



CBIC
2019



21 **MFLU.**
YEARS

CONFERENCE PROCEEDINGS

**COSMETIC AND BEAUTY
INTERNATIONAL CONFERENCE 2019**
: SUSTAINABLE COSMETIC & BEAUTY INNOVATIONS



7-9 OCTOBER 2019
SCHOOL OF COSMETIC SCIENCE
MAE FAH LUANG UNIVERSITY
CHIANG RAI, THAILAND

President's Welcome Message



On behalf of Mae Fah Luang University, I would like to express a very warm welcome to all participants of Cosmetic and Beauty International Conference 2019.

The year 2019 is yet another special year for Mae Fah Luang University (MFU). It is now the 21st anniversary of MFU establishment. Since 1998, MFU has been operating under core philosophy of Her Royal Highness Princess Srinagarindra, to sustain her legacy of nurturing nature and to further develop people in rural areas.

The School of Cosmetic Science is one of Thailand's leading school in the field. It is the 1st established School in Thailand to fully provide courses solely on Cosmetic science. They have many academic work and also cosmetic products and patents that were translated from their research. And in this conference, I am glad to see the sustainable topic being brought up. Because as you all know, the world is changing and resources are becoming scarce. So shifting the paradigm towards a more ecofriendly industry is a certain interest for everyone, also the cosmetic field. It would be pleasant for both humans and the environment to be pretty at the same time.

I believed that this conference will be a great platform for participant to connect and form future collaboration within the research of cosmetic science fields. The network of scientists is a valuable asset that helps the development science advancement in your fields. It is clear from the number of participants and the number of paper submitted to the conference, that you all have made great progress in field of work and I am confident that you will have a successful conference.

Lastly, I would like to thank you, all of the participants for attending this event. And I hope you all a successful meeting and enjoy the many opportunities and beauty that Mae Fah Luang University has to offer.

Sincerely Yours,

Assoc. Prof. Chayaporn Wattanasiri, Ph.D.

President of Mae Fah Luang University

Conference Chair's Welcome Message

Dear Friends and Colleagues,

On the behalf of the Cosmetic and Beauty International Conference 2019 organizing committee, I am honored and delighted to welcome you all to this special event that is organized by School of Cosmetic Science, Mae Fah Luang University, Chiang Rai, Thailand. This conference highlights the utilization of knowledge for creation of sustainable cosmetic and beauty innovation. It consists of several interesting talks by famous keynote speakers and a handful of oral and poster research presentations from the participants. In addition, there are also workshops that related to make-up trend and technique, fragrance creation and innovation regarding cosmetic ingredients that could provide up-to-date knowledge to you.

We encourage all participants to take this opportunity to strengthen their knowledge in the fields, share their ideas and collaborate with other researchers from around the world in this extraordinary CBIC2019 event.

I would like to take this opportunity to thank our delightful sponsors for their great collaboration and supports; to the speakers for sharing their valuable experiences; to the reviewer for their scientific supports and to all the committee for their hard work in supporting and organizing this event very well.

Lastly, I would like to sincerely thank all of the conference participants and hope you have a memorable time here in the beautiful Northern City of Thailand, Chiang Rai, and in one of the most beautiful university in the world, Mae Fah Luang University.



Best regards,

Assist. Prof. Phanuphong Chaiwut, Ph.D.

Conference Chair, CBIC2019 Organizing Committee

Dean, School of Cosmetic Science

Mae Fah Luang University
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Development of carboxymethyl chitosan hydrogel patch containing sodium ascorbyl phosphate as skin care cosmeceutical delivery system

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Abstract

This study was aimed to develop carboxymethyl chitosan (CMCS) hydrogel patch containing sodium ascorbyl phosphate (SAP), and evaluate the stability as well as clinical evaluation. CMCS was combined with copolymers including methyl cellulose, hydroxyethyl cellulose, and ethyl acrylate-methyl methacrylate copolymer dispersion. The various ratios of CMCS and each copolymer were set up together with a combination of plasticizers (propylene glycol, triethyl citrate and glycerin) for preparing hydrogel patches by casting method. The physical appearances, mechanical properties, and stability of the hydrogels were characterized in various conditions. The results showed that formulation D3 (polymers in the ratio of 4:0.5:0.5:1 and plasticizers in the ratio of 2:1:1) containing 1% SAP (D3SAP) that presented the most suitable physical appearances, the tensile strength was 2.62 N/mm², elongation at break was 94%, and adhesion was 47.58 mm. After storage at 4°C, 45°C, room temperature with and without light for 90 days and also by heating-cooling cycling, it was not significantly different from day 0 analyzed by t-test ($p < 0.05$). In addition, the D3SAP also exhibited a higher *ex vivo* permeation of SAP than conventional gel (1% SAP) in the skin layers and receiving chamber from pig skin model. The amount of SAP in stratum corneum, viable epidermis and dermis of the D3SAP were 12.60±0.18 % and 22.94±0.22 %. The percentage cumulative amounts of SAP of D3SAP in receptor chamber after 8 h as 16.43±1.92. The D3SAP patch or commercial patch was applied on the forearm of 30 volunteers. Skin moisture, pigment and wrinkles parameters were analyzed after applying for 30 days. Skin irritation was also tested on the back. The skin moisture, pigmentation, and wrinkles were significantly different after applying the D3SAP ($p < 0.05$) and no skin irritation. Therefore, the CMCS hydrogel patch containing 1% SAP could be a promising cosmeceutical delivery system for skin care.

Keywords: Hydrogel patch; Carboxymethyl chitosan; Sodium ascorbyl phosphate; Cosmeceutical delivery system; Skin care

1. Introduction

Transdermal delivery system can increase the permeation of an active ingredient through the skin. Different strategies have been proposed to achieve efficient delivery system for cosmetic formulations. Hydrogel patch has been considered as a good choice for this purpose because of the three dimensional, hydrophilic and cross-linked networks of polymers (Patravale and Mandawgade, 2008). It is biocompatible, low-toxicity and able to hold a large amount of water when fully hydrated. Hydrogel is designed for many advantages over conventional cosmetics by reducing the frequency of application and delivering the active ingredients into a specific area of the skin. The occlusive effect of hydrogel can improve the moisture of stratum corneum, increase the delivery of active ingredient through the skin (Peppas *et al.*, 2000; Sang *et al.*, 2013).

Polymers are the backbone of hydrogel preparations. Suitable hydrogel is formulated from biocompatible polymers such as natural polymers or modified natural polymers rather than synthetic polymers. Chitosan is a naturally derived cationic polymer used for hydrogel preparation because of its unique properties such as biodegradability, biocompatibility, nontoxicity and also a good film former (Patravale and Mandawgade, 2008; Boriwanwattanak *et al.*, 2008). Due to their hydrophilic nature and great solubility in acidic medium, chitosan hydrogel exhibits low mechanical properties and limited ability to control the release of active ingredients into the skin (Marguerite, 2006). Therefore, a water-soluble chitosan derivative has been synthesized by carboxymethylation reaction to obtain carboxymethyl chitosan (Giri *et al.*, 2012; Jimtaisong and Saewan, 2014). It shows the similar properties to chitosan but is more suitable for use in cosmetic formulations. L-ascorbic acid (vitamin C) is a strong antioxidant and well-known as a natural controller of melanin formation (Choi *et al.*, 2014). It interferes the melanin production in melanogenesis process by interacting with copper ions at the tyrosinase enzyme activity site and reducing dopaquinone formation. Tyrosinase is a copper containing glycoprotein which is the rate limiting enzyme in the melanogenic pathway and the lack of the copper resulting to its inactive intermediates (Marta *et al.*, 2005). Philippe *et al.* (2003) reported that the topical application of 5% ascorbic acid could be used for prevention and correction of skin aging in 20 female volunteers. However, use of ascorbic acid in cosmetic products is limited by its low stability due to its be simply oxidized and inactivated when exposed to light. Previous study also showed that sodium ascorbyl phosphate (SAP) and magnesium ascorbyl phosphate are more stable than ascorbyl palmitate and may be easily used in cosmetic products (Segall and Moyano, 2008). SAP is a hydrophilic derivative of ascorbic acid which has chemically modified structure (Spiclin *et al.*, 2003). Phosphate group in the SAP structure is located in the second position on the cyclic ring which protects the enediol system of the molecule against oxidation, so its salts cannot act as an antioxidant agent to stabilize formulations (Hira *et al.*, 2016). From technical data of SAP described by BASF, it indicated that SAP is cleaved enzymatically in the skin to release active ascorbic acid and protects the cells against damage caused by free radicals. In previous study, multiple emulsions loaded the combination of ascorbyl palmitate and SAP could reduce facial skin melanin and erythema (Hira *et al.*, 2016). Consequently, a stable ascorbic acid derivative as SAP has been widely used for cosmetic preparation. Therefore, this study aims to develop hydrogel patch using CMCS as a polymer base containing SAP as active ingredient. The SAP hydrogel patch was investigated for physical properties, mechanical properties and also its stability. In addition, *ex vivo* skin permeation, primary skin irritation and clinical trial test of carboxymethyl chitosan hydrogel patch containing sodium ascorbyl phosphate were also investigated.

2. Materials and Methods

2.1 Materials

Carboxymethyl chitosan derived from shrimp with a molecular weight in the range of 10,000-30,000 Da and higher 90% degree of deacetylation was obtained from Associated Professor Dr. Pornchai Rachtanapun, Faculty of Agro-industry, Chiang Mai University, Chiang Mai, Thailand. Sodium ascorbyl phosphate was purchased from Hoffmann-La Roche Inc. (Germany). Methyl cellulose 4000 (MC), hydroxyethyl cellulose (HEC), propylene glycol (PG), glycerin (GC), acetonitrile, and sodium lauryl sulfate (SLS) were purchased from RCL Labscan Co., Ltd. (Bangkok, Thailand). Triethyl citrate (TEC) was purchased from Merck Co., Ltd. (Germany). Ethyl acrylate-methyl methacrylate copolymer dispersion (Ea) was purchased from Evonik nutrition and care GmbH (Germany). Finn chambers[®] was purchased from SmartPractice Phoenix, AZ. (U.S.A.).

2.2 Preparation of carboxymethyl chitosan hydrogel patches

The hydrogel patches were prepared by mixing of polymers (CMCS, MC, HEC and Ea) and plasticizers (PG and TEC) in various ratios using casting technique. The components of each formulation are shown in Table 1. The plasticizer blend was fixed into the formulation to control the elasticity of hydrogel patch. Firstly, CMCS was dispersed in deionized water and then other polymers were added to the formulation. The polymers and plasticizer were mixed with a magnetic stirrer for 1 h and air bubbles were removed by sonication at room temperature for 15 min. The formulation was poured on a smooth surface and dried at 45°C in a hot air oven for 8 h. Hydrogel patches with good physical appearance were selected for further characterization and loaded with 1% SAP by dissolving in DI water and adding into the base formulation.

Table 1 Compositions of hydrogel patches with different ratios of polymers and fixed plasticizers.

Formulation	A1	A2	A3	B1	B2	B3	C1	C2	C3	D1	D2	D3
Polymer ratios	1	2	4	1	2	4	1	2	4	1	2	4
(CMCS: MC: HEC: Ea)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
	1	1	1	1	1	1	1	1	1	1	1	1
Plasticizer (%)												
PG	5	5	5	5	5	5	10	10	10	5	5	5
TEC	5	5	5	-	-	-	10	10	10	5	5	5
GC	-	-	-	5	5	5	10	10	10	10	10	10

CMCS = carboxymethyl chitosan, MC = methyl cellulose 4000, HEC = hydroxyethyl cellulose, Ea = ethyl acrylate-methyl methacrylate copolymer dispersion, PG = propylene glycol, TEC = triethyl citrate and GC = glycerin.

2.3 Characterization of hydrogel patches

2.3.1 Physical appearances

Physical appearances of the hydrogel patches such as color, transparency and flexibility were assessed by visual inspection.

2.3.2 Mechanical properties

2.3.2.1 Tensile strength and percentage of elongation at break

A Texture analyser (TA.XT. plus, Stable micro systems Ltd., Surrey GU7 1YL, UK) was used for measuring ultimate tensile strength and percentage of elongation at break of the hydrogel patches. The hydrogel patch (20 mm×50 mm) was fixed between two clamps (50 mm) of the apparatus. The rate of clamp separation was 20 mm·min⁻¹. Each formulation was tested in triplicate. The tensile strength and percentage of elongation at break were calculated following these equations (Preeti and Keshavayya, 2010).

$$\begin{aligned}\text{Tensile strength} &= \text{Breaking force (N)} / \text{Cross-sectional area of sample (mm}^2\text{)} \\ \text{Elongation at break (\%)} &= [\text{Increase in length at breaking point (mm)} / \text{Original length (mm)}] \times 100\end{aligned}$$

2.3.2.2 Adhesion

Adhesion of hydrogel was determined by texture analyzer. The adhesive force was measured ten times using a holder of 50 mm diameter at 25°C, 55% relative humidity. The contact time of the holder and the sample surface was set to 1 sec (Preeti and Keshavayya, 2010).

2.4 Stability of the hydrogel patches

Stability of hydrogel patch and CMCS hydrogel patch containing SAP was studied by storage under accelerated conditions including heating cooling cycling (45°C 48 h and 4°C 48 h for 6 cycles), 4°C, 45°C, and room temperature (30±2°C) with 75% humidity for 90 days. Thereafter, the tested hydrogel patches were characterized for physical and mechanical properties. The SAP content in the patch was also determined by high performance liquid chromatography (Boriwanwattanakarak *et al.*, 2008).

2.5 High performance liquid chromatography analysis of SAP content in hydrogel patch containing SAP

The patch was cut into 10×10 mm² rectangles and was accurately weighed and dissolved in DI water. Then, it was stirred with a magnetic stirrer at room temperature (25±2°C) for 24 h and then sonicated in an ultrasonicator bath at room temperature (25±2°C) for 2 h. The obtained solution was filtered, and the amount of SAP was determined by HPLC using Agilent HP 1100 (Hewlett Packard, Waldbronn, Germany) and reverse phase C-18 column (Inertsil ODS-35 µm 4.6×250 mm). The mobile phase consisted of acetonitrile and 0.3 M phosphate buffer pH 4 (ratio of 40:60), the flow rate 0.8 mL/min, injection volume 20 µL and UV detection was set at 258 nm. Chemical content analysis was performed in triplicate (Spiclin *et al.*, 2003).

2.6 Ex vivo skin permeation study of hydrogel patch containing SAP

The skin permeability of SAP from CMCS hydrogel patch containing SAP (D3SAP) and conventional gel (CG) in the same concentration of SAP were determined using Franz-diffusion cells. Stillborn dorsal piglet skin was used in the experiment. It was removed fat and was equilibrated in PBS solution pH 6.0 before experiment and mounted on Franz-diffusion cell. The D3SAP was cut into the circular shape (diameter 4 cm) weight 0.56 g and placed on the piglet skin while the CG was weighted 1 g and added into the donor chamber. PBS buffer solution volume of 13 mL was controlled at 32±0.5°C by the flow water in tube and under stirring at 500 rpm in the receptor chamber. One milliliter of receptor solution was collected at various times (1, 3, 6 and 8 h) and was replaced with the same volume of fresh buffer. The sample from the receptor was analyzed for the SAP content by HPLC. After 8 h, the dorsal piglet skin was rinsed with a PBS buffer. The stratum corneum was separated from the skin using

a tape stripping technique and the viable skin layer was cut into small size. The tapes and the viable skin layer were separately extracted with deionized water using ultrasonic bath for 15 min and then the SAP content was analyzed by HPLC (Boriwanwattanak *et al.*, 2008; Spiclin *et al.*, 2003; Preedalikit, 2012). The permeation of SAP from D3SAP was calculated and compared with SAP in CG.

2.7 Primary skin irritation testing of hydrogel patches containing SAP in human volunteers

The human protocol for efficacy testing was approved by the ethics committee of the Faculty of Pharmacy, Chiang Mai University (permission number 019/2561). The design of this study was a randomized and controlled trial. One percent sodium lauryl sulfate (SLS) was used as a positive control and untreated skin was used as a negative control. Each sample was applied to a Finn chamber[®] and placed on the back of 30 healthy volunteers age between 20-60 years old with no dermatitis or allergy history. The Finn chambers[®] was removed after 48 h and the back was cleaned with water. The tested areas were observed for erythema and edema reactions at 1, 24, 48 and 72 h after patch removal. The skin reactions were scored based on the Draize scoring system and calculated the primary irritation index (PII) to classified the degree of irritation (OECD guildlines, 2002).

2.8 Efficacy testing of hydrogel patches containing SAP in human volunteers

The human protocol for efficacy testing was approved by the ethics committee of the Faculty of Pharmacy, Chiang Mai University (permission number 019/2561). Thirty healthy volunteers, 20-60 years old with no dermatitis and no history of allergy were enrolled after signing the informed consent. The hydrogel patches were applied for 20 min once a day. On the left forearm, the D3SAP was placed on the area 2 inches from the elbow where the commercial anti-aging and skin whitening hydrogel patch (commercial A) was placed on the area 2 inches from the wrist. On the right forearm, the untreated skin at the same area as samples were used as control (untreated skin for D3SAP, untreated skin for commercial A). Moisturizing efficacy was evaluated by Corneometer[®] CM 825, skin pigmentation was evaluated by Mexameter[®] MX 18 and skin wrinkles was tested by Skin-Visiometer[®] SV 700 at before and after 30 days of application (All instruments supplied by Courage + Khazaka electronic GmbH, Cologne, Germany). The percentage efficacy of hydrogel patch formulations was calculated at day 14 and day 30 by comparing to baseline following equation: % Efficacy = [(After-Before) / Before] × 100

3. Results and discussion

3.1 Preparation and characterization of hydrogel patches properties

Various composition of hydrogel patches were prepared and then characterized as follow.

3.1.1 Physical appearances

Various types of polymers (CMCS, MC, HEC and Ea) at different ratios were combined to optimize for the best composition based on physical appearances. Their physical appearances presented the homogeneous and smooth surfaces. The formulation A1-A3 showed transparency, thin film with lower flexibility than other formulations due to the property of MC and the lowest plasticizers content. In contrast, the formulation B1-D3 consisting of glycerin combined with PG and TEC were slightly opaque and more flexible. Therefore, the flexibility of the patch depended on the ratio of polymers and plasticizers. The formulation consisting of CMCS, MC, HEC and Ea in the ratio of 4:0.5:0.5:1 (formulation D3) showed the most flexibility due to the high percentage of CMCS which potentially

increase the number of the hydrogen bonds with water in polymer network. This along with the higher level of plasticizers may lead to a more flexible and soft polymeric structure of the patch (Boriwanwattanakarak *et al.*, 2008; Preedalikit, 2012).

3.1.2 Mechanical properties

3.1.2.1 Tensile strength and percentage of elongation at break

Because of the good physical appearances of formulation D1-D3 including color, thickness, transparency and flexibility, they were selected to determine their mechanical properties. Strength and elasticity of the patches were determined by the texture analyzer in terms of tensile strength and percentage of elongation at break. The results are shown in Figure 1. Formulation D1 had a higher tensile strength (3.96 ± 0.14 N/mm²) than formulation D2 and formulation D3. The formulation D1 consisted of CMCS, MC, HEC and Ea in the ratio of 1:0.5:0.5:1. This may lead to bridges among the polymer chains via hydrogen bonding that increased the mechanical strength. However, formulation D3 with higher ratio of CMCS, showed the highest percentage of elongation at break and presented the most flexibility. This may be due to the more hydrophilicity of CMCS leading to a more flexibility network structure (Jimtaisong and Saewan, 2014; Guo *et al.*, 2014).

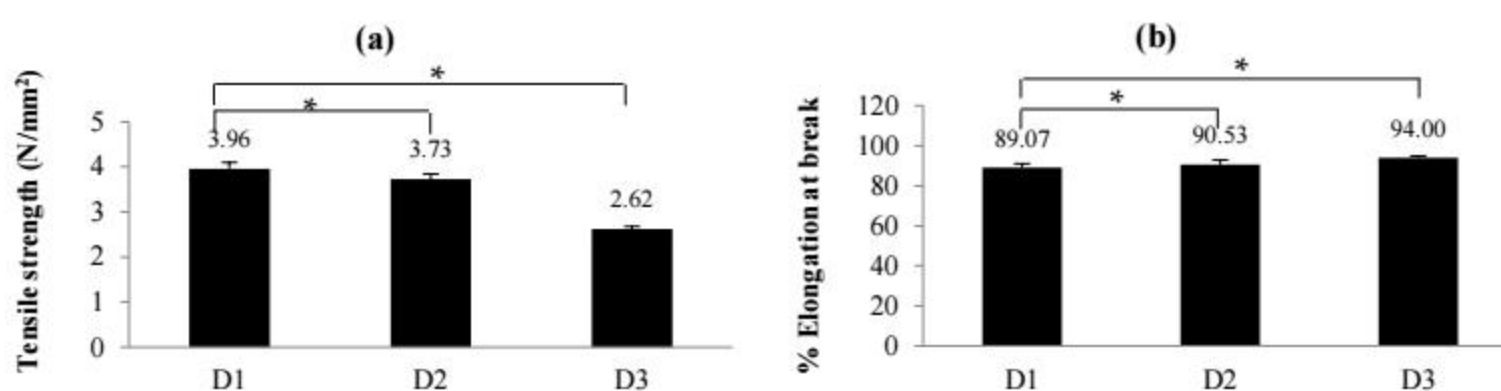


Figure 1 Mechanical properties of the hydrogel patches formulation D1-D3: (a) Tensile strength (N/mm²); and (b) percentage of elongation at break. Each value represents the mean $\pm$ s.d. (n=3). * indicates a significant difference of each formulation ($p < 0.05$)

3.1.2.2. Adhesion

In the measurement using texture analyzer, the distance value between the probe and the patch is a parameter used as an indicator of the adhesion property of the CMCS hydrogel patches. The patch was stuck between the stage and measuring probe. When the probe contacts with the patch surface a force of 10 N was applied. The pullback distance of the probe was then measured. The longer the distances were more the adhesiveness of the patch. The adhesion of the formulation D1-D3 increased with the increasing ratio of CMCS. The formulation D3 with the highest CMCS content showed the highest adhesion followed by formulation D2 and D1, respectively (Figure 2). CMCS is a polymer base with good bioadhesive property due to the interaction between its carboxyl groups and negative charge on the skin. The formulation containing a higher ratio of CMCS showed a lower tensile strength (Figure 1a) but resulted in the increasing of adhesion and percentage of elongation at break. The adhesion of the CMCS hydrogel patches also related to the amount of plasticizers. The increasing amount of plasticizers from formulation A to formulation D also increase the adhesion leading to better

adhesiveness but decreased the tensile strength of the patch (data not shown). Therefore, formulation D1-D3 with 20% of plasticizers (5% PG, 5% TEC and 10% GC) were focused. The increasing flexibility of CMCS hydrogel patches could improve the contact between the patch and the skin. Finally, the formulation D2 and D3 were selected for loading with 1% SAP and further investigated due to the optimum of tensile strength, flexibility and adhesion.

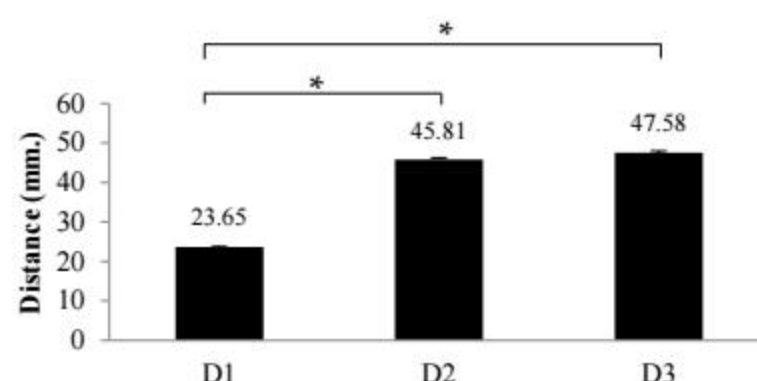


Figure 2 Adhesion of the hydrogel patch formulation D1-D3. Each value represents the mean $\pm$ s.d. (n=3). * indicates a significant difference of each formulation ($p < 0.05$).

3.2 Stability of carboxymethyl chitosan hydrogel patch containing sodium ascorbyl phosphate

The CMCS hydrogel patch formulation D2 and D3 containing SAP (D2SAP and D3SAP) were stored under various conditions to study the stability. It was found that both formulations were stable in all conditions as shown in Figure 3. The tensile strength of the D2SAP at room temperature significantly increased but it was significantly decreased compared to day 0 at 45°C ($p < 0.05$). The tensile strength of the D2SAP increased from 4.14 ± 0.06 N/mm² (day 0) to 4.66 ± 0.06 N/mm² after storage at room temperature for 90 days and decreased to 3.73 ± 0.11 N/mm² after storage at 45°C for 90 days. The percentage of elongation at break was related to the results of tensile strength. The percentage of elongation at break of the D2SAP at room temperature and 45°C showed significant difference from day 0 ($p < 0.05$). The percentage of elongation at break of the D2SAP increased from 95.80 ± 1.20 to 99.27 ± 1.55 after storage at room temperature for 90 days and decreased to 90.53 ± 2.47 after storage at 45°C for 90 days. The adhesion of the D2SAP was significantly different from day 0 ($p < 0.05$) after storage at 4°C and 45°C. The adhesion of the D2SAP increased to 24.50 ± 0.40 mm and 25.37 ± 0.40 mm after storage at 4°C and 45°C for 90 days, respectively. The SAP content of the D2SAP presented a significant difference from day 0 ($p < 0.05$) after storage at 45°C. In contrast, the D3SAP was more stable than the D2SAP for all tested parameters after 90 days that showed no significant difference from day 0 ($p < 0.05$). This may be due to the higher ratio of CMCS in the D3SAP leading to enhanced film forming ability and more flexible gel network that was better tolerated to temperature change (Boriwanwattanak *et al.*, 2008; Parente *et al.*, 2015).

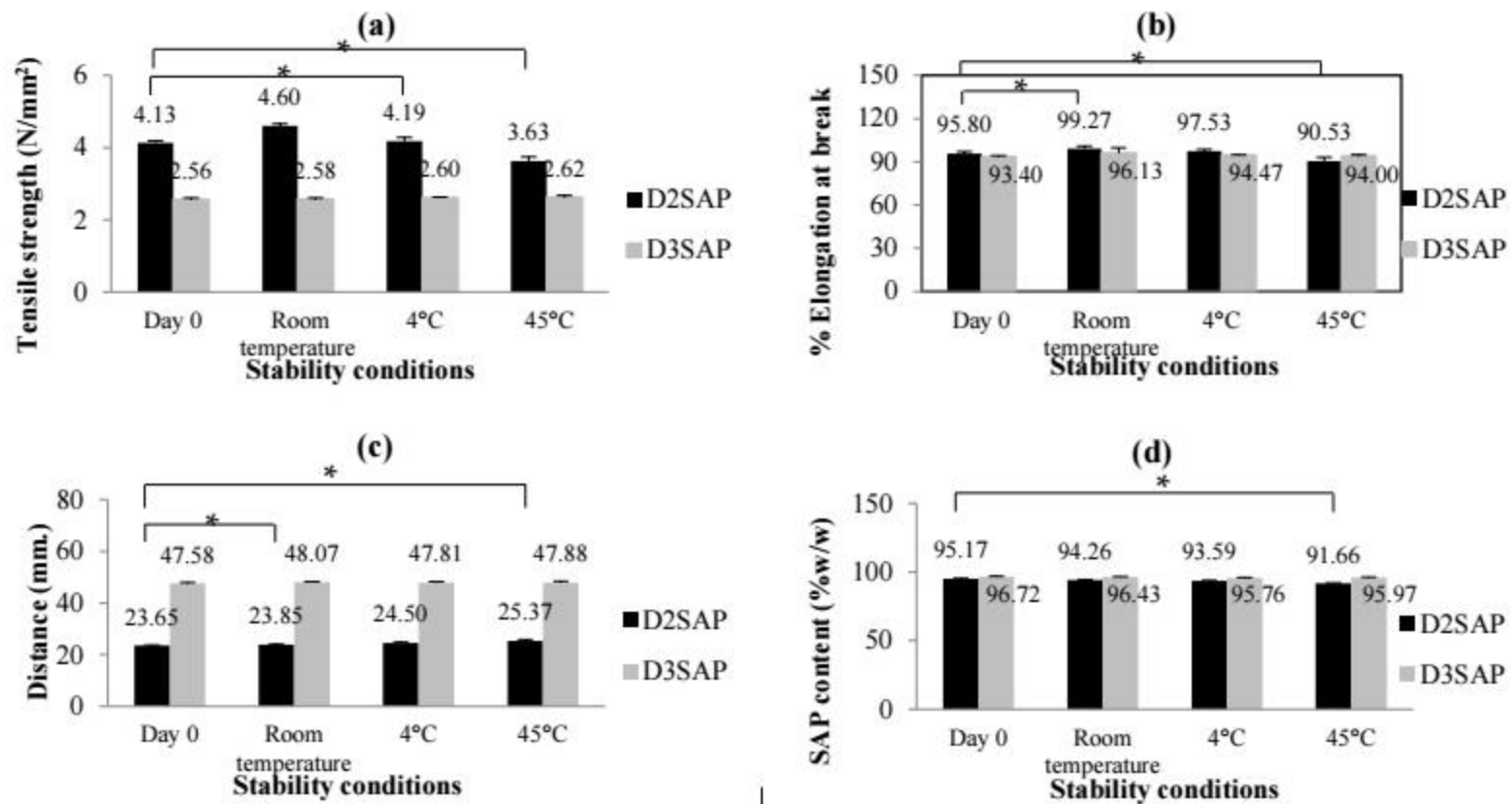


Figure 3 Characterization of carboxymethyl chitosan hydrogel patches containing sodium ascorbyl phosphate after storage at different conditions for 90 days: (a) tensile strength; (b) percentage of elongation at break; (c) adhesion property; and (d) SAP content. Each value represents the mean $\pm$ s.d. (n=3). * indicated a significant different from day 0 of each formulation ($p < 0.05$)

3.3 *Ex vivo* permeation of hydrogel patch and gel containing sodium ascorbyl phosphate

Ex vivo permeation of SAP from the D3SAP (1% SAP) and the conventional gel or CG (1% SAP) were evaluated in terms of the cumulative amount of SAP in stratum corneum, viable epidermis and dermis and receptor solution at 1, 3, 6 and 8 h. The skin permeation was studied using Franz diffusion cells, the patch or conventional gel was mounted over newborn dorsal pig skin as mentioned above that has been reported to represented similar structural and biochemical characteristics to human skin (Avon *et al.*, 2005; Tory *et al.*, 2001; Ana and Frederick, 2009). The use of human skin was limited and depended on the international ethical considerations, so animal skins were used as a replacement for human skin. Previous study reported that the pig skin was a good model to replace of human skin in the permeation study because the time correlations permeated between pig and human skins were not significantly different (Ana and Frederick, 2009). Francesco *et al.* (2007) also mentioned that stratum corneum of pig skin is the most similar to human stratum corneum in term of lipid composition, the thickness of newborn pig stratum corneum is considerably thinner than adult pig and is more similar to that of human skin. Therefore, the newborn pig skin was selected for this study. The human skin pH is normally in the values of 4-6 and also SAP is the most stable at pH 6.0 (Rippke *et al.*, 2002; Saba *et al.*, 2013). So, phosphate buffer was used as a medium. The results are shown in Figure 4. The D3SAP and the CG exhibited the percentage cumulative amounts of SAP in receptor chamber after 8 h as 16.43 ± 1.92 and 7.60 ± 1.08 , respectively. The amount of SAP in stratum corneum, viable epidermis and dermis of the D3SAP were 12.60 ± 0.18 % and 22.94 ± 0.22 % whereas the CG showed as 0.27 ± 0.03 % and 2.57 ± 0.29 %. The results indicated that the percentage amount of SAP permeated into skin layers from the D3SAP was higher than that of the CG. This might be due to the polymer network in the patch and penetration enhancing property of propylene glycol in the formulation, while the conventional gel

containing only gelling agent and water. Moreover, possible reason was the strong interaction between cationic of CMCS and hydroxyl groups in HEC that can form network structure and may acts as a controllable release of SAP. Furthermore, the amphoteric charge of carboxymethyl chitosan helps to deliver SAP into the skin (Jimtaisong and Saewan, 2014). The results correspond with previous studies reporting that polymer in the formulation improved active ingredient delivery (Spiclin *et al.*, 2003; Guo *et al.*, 2014). In addition, the penetration enhancer can disturb the stratum corneum barrier and increase the diffusion coefficient of active substance into the skin.

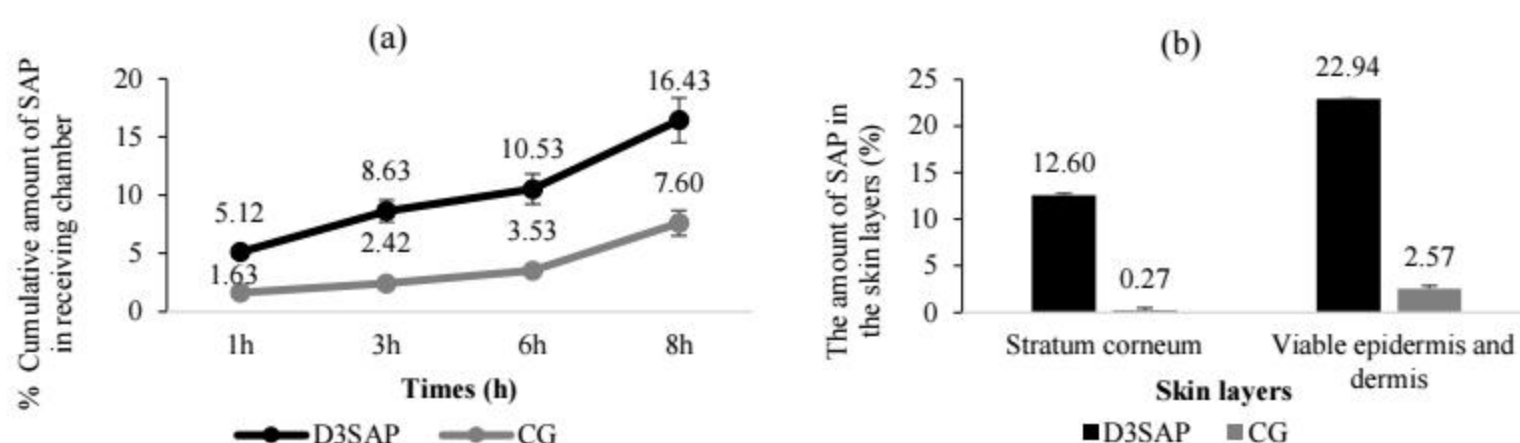


Figure 4 Percentage amount of SAP permeated through dorsal piglet skin from the D3SAP and the CG after 8 h.: (a) the percentage of cumulative amount of SAP in receiving chamber at various time; and (b) the amount of SAP (%) in stratum corneum, viable epidermis and dermis. Each value represents the mean $\pm$ s.d. (n=3).

3.4 Primary skin irritation test of hydrogel patch containing sodium ascorbyl phosphate in human volunteers

Primary skin irritation was tested on 30 human volunteers using Finn chambers[®]. The score of skin reaction and the type of skin irritation were classified by the Draize scoring system and primary index irritation (PII) values. The CMCS hydrogel patch formulation D3 containing SAP (D3SAP) induced a score of 0.5 skin reaction only in one volunteer. The primary index irritation value of positive control (1% w/v SLS) was classified as slightly-irritation (PII=0.85) whereas the PII value of negative control (bare skin) showed non-irritation (PII=0). According to all data, the primary index irritation value of the D3SAP was classified as non-irritating (PII=0.02), indicating that it is safe to apply on the skin.

3.5 Efficacy test of hydrogel patch containing sodium ascorbyl phosphate in human volunteers

The efficacy of hydrogel patch formulation D3 containing sodium ascorbyl phosphate was measured on human volunteers using the Corneometer[®], Mexameter[®] and Skin-Visiometer[®]. The skin moisture content, melanin content, erythema and surface texture of treated skin (left forearm) and untreated skin (right forearm) were compared before and after 30 days of patch application. The Corneometer[®] results demonstrated that the skin surface moisture content was significantly increased after using the D3SAP for 14 and 30 days whereas no change occurred in the untreated skin. The moisture content of D3SAP was also increased more than commercial A, as shown in Table 2. The Mexameter[®] results showed that skin pigmentation (melanin) and erythema were significantly

decreased after using the D3SAP for 14 and 30 days whereas no change occurred in the untreated skin. Moreover, the D3SAP could decrease melanin content more than the commercial A (Table 2). For skin wrinkles reducing capacity, the Skin-Visiometer® results showed that the D3SAP could be significant decrease in skin wrinkles after 14 and 30 days whereas no change occurred in the untreated skin (Table 3). The D3SAP was more effective than the commercial A. From these results, it can be concluded that the hydrogel patch formulation D3SAP exhibited good skin moisturizing, skin brightening and skin wrinkle reducing effects. In addition, the D3SAP patch that showed good adhesion and better deliver SAP into the specific area from the above mentioned permeation study affect to increase its effectiveness compared with day 0 and untreated skin (Jimtaisong and Saewan, 2014; Hira *et al.*, 2016).

Table 2 Efficiency of skin moisture and skin pigmentation in 30 human volunteers after application of samples for 14 and 30 days. % efficacy was calculated from the mean value (n=30) of before (day 0) and after (day 14 or day 30).

Treatment area	Day	Efficiency (%)		
		skin pigmentation		Skin moisture
		Melanin	Erythema	
Untreated skin for D3SAP	Day 14	-1.28	-0.35	-1.28
	Day 30	-2.65	-4.55	1.77
D3SAP	Day 14	-4.68*	-8.39*	14.93*
	Day 30	-7.84*	-10.74*	19.54*
Untreated skin for A	Day 14	-1.35	-0.77	-0.80
	Day 30	-1.71	-0.93	2.44
Commercial A	Day 14	-2.03*	-3.48*	9.46*
	Day 30	-5.55*	-7.10*	10.99*

* indicated a significant difference from untreated skin ($p < 0.05$).

Table 3 Efficiency of skin wrinkle parameters (R3, R5, surface, and volume) in 30 human volunteers after application of samples for 14 and 30 days. % efficacy was calculated from the mean value (n=30) of before (day 0) and after (day 14 or day 30).

Treatment area	Day	Efficiency of skin wrinkle parameters (%)			
		Roughness		Surface (%)	Volume (mm ³)
		R3	R5		
Untreated skin for D3SAP	Day 14	-2.39	-4.31	-3.07	0.19
	Day 30	-5.87	-8.61	-5.05	-4.64
D3SAP	Day 14	-17.66*	-20.28*	-10.11*	-15.77*
	Day 30	-26.36*	-26.22*	-12.28*	-18.00*
Untreated skin for A	Day 14	-4.07	-5.12	-5.04	1.78
	Day 30	-7.10*	-5.43	-6.64	-2.02
Commercial A	Day 14	-21.30*	-15.57*	-8.28*	-12.75*
	Day 30	-27.59*	-21.16*	-12.09*	-15.27*

* indicated a significant difference from untreated skin ($p < 0.05$).

4. Conclusion

In this study the carboxymethyl chitosan hydrogel patch containing sodium ascorbyl phosphate was successfully prepared using carboxymethyl chitosan, methyl cellulose 4000, hydroxyethyl cellulose and ethyl acrylate-methyl methacrylate copolymer dispersion in the ratio of 4:0.5:0.5:1 with 5% propylene glycol, 5% triethyl citrate and 10% glycerin as plasticizers. The hydrogel patch showed good stability on physical, mechanical and chemical properties after storage under various conditions. The *ex vivo* permeation data exhibited that the amount of SAP permeated from the hydrogel patch to the stratum corneum, viable epidermis and dermis and also in receiving chamber were higher than that from conventional gel. The developed CMCS hydrogel patch demonstrated novel properties due to its high water solubility at neutral pH, improved moisture-retention ability, bioadhesion and increased permeation of SAP into the skin layer. Moreover, efficacy testing of the carboxymethyl chitosan hydrogel patch containing SAP exhibited a good skin moisturizing, whitening, and wrinkle reducing effects in 30 human volunteers after 30 days of once daily application. The hydrogel patch also showed no skin irritation when tested on human volunteers. These results could be concluded that the CMCS hydrogel patch containing SAP is a promising anti-aging, skin moisturizing and whitening cosmeceuticals.

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Development of watermelon extract loaded nanostructure lipid carriers**Jutiporn Sirikhet¹, Wisinee Chanmahasathien¹, Araya Raiwa¹ and
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Abstract

Nanostructured lipid carriers (NLCs) are delivery system providing many advantages for enhancing stability and prolonging half-life of natural active substances against environmental. In the present work, watermelon extract (WE) was used as a source of lycopene. Lycopene is deep-red color compound occurring in plants that exhibits high antioxidant activity. However, heat, light, oxygen and different environmental conditions are factors that affect isomerization and auto-oxidation of lycopene. The aim of this study was to develop appropriate NLCs system for loading watermelon extract. NLCs formulations were prepared by high pressure homogenizer. They were composed of cocoa butter as solid lipid, grape seed oil as liquid lipid, and the mixture of Span[®] 80 and Tween[®] 80 was used as emulsifier. The result showed that NLCs formulation with 10% (w/w) total lipids (solid lipid: liquid lipid ratio of 4:1) and 7% emulsifier showed small particle size (106.80 ± 0.70 nm) and narrow polydispersity index (PDI) value (0.122 ± 0.004). After storage under heating-cooling condition, the physical appearance did not change. Therefore, it was selected for loading watermelon extract. The WE-loaded NLCs showed average size as 113.23 ± 1.76 nm, PDI value as 0.129 ± 0.06 and zeta potential as -20.23 ± 0.76 mV, respectively. The entrapment efficiency was 88.46%. The special structure of the lipid matrix could enhance a high loading capacity, especially for lipophilic component. Thus, the WE-loaded NLCs exhibited good properties that could be useful for further application in cosmetic products.

Keywords: Lycopene; Nanostructure lipid carriers; Watermelon extract; High pressure homogenizer

1. Introduction

Watermelon is a valuable natural source of antioxidants that compose of lycopene, ascorbic acid and citrulline. These functional ingredients help to protect chronic health problems such as cancer and cardiovascular disorders (Zhang and Hamauzu, 2004; Omoni and Aluko, 2005). Lycopene is characterized by its distinctive red color in fruits and vegetables (Mutanen and Pajari, 2011). Additionally, watermelon is a rich source of β -carotene that acts as an antioxidant and precursor of vitamin A. Watermelon was chosen in this research because it contains higher lycopene content than tomato (4.81 and 3.03 mg/100 g, respectively) (Perkins-Veazie and Collins, 2004). In addition, it is harvested in all seasons, inexpensive and easily found in general market. Because of the benefits of

lycopene, it can be used as an active ingredient in cosmetics and food industries. Lycopene is stable in fresh fruits during mild thermal treatment. However, all-*trans*- isomer of lycopene is sensitive to isomerization into *cis*-isomer, oxidation and highly degradation process (Regier *et al.*, 2005). The most important factors of lycopene degradation are heat, light, and oxygen. Moreover, reaction medium and environmental conditions are also affected to stability of lycopene (Zechmeister *et al.*, 1943). Therefore, these effects can reduce health benefits and bioactivity of lycopene.

Nanostructured lipid carriers (NLCs) are an alternative formulation to improve lycopene stability due to many advantages over other traditional carrier systems. It is simple to prepare and available for a big scale production. It shows occlusive effect and increase the absorption of active compound into the skin. It is very useful for protecting photo-labile agent from light. All compositions are not harmful to the body. Moreover, it can be used in many topical dosage forms such as gel, cream, lotion, and ointment. Hence, NLCs is the suitable carrier for improving stability of lycopene (Riangjanapatee and Okonogi, 2012). The aim of this research was to develop appropriate NLCs system for loading watermelon extract.

2. Materials and Methods

2.1 Development and characterization of NLCs formulations

NLCs formulation composed of mixture of solid and liquid lipids. Cocoa butter was used as solid lipid and grape seed oil was used as liquid lipid. Span[®] 80 and Tween[®] 80 were used as emulsifier. NLCs formulations were prepared by high pressure homogenizer based on the previous method with some modifications (Riangjanapatee and Okonogi, 2012). The NLCs formulations were developed by varying total lipid, liquid lipid to solid lipid ratio and concentration of emulