Goossens	Hed Chone	18.53±0.29
Russula emetic (Schaeff) Pers.	Hed Nam Mak	17.28±0.03
Russula nigricans (Bull.) Fr.	Hed Tan	17.22±0.16
Russula cyanoxantha (Schaeff) Fr.	Hed Nar Lare	16.51±0.19
Auricularia auricular (Hook.) Underw.	Hed Hoo Noo	15.64±0.21
Cantharellus cibarius Fr.	Hed Mun Poo	13.83±0.08
Amauroderma subresinosum (Murrill) Corner.	Hed Zin	12.42±0.04
Mycoamaranthus cambodgensis (Pat.) Trap.	Hed Hum Fran	8.19±0.08
Russula foetens (Pers.) Fr.	Hed Poong Moo	7.46±0.08

2. Amount of crude fiber

The crude fiber found in *L. edodes* was 38.00±7.21 %w/w. From this current study, the crude fiber contents obtained from the 3 edible wild mushrooms which have the highest content were *R. aeruginea, M. cambodgensis* and *A. subresinosum* (48.00±15.01, 42.67±17.33 and 34.00±1.15 %w/w, respectively). Whereas, the lowest content was found in *T. globules* (7.33±0.67 %w/w). The results are shown in Table 2. The results were different from those result reported by Srikram and Supapvanich in 2016 [28], using the same method in the same mushroom. This may be due to the sample mushrooms used from different sources

.

Table 2: Crude fiber contents in *L. edodes* and some edible wild mushrooms.

Mushrooms (Scientific name)	Local Thai name	Amount of crude fiber (%w/w; Mean±SEM, n=3)
Lentinus edodes (Berk.) Sing.	Hed Hom	38.00±7.21
Russula aeruginea Lindbl.	Hed Din	48.00±15.01
Mycoamaranthus cambodgensis (Pat.) Trap.	Hed Hum Fran	42.67±17.33
Amauroderma subresinosum (Murrill) Corner.	Hed Zin	34.00±1.15
Cantharellus cibarius Fr.	Hed Mun Poo	30.67±0.67
Termitomyces clypeatus Heim	Hed Chone Pluag Jig	30.00±5.09
Boletus colossus Heim.	Hed Peung	23.00±0.01
Amanita hemibapha (Berk. & Broome) Sacc.	Hed Ra York Luang	23.00±0.01
Auricularia auricular (Hook.) Underw.	Hed Hoo Noo	21.33±0.67
Russula cyanoxantha (Schaeff) Fr.	Hed Nar Lare	19.33±1.76
Lentinus squarrosulus Mont.	Hed Khon Kaw	18.89±1.11
Russula foetens (Pers.) Fr.	Hed Poong Moo	16.67±1.76
Boletellus emodensis (Berk.) Sing.	Hed Peung Nok Yoong	13.33±1.92
Amanita vaginata var. vaginata (Bull. & Fr.) Vitt.	Hed Sai Deun	12.11±2.22
Russula virescens Fr.	Hed Klai	12.00±1.15
Russula emetic (Schaeff) Pers.	Hed Nam Mak	10.67±2.40
Russula lepida Fr.	Hed Kho	8.00±2.00
Boletus griseus Frost.	Hed Peung Karm	7.78±1.11
Termitomyces globules Heim & Goossens	Hed Chone	7.33±0.67

3. Amount of β-glucan

The Megazyme Test Kit was used to determine β-glucan content in 20 edible wild mushrooms and *L. edodes*. The principle of this method is using specific enzymes for cutting glucose from total glucan and alpha-glucan branch to single molecules of D-glucose. Then, determine the amount of D-glucose, where D-glucose represents the amount of glucan. Subtract the total glucan content by the amount of alpha-glucan is the amount of beta glucan. Percentages of beta-glucan content per g dry weight of mushrooms (% w/w) are shown in Table 3. The β-glucan content in shiitake mushrooms, which is well-known and popular, was 26.68±1.01 %w/w. *R. foetens*, *M. cambodgensis* and *A. subresinosum* had the highest contents of β-glucan compared to others (43.83±0.28, 41.52±1.47 and 36.61±3.85 %w/w, respectively). The β-glucan content in these 3 edible wild mushrooms has not been reported. However, the beta-glucan content in some edible wild mushrooms used in current study has been determined by Boonyanuphap and Hansawasdi in 2011 [30], for example, *B. colossus* (0.03 %w/w), *R. emetic* (0.10%w/w), *R. cyanoxantha* (0.29 %w/w), *A hemibapha* (0.05 %w/w) and *T. microcarpus* (0.08 %w/w). The β-glucan contents obtained are different, might from the different of sample source and methods used to determine. However, the amount of β-glucan in Shitake mushroom obtained from this study (26.68±1.01 %w/w) is consistency with data of Sari et al. (2017) [29] studied in cultivated Shitake mushroom (25.31 %w/w). Bak et al. (2004) [31] also reported that the variance in β-glucan content dependent on the cultivar. The data are shown in Table 3.

Table 3: β-glucan contents in *L. edodes* and edible wild mushrooms.

Mushrooms (Scientific name)	Local Thai name	Amount of β-glucan (%w/w; Mean±SEM, n=3)
Lentinus edodes (Berk.) Sing.	Hed Hom	26.68±1.01
Russula foetens (Pers.) Fr.	Hed Poong Moo	43.83±0.28
Mycoamaranthus cambodgensis (Pat.) Trap.	Hed Hum Fran	41.52±1.47
Amauroderma subresinosum (Murrill) Corner.	Hed Zin	36.61±3.85
Russula emetic (Schaeff) Pers.	Hed Nam Mak	28.09±2.35
Russula virescens Fr.	Hed Klai	24.76±1.02
Auricularia auricular (Hook.) Underw.	Hed Hoo Noo	24.67±1.59
Lentinus squarrosulus Mont.	Hed Khon Kaw	23.98±0.27
Russula cyanoxantha (Schaeff) Fr.	Hed Nar Lare	23.79±0.55
Russula aeruginea Lindbl.	Hed Din	21.60±1.39
Russula lepida Fr.	Hed Kho	18.27±0.57
Russula nigricans (Bull.) Fr.	Hed Tan	15.87±1.56
Amanita hemibapha (Berk. & Broome) Sacc.	Hed Ra York Luang	14.52±0.07
Cantharellus cibarius Fr.	Hed Mun Poo	13.81±0.15
Termitomyces clypeatus Heim	Hed Chone Pluag Jig	13.03±1.34
Boletellus emodensis (Berk.) Sing.	Hed Peung Nok Yoong	12.52±0.43
Boletus griseus Frost.	Hed Peung Karm	11.76±0.33
Boletus colossus Heim.	Hed Peung	11.03±1.76
Amanita vaginata var. vaginata (Bull. & Fr.) Vitt.	Hed Sai Deun	10.37±0.34
Termitomyces globules Heim & Goossens	Hed Chone	7.45±0.69
Termitomyces microcarpus (Berk. & Broome) R. Heim	Hed Chone Khaw Tog	6.01±0.61

4. Anti-oxidative activity

Study of anti-oxidative activity was focused on *A. subresinosum*, *R. foetens* and *M. cambodgensis*, because they have higher β-glucan contents compared with the other edible wild mushroom in this study. The DPPH, ABTS, FRAP and FTC assay were conducted to evaluate this study.

a. Scavenging activity by DPPH and ABTS assay

In DPPH assay, IC_{50} value was used to determine the anti-oxidative activity and trolox was used as a positive control. The results found that, *A. subresinosum* has the highest anti-oxidative activity (123.57±2.05 µg/mL), followed by *R. foetens* and *M. cambodgensis* (245.50±7.23 and 770.44±28.72 µg/mL, respectively). For ABTS assay, the TEAC value was used to measure anti-oxidative activity and trolox also used as positive control. The high value of TEAC represents the high potent of anti-oxidative activity. *A. subresinosum* has the highest TEAC value (0.50±0.07 mM trolox/mg compound), followed by *R. foetens* and *M. cambodgensis* (0.20±0.04 and 0.07±0.01 mM trolox/mg compound, respectively). The results shown in Table 4, indicated that *A. subresinosum* is the good free electron scavenger. From review literature, IC_{50} and TEAC value of these 3 mushrooms have not been reported, but the IC_{50} from DPPH assay of the other wild mushrooms were examined and found that IC_{50} value have similar range between 100–1,000 µg/mL.

b. FRAP (Ferric Reducing Antioxidant Power) assay

The FRAP value was used to measure anti-oxidative activity. The high FRAP value shows the high anti-oxidative activity. The results of this study found that *M. cambodgensis* had the highest of FRAP value (263.58±63.83 mgFe^{2+}/mg test compound), and 170.17±41.71 and 39.56±9.23 mgFe^{2+}/mg test compound were found in *A. subresinosum* and *R. foetens*, respectively as shown in Table 4. From these result showed that *M. cambodgensis* has the highest of electron donating activity compared with other two mushrooms. The *M.*

cambodgensis has been reported that it has anti-oxidative activity. In addition, The FRAP value of *M. cambodgensis* was expressed as TAC value of 0.44±0.22 millimoles Trolox equivalents per 100 g dry weight. [28] Although, cannot compared the values with this value obtained from the current study. FRAP assay was used to confirm the anti-oxidative activity. However, the other two mushrooms have not been reported.

Table 4: Anti-oxidative activity studied by DPPH and ABTS assay and FRAP assay of *A. subresinosum, R. foetens and M. cambodgensis*, n=3.

Test compounds	DPPH assay IC_{50} (µg/mL, Mean±SEM)	TEAC (mM Trolox/mg compound, Mean±SEM)	FRAP value (mg Fe^{2+}/mg compound, Mean±SEM)
Trolox	4.72±0.08	-	-
A. subresinosum	123.57±2.05	0.50±0.07	170.17±41.71
R. foetens	245.50±7.23	0.20±0.04	39.56±9.23
M. cambodgensis	770.44±28.72	0.07±0.01	263.58±63.83

c. Anti-lipid peroxidative effect by FTC (Ferric thiocyanate) assay

In this study, trolox was used as a standard substance and the control group gave a positive result. The results are shown in percentage of increased free radicals compared to the first day (Day 0). In the control group, the percentage of free radicals increased continuously from day 0 to day 6. But in those with trolox, the percentage of free radicals could be significantly reduced compared with the control group. The decrease in the amount of free radicals means that the extract has anti-peroxidation activity. The addition of *M. cambodgensis, A. subresinosum* and *R. foetens* extracts in those significantly reduced the percentage of free radicals from Day 0 to Day 6 (p value < 0.05). Especially, *M. cambodgensis* extract has anti-lipid peroxidation similar to trolox. The anti-lipid peroxidation of these three mushroom extracts have not been reported. However, the anti-lipid peroxidative effects of other mushrooms, such as *Dictyophora industata*, *Hypsizygus marmoreus*, *Lentinus edodes*, *Pleurotus eryngii*, *Hericium erinaceus*, *Auricularia auricula*, etc., have been reported by Li et al in 2012. The results are shown in Figure 1.

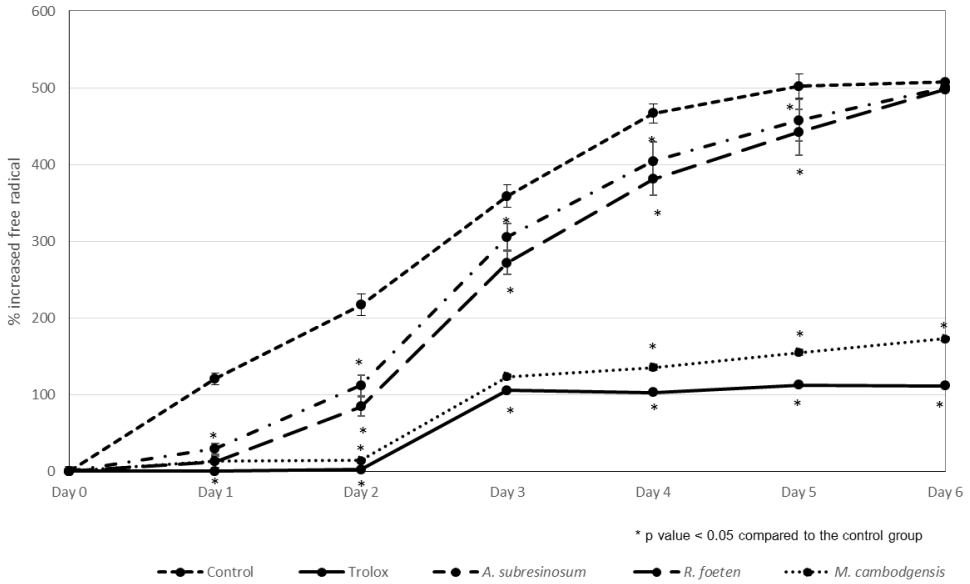

Figure I: Percent of lipid peroxidation increased compared to day 0 when studied with FTC assay, n=3.

Conclusion

The purpose of this research was to determine the types of edible mushrooms in Ubon Ratchathani province with the highest levels of protein, and β-glucan compared to shiitake mushrooms. *A. vaginata, R. delica and R. aeruginea* have protein content with more than 20.00 %w/w. The edible wild mushrooms containing most crude fiber are *R. aeruginea, M. cambodgensis, A. subresinosum, C. cibarius and T. clypeatus* which had fiber content of more than 30 %w/w. The 9 species edible wild mushrooms which had β–glucan contents more than 20.00 %w/w are *R. foetens. M. cambodgensis, A. subresinosum, R. emetic, R. virescens, A. auricular, L. squarrosulus, R. cyanoxantha and R. aeruginea*, respectively.

The anti-oxidative activity study of *A. subresinosum, R. foetens* and *M. cambodgensis* water extract using DPPH, ABTS and FRAP, found that *A. subresinosum* is good free electron scavenger, while *M. cambodgensis* is good electron donator. And *M. cambodgensis* extract has high anti-lipid peroxidation activity.

Acknowledgement

This study was supported by a grant from the Maintenance Support Arts and Culture (MSAC) Ubon Ratchathani University. The research facilities were provided by the Faculty of Pharmaceutical Sciences, Ubon Ratchathani University, Thailand.

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Optimization of Ultrasonic Treatment Assisted Acid Hydrolysis of Cassava Pulp for Reducing Sugar Production using Response Surface Methodology

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Abstract

Cassava pulp (CP) is a major biomass from cassava starch manufacturing used as a biomaterial for production of value-added products via fermentation of sugars. Ultrasonic treatment was used to improve the hydrolysis of polysaccharides into monomeric sugars. The effect of ultrasonic treatment on production of reducing sugar from CP was studied. Cassava pulp was mixed with 1% w/v hydrochloric acid (HCl) in the ratio of 1 to 10, and then ultrasonication was applied for 0, 1, 2 and 3 h. Moreover, response surface methodology was used for optimization of reducing sugar production from CP. The effects of ultrasonication time (0, 1.5 and 3 h), temperature (80, 100 and 120°C), HCl concentration (0, 1 and 2%, w/v) and reaction time (15, 30 and 45 min) were studied using Box-Behnken design. The yield of reducing sugar from ultrasonic treatment with 1% (w/v) HCl for 3 h at 50°C was increased in 2.01 folds compared to the treatment without ultrasonication. The highest reducing sugar yield of 0.44 g/g was obtained from the experimental design (ultrasonic treatment for 0.6 h followed by 2% of HCl hydrolysis at 120°C for 45 min), which was 36 folds higher than the control (12.15 mg/g). These results indicated that the model obtained through response surface methodology was suit to predict the optimum hydrolysis condition, and there was a potential of using ultrasonication assisted acid hydrolysis of CP to increase reducing sugar yield.

Keywords: Cassava pulp, Acid hydrolysis, Ultrasonication, Reducing sugar

Introduction

Cassava (*Manihot esculenta* Crantz) pulp (CP) is the solid residues produced in large amounts as a by-product of starch manufacturing. In Thailand, approximately 30 million tons of fresh cassava tubes are harvested in 2012-2015 (Office of Agricultural Economics, Ministry of Agriculture and Cooperatives), and generated about 7% of CP corresponding to 2.1 million tons per year [1]. Normally, cassava pulp is dried, and used as a low value animal feed or fertilizer [1, 2], which has a relatively low value. Therefore, application of CP as a fermentation substrate not only produces the value-added products, but also solves the problem for the cassava starch manufacturing. According to Sriroth et al. [2], an amount of starch and cellulose about 60% (on a dry weight basis) was contained in the CP. Therefore, the important step is hydrolysis into fermentable sugars which can be further fermented to value-added products, such as lactic acid [3], ethanol [4, 5, 6] and hydrogen [7].

Hydrolysis of polysaccharides in cassava pulp has been investigated by several methods such as acid hydrolysis [3, 5, 7] and enzymatic hydrolysis [4, 8]. Acid hydrolysis presents advantages over the enzymatic process are the shorter reaction time [9] and low costs in raw materials [10]. Acid hydrolysis can be accomplished by using strong acids at low temperature (20-40°C) and diluted acids at high temperature (100-250°C). The weak acids hydrolysis was attractive because there was a lower degradation products obtained in process than strong acids [9]. Diluted acid hydrolysis has been carried out in lignocellulosic based biomass such as *Roystonea oleracea* palm leaves [11], eucalyptus chips [12] and palm empty fruit bunches [13], and starch based biomass such as cassava pulp [3, 7], cassava peel [5] and yam (*Dioscorea* spp.) [14]. Diluted acid hydrolysis of cassava pulp was conducted at high temperature (100-180°C) [3, 5, 7, 15]. The need to carry out hydrolysis at relatively high temperature was considered as the prime disadvantage of acid hydrolysis.

In order to overcome the problems associated with the high temperatures necessary when acid is used in hydrolysis of biomaterial, an alternative approach consists in the use of ultrasound technology. The reaction rates and mass transfer are improved by using ultrasound, which can be considered as a source of agitation and heating. The formation of microbubbles was occurred during ultrasonic treatments leading to the cavitation, which involves at the

solid-solvent interface [14]. The application of ultrasonic treatment may significantly increase the conversion of starch materials to glucose due to facilitating the disintegration of starch granules, exposing a larger surface area to hydrolysis [16, 17, 18]. Some researchers have been done of using ultrasonic treatment combined with acid hydrolysis for sugar production from biomaterials such as, *Roystonea oleracea* palm leaves [11] yam (*Dioscorea* spp.) [14] and cassava pulp [18], which were conducted at low temperature (50-80°C). Moreover, the fermentable sugar yield were increased after the combined treatment. Hence, ultrasonic treatment technology has been selected to decrease the operating temperature of dilute acid hydrolysis, and to improve the production of reducing sugar in the present work.

However, each condition is different operated under optimum condition. For this reason, response surface methodology (RSM), is a statistical and mathematical technique, was used for optimizing the processes of a variable, offering a large amount of information for a small number of experiments, and reducing the reaction time [19]. To find the optimal conditions for variables, RSM was employed to evaluate the effects of ultrasonication time, temperature, acid concentration and reaction time on reducing sugar production.

Materials and methods

1 Sample preparation

Cassava pulp (CP) was obtained from Eiamburapa Co., Ltd. in Sakaeo province, Thailand. CP was dried in hot air oven at 50°C for 48 h. Then, it was ground to powder and kept in plastic bag until used. The chemical compositions of CP, given on dry weight basis, were as follows: 7.93% moisture content, 1.65% ash, 1.80% protein, 15.76% cellulose, 7.38% hemicellulose and 1.78% lignin [20, 21].

2 Ultrasonic treatment on reducing sugar production

Experiments were carried out in an ultrasonicator with temperature control. The ultrasonicator has an operating frequency of 40 kHz and a maximum electrical power output of 500 W. The CP was treated with 1% (w/v) of hydrochloric acid at the solid-liquid ratio of 1 to 10 (w/v), and placed in the ultrasonicator filled with water for various times (0, 1, 2 and 3 h) at 50°C. The experiments were done in triplicate. The effect of ultrasonication time on the amount of reducing sugar was evaluated. After ultrasonication, the treated CP was centrifuged at 5000 rpm for 15 min and the supernatants were collected for reducing sugar determination.

3 Box-Behnken design (BBD) for optimization of ultrasonic treatment assisted acid hydrolysis

For enhancement of reducing sugar yield from hydrolysis of cassava pulp, the ultrasonic treatment combined with acid hydrolysis condition were optimized using response surface methodology (RSM). RSM is a mathematical and statistical techniques for designing experiments, developing models, analyzing the effects of variable, and optimizing the process variables. It has advantages in terms of reductions in the number of experiments and reduced time requirements from over all analysis, and has been successfully applied in the field of biotechnology.

In this study, the effects of four variables (ultrasonication time, temperature, HCl concentration and reaction time) on the response (reducing sugar yield) was studied. The levels of the variables were selected based on previous publications of ultrasonic treatment [11, 14, 18] and diluted acid hydrolysis [3, 5, 7, 15]. The Box-Behnken design (BBD) with four factor and three levels (-1, 0 and +1) was used for optimization of ultrasonic treatment combined with acid hydrolysis (Table 1). The experiments were design using Design Expert® 6.0.2 software resulting in 29 experimental runs (Table 2). The CP was treated with hydrochloric acid at the solid-liquid ratio of 1 to 10 (w/v), placed on ultrasonicator and further hydrolyzed in autoclave according to the experimental conditions in Table 2. After hydrolysis, the mixture was centrifuged at 5000 rpm for 15 min and the supernatants were collected for reducing sugar determination. The experiments were carried out in triplicate. The reducing sugar yield was considered as response of operating factors. The four factors were designated as X_1 (ultrasonication time), X_2 (temperature), X_3 (HCl concentration) and X_4 (reaction time). The response was fitted to the following second-order polynomial model (Eq. 1).

$$Y = \beta_0 + \sum_{j=1}^{k} \beta_j X_j + \sum_{j=1}^{k} \beta_{jj} X_j^2 + \sum_{i<j}^{k} \sum_{j=2}^{k} \beta_{ij} X_i X_j \qquad \text{Eq. (1)}$$

where Y is the predicted response variable, β_0, β_j, β_{jj}, and β_{ij} are regression coefficients for intercept, linear, quadratic and interaction terms, respectively, and X_i and X_j are the variables. The response surfaces of the factors were analyzed using Design Expert software Version 6.0.2. The fit of the model was evaluated by the determination of R^2 and adjusted R^2 coefficient. The validation of the model optimum value of selected variables was obtained by solving the regression equation using Design Expert® 6.0.2. The predicted optimum value was confirmed by experiment using the selected optimum values of the four variables.

Table 1 Process variables used in Box-Behnken design with actual factor levels corresponding to code factor levels

Variables	Codes	unit	Actual factor levels at coded factor levels		
			-1	0	1
Ultrasonication time	X_1	hours	0	1.5	3
Temperature	X_2	°C	80	100	120
HCl concentration	X_3	%, w/v	0	1	2
Reaction time	X_4	min	15	30	45

4 Analytical method

After the completion of the experiments, the samples were centrifuged at 5000 rpm for 15 min, and determined by 3, 5-dinitrosalicylic acid (DNS) method using glucose as standard [22]. The samples of 0.25 ml were place in test tube, and 0.5 ml DNS solution were added into the supernatants. The tubes were placed in boiling water for 5 min. Then, the reaction mixtures were cooled to room temperature and mixed with 5 ml distilled water. After that, the reaction mixtures were measured absorbance at OD 540 nm. The concentration of reducing sugar was calculated by substituting the absorbance of the mixture into the equation obtained from standard curve. Reducing sugar yield was expressed as gram of sugar (glucose equivalent) per gram of dry material by the following equation:

$$Reducing\ sugar\ yield = \frac{Amount\ of\ reducing\ sugar\ produced}{Amount\ of\ initial\ substrate} \quad \text{Eq. (2)}$$

5 Statistical analysis

All the experiments were carried out in triplicate. The analysis of variance (ANOVA) for Box-Behnken design was performed using Design Expert software Version 6.0.2. Analysis of variance (ANOVA) for other experiments was done using SPSS Version 17 (statistical analysis system). Values of P<0.05 were considered as a statistical significance.

Results and Discussion

1 Preliminary study of ultrasonic treatment on reducing sugar production

As shown in Figure 1, an increase in ultrasonication time from 0 to 3 h at 50°C increased the yield of reducing sugar from 6.04 to 12.15 mg/g, which were 2.01 folds of reducing sugar yield. This result might be explained that the coarse particles of CP were disintegrated into finer particles after ultrasonic treatment leading to an increase in the surface area of CP particles for hydrolysis and release of starch from the material [16, 23]. This result was agreed by Simanjuntak et al. [18] who found that very evident disrupted surface structure and transforming the surface into porous structure was found in CP after ultrasonic treatment for 3 h leading to increased efficiency of hydrolysis process. Moreover, Lomboy et al. [24] reported that the ultrasonic treatment could degrade the crystalline structure by mechanical shock, resulting in reduction of particle size, increase in surface area and sugar conversion. However, the reducing sugar yield of 12.15 mg/g was low and still far from industrial purposes. To increase the reducing sugar yield, the combination hydrolysis process of cassava pulp was desired. Dilute acid hydrolysis process has been carried out in both of lignocellulosic material [11, 12] and starch based material [3, 5, 7, 14]. Nevertheless, the optimum condition of ultrasonic treatment and acid hydrolysis was different. For this reason, optimization of

ultrasonic treatment combined with acid hydrolysis using response surface methodology was studied.

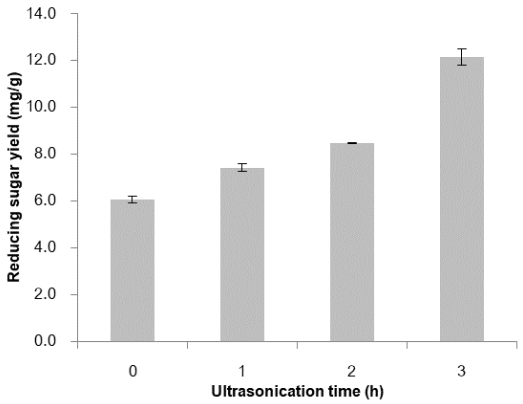

Figure 1 Reducing sugar yield of CP after ultrasonic treatment assisted with 1% (w/v) of hydrochloric acid at 50°C. Values in data bars are mean ± SD (n = 3).

2 Modelling of ultrasonic treatment assisted acid hydrolysis for reducing sugar yield

The optimal conditions of the factors and the effects of their interactions on reducing sugar yield were determined using BBD. The responses at various hydrolysis conditions are presented in Table 2.

Table 2 Process variables used in the Box-Behnken design with coded factor levels corresponding to actual factor levels

Run	Variables								Reducing sugar yield (mg/g CP)
	Actual				Code				
	X_1	X_2	X_3	X_4	X_1	X_2	X_3	X_4	
1	0	80	1	30	-1	-1	0	0	6.42 ± 1.05
2	3	80	1	30	1	-1	0	0	8.90 ± 1.33
3	0	120	1	30	-1	1	0	0	326.73 ± 2.66
4	3	120	1	30	1	1	0	0	320.28 ± 2.80
5	1.5	100	0	15	0	0	-1	-1	8.71 ± 1.13
6	1.5	100	2	15	0	0	1	-1	7.12 ± 0.74
7	1.5	100	0	45	0	0	-1	1	4.66 ± 2.21
8	1.5	100	2	45	0	0	1	1	170.77 ± 13.02
9	0	100	1	15	-1	0	0	-1	3.21 ± 0.40
10	3	100	1	15	1	0	0	-1	6.05 ± 0.58
11	0	100	1	45	-1	0	0	1	87.21 ± 9.22
12	3	100	1	45	1	0	0	1	100.51 ± 10.97
13	1.5	80	0	30	0	-1	-1	0	2.62 ± 0.67
14	1.5	120	0	30	0	1	-1	0	10.95 ± 0.97
15	1.5	80	2	30	0	-1	1	0	20.21 ± 3.40
16	1.5	120	2	30	0	1	1	0	321.60 ± 0.33
17	0	100	0	30	-1	0	-1	0	7.81 ± 0.03
18	3	100	0	30	1	0	-1	0	1.85 ± 0.14
19	0	100	2	30	-1	0	1	0	43.75 ± 0.73
20	3	100	2	30	1	0	1	0	54.75 ± 1.35
21	1.5	80	1	15	0	-1	0	-1	4.00 ± 0.16

Run	Variables								Reducing sugar yield (mg/g CP)
	Actual				Code				
	X_1	X_2	X_3	X_4	X_1	X_2	X_3	X_4	
22	1.5	120	1	15	0	1	0	-1	324.53 ± 1.00
23	1.5	80	1	45	0	-1	0	1	19.92 ± 7.23
24	1.5	120	1	45	0	1	0	1	327.88 ± 1.89
25	1.5	100	1	30	0	0	0	0	25.97 ± 2.71
26	1.5	100	1	30	0	0	0	0	28.69 ± 7.44
27	1.5	100	1	30	0	0	0	0	25.20 ± 5.30
28	1.5	100	1	30	0	0	0	0	21.66 ± 3.71
29	1.5	100	1	30	0	0	0	0	24.84 ± 7.76

X_1: ultrasonication time; X_2: temperature; X_3: HCl concentration; X_4: reaction time

A second-order polynomial model was fitted to the experimental data and constructed using Design Expert software. The quadratic model for reducing sugar yield, in coded factors, was developed by the following equation (Eq. 3).

$$Y = 25.27 + 1.43X_1 + 130.83X_2 + 48.47X_3 + 29.78X_4 + 17.03X_1^2 + 107.85X_2^2 - 22.06X_3^2 + 29.17X_4^2 - 2.23X_1X_2 + 4.24X_1X_3 + 2.62X_1X_4 + 73.27X_2X_3 - 3.14X_2X_4 + 41.92X_3X_4 \quad \text{(Eq. 3)}$$

The main effect of the factor was displayed in coefficient in model equation. As shown in Eq (3), the main effect of 1.43, 130.83, 48.47 and 29.78 was obtained from ultrasonication time, temperature, HCl concentration and reaction time, respectively. Moreover, these effects were positive effect on reducing sugar yield. Furthermore, the magnitude of coefficients of temperature (130.83) was larger than the coefficients of ultrasonication time (1.43), HCl concentration (48.47) and reaction time (29.78) indicating that temperature was the most significant effect on reducing sugar production. As also reported by Zhang et al. [25] who found that temperature showed the surpassing effect comparing to acid concentration and reaction time.

Table 3 shows the ANOVA for the response surface model. The significance of the model is confirmed by low probability values (*P*-value <0.05). The *F*-value (10.44) with a low probability value (*P* <0.0001) demonstrated that the quadratic model of reducing sugar yield was highly significant. The accuracy of the model was checked by the correlation coefficient (R^2). The R^2 value of reducing sugar yield was 0.9126. This result explained that the model is statistically significant and only 8.74% of the total variations is not defined by the regression. The adequate precision of 11.40 was high compared to the desirable value (greater than 4) [26]. For this reason, this model can be used to navigate the design space.

Table 3 The ANOVA for the quadratic model of Box-Behnken design

Source	Sum of squares	Degree of freedom	Coefficients	Mean squares	*F*-value	*P*-value
Model	362150.45	14		25867.89	10.44	< 0.0001*
X_1	24.67	1	1.43	24.67	0.01	0.9219
X_2	205382.21	1	130.83	205382.21	82.92	< 0.0001*
X_3	28189.04	1	48.47	28189.04	11.38	0.0045*
X_4	10640.78	1	29.78	10640.78	4.30	0.0571
X_1^2	1881.29	1	17.03	1881.29	0.76	0.3982
X_2^2	75450.96	1	107.85	75450.96	30.46	< 0.0001*
X_3^2	3155.42	1	-22.06	3155.42	1.27	0.2780
X_4^2	5518.29	1	29.17	5518.29	2.23	0.1577
X_1X_2	19.93	1	-2.23	19.93	0.01	0.9298
X_1X_3	72.00	1	4.24	72.00	0.03	0.8671
X_1X_4	27.37	1	2.62	27.37	0.01	0.9178

Source	Sum of squares	Degree of freedom	Coefficients	Mean squares	*F*-value	*P*-value
X_2X_3	21471.76	1	73.27	21471.76	8.67	0.0107*
X_2X_4	39.54	1	-3.14	39.54	0.02	0.9013
X_3X_4	7030.70	1	41.92	7030.70	2.84	0.1142
Residual	34677.98	14		2477.00		
Cor. Total	396828.43	28				
Intercept			25.27			
R^2 = 0.9126						
Adj-R^2 = 0.8252						
Adequate precision = 11.40						

* Significant at $P<0.05$; X_1 = ultrasonication time; X_2 = temperature; X_3 = HCl concentration; X_4 = reaction time

The optimal value of each factor was studied by point prediction function of Design Expert software version 6.0.2. The fitted response surface plot was generated using equation model (Eq. 3). The maximum reducing sugar yield of 463 mg/g was predicted at the optimum condition of ultrasonic treatment at 0.6 h, hydrolysis temperature at 120°C, HCl concentration of 2% (w/v) and reaction for 45 min.

3 Response surface methodology analysis

The model could be represented in response surfaces to explain the interaction among the four variables and to determine the optimum level of each variable for maximum response of reducing sugar yield. Response surface plot (Figure 2) was generated based on Eq. (3). The interactions of two variables were represented in the three dimensional surface plot, while the other variable was held at the optimum level.

An increase in reducing sugar yield of 2.01 folds was found in the increasing of ultrasonication time from 0 to 3 h at 50°C of CP with 1% HCl concentration as a catalyze (Figure 1). By the way, ultrasonication time was not significant during combined with acid hydrolysis as shown in Table 3 as well as Figure 2. This result might be due to the surpassing effect of temperature (130.83) which was more than ultrasonication time (1.43) leading to low efficiency of ultrasound technology. Even though the effect of ultrasonic treatment in the combined method on reducing sugar production in this study was not significant in the experiment, ultrasonic technology be able to combine with acid hydrolysis in other publications [11, 14, 18]. They performed the ultrasound-assisted acid hydrolysis at the low temperature (<80°C). This result may be concluded that ultrasonic treatment displayed the significant effect during acid hydrolysis at the low temperature (<80°C).

As shown in Table 3 and Figure 2d, HCl concentration and temperature displayed the significant ($P<0.05$) influence on reducing sugar yield. An increase in HCl concentration from 0 to 2% (w/v) during hydrolysis resulted in increasing reducing sugar yield (Figure 2b, 2d and 2f). This result can be explained that more glucose was released from starch and cellulose at high acid concentrations because more H^+ ions led to a higher degradation rate of the lignocellulosic material and starch presenting in the CP [3]. Moreover, an increase in temperature from 80 to 120°C increased the yield of reducing sugar (Figure 2a, 2d and 2e). This result also found by Chavan et al. [27] and Amadi et al. [28] who observed that an increase in temperature resulted in increasing of released glucose from starch based biomaterials.

Confirmatory experiment was conducted to validate the predicted reducing sugar yield with correlate to the optimum condition. The average reducing sugar yield of 440 mg/g CP corresponding to concentration of 44 g/l was obtained. These values are in good agreement with the predicted values considering a range of 95% confidence level. The predicted reducing sugar yield was slightly higher than the observed reducing sugar yield with an error of 4.97%. These values are in good agreement with the predicted values considering a range of 95.03% confidence level. The difference between predicted value and observed value during optimization process was also found by many researchers [25, 29, 30, 31, 32]. In the acid hydrolysis of CP, the optimal condition obtained by difference researchers closely resembles the optimum condition obtained in the present study. A high glucose concentration (100 g/l) was obtained from the hydrolysis with 1 N HCl at 121°C for 15 min [3]. Phowan and Danvirutai [7] obtained the maximum yield of 27.4 g/l of total sugar after hydrolysis

with 0.5% H_2SO_4 for 30 min at 121°C. While, Simanjuntak et al. [18] found the highest reducing sugar of 0.8 g/l at adjusted pH 2.0 with H_2SO_4 for 90 min at 80°C. The varying yield of sugar might be attributed to factors such as hydrolysis conditions like acid concentration, temperature and reaction time.

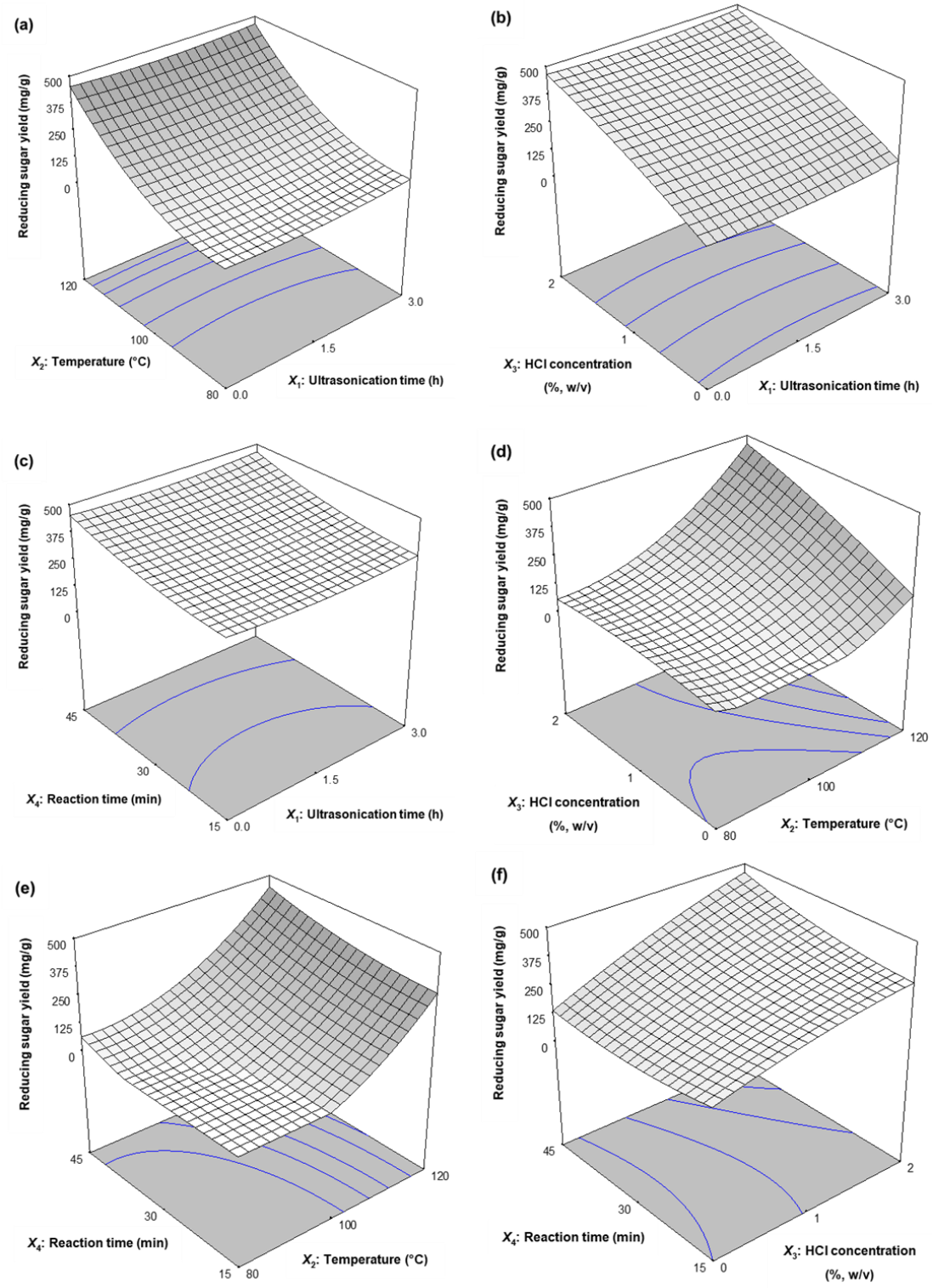

Figure 2 Response surface plot showing interactive effects between **(a)** ultrasonication time and hydrolysis temperature at HCl concentration of 2% (w/v) for 45 min, **(b)** ultrasonication time and HCl concentration at 120°C for 45 min, **(c)** ultrasonication time and reaction time at HCl concentration of 2% (w/v) at 120°C, **(d)** temperature and HCl concentration for 45 min with ultrasonication for 0.6 h, **(e)** temperature and reaction time at HCl concentration of 2% (w/v) with ultrasonication for 0.6 h, and **(f)** HCl concentration and reaction time at 120°C with ultrasonication for 0.6 h on reducing sugar yield (mg/g).

Conclusions

Ultrasonication combined acid hydrolysis of CP is more effective than acid hydrolysis alone for reducing sugar production under the low severe condition (2% of HCl at 50°C), which was increased in 2.01 folds. The effect of ultrasonication was covered by the influence of high temperature under the harsh condition. The highest reducing sugar yield of 440 mg/g was obtained from the experimental design (ultrasonic treatment for 0.6 h followed by 2% of HCl hydrolysis at 120°C for 45 min), which was 36 folds higher than the control (12.15 mg/g).

Acknowledgements

The authors gratefully acknowledge Faculty of Agricultural Technology, Burapha University (Grant No: 001/2559) for financial support, and would like to acknowledge Asst. Prof. Dr. Piaw Phatai from the Department of Chemistry, Faculty of Science, Udon Thani Rajabhat University for his constructive comments.

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The Effect of *Thunbergia laurifolia* Lindl. in Layer Dietary Supplementation on Productive Performance and Egg Quality

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Abstract

The experiment was conducted to study the effect of *Thunbergia laurifolia* Lindl. (Rang Chuet) supplementation in layer diets on the productive performance and egg quality. The ISA brown laying hens were randomly divided into 2 groups of control diet and 2% *T. laurifolia* supplemented diet with 4 replications in each group, 25 hens per replicate. The diets were fed *ad libitum* and continuous period of 8 weeks. The number of eggs per day, egg weight per day and feed were recorded and analysis as productive performance. The egg sample was collected every 2 weeks for evaluation of egg quality. The results demonstrated that dietary supplementation with 2% *T. laurifolia* showed higher egg production than the dietary control overall the experimental period. Nevertheless, *T. laurifolia* supplementation was not significantly different in average egg weight, average feed intake, feed conversion ratio, eggshell and egg quality compared with the control diet. However, the feed conversion ratio in supplemented group was slightly greater than the control group with no significant. In conclusion, supplementation with 2% *T. laurifolia* leaves in layer diet can improve the egg production and had no adverse effect on egg quality.

Keywords: *Thunbergia laurifolia* Lindl., layer diet, productive performance, egg quality

Introduction

Layer chicken (*Gallus gallus domesticus*) is an important livestock in Thailand. The egg quality and productive performance were considered in layer production to supply the egg consumption. Therefore, the feed additive and antibiotic drugs use in disease prevention, treatment and improve the hens production are generally found in layer farm, but residual drug in egg [1-4] may occur and impact to the costumers. In recently, herbs or medicinal plants are an alternative use to improve the egg quality and production in layer [5-8], it solves the drug residual problems. In addition, the medicinal plants can enhance the animal health [9-10] resulting in decreasing of drug use in farm.

. *Thunbergia laurifolia* Lindl., locally known as “Rang Chuet” is a medicinal plant in Thai traditional medicine used as an antidote and detoxifying for several poisonous agents [11-13]. This plant is also used to antipyretic, anti-inflammatory, antioxidant and free radical scavenging activity [14-17]. The leaves and flowers of *T. laurifolia* have been found to consist of phenolic compounds, including apigenin, apigenin glucosides, delphinidin, protocatechuic and chlorogenic acid [18]. The phytochemical studied of *T. laurifolia* leaves were revealed the composition of two novel iridoid glucosides and seven known compounds such as grandifloric acid and apigenin [16]. The water extract of *T. laurifolia* leaves showed the highest total phenolic content (TPC) and free radical scavenging property when compared with ethanol and acetone extracts [18-20]. However, *T. laurifolia* leaves extract by using 50% ethanol showed the protective effect to against lead toxicity in the Nile tilapia [21].

The previous studied of *T. laurifolia* crude powder supplementation in broiler diet indicated that the basal diet supplemented with 2% *T. laurifolia* had no effect on feed intake and growth performance in broiler. Moreover, supplementation with 2% *T. laurifolia* and 2% *T. laurifolia* plus 1% yeast glucomannan can decrease the adverse effect of mycotoxins contamination in feed with increasing antioxidant enzyme activity and improving nutrient digestibility [22]. Thus, this study aimed to investigate the effect of *T. laurifolia* supplementation in layer diet on the productive performance and egg quality.

Methodology

Experimental birds and management

The ISA Brown laying hens at age of 38 weeks were used in this study, 200 hens were randomly divided into 2 groups. There were diet control and diet supplemented with 2% *T. laurifolia* (TL) groups, each group

consists of 4 replicates with 25 hens per replication. The experiment was conducted in an open housing system with the battery cages, nipple and feed trail. The industrial fans were used to improve the housing temperature and ventilation. The cages were reared 2 hens in each, feed and water were given *ad libitum* throughout the experimental period (8 weeks). All hens received a total of 15 hours of light per day. The experimental protocol was approved by the Animal Ethics Committee of Burapha University.

Basal and experimental diet

The commercial feed of the laying hen period was used as basal diet. For preparing the experimental diet, fresh of *T. laurifolia* were obtained in Sakaeo, Thailand. The leaves were washed and dried in oven at 60°C for 24 hours, then grounded using cutting mill. *T. laurifolia* powder was added into the basal diet at 2% proportion. The basal and experimental diets were proximately analyzed by AOAC method [23].

Data recording and sample collection

Hen-day egg production and egg weight were daily recorded, whereas the feed intake was weekly measured and converted to daily values. For evaluation of egg quality, 13 eggs from each replication of both groups were collected every 2 weeks. The yolk color, egg weight, albumen height and Haugh unit were measured using by Egg multi-tester (EMT7300, Robotmation Co. Ltd., Japan). Two points of egg shell in each sample were measured the thickness using a micrometer and the color by using QCR-shell color reflectometer (TSS, York, UK).

Statistical analysis

The data was represented in mean values with standard deviation. The differences of variables between groups were determined by t-test using the SPSS statistic program.

Results

Chemical analysis of diets

In table 1, the chemical analysis results of nutritional values in dietary supplementation with 2% *T. laurifolia* was similar to the dietary control. The gross energy in control and *T. laurifolia* supplemented diets were 3917.6 and 3895.1 kcal/kg, respectively.

Table 1 Nutrient composition of control diet and *T. laurifolia* (TL) supplemented diet by proximate analysis.

Chemical analysis (%)	Control diet	2%TL diet
Crude protein	19.53	19.18
Ether extract	3.86	3.91
Crude fiber	2.90	3.13
Ash	11.25	11.52
Moisture	3.67	2.98
Calcium	3.91	3.81
Phosphorus	0.76	0.73
Gross energy (kcal/kg)	3917.6	3895.1

Egg production and egg weight

During 8 weeks of the experimental period, hen-day egg production of the control group was 83.4-86.9%, whereas the *T. laurifolia* supplemented group was 87.6-92.3%. These results indicated that dietary supplementation with *T. laurifolia* leaves showed higher egg production than the dietary control in every week throughout the experimental period (Figure 1). However, there was no significant difference in average egg weight between groups.

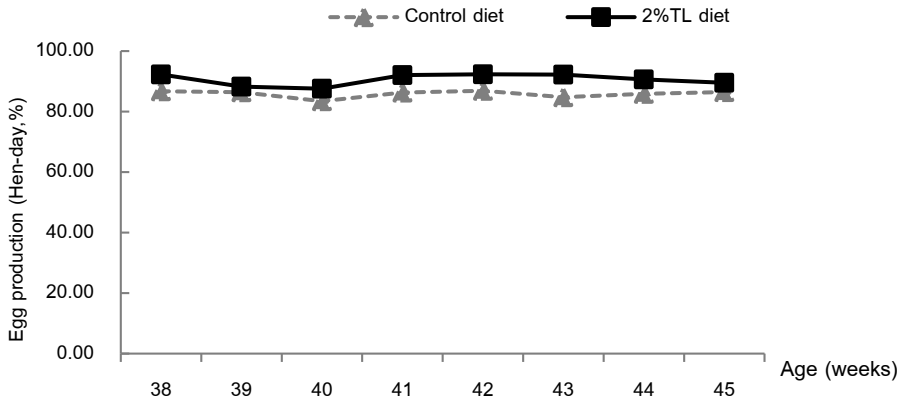

Figure 1 The percentage of hen-day egg production from control diet and *T. laurifolia* (TL) supplemented diet groups.

Feed intake and feed conversion rate

The average feed intake of layer hen in control and *T. laurifolia* supplemented groups was no significant difference. Although, the feed conversion to 1 kilogram of egg weight in 2% *T. laurifolia* supplemented group (1.89±0.20) was better than control group (2.00±0.26) but there was no significant difference (Table 2).

Egg quality

In table 2, dietary addition of 2% *T. laurifolia* had no significant effect on egg shell color, egg shell thickness, yolk color, egg weight, albumen height and Haugh units compared with the dietary control.

Table 2 Effect of *T. laurifolia* (TL) supplementation in diet on productive performance and egg quality of laying hens

Item	Control diet	2%TL diet	P-value
Productive performance			
Egg weight (g)	61.85 ± 1.91	61.42 ± 1.04	0.71
Feed intake (g/hen/day)	105.35 ± 10.62	104.95 ± 9.72	0.94
Feed conversion per 1 kilogram of egg	2.00 ± 0.26	1.89 ± 0.20	0.37
Egg quality			
Shell color	29.93 ± 1.01	29.69 ± 0.71	0.71
Shell thickness (mm)	0.343 ± 0.007	0.349 ± 0.012	0.47
Yolk color	13.80 ± 0.05	13.72 ± 0.11	0.25
Egg weight (g)	56.51 ± 0.97	56.53 ± 0.75	0.98
Albumen height (mm)	6.91 ± 0.28	6.91 ± 0.27	0.98
Haugh units	82.95 ± 2.00	83.34 ± 1.70	0.78

Discussion and conclusion

The chemical values of fiber, protein, fat, carbohydrate and ash in *T. laurifolia* leaves were 16.82, 16.70, 1.68, 46.01 and 8.79%, respectively [24]. The experimental diet in this study was 2% *T. laurifolia* leaves supplemented basal diet. The chemical analysis of experimental diet showed the similar level of nutritional values compared with basal diet, especially the crude protein, crude fiber and gross energy. Thus, there was no effect on the average feed intake and average egg weight between both groups. This result correlated with previous study that supplementation with 2% *T. lauriforia* leaves in the broiler diet had no effect on feed intake and growth performance [22].

T. laurifolia leaves consist of several bioactive compounds such as sterols, phenolics [18-25], carotenoids [25], glycosides [19, 25-26], and unclassified steroids [25]. The plant has been used as a folk medicine for detoxification, anti-inflammation, antioxidant and antipyretic. The aqueous extract from *T. laurifolia* leaves was reported to reduce the inflammatory cells and improve liver function in hamsters treated with liver fluke or administration with N-nitrosodimethylamine [10]. In addition, ethanolic extract of *T. laurifolia* leaves had efficiency to reduce acute and

chronic inflammation [14]. Moreover, the phenolic compounds in *T. laurifolia* leaves had an antioxidant effect and inhibited oxidative stress [20, 25, 27]. According to this medicinal plant efficiency, the egg production in 2% *T. laurifolia* supplemented group was higher than control group throughout the experimental period. Although, *T. laurifolia* supplemented group showed the better in feed conversion ratio than control group, there was no statistical difference. This result might due to the effect of *T. laurifolia* compounds such as phenolics, which improve the animal health and productive performance in layer hens.

The nutritional composition in diet and feed intake generally play an important role to the egg quality. In this study, supplementation with 2% *T. laurifolia* in layer diet had no effect on egg quality. The similar values of dietary nutrition and feed intake in both groups led to the similar results in egg weight, albumen height and Haugh units as well as shell thickness which depends on calcium carbonate in the diet [28]. Layer genetic and housing environment generally affect to shell color [29-31]. There was no different in genetic and environment in this study. The yolk color is dependent on the concentrations of carotenoids and xanthophylls in diet, and hen ability in absorption and utilization. Although, *T. laurifolia* leaves was reported to contain the carotenoids [25], addition of 2% *T. laurifolia* leaves in this study might have not enough pigment resulting in no significant difference in yolk color.

The results of this study demonstrated that 2% *T. laurifolia* leaves can be supplemented in layer diet and improve the egg production without the adverse effect on egg quality.

Acknowledgements

This work was financially supported by the Faculty of Agricultural Technology, Burapha University, Sakaeo Campus, Sakaeo, Thailand.

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Effects of Condensed Tannin Compound from Fresh Neem Foliage *Azadirachta indica* A. Juss. Var. Siamensis Valeton on Rumen Fermentation, and Nematode Eggs in Goat Diets

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Abstract

The objective of this study was to determine the effects of neem (*Azadirachta Indica* A. Juss. Var. Siamensis Valeton) foliage in meat goat diets on rumen fermentation and productive performances. Four crossbred meat goats (Native x Anglo Nubian) of 6 months old with an average body weight (BW) of 21.3 ± 0.8 kg, were given 4 different neem foliage levels over 4 different time periods, using Latin Square Design experimental protocol. Each period was 28 d, including a 21d adjustment period, followed by 7 d measurement period. The isolated condensed tannin (CT) extract from fresh neem foliage was approximately 8.60-9.05% of total fresh neem foliage weight. The diet treatments consisted of control feed (isonitrogenous 16%CP and isocaloric (3.6 Mcal/kg DM); 1%BW) (control), supplemented with 15%, 30% and 45% neem foliage of total feed respectively. All goats received *ad libitum* corn silage and were individually fed each treatment. The results showed that dry matter intake (DMI), BW change, digestibility, rumen pH, ammonia nitrogen, and volatile fatty acids were unaffected by dietary treatments. However, ruminal bacteria populations in goats fed 30% neem foliage gradually increased at 2 and 6 hours after feeding compared with the control group and goats fed 15% neem foliage. Protozoa populations at 2, 4 and 6 hours of goats fed 30% neem foliage were decreased after feeding compared with the control group, while goats fed 15% and 45% neem foliage had no significant change. Gastrointestinal parasite eggs were found in both the control group and goats fed 15% neem foliage, of 90.3% with 78.1% of total infection rate. Worm egg output was reduced by 58.4% and 59.1% in goats receiving 30% and 45% supplemented feeds, respectively. In conclusion, CT extract from neem foliage may have an important beneficial health effect by reducing gastrointestinal parasite load in goats. Therefore, it is suggested that meat goat diets should be supplemented with 30% neem foliage to increase ruminal, decrease protozoa populations and excreted faecal nematodes eggs.

Keywords: *Azadirachta Indica* A. Juss. Var. Siamensis Valeton*, neem foliage, meat goat, rumen fermentation, nematode eggs*

Introduction

Neem (*Azadirachta Indica* A. Juss. Var. Siamensis Valeton*)* is the one well known edible plants of southeastern Asia part of India, Nepal and also in Thailand. The chemical group is called the botanical insecticides, which does not at all relate to their chemistry, but just to the fact that they are plant-derived. In neem, there are various active ingredients, the most important being chemicals called azadirachtins. This product made from neem tree was used as a traditional medicine properties [1] for Anthelmintic, antifungal, antidiabetic, antibacterial, antiviral, contraceptive, and sedative [2]. Neem was reported to have high nutritional value and contain condensed tannins (CT) compound, phenolics and flavonoids [3]. However, little is known about factors regulating its expression and CT activity effects from fresh neem foliage on rumen fermentation and nematode eggs in goats. Parasitic nematodes of the digestive tract in goats have been reported that are a main issue adversely affecting health and they remain a primary constraint to achieving full goat productivity resulting in the subsequent economic losses especially in tropical countries. In the past, Thai farmers are often use of drugs (anthelmintics) to kill the parasites, one of the usual toxic modes employed to control gastrointestinal nematodes (GIN) [3]. However, repeated use of anthelmintics represents a high risk to goats' natural immunity and there is need to find safely alternative solutions to reduce the incidence of chemical drugs resistance and reliance on chemotherapy. There is a requirement for locally forage available, substitutive feed resources which are used for the enhancement of ruminant's production especially in tropical regions. Recent studies revealed that efficient supplementation of local plants mainly comprising CT affectively reduced nematode

egg counts by means of starvation, paralysis, and expulsion of the parasite by interfering with parasite egg hatching for meat goats [3] in Nakhon Ratchasima, Thailand and also anthelmintic effects from diverse plant sources in parasite *Ascaris suum* by reduced migratory ability of newly hatched third-stage larvae and reduced motility and survival of fourth-stage larvae recovered from pigs [4]. Supplementation of tropical roughages with foliage's, is a promising way of consist phenolic secondary plant products, such as tannins. It has been reported that tanninferous trees in tropical plants ranging in low concentrations range from (usually less than 50 g CT/kgDM which ruminants can use as effective utilization of forage, due to they can provide significant protein supplements due to increase the flow of protein compounds and increasing the supply of amino acid through rumen to the small intestine thereby escaping microbial fermentation [5-6]. Although local feed containing CT has been reported useful with positive effects on nutritive value [7], there is a greater than ever awareness for the requirement to study the specifics for goats in regards to these interactions and the effects of these compounds on goat diet and production. Currently, such studies on the interactions between nematode infections and tanniferous plants remain limited. It is therefore critical that proper techniques be developed and used to evaluate and enhance feeding value of tannin-containing foliage's and by-products. The aim of this study was to evaluate the feeding value of tannin-containing neem foliage in meat goat diets in order to improve the rumen ecology and the associated effects on number of nematode eggs populations.

Methodology

Neem foliage collection and condensed tannin extract preparation

Fresh foliage's of *Azadirachta Indica* A. Juss. Var. Siamensis Valeton neem were randomly collected from community forest in Suranaree University of Technology, Suranaree district, Nakhon Ratchasima, Thailand (Fig. 1A). The neem foliage was sliced and dried by using hot air oven at 45ºC until dried (Fig. 1B). The dried neem was analyzed chemical compositions by proximate analysis: DM, ash, crude protein (CP), ether extract (EE), crude fiber (CF) (AOAC, 1990) [8]. Neutral detergent fiber (NDF) and acid detergent fiber (ADF) were determined using the method described by Van Soest et al. (1991) [9], adapted for Fiber Analyzer. The NDF analysis used α-amylase, sodium sulfite in the neutral detergent solution. Both NDF and ADF are expressed inclusive of residual ash (Georing and Van Soest, 1970) [10]. All chemical components were expressed on DM basis. As per data described previously, energy values were to be evaluated using equations recommended by NRC (2001) [11] for formulated mixing of the concentrate ingredients. Obtained analyzed data was then to be calculated for nutrient intakes of DM, CP, EE, NDF and ADF(g/d).

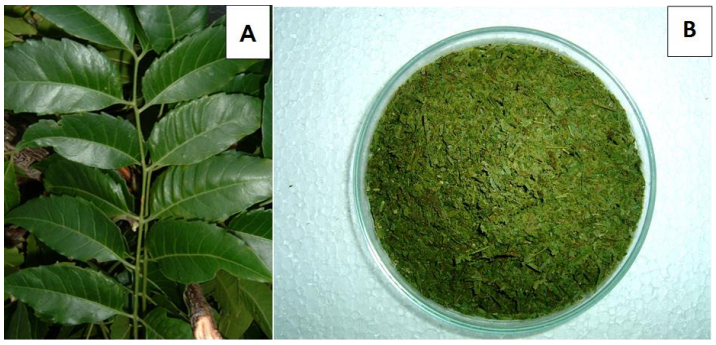

Figure 1. The outside nature (A) appearances of fresh foliage's of *Azadirachta Indica* A. Juss. Var. Siamensis Valeton neem and a cut-off of dried neem foliage (B)

The measurement of purification of CT extract from *Azadirachta indica* A. Juss var. Siamensis Valeton: the method used was that of Dazell and Kervin [12] with modified by Lopez [13]. The fresh neem foliage was cut into small pieces and then kept in the dark chamber at room temperature before freeze drying for 3 d until dry. Triplicate 200 mg samples of finely ground (1 mm sieve) freeze-dried plant material were placed in 50 ml Nalgene vials with 10 ml of aqueous acetone (Analar grade) containing 0.1% ascorbic acid by mixing of acetone, deionized water (70:30, v/v) and sonicated (ultrasonic water bath (Branson 3210)) for 15 min in an ice bath for 3 times (each using of 10 min and breaking for 5 min). Dried samples with 70-act were centrifuged at 3500 g for 10 min, 3 times at 4^{o}C. After 15

min shaking, the samples were then washed with diethyl ether twice times. An equal volume of 40% methanol was then added and the solution purified by adsorption chromatography on Sephadex LH-20 packed in the column using 40% methanol and 80% aqueous acetone overnight. After combining these fractions and adding acetone, they were then rotary evaporated to a small volume and frozen overnight prior to freeze-drying. The final product of purified CT was measured the absorbance of the color at a wavelength of 550 nm using spectrophotometer as shown in Fig. 2C. The value of roughly CT (DM basis) concentration is calculated by the formula: % CT = (value 550 nm absorbance x 78.26 x dilution factor)/(%DM). Dilution factor = 0.5 ml/(volume of extract taken).

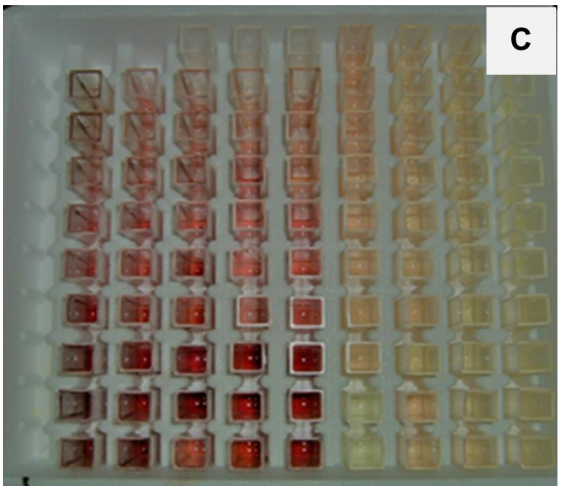

Figure 2. The standard with the final product of purified CT from fresh foliage's of *Azadirachta Indica* A. Juss. Var. Siamensis Valeton neem was measured the absorbance of the color at a wavelength of 550 nm using spectrophotometer (C)

Animals, experimental design, and diets

All experimental procedures were conducted following the Ethical Principles and Guidelines for the Use of Animals issued by National Research Council of Thailand. Four crossbred (Native x Anglo-Nubian) male goats, averaging 6 months old and 21.3±0.8 kg of live weight were used in 4x4 Latin Square Design (LSD). Each goat was treated with vitamins A, D_3 and E before start the experiment. Goats were screened for a parasite egg first from faecal collecting (per rectum) and then the gastrointestinal nematode eggs were examined by the McMaster egg counting technique [14]. Goats were examined nematode eggs, all arranged into 4 experimental periods (28 d per period).

Concentrated feed was balanced protein (isonitrogenous; 16% CP) and *ad libitum* corn silage as a main roughage source. They had free access to water and mineral block, and they had enough space to walk. Treatments were composed of the basal diet without neem foliage supplement (16%CP; 1%BW) (control), or diets of (DM basis) supplemented with 15% neem foliage (30 g neem foliage/head/day (approximately 2.25% CT), or 30% neem foliage (60 g neem foliage/head/day (approximately 4.50% CT) and 45% neem foliage (90 g neem foliage/head/day (approximately 6.75% CT). After concentrated and dried neem foliage mixing, the all treatments were kept to room temperature, re-bagged, and kept in the storeroom close the goats' shed for further use. Feed was offered daily rations as equal meals twice a day at 06:00 and 17:00 h. The individually, goats live body weights were recorded weekly on 5 consecutive days immediately before the morning feeding and after feeding in each period until the end of the experiment. Feed offered and refused were weighed daily prior to the morning feeding to determine the daily DMI. Before and after feeding, feed samples, faeces and urine were separately collected during the last period for chemical compositions analysis by total collection method. Faeces were calculated by the method of Schnieder and Flatt [15]. Urine was analysed for nitrogen by Kjedahl method (AOAC, 1990). DMI and digestibility of nutrients were calculated.

Rumen microbial populations

At the end of each period, a sample of rumen fluid contents approximately 200 ml, as representative of the total ingesta was collected at 0, 2, 4, and 6 h-post feeding by suction pump technique. Rumen fluid contents

contained with large pieces of particulate matter was pulverized by using filtered through four layers of cheesecloth, and pH were measured using a portable pH meter (TESTEK INC instruments ISFET pH meter, USA) immediately Rumen fluid was fixed with 10% formalin solution (1:9 V/V, rumen fluid: 10% formalin), the total direct feasible count cells of protozoa and bacterial populations on hemocytometer (Boeco, Hamburg, Germany) was established by Galyean [16]. The residues sample was prepared for later analyses of volatile fatty acids (VFAs) (acetic acid (C_2), propionic acid (C_3) and butyric acid (C_4) by gas chromatography; Hewlett Packard GC system HP 6890) and NH_3-H refined method of Bromner and Keeney [17].

Counting of nematode eggs number

The fecal samples were collected via the rectum from goats. All fecal samples were examined the parasite eggs using the simple sedimentation technique by 0.85% normal saline. Faeces is mixed with saturated saline immediately and then after 30-45 min, parasite eggs were observed floating, the cover slip was placed on a glass slide. Eggs were counted by microscopic scale.

Statistical analysis

Data obtained from the experiment will be subjected to ANOVA according to a LSD using the General Linear Models, procedure of the Statistical Analysis System SAS software [18]. Duncan's Multiple Range Test will be used to compare means of the difference between treatments with the following Principles and Procedures of Statistics [19]. A significance level of $P<0.05$ will be used.

Results

Chemical composition of experimental feeds and diets is showed in Table 1. Crude protein (CP) and isolation CT extract from fresh neem foliage was approximately 19% CP and 8.60-9.05% CT respectively. The result revealed that dry matter intake, average daily gain, digestibility of intake, rumen pH, ammonia nitrogen and concentrations of VFAs were unaffected ($P>0.05$) by 4 dietary treatments (Table 2). Based on this result, neem foliage a good chemical composition of dietary treatment with had no negative effect after feeding by moderate concentration, therefore offer using as alternative protein source for meat goat diets than relying on the more expensive soybean meal. However, total bacterial populations in rumen fluid of supplemented with 60 g neem foliage/ head/ day (third group) showed tendency to increase at 4 and 6 h ($P<0.05$) after post feeding compared with the control group and second group (Fig 3). Protozoa populations of third group were decreased ($P<0.05$) based on 4 and 6 h compared with the control group while the second and fourth group were not difference in experimental group (Fig 4). The populations of ruminal bacteria at the same sampling period of supplemented with 60 and 90 g neem foliage/ head/ day increased significantly when compared with control and second group, whereas protozoa populations was the lowest of fourth group when compared with control. The number of nematode eggs of treatments supplemented with 30% and 45% neem foliage were significantly decreased ($P<0.05$) at 2 to 3 weeks in the first period compared with that of control and supplemented with 15% neem foliage (Figure 5). Thereafter, the nematode eggs number both dietary treatments increased again at 4 and 7 weeks range from 2500 eggs per gram (EPG) to 3300 EPG and decreased again in 8, 12, 13, 14, 15 and 16 weeks respectively. There were significant differences between 1 week and 3, 6, 8, 12, 13, 14, 15 and 15 weeks.

Table 1. Chemical composition (% DM) and composition of individual feeds

Item	Experimental feed					
	Concentrate	Treatment 2 (2.25%CT)	Treatment 3 (4.50%CT)	Treatment 4 (6.75%CT)	Neem foliage	Corn silage
DM	94.7±0.04	94.5±0.06	93.5±0.02	94.1±0.05	36.3±0.03	28.7±0.12
CP	14.3±0.08	14.4±0.04	14.6±0.06	14.5±0.00	19.2±0.07	8.4±0.07
EE	1.2±0.03	1.3±0.01	1.2±0.05	1.2±0.04	1.7±0.03	1.2±0.02
Ash	8.2±0.09	8.4±0.06	8.2±0.08	8.3±0.06	2.0±0.04	2.6±0.07
CF	4.3±0.06	4.4±0.04	4.4±0.03	4.5±0.08	48.2±0.12	38.5±0.11

Item	Experimental feed					
	Concentrate	Treatment 2 (2.25%CT)	Treatment 3 (4.50%CT)	Treatment 4 (6.75%CT)	Neem foliage	Corn silage
NDF	36.3±0.14	36.5±0.25	36.6±0.22	36.8±0.16	49.3±0.22	63.2±1.74
ADF	9.3±0.81	9.4±0.75	9.7±0.73	10.1±0.67	37.1±0.63	44.6±0.52
%						
CT	0[a]	2.20-2.32[b]	4.16-4.33[c]	6.65-6.85[c]	8.8±1.32	-

DM= Dry matter, CP= crude protein, EE= ether extract, CF= crude fiber, NDF = neutral detergent fiber, ADF = acid detergent fiber and CT= condensed tannins

Mean ± standard error (SE)

[a, b, c] Mean within row which different superscripts are significant difference ($P<0.05$)

Table 2. Effects of dietary levels of neem foliage on nutrient intakes, crude protein and live weight change in goats

Item	Control[1]	Treatment 2 (2.25%CT)	Treatment 3 (4.50%CT)	Treatment 4 (6.75%CT)	SEM
Concentrate DM intake/d					
g/day	304.9	309.0	301.2	302.8	7.85
%BW	1.6	1.5	1.5	1.6	0.05
g/kgW$^{0.75}$	32.6	31.8	31.1	32.9	1.28
Roughage DM intake/d					
g/day	192.0	197.7	198.9	185.7	10.02
%BW	1.0	0.9	1.0	1.0	0.04
g/kgW$^{0.75}$	20.4	20.2	20.5	20.1	0.27
Total DM intake/d					
g	496.8	506.8	500.0	488.6	13.53
%BW	2.5	2.4	2.4	2.5	0.08
g/kgW$^{0.75}$	53.0	52.0	51.5	53.0	1.21
LW gain, g/d	55.2	55.7	56.5	56.3	0.8
pH (h, Post-feeding)					
0	7.2	6.9	7.1	7.2	0.13
2	6.5	6.6	6.6	6.6	0.18
4	6.9	6.7	6.7	6.7	0.18
6	7.2	6.8	6.8	6.8	0.18
NH_3-N, mg%					
0	7.7	7.8	7.6	7.5	0.25
2	9.7	9.4	9.6	9.8	0.11
4	12.2	12.6	10.8	12.5	0.95
6	8.7	8.4	8.6	8.8	0.14
Acetic acid, mol/100 mol	75.6	75.7	76.1	76.2	0.44
Propionic acid, mol/100 mol	17	16.7	16.3	16.3	0.26

Butyric acid, mol/100 mol	7.4	7.6	7.6	7.5	0.29

BW = body weight, $BW^{0.75}$ = metabolic body weight, ADG = average daily gain, SEM: standard error of mean

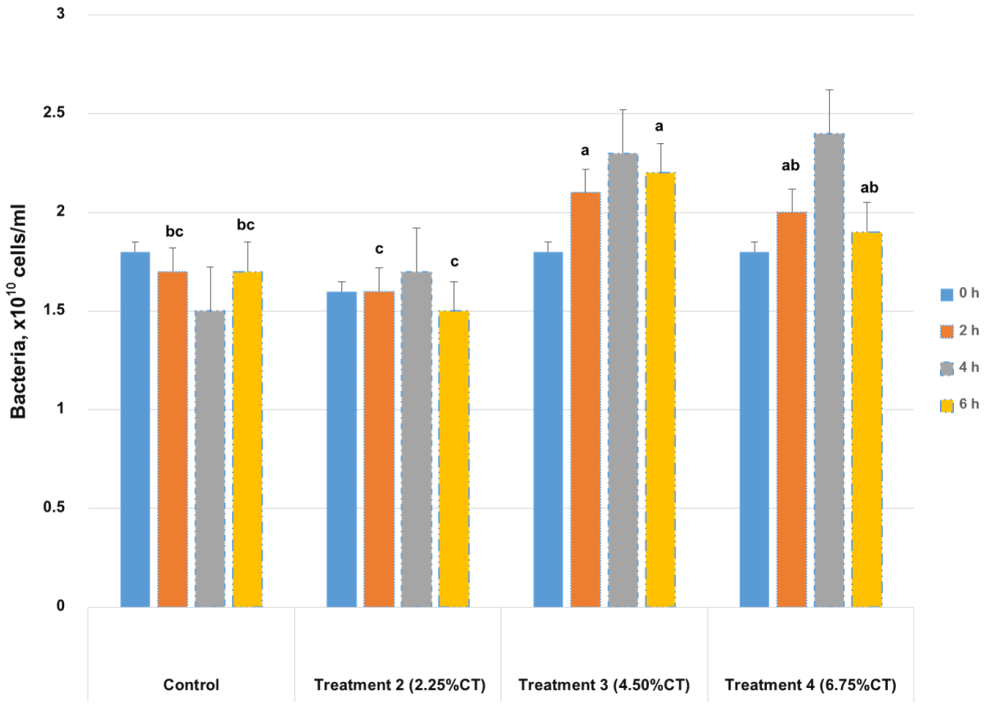

Figure 3. Effect of dietary treatments on total bacterial population in rumen fluid. Values are expressed as mean ± standard error of the means (n = 4). Differences between values with different letters within treatment (^{a,b,c}) are significant (P<0.05).

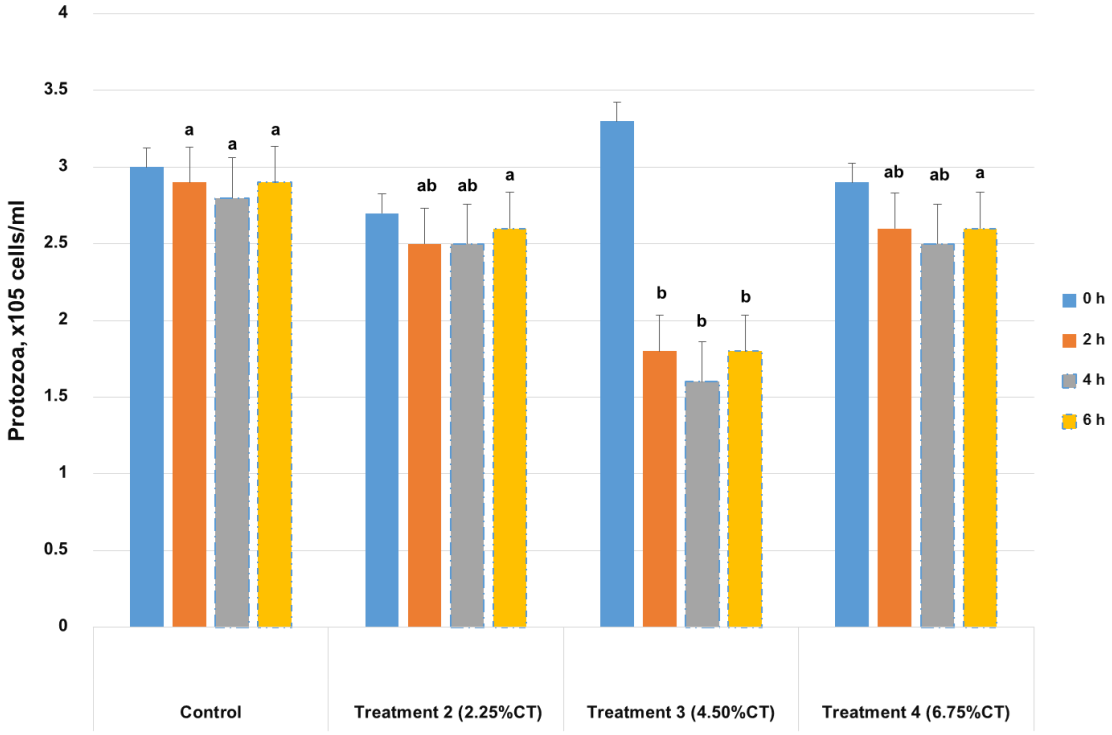

Figure 4. Effect of dietary treatments on protozoa population in rumen fluid. Values are expressed as mean ± standard error of the means (n = 4). Differences between values with different letters within treatment (^{a,b}) are significant (P<0.05).

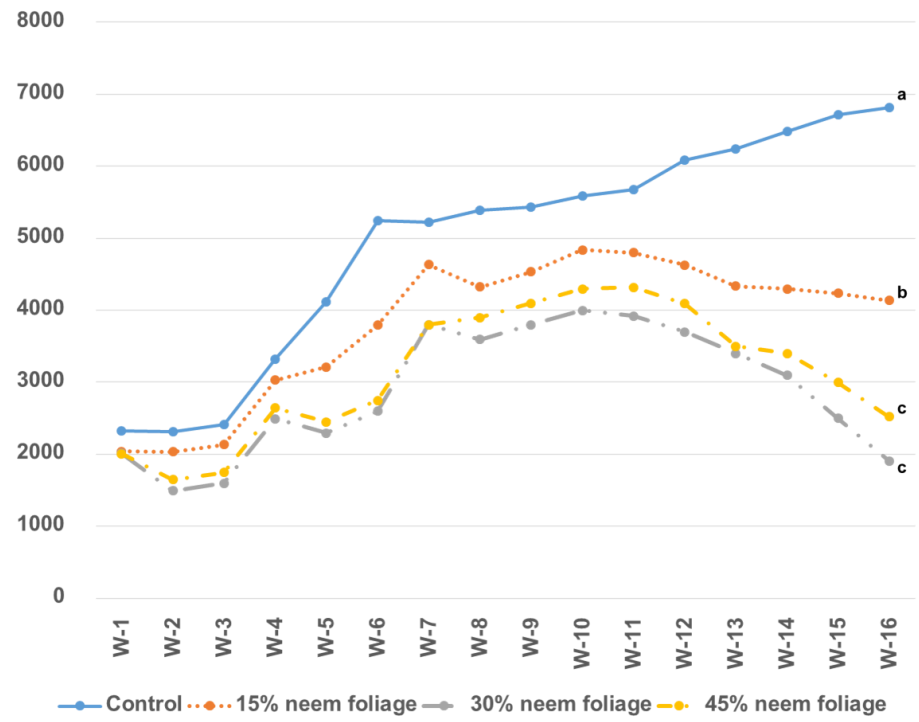

Figure 5. Effect of fresh neem foliage on nematode eggs (Egg per Gram; EPG) in Goat Feces. Values are expressed as mean ± standard error of the means (n = 4). Differences between values with different letters within treatment (^{a,b,c}) are significant (P<0.05).

Discussion and Conclusion

The analyzed values of chemical composition of the feed (control concentrate (16%CP), is in the range reported in the case of Suranaree University of Technology's standard recording. Large variations in chemical composition of feeds (corn silage and neem foliage) reflect the differences in breeds, harvesting processes, the cutting interval and/or cutting height, soil types, fertilizer applications, season and location etc. The present study clearly indicates that neem foliage could be effectively replace other high cost protein main source which more expensive such as soybean meal which that beneficial to meat goat's dietary due to its crude protein content exceeds between 18-20% of DM. These results support the many previous studies in which forage supplemented with tropical foliage's or legume, shrubs that contains high CP content are a promising way of alleviating N deficiencies. The neem foliage was used as a source of CT compound, the neem foliage was rather safe concentrate level in CT (8.60-9.05%); in contrast, the supplementation of neem foliage was chosen to increase the total CT content intake as the level of neem foliage supplementing in treatment diets increased. The average CT content of neem foliage was 8.83±1.23% (DM basis) which yields CT at 30, 60 and 90 g/h/d neem foliage to increase respective CT of 2.25, 4.50 and 6.75 %CT respectively. Although the moderate levels of CT activity (2 to 4% DM) were found to be a potential ruminants feed in the tropics [20], to reduce ruminal protein digestion and ruminal bacterial activity by forming a pH-reversible bond with soluble proteins [21], CT its possible detrimental effects to suppress on feed intake by binding proteins, cellulose and others minerals and therefore decreasing utilization by ruminants. The present study revealed that the CT by neem foliage was slightly concentrated and used appropriate levels does not appear to have undesirable effects in animal productive performances and induction of metabolic disorders. These study levels of CT derived from neem foliage ingestion are safe range for goats.

Due to rumen-pH showed tendency to decline after post feeding, the ratio of C_2/C_3 was significantly decreased at 4 until 6 h compared with that of 0 h. C_3 increased, while C_2 decreased compared with at 0 h. In term of productivity bacteria and protozoa populations in rumen, protozoan populations and nematode eggs of goats fed 30% neem foliage were decreased. These results supported the findings of previous studies which reported condensed tannins as anti-protozoan thereby enabling

increased roughage utilization which, in turn, through the process of carbohydrate digestion by microbial activity, increased the production of C_3. These studies also indicated a lowering of pH values after improved C_3 which resulted in unsuitable conditions for cellulolytic bacteria and an associated decrease producing in C_2. Benefits are available in terms of efficiency in increasing number of bacteria, and can be captured by altering the animal feeding practices. The results showed that bacterial populations in goats fed 30% neem foliage were significantly increased. Generally, C_2 and C_4 levels depend on protozoan populations because the end products of rumen fermentation by protozoa are acetate and butyrate. As mentioned, within the rumen ecosystem, live bacteria and protozoa share a symbiotic relationship. Bacteria counts will increase subsequent to any reduction in protozoan population. Some properties of CT may possibly be anti-protozoan due to the fluid or via its capacity to alter the cell membrane permeability[22] thereby disintegrating the protozoan cell membrane. In fact, the mechanisms of the activity on nematode eggs excretion by CT derived from dietary treatments supplemented neem foliage is not certain however, main hypotheses have been proposed to elucidate the effect of condensed tannins against gastrointestinal nematodes in ruminants. The assertion that tannins may have inherent anthelmintic properties which influence key biological processes is much better supported by research. The results from multiple *in vitro* assays and, importantly, from *in vivo* studies in ruminants (sheep and goat) in which host immune responses were not influential lends weight to the principle concept [3, 23-24]. However, little is known about factors regulating its expression and the actual mechanisms activity for the anti-parasitic activity remain obfuscated and may differ depending on the parasite type, its stage of development and the specific biochemical characteristics of the plant species[22]. Based on the experimental results, it can be determined that there is potential for the effective use of local neem foliage plants as anthelmintics in gastrointestinal nematode infection and defaunation in goats adapted to quantity of neem foliage feeding. The mode of action between neem foliage and nematode eggs excretion is less clear and any negative effects resulting from longer term feeding would still require further investigation.

In conclusion, neem contains CT as a secondary plant compounds which could either improve rumen ecology and/or affectively reduced the number of nematode egg counts by means of starvation, paralysis, and expulsion or digestion of the parasite for goats. In goats on the test diets, CT activity of neem foliage and other compounds on the number of larvae will be further studied. These results can support the use of neem foliage can be used as a nitrogen nutritional source since its high CP content and also as a good chemical constituents of tannin activity that may be potential source of natural products.

Acknowledgements

Financial support was provided by the Institute of Research and Development, Suranaree University of Technology, Thailand.

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Effect of Papaya on Alpha Glucosidase and Pancreatic Lipase Activity and Small Intestinal Morphology in High-Fat Diet-Induced Obesity Rats

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Abstract

This study focused on evaluating the effect of papaya on alpha glucosidase and pancreatic lipase activity *in vitro* and the small intestinal morphology in high-fat diet-induced obese rats. *In vitro*, we used alpha glucosidase and pancreatic lipase inhibition assay. *In vivo*, twelve Sprague Dawley rats were randomly divided into two groups, and one group fed with a high-fat diet to induce obesity, with the other group fed a normal diet as the control. After 8 weeks on the high-fat diet, this group was further divided into three sub-groups: high fat diet (HFD group), high fat diet + papaya juice 0.5 ml/100g BW (HFL group), and high fat diet + papaya juice 1.0 ml/100g BW (HFH group). Papaya juice was administered orally to the rats in the HFL and HFH groups and the same amount of water was administered orally to the rats in the control and HFD groups. Our results showed that alpha glucosidase and pancreatic lipase inhibition effect of 100% of papaya was the most effective inhibitor. In an *in vivo* study, the HFD groups rats showed increased villi length and numbers of goblet cells, whereas the rats in the both HFL and HFH groups had significantly decreased villi length and goblet cells number. Our results clearly showed that papaya is an effective inhibitor of alpha glucosidase and pancreatic lipase and induced a reduction in the villi length and goblet cells. We can confidently conclude that papaya is a beneficial natural product for the treatment of obesity.

Keywords: Papaya, Alpha glucosidase, Pancreatic lipase, Obesity, Small intestine morphology

Introduction

Obesity is a significant public health problem worldwide which is caused by an imbalance between energy intake and energy expenditure. It is also associated with the disturbance of food intake regulation (Moro & Basile, 2000). Obesity is characterized by the excessive fat accumulation in the body that results in a body mass index (BMI) greater than or equal to 30 kg/m^2. It is associated with an increased risk factor for metabolic diseases such as dyslipidemia, diabetes mellitus, cardiovascular disease and metabolic syndrome. In 2014, the World Health Organization reported that 13% of the world's adults aged 18 years and over were obese (11% of men and 15% of women).

There are medications, such as orlistat, which can support therapeutic measures against obesity. One therapeutic method for treating obesity is to decrease postprandial glycemia by the inhibition of carbohydrate-hydrolyzing enzymes, including alpha-glucosidase, and also, by inhibiting pancreatic lipase, delaying the digestion and absorption of fat (Mukherjee, 2003).

Extracts from plants or herbs have been used historically and traditionally in natural medications for the prevention and treatment of many chronic diseases such as obesity. Herbal medicines have fewer side effects than pharmacological drugs (Moro & Basile, 2000). Our objectives were to ascertain the effects of papaya juice on obesity. *Carica papaya* Linn. (family: Caricaceae) is a tropical tree widely grown in many countries in the tropical regions of the world. It is a nutritious fruit that contains a lots of vitamins and minerals (Saeed et al., 2014). Many biologically active phytochemicals from different parts of papaya trees (seed, leaf, root and fruit) have medicinal properties. Some of these parts are known to be diuretic activity, wound healing activity, anti-inflammation, anti-hypertension and hypoglycemic activity (Vij & Prashar, 2015). However, little has been published on an anti-obesity property of papaya, which was the focus of this study, specifically the effects of papaya on alpha glucosidase and pancreatic lipase inhibition and on the histology of the small intestine in high-fat diet-induced obesity in rats.

Methodology

Fruit material

Papaya fruits were purchased from a supermarket in Meuang, Phitsanulok, Thailand. The fruit were selected based on uniformity of shape and size, and external color level indicating ripeness. Papaya juice was prepared daily. Briefly, the peel and seeds were removed, cut into pieces, weighed and crushed into a beaker to make papaya juice.

Alpha-glucosidase inhibition

To determine the inhibitory effect of papaya juice on alpha-glucosidase, a 50 µl phosphate buffer (pH 6.8) was mixed with 18 µl alpha-glucosidase inhibitor (acarbose and papaya) and 2 µl alpha-glucosidase. The reaction mixture was incubated at 37°C for 20 min before adding 50 µl of sodium carbonate. The absorbance was measured at 405 nm in a microplate reader. The results were expressed as the percentage inhibition calculated using the following:

Inhibitory activity (%) = $(A_{blank} - A_{sample})/A_{blank} \times 100$

where, A_{blank} is the absorbance of the control and A_{sample} is the absorbance of the papaya juice

Pancreatic lipase inhibition

To determine the inhibitory effect of papaya juice on pancreatic lipase, an 80 µl assay buffer was mixed with 10 µl pancreatic lipase inhibitor (orlistat and papaya), 30 µl pancreatic lipase and 90 µl 4-nitrophenyl laurate (substrate). The reaction mixture had incubated at 37°C for 2 hr. before being centrifuged at 16,000 rpm for 2.5 min. The absorbance was measured at 400 nm in a microplate reader. The results were expressed as percentage inhibition, which calculated using the formula below,

Inhibitory activity (%) $= A_{blank} - A_{sample})/A_{blank} \times 100$

Where, A_{blank} is the absorbance of the control and A_{sample} is the absorbance of the papaya juice

Animals and experimental protocol

Male Sprague Dawley (SD) rats were purchased from the National Laboratory Animal Center, Mahidol University. Rats were keep in controlled temperature (22±1°C) with relative humidity of 55±10%. Rats were on a dark-light cycle for 12-12 hr. Diet and water were provided *ad libitum*. After 1 week, rats were fed with difference diets and divided randomly into four groups. Group 1: control, Group 2: high fat diet (HFD), Group 3: HFD + papaya juice 0.5 ml/100g BW (HFL), and Group 4: HFD+ papaya juice 1.0 ml/100g BW (HFH). Group 1 rats were fed with normal diet. Group 2,3 and 4 received HFD for 12 weeks. In addition, group 3 and 4 rats received papaya juice from the 8th to 12th week. The duodenal segment of small intestine was removed and placed in 10% formaldehyde for histological study. All the animal protocols were approved by the Naresuan University Animal Care and Use Committee (NUACUC), Phitsanulok, Thailand prior to starting the experiment.

Histological analysis of small intestine

Duodenum was fixed in 10% neutral buffered formaldehyde. Tissues were processed in a paraffin automatic processor and embedded in paraffin. Consecutive 5 µm paraffin sections were made and stained with hematoxylin and eosin (H&E). After staining, histopathological features were assessed under a light microscope.

Statistical analysis

Data are expressed as mean±standard error of the mean. To determine differences between the groups, the data were compared using one-way ANOVA with Tukey's Multiple Comparison Test.

Results

Effect of papaya on pancreatic lipase and alpha glucosidase activity

Figure 1A shows the percentage inhibition of papaya and figure 1B shows the percentage orlistat inhibition of on the pancreatic lipase. Both showed a dose dependent pattern. The percentage inhibition of 25% juice was 17.10±2.95%, 50% juice was 23.49±1.35% and 75% juice was 30.25±5.22%. The highest inhibition was found when treating with 100% juice which demonstrated inhibition of 40.37±3.87%. The pancreatic lipase inhibition effect of 100% juice was similar to that of 500 nM orliastat.

Figure 1C shows the percentage inhibition of papaya and Figure 1D shows the percentage acarbose inhibition of on the alpha glucosidase activity. The inhibition of papaya juice on alpha-glucosidase exhibited a dose dependent manner. The percentage inhibition of 25% juice was 52.52±6.46%, 50% juice was 69.41±14.11% and 75% juice was 92.73±2.05%. Interesting that 100% juice completely inhibited alpha glucosidase by 106.01±7.14%, which was more effective result than acarbose.

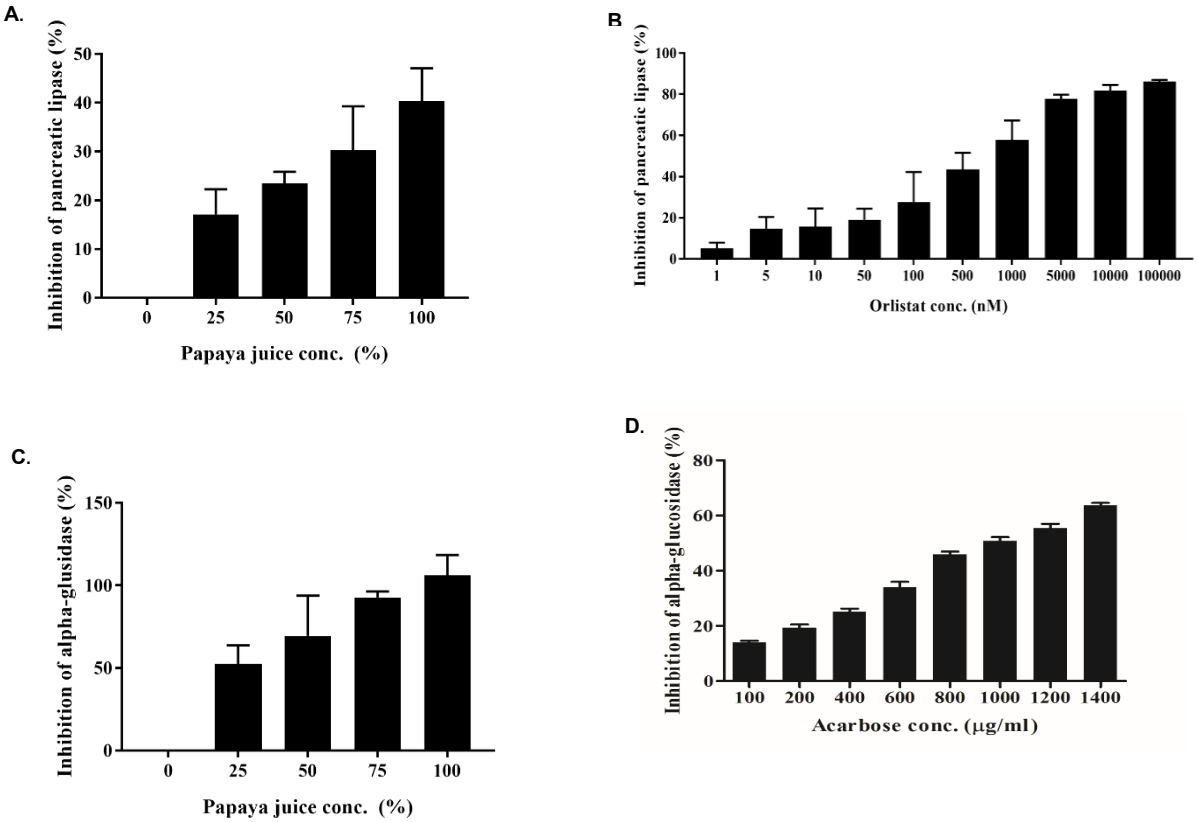

Figure 1 Effect of papaya (A) and orlistat (B) on pancreatic lipase inhibition and effect of papaya (C) and acarbose (D) on alpha-glucosidase inhibition.

Effect of papaya on duodenum morphology

Figure 2 shows photomicrograph of the duodenum sections of all experimental groups, figure 3 shows the villi length and figure 4 shows the number of goblet cells per villus in the duodenum of the rats. The duodenum villi of rats in the HFD group (1006.07±102.39μm) were significantly longer than those of the control (795.88±33.94). In addition, the animals with HFD plus 0.5 or 1.0 ml/100 g BW of papaya (783.48±73.99, P<0.05 and 708.11±95.63, P<0.01, respectively) had the decreased villi length in the duodenum when compared with the HFD group. The rats fed with HFD had increased villi length while in those fed with papaya juice the villi length returned to normal, equal to the control group. Furthermore, the number of goblet cells per villus in the HFD group (10.20±0.81) was significantly higher compared with the control group (7.47±0.50). The number of goblet cells in the duodenum in the HFD plus 0.5 and 1.0 ml/100 g BW of papaya (7.60±0.62, P<0.05 and 7.20±0.60, P<0.01, respectively) were significantly decreased when compared with the HFD group.

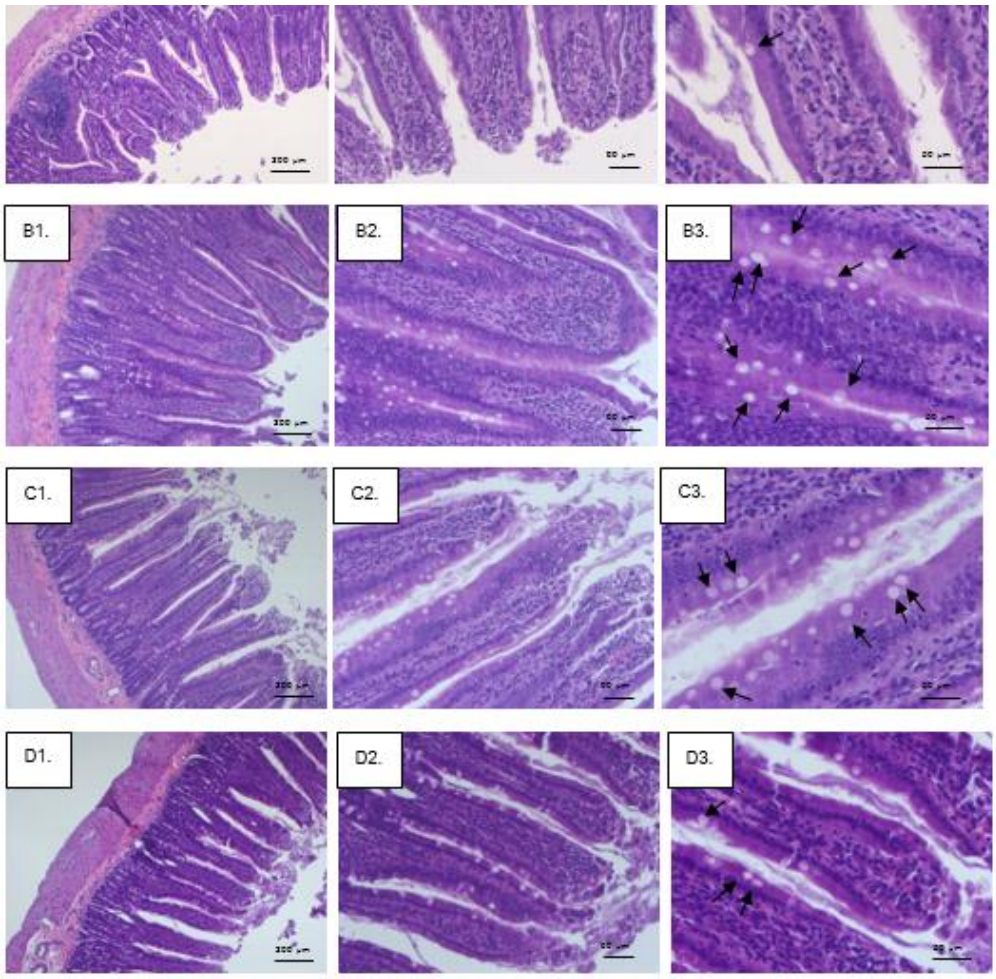

Figure 2 Effect of papaya on morphology of villi and cells in the duodenum. The duodenum was stained with hematoxylin and eosin (H&E). A; control group, B; HFD group, C; HFL group and D; HFH group. 1, 2 and 3 refer to 4X, 10X and 20X magnification. Black arrow indicated the goblet cells.

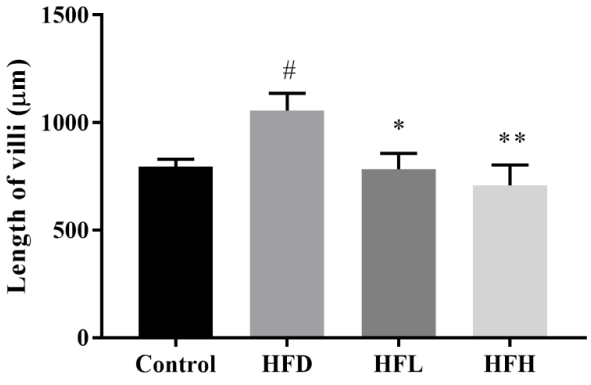

Figure 3 Effect of papaya on the length of duodenal villi. All data were analyzed using one-way ANOVA with Tukey's Multiple Comparison Test. # $p<0.05$ compared with that of the normal diet group,
* $p<0.05$ and ** $p<0.01$ compared with that of the HFD group.

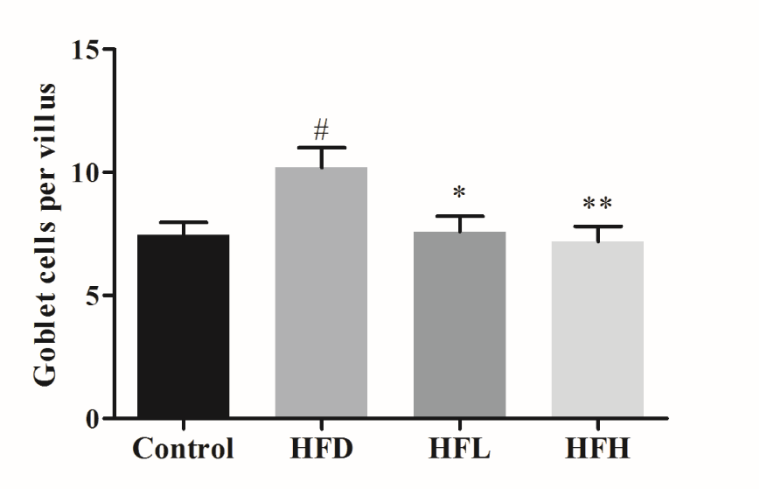

Figure 4 Effect of papaya on the goblet cells. All data were analyzed using one-way ANOVA with Tukey's Multiple Comparison Test. # *p<0.05* compared with that of the normal diet group, * *p<0.05* and ** *p<0.01* compared with that of the HFD group.

Discussion and conclusion

Obesity is an increasing health problem in many countries, particularly those where incomes are rising, leading to significant changes in diet and lifestyle. Obesity is characterized by excessive body weight caused by excessive accumulation of body fat. The enzyme responsible for the hydrolysis of 50% to 70% of total dietary fats is pancreatic lipase (Birari & Bhutani, 2007). One therapeutic method for treating obesity is to delay digestion and absorption of fat through inhibition of pancreatic lipase. orlistat, an inhibitor of pancreatic lipase, is the most common anti-obesity drug. Previous studies have shown that orlistat inhibits pancreatic lipase activity by 95.7% at a concentration of 250 µg/ml (Zhang et al., 2008). The other researcher has reported that orlistat is a pancreatic lipase inhibitor with IC_{50} values of 0.29 mg/ml (Liu et al., 2013). However, orlistat has gastrointestinal side effects such as fat malabsorption and steatorrhea (Ballinger & Peikin, 2002). Other studies have identified the polyphenolic compounds in herbal plants that manifest inhibitory activity on pancreatic lipase (Liu et al., 2013; Moreno, Ilic, Poulev, & Raskin, 2006; Nakai et al., 2005; Ono, Hattori, Fukaya, Imai, & Ohizumi, 2006; Sharma, Sharma, & Seo, 2005).

In our study, we have demonstrated that papaya juice inhibited pancreatic lipase activity in a dose-dependent manner at the concentrations of 25%, 50%, 75% and 100%. Pancreatic lipase plays a key role in the digestion and absorption of triglycerides into monoglycerides and fatty acids (Belfeki, Mejri, & Hassouna, 2016), and another study showed that the inhibition of pancreatic lipase in hyperlipidemia rats exhibited decreased triglyceride levels (Liu et al., 2013). This demonstrates that by inhibiting pancreatic lipase, plasma triglyceride levels are reduced. In our study, we found that papaya juice reduced the elevation of plasma triglyceride levels (unpublished data) which suggests that papaya decreased triglyceride levels in the high-fat diet-induced obesity rats by inhibiting the pancreatic lipase activity.

A current therapeutic approach in treating obesity- associated diabetes is through decreasing hyperglycemia by retarding the absorption of glucose by inhibiting carbohydrate- hydrolyzing enzymes, such as alpha-glucosidase. In this context, many researchers have studied the inhibitory effects of various medicinal plants on alpha glucosidase (Liu et al., 2013; Majouli et al., 2017; Yang et al., 2017). The results from the present study show that papaya does inhibit the alpha-glucosidase activity in a dose-dependent manner at concentrations of 25%, 50%, 75% and 100%. However, inhibitory effect of papaya on the alpha glucosidase activity *in vitro* could not decrease the elevation of plasma glucose levels (unpublished data). Our histological study of the duodenal cells of obese rats showed increased villi length and goblet cells when compared with rats fed with a normal diet. This result was similar with the previous study showed longer villi length in obese mice (Mao et al., 2013) It is clear that the villi length on the duodenum in high-fat diet rats increased due to the intake of luminal nutrients and an increase in intestinal absorption in obese mouse models (Mayer & Yannoni, 1956). In addition, previous study showed that the total

number of cells per villus were increased in mice fed high-fat diet (de Wit et al., 2008). Interestingly, our study found that papaya reduced the villi length and goblet cells in the duodenum in high-fat diet-induced obesity rats.

Therefore, obese individuals have long villi. Effect of papaya is to decrease villi length which can help to decrease the surface area in the small intestine and thus reduce the absorption of nutrients into the circulation. Our results suggest that papaya is effective against obesity. Furthermore, our result showed that HF increased goblet cells whereas papaya reduced the number of goblet cells. Goblet cells are responsible for the secretion of mucin to protect the intestinal cells from irritating or inflammation (Specian & Oliver, 1991). The increased number of goblet cells is associated with the inflammation in mice fed with various concentration of high-fat diet (Benoit et al., 2015). From our present study, papaya might have some protective effects against inflammation in small intestine induced by high-fat diet, resulting in the decreased numbers of goblet cells as shown in the papaya treated groups.

In conclusion, the results achieved in our study strongly indicate that papaya is an effective inhibitor of alpha glucosidase and pancreatic lipase. As obesity is associated with the alteration of intestinal morphology such as increased villi length and numbers of goblet cells on the duodenum, we have demonstrated that papaya reduces the elevation of villi length and goblet cells, thereby acting against obesity. Our conclusion is that frequent and regular consumption of papaya will have beneficial effects for the treatment of obesity.

Acknowledgments

This research was partially supported by grant from Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand and Thailand Research Fund (RDG5820017). Thank you to staffs of the Department of Physiology and Anatomy, Faculty of Medical Science, Naresuan University, Phitsanulok, Thailand for providing equipment and technical assistance. Many thanks to Mr. Roy Morien of the Naresuan University Language Centre for his English editing assistance in this document.

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Development of Low Saturated Fat Chocolate Cakes by replacing Butter With Soybean Oil

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Abstract

This paper aimed to investigate the possibility of replacing butter with soybean oil, which is low in saturated fats and a good source of plant sterol. The latter has been incorporated in various functional foods targeted to lower cholesterol level. First, quality of cakes with butter being replaced by the oil (25%, 50wt%, 75wt% and 100% by weight) was investigated in terms of physical appearance, volume, porosity, texture (stress-strain test) and a sensory test. The results showed the optimal replacement at 25%. Nevertheless, the cakes made with this amount of butter replacement had smaller volume, less number of bubbles trapped in the cake batter, crispier texture and were harder than the all-butter cake. Hence, improvement of qualities of the cakes made at this percentage of oil replacement was investigated initially by varying mixing speeds (90-220 W) and emulsifier addition (UFM SP). The mixing speed could improve amount of air incorporated in the cake batter whereas the emulsifier could stabilize the cake batter by improving the mixing of the liquid oil phase and the other ingredients which were more solid. The results demonstrated that a combination of optimized mixing speed and emulsifier addition could improve the oil cake to have similar physical properties to the all-butter cake. As baking temperature could have effects on texture of the cake, temperature effect on rheology of cake batters was also studied. The results suggested that baking temperature and baking time could be modified to improve quality of the oil cake. Although the modification to the baking temperature and the baking time was not included in this work, statistical study of the sensory evaluation of the cakes (texture and mouthfeel) showed that the oil cake with the modified mixing speed and the emulsifier addition were equally accepted as the all-butter cake (α = 0.05). Another concern of the oil cake was the unpleasant odour associated with soy bean oil. Nevertheless, the statistical study of the sensory evaluation also demonstrated that the oil cake with cake icing was indifferent from the all-butter cake with the same type and amount of icing (α = 0.05).

Keywords: Cake, Emulsifier, Rheology, Sensory Test, Soybean Oil

Introduction

In the functional food industry, modification of cake recipes has been done by replacing butter with unsaturated oils and fiber addition. Some of the cake ingredients are also replaced to target consumers with food allergy (e.g. eggs and gluten). Hydrogenated shortenings are used to replace butter in cake production as it can provide desirable attributes. However, being saturated, shortenings tend to raise the level of blood cholesterol and increase the risk of cardiovascular disease [1]. Hence, replacing butter with some unsaturated oils to avoid the use of shortenings have been considered [2-3]. Vegetable oils are unsaturated and also provide essential fatty acids such as linoleic acid [2]. Although vegetable oil may be used in baking specific types of cakes such as chiffon cake, vegetable oil is not included in the recipes of batter-type cakes such as butter cakes. Moreover, substituting butter with all liquid oil in conventional preparation of cakes as the final cakes will have a relatively low volume and an undesirable oily or glossy appearance [4]. This is because butter and shortening in cakes helps entrapping air bubbles into the cake batter during mixing and helps to leaven the finished product [3-4]. Moreover, shortening tenderizes the crumb, gives moistness to the product and enhances mouthfeel [4-5]. Attempts have been made in lower amount of butter in cake baking [5-7]. Studies of replacing butter or other shortening such as margarine with healthy oils have been reported. Matsakidou et al. [8] studied replacing margarine with olive oil in preparation of madeira cake and found that the cake made by replacing margarine with olive oil at 40% by weight had different physical characteristics from the cake made by margarine only. Nevertheless, consumer's acceptance of the two cakes was comparable. Sowmya et al. [2] studied physical characteristics of cakes made by replacing butter with sesame oil with various ratios. It was discovered that replacing butter with oil had adverse effects on cake characteristics. Nevertheless, most of the modifications made to reduce amount of butter in cake baking involve only varying amount of oil replacing in the recipe and not much has been reported on the modification

of production process. The most important step of cake baking is preparing the cake batter [9]. This is when air cells are trapped in the batter resulting in smooth texture and rising of the cake. An amount of the air cells in the cake batter depends on the design of the beater, and the viscosity and the surface tension of the batter [10]. Rheological properties of the batter also play an important role in holding the air bubbles during cake baking [11-12]. According to the literature, final bakery product qualities are related to rheology of the product mixture before baking [13-15].

Soy bean oil is a triglyceride which has an average composition of 15% stearic acid, 25% oleic acid, 51% linoleic acid and 9% linolenic acid [16]. With 51% of linoleic acid, soy bean oil is one of the sources of linoleic acid which is essential nutrient of human [17]. Although there have been no conclusive results regarding health effects of linoleic acid [18-20], one nutritional interest of soy bean oil comes from its high content of plant sterol [21]. Plant sterols are structural analogues of cholesterol, which occur naturally in all plant-derived foods [21-23]. Because of the structure of plant sterols, they inhibit intestinal cholesterol absorption. Lowering LDL cholesterols effects of plant sterols have been extensively reported [22, 24-26]. However, daily intakes of plant sterols from natural food sources are not sufficient to substantially lower LDL-cholesterol [23, 26]. Consequently, there have been growth of functional foods containing plant sterols designed to lower risk of coronary disease [23, 26].

The aims of this study, hence, were to examine the effects of replacing butter with soy bean oil in a conventional butter cake recipe (chocolate cake) on the quality and sensory characteristics (appearance, texture, flavour, and overall acceptability) of the final cakes. The results obtained were then analysed to identify any modifications required to the production process. As it has been reported that cake quality could be improved with modification of baking temperature [27], rheological behaviours of the cake batters during heating were also studied. Transition in normal force applied on the cake batters during heating would signify temperature at which the cake batter started to change its phase from gel to solid [28]. Soybean oil was selected as it has balanced fat profile, low saturated fat, high amount of plant sterol [21] and no specific odour unlike other healthy oils such as sesame oil, coconut oil and virgin olive oil. However, the lower amount of butter used may make cake odour less pleasant to consumer's perception. Hence, chocolate recipe was chosen in this study; it was expected that the chocolate odour may improve odour of the finished cakes without using any additives or artificial chemicals.

Methodology

Materials

Cake flour (United flour mall public co. Ltd., Thailand), unsalted butter (Allowrie, Australia), soybean oil (Morakot, Thailand), baking powder, chocolate powder, sugar, evaporated milk (Carnation, Thailand) cashew nuts, and cake emulsifier (SP, UFM, Thailand) were purchased from local supermarket.

Cake formulation

The cake recipe studied was based on chocolate cake recipe with a control cake made from 110 g flour, 110 g sugar, 110 g butter, 100 g eggs, 14.75 g cocoa powder, 10 g baking powder and 13.6 g evaporated milk. This study was divided into 2 phases: (i) preliminary phase where effects of varying percentage of oil replacement were investigated and (ii) modification phase where the results in the preliminary phase were used to improve qualities of the cake.

During the preliminary phase, butter was replaced by soy bean oil by weight at 25%, 50%, 75% and 100% (no butter). The amount of soybean oil and butter in these cakes and their codes are summarized in Table 1.

Table 1: Cake formulations studied and their codes

Replacement level (%)	Amount of butter (g)	Amount of vegetable oil (g)	Codes
0 (Control cake)	110	0	C
25	82.5	27.5	O25
50	55	55	O50
75	27.5	82.5	O75
100	0	110	O100

During the modification phase, cake emulsifier was added to cake batters at the amount as specified by the manufacturer (5.5 g). Finally, when cake processing was modified and the modification that resulted in the oil cake with physical properties closest to the control cake, this oil cake sample and the control cake sample were covered with chocolate icing to simulate the real products. The recipe for the icing was: 150 g dark chocolate (50% cocoa solid), 30 g butter, 100 ml evaporated milk and 100 g cashew nuts.

Cake Processing

Preliminary phase

Cake batter was prepared using creaming method. Dry ingredients which were flour, cocoa powder, and baking powder were sifted together. Then in a separate container, the shortening (oil, butter) and the sugar were creamed using a hand mixer (Sharp R267, 180 W, 5 speeds, Thailand) for 8 minutes at speed 4. Beaten eggs were added and mixed at speed 3 for 2 minutes. The dry ingredients were then added and the mixture was mixed at speed 2 for 2 minutes. Finally, milk was added and mixed at speed 3 for 1 minute. The batter was placed into an aluminium butter-coated tin (27.5 cm diameter and 7.7 cm height). The samples were baked at 170°C in an electric oven (Homemate, HA 3086, 230 W, Vietnam) for 30 minutes. One sample was baked each time at the same location (in the centre of the middle tray). After baking, cakes were removed from the tins, and cooled at room temperature for 1 hour before any physical measurements. For sensory tests, cakes were stored in air-tight containers (after cooled down to room temperature).

Modification phase

Results from the preliminary phase suggested that less air bubbles were trapped in the oil cake batters. Hence, in the modification phase, modification to mixing process was made to increase bubbles invigorated in cake batter by: (i) the use of emulsifier; (ii) varying mixing speeds and (iii) a combination of both. Three mixing speeds were investigated, mixing speed 2 (120 W), 3 (150 W) and 4 (180 W). Although, the hand mixer used can be set up to 5 speeds, it was not possible to make homogeneous batter with speeds 1 (90 W) and 5 (220 W). This was because speed 1 was too slow and oil would separate out from cake batter. Speed 5, on the other hand, was too fast and some dry ingredients tend to stick to a mixing bowl making lumps in the cake batter. In addition, from the literature review medium speeds were recommended [9]. Volumes of bubbles in cake batters were also estimated to investigate effects of mixing speeds on amount of air that could be incorporated in cake batters during mixing. Cake batter was placed inside a glass tube to a marked level and the sample was centrifuged (5000 rpm). After centrifugation, cake batter volume decreased; the top level of the cake batter was marked. The glass tube was then cleaned and water was used to find the difference in volumes before and after the centrifugation. The difference in volumes was used to quantify total volume of bubbles captured within the batter.

With oil present in the cake batter studied here, it was assumed that the baking temperature could also be changed. Hence, a rheometer (KNX2100, Malvern, Germany) was used to investigate a normal force applied to a cake batter sample in a temperature profile related to cake baking as reported in [28]. This result then was used to discuss possibility of changing baking temperature. The rheometer was also used to investigate viscosity of cake batters using the shear stress-shear rate measurements.

Measurement of the cake characteristics

All cake characteristics were measured in triplicates. Following characteristics were measured:

Moisture content

Moisture content was determined by measuring the moisture loss due to drying based on method given in [29]. Cake samples (80 g, left to cool down in at room temperature for 1 hour and then kept in an air-tight container) were dried in the oven at 170^{o}C for 55 minutes. The water content (W) was evaluated by the weight loss divided by the original sample weight.

The cake volume (V) was estimated using equation (2) where V_t and V_b were estimated by modifying the rapeseed displacement method reported by Lebesi and Tzia [29]. Glass beads (diameter~2 mm) were used instead of rapeseeds because they are easier to find, have a similar diameter and both rapeseeds and the glass beads have round shapes. V_t. is volume of the glass beads (measured using a graduated cylinder) that could fill up an empty cake tin whereas V_b is volume of the glass beads that could fill up the cake tin with the cake placed inside the tin.

$$V = V_t - V_b \quad (2)$$

Cake rising ratio (*R*) [27] was estimated by:

$$R = \frac{h_1}{h_2} \quad (1)$$

where h_1 is the height of the cake at the centre and h_2 is the height of the cake at the edge (Shown in Fig. 1)

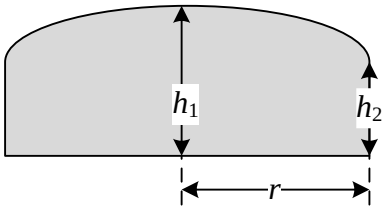

Fig. 1: Measurements of the heights of cake samples: at the centre - h_1, at half radius (r) - h_2 and at the edge - h_3.

Cake compressibility

Universal Testing Machine (Instron 5566, Max load 1000 kg, USA) was used to investigate the compressibility of the cake samples (the entire cake was used in each measurement). A controlled compressive extension mode was used and the machine recorded the load that gave the required extension. In the preliminary stage, measurement was taken at 10% and 50% compression at a speed of 5 mm/min. In the modification stage, measurements were taken at smaller strains at three compressive extensions (5, 10, 15 mm) for more detailed measurements of the cake compressibility.

Porosity

Digital pictures of a cross-sectional area of each cake samples (4 cm x 5 cm) were taken and number of pores was determined using Image-Pro Plus Software. Microscopic pictures of the samples were also taken to investigate the texture at a microscopic level.

Sensory Evaluation

Sensory tests were conducted at the end of the preliminary stage and the end of the modification stage. At the end of the preliminary stage, cake samples C, O25, O50, O75, and O100 were tested by 100 panellists recruited among staff and students of faculty of Engineering, Thammasat University. Cake samples were coded with random numbers and were evaluated for odour, flavour, texture appearance, and mouthfeel of decomposition (as described by in [30]). This result together with other measured cake characteristics then were used to identify the modification required in the modification stage.

Sensory tests at the end of the modification stage were conducted using 200 panellists recruited among staff and students of faculty of Engineering, Thammasat University. Initially, the panellists were asked to evaluate cakes prepared using varying mixing speeds, flavour, texture, appearance, and mouthfeel of decomposition. The result of this sensory test was used to find the mixing speed used in mixing of the batter that gave the most accepted oil cake. Then oil cakes were prepared using this optimal mixing speed with and without emulsifier added. After that these cakes were tested by the 200 panellists to identify the best modification method (combination of varying mixing speeds and emulsifier addition). Finally, all cake samples made from the modified processes were covered with icing to simulate the real products and the panellists were asked to select the sample they like the most.

Results

Preliminary stage

Moisture Content

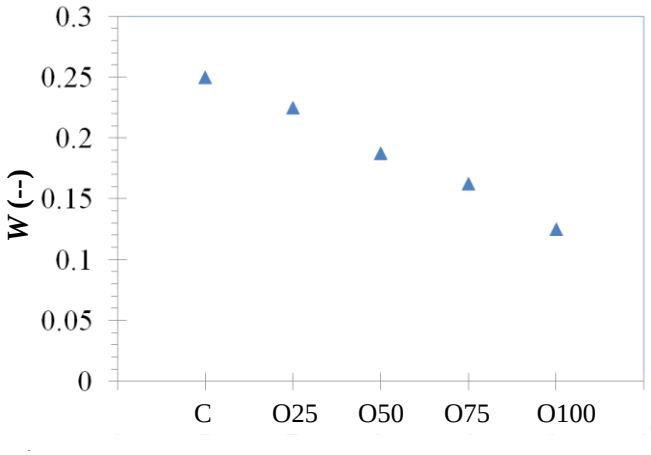

Fig. 2: Moisture content (*W*) of various cake samples (error bars are too small to be shown).

Weight of all cake samples was similar in the range of 440-450 g. The difference in weight was in accord with the moisture contents measured where the heavier cake sample had more moisture content. Moisture content of the cake samples, *W*, are shown in Fig. 2. As expected, sample C contained the most moisture as butter is known to be one of ingredients added to yield moist cakes [9]. In addition, a clear linear relationship between *W* and oil content can be observed from Fig. 2.

Rising ratio and cake volumes

According to Fig. 3, the rising ratio (*R*) increases slightly with increasing oil content. The values of *R* higher than 1 are desirable in cake baking as they show dome-shaped contour [27]. Nevertheless, it cannot be concluded that oil-replaced cakes were better than the all butter cake (sample C). This is because the higher *R* values were only due to the low h_2 measured from the samples O50, O75 and O100; these samples showed almost no rising at the edges, *i.e.*, h_2 values of baked cakes were similar to that of unbaked ones. On the other hand, the middle part of samples O50, O75 and O100 rose to similar extent of the control sample (sample C). It has been reported that the values of *R* that are larger than 1 significantly would result in cake collapsing on cooling (dimpled cake) and/or cracking at the top [29-30]. Such instability in cake shapes were also observed from samples O50, O75 and O100 where fluctuations in *R* (shown by error bars) were seen. Cracks at the top surface of cakes were also found with sample O100. For these reasons, oil-replaced cakes were not at all comparable to all butter cake (sample C). Volumes (*V*) measured also agree with *R* results (Fig. 3). As aforementioned, an increase in *R* values of samples O50, O75 and O100 was because these cakes almost did not rise at edges. Hence these cakes showed less volume. The large variation in R observed from samples O50, O75 and O100 also suggests instability of these cake batters.

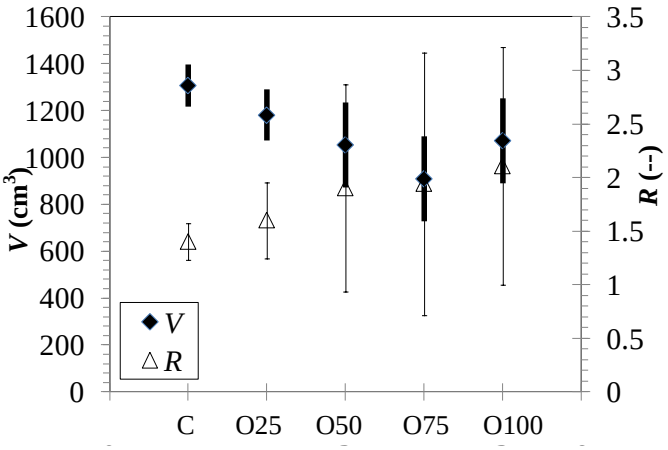

Fig. 3: Rising ratio (*R)* and volumes (*V*) of final cakes; error bars: *R* – thick line; *V* – thin line.

Porosity

Fig. 4 illustrates pores observed in cake samples. Pore characteristics of sample C differ clearly from other samples with large and well-defined-shaped pores distributed evenly among smaller pores. Samples O25, O50 and O75 showed similar textures with rather small pores compared to the sample C whereas sample O100 showed not well-defined pore shapes. These results illustrate that oil replacement adverse the stabilization of air cells trapped during batter mixing, even at only 25% replacement.

Number of pores counted by Image-Pro Plus software (Fig. 6) showed agreeable results to the Fig. 4 where number of pores decreases with increasing oil content. It should be noted that number of pores of the sample O25 is similar to that of the control sample. As number of pores are literally number of air bubbles trapped within the cake batter, the number of pores depend on the physical properties of the batter (*e.g.* elasticity) [9]. Hence, it could be concluded that replacing butter with oil at only 25% did not significantly affect the ability of the batter to hold bubbles during baking. Nonetheless, at microscopic level, the texture of the solid parts of all cake samples is similar (Fig. 6).

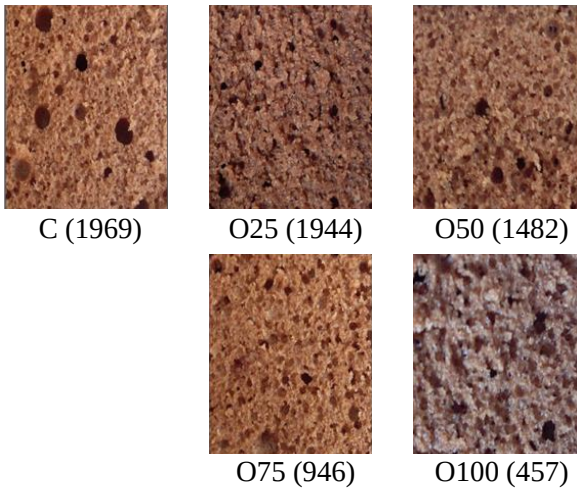

Fig. 4: Porosity of cakes (number of pores in the brackets)

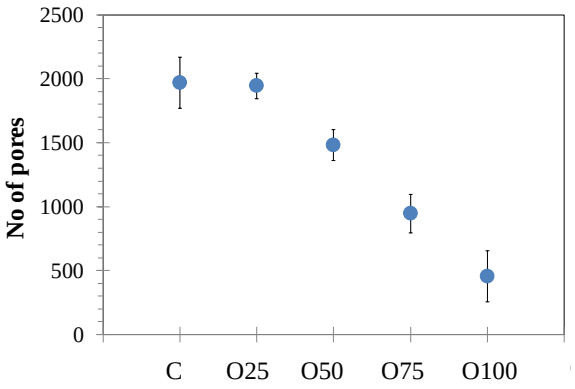

Fig. 5: Number of pores of the cake samples.

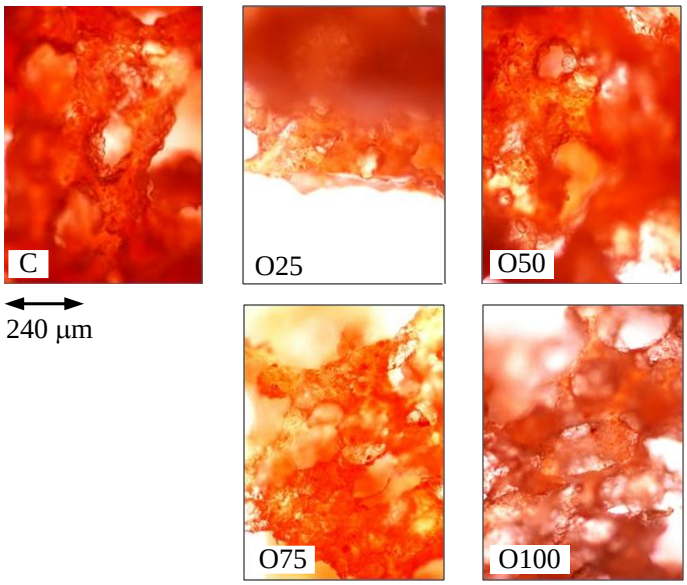

Fig 6. Micrographs of cake samples

Compressibility

Fig. 7 depicts compressibility test results of cake samples where samples O50 require the largest compression load before failure could be observed. The results at 10% and 50% strains (not shown) showed also the same trend as that in Fig. 7. Observing the samples after failure, it could be concluded that samples O50, O75 and O100 showed behaviours of brittle materials; clear breaking was seen particularly on the sides of cake samples. Samples C and O25, on the other hand, showed ductile behaviours where samples after the tests looked like smashed cakes without the sides bulging out. Moreover, after being compressed even at 50% strain, samples C and O25 were considerably thicker than the other samples.

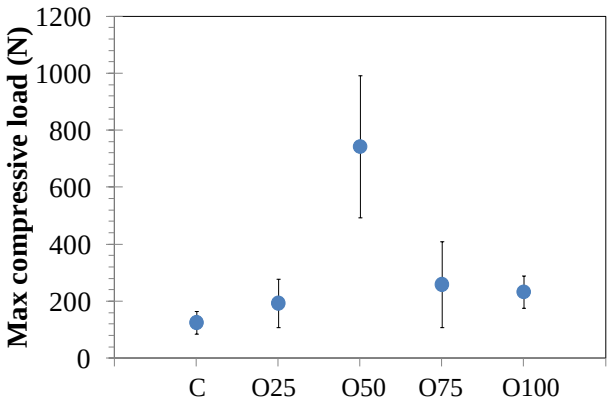

Fig. 7: Maximum compressive load of the studied cake samples.

Consider cake characteristics namely volume, and rising ratio, similar trend is observed from both characteristics. A linear relationship between the characteristics and oil content is observed initially and such relationship breaks off when the oil content is larger (replacement percentage larger than 50%) (Fig. 3) One explanation is that these properties are controlled by viscosity of cake batters. It has been reported that the cake batter viscosity is one of the factors controlling the final volume of the cake [13, 31]. Moreover, the rising rate of bubbles in the cake is commonly in inverse proportion to the viscosity [29]. Hence, lower viscosity due to an increase in oil content would result in fewer bubbles retained (less porous) and less voluminous cakes. This assumption was confirmed by the viscosity of cake batters measured using a rheometer. Fig. 8 shows shear stress-shear rate curves of the cake batters where viscosity could be extracted from slopes of the curve. The figure shows that viscosity of O25 cake batter was similar to that of the C cake batter at all shear rates.

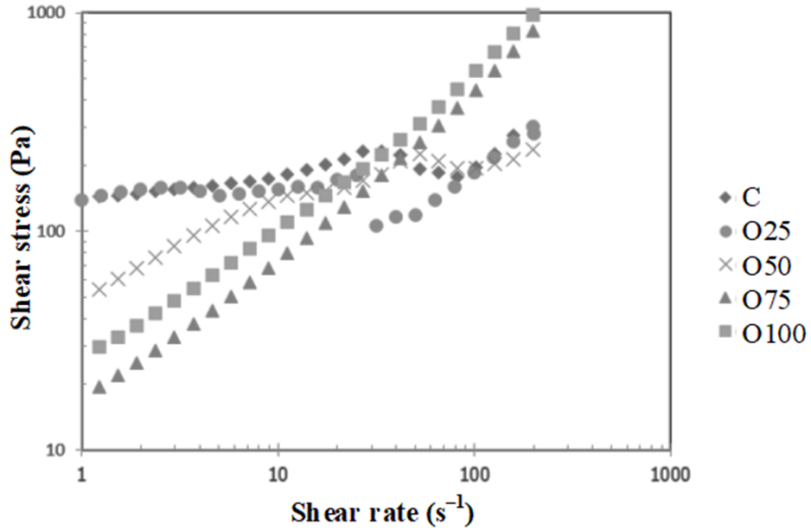

Fig. 8: Shear stress vs. shear rate of cake batters.

With aforementioned explanation, it is expected that the relationship between numbers of pores *vs*. the different oil contents would be similar to the trends observed for *V* and *R*. Nonetheless, Fig. 5 contradicts this expectation. This could be because the number of pores was reported in regardless of the sizes. For instance, number of pores counted from sample C and sample O25 were similar (Fig. 5) but there were less large bubbles in the latter (Fig. 4). Moreover, as discussed previously that only certain shapes of bubbles could be counted. This would be a reason why dramatically drop of number of pores in sample O100 was observed.

Sensory tests

In initial sensory test where all cake samples were tested, it was found that most panellists (50%) chose the all butter cake (Sample C). In contrast, the all oil cake (Sample O100) was the least favourite (7%) whereas the other cake samples were selected by similar number of panellists. The common reasons given by the panellists who chose sample C were the best texture and odour. The second sensory test was then conducted using only the samples that share similar popularity namely O25, O50, and O 75. From this test, O25 was selected the most (53.7%) and the popularity decreases with the increased oil content. Samples O50, and O75 were selected by 31.5% and 14.8% of panellists respectively. Statistical analysis showed that popularities of the three samples were different (α = 0.05). From both sensory tests, the sensory evaluation in terms of odour, texture (mouthfeel and visual observation), colour and taste, varied slightly between samples; on average, it can be concluded though that the sensory quality of cakes decrease with increasing oil content (Fig. 8). Base on all results, 25% of oil replacement was chosen for further modifications.

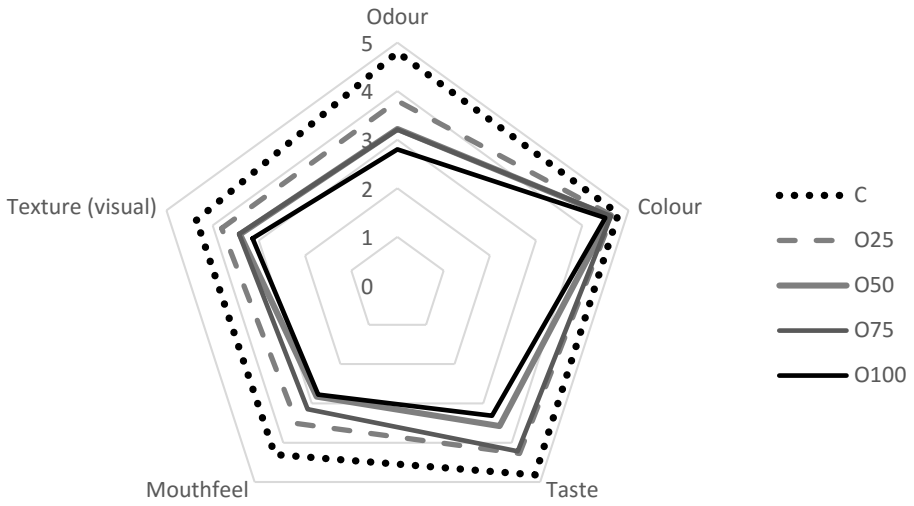

Fig. 9: Sensory tests of the studied cake samples

Modification stage

Optimization of mixing speed

As the resulting cakes made by using mixing speeds 2, 3, and 4 were similar; their volumes, rising ratios, and numbers of pores were similar (data not shown here). The two extreme mixing speeds, speeds 2 and 4, were selected for further investigation. The cake samples are coded Os2 and Os4 respectively.

Effects of mixing speed were investigated further as the higher speed was assumed to help trapping air into cake batter. So the amount of air bubbles in cake batter was measured. It was surprised to see that amount of air bubbles in all cake batters (including all butter cake) were similar except those made at mixing speed 4, where the amount of bubbles was almost tripled (Table. 2). So the assumption about the mixing speed is correct. However, comparing the resulting cake volumes and amounts of bubbles in cake batters, it could be concluded that the two variables were not closely related.

Table 2 Characteristics of cakes made by varying speeds and addition of emulsifier: Os2 and O24 are the cakes made by mixing at speeds 2 and 4 respectively whereas Os2E and Os4E are the cakes made with addition of emulsifier and mixing speeds 2 and 4 respectively.

Characteristics	**Control**	**Os2**	**Os4**	**Os2E**	**Os4E**
Rising ratio (*R*)	1.61±0.98	1.73±0.11	1.64±0.10	2.03±0.10	1.98±0.10
Volume (*V*)	1220±100	1160±150	1290±160	1330±130	1280±150
Bubbles (%v/v)	0.00014±0.00001	0.00024±0.00001	0.00097±0.00002	0.00027±0.00001	0.00098±0.00001
Moisture content (*W*)	0.27±0.002	0.23±0.002	0.24±0.002	0.25±0.002	0.25±0.002

Effects of emulsifier

To study effects of emulsifier, emulsifier was added to cake batters mixed at speeds 2 and 4 respectively; these samples are coded as Os2E and Os4E. According to Table 2, the Os2E and Os4E cakes showed similar rising ratio (~2, compare to 1.6 of control cake). However, addition of emulsifier could improve the volume of Os2E significantly. Without the emulsifier, the volume was 1160 but when the emulsifier was added, the cake volume was 1330. It was also observed that emulsifier addition could improve moisture content of oil cakes to the amount close to that of all butter cake.

Nevertheless, from compression test results, it was found that Os2E and Os4E were softer compared to all butter cake as shown in Table 3. According to the table, forces required to deform Os2 sample (this is O25 in the preliminary test) were the largest compared to other samples. This suggests that Os2 had the hardest texture

and was harder compared to the control cake. Since, Os4E was the softest with other properties not significantly different from the control cake (Table 2), the mixing speed 4 with addition emulsifier was selected as the best mixing condition for the cake with 25% butter replaced by oil.

Table 3 Compressive load (N) results

Samples	Compressive Extension		
	5 mm	10 mm	15 mm
Control	5.35±2.55	17.04±3.55	28.44±4.65
Os2	8.68±2.67	26.25±3.54	50.22±4.70
Os4	4.66±2.65	10.1±3.55	17.24±4.71
Os2E	3.57±2.60	8.72±3.60	15.84±4.75
Os4E	2.57±2.61	6.86±3.59	12.65±4.70

Sensory Test

Sensory tests were made on Os4E cake and the control cake; panellists were asked to evaluate the cakes based on odour, texture (visual observation), mouthfeel texture, colour and taste. Fig. 9 shows that Os4E cake was comparable to the all butter cake except for their odours. This was because Os4E cake sample had only a hint of oil smell.

Since most chocolate cake is covered with icing, another sensory test was conducted by putting icing on the all butter cake sample and Os4E sample. The panellists then were asked to select only one cake and it was found that the numbers of panellists choosing all butter cake and Os4E cake were similar (58% and 42%) and they are not different statistically (Paired difference test, α = 0.05). Hence, the less desirable odour of Os4E could be made insignificant by the chocolate icing.

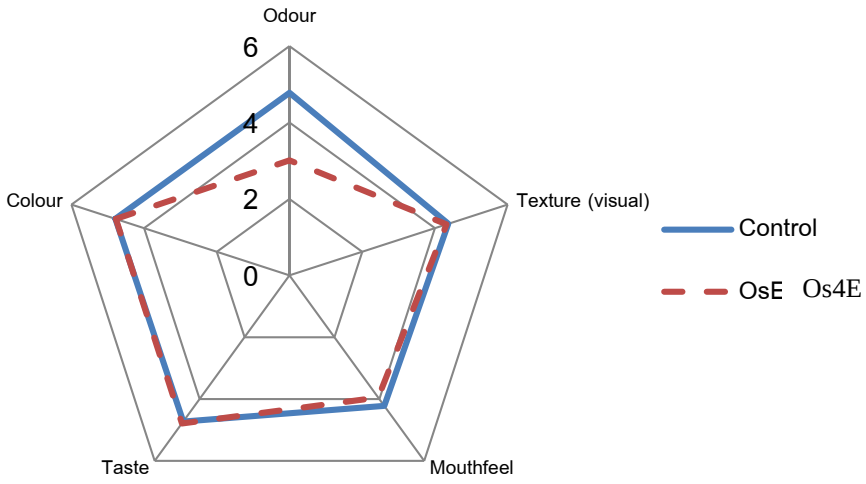

Fig. 10 Sensory tests of the control cake and modified cake (Os4E)

Possibility of modifying baking temperature

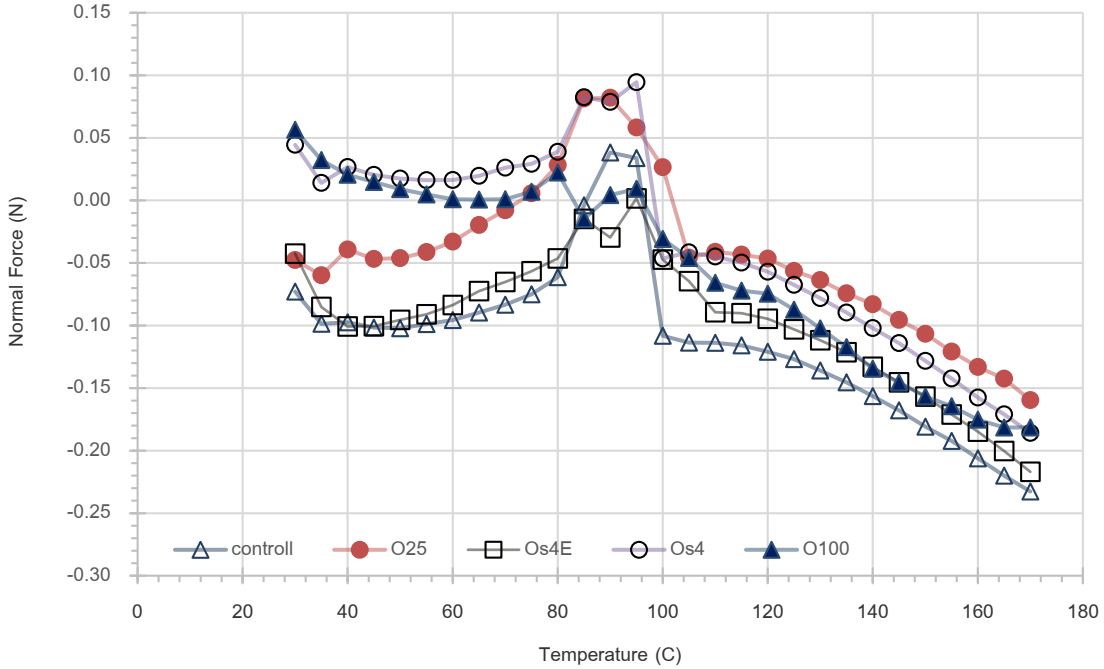

Fig. 11 Normal force measured by a rheometer during a temperature profile. Control-all butter cake; O25-cake with 25% butter replaced by oil; Os4-cake with 25% butter replaced by oil with mixing speed 4; Os4E-Os4 with emulsifier addition; O100-cake with 100% butter replaced by oil.

Normal forces applied onto cake batters under heating (temperature gradient in the range used in cake baking [27]) were measured to investigate transition in rheological behaviours during baking (Fig 11). All samples showed peaks around 85-95°C. The peaks correspond to the transition from being viscous to more crystallize that could be attributed to a combination of starch gelatinization and egg protein denaturation [15]. Comparing results of O25 and Os4, it can be concluded that mixing speed has little effect on such transition. Os4E had similar response to temperature to the control sample. This is probably why Os4E showed the closest properties to the control cake. The difference is that two peaks were observed from Os4E sample and they were lower than the peak observed with the control sample. This could be the reason why Os4E showed lower compressive strength compared to the control cake. Another difference is that on Os4E sample responded faster to the increasing temperature comparing to the control cake as the normal load increased faster at the beginning. This could be due to better heat transfer or simply different response due to different composition. This suggests possibility of changing baking temperature and baking time to improve quality of the resulted Os4E cake. This could be an area of future work.

Discussion and conclusion

Possibility of butter reduction in cake baking was investigated. First, cake characteristics and sensory tests were conducted on chocolate cake samples to investigate the effect of replacing butter with vegetable oil in all butter cake recipe. The cake characteristics measured were rising ratio, volume, number of pores and compressive strength of cake samples. It was found that these characteristics of oil-replaced cakes were not comparable to the butter cake and the difference tended to increase with increasing oil contents. This is in accord with sensory test where the butter cake was scored the highest in all aspects (odour, colour, taste, mouthfeel, texture (visual)). Nevertheless, the sample O25 showed characteristics closest to those of butter cake so it was selected for improvement. According to the sensory test, mouthfeel and odour of O25 had to be improved.

Hence, in the second part of the investigation, modification of cake making process was studied focusing at improving mouthfeel of the cake. According to the results in the preliminary stage, amount of bubbles in cake batters during baking seemed to be the major cause of difference in properties of O25 cake compared to the butter cake. Therefore, effects of mixing speed and emulsifier addition

were explored. It was found that mixing speed could increase the amount of air incorporated in cake batter However, the increased air bubbles had no significant effect on the quality of the resulted cake. This could be because oil cake batters were less viscous compared to the butter cake batter. Consequently, the incorporated air bubbles could not be retained during cake baking [12, 28, 31]. Emulsifier addition, on the other hand, could improve cake's qualities (compressive strength, moisture content, volume, rising ratio). This could be because the added emulsifier helped stabilize the cake batter [10, 14]. The optimized condition of mixing was found to be using high mixing speed with emulsifier addition (Os4E sample).

The possibility of changing baking temperature was also investigated using rheological response of cake batter to temperature. It was observed that the response of Os4E sample was closely similar to that of the control cake batter. As final bakery product qualities are related to rheology of the product mixture before baking [13-15], the similar rheological response to temperature of Os4E and control batters could explain the similar characteristics of the Os4E and control cakes. Nevertheless, the slight difference in the rheological response to temperature suggests possibility of changing baking time and baking temperature in the future work.

Acknowledgments

This research was supported by research grant from Thammasat University. Advice and suggestions from Assoc.Prof.Dr. Cattaleeya Pattamaprom are also greatly appreciated.

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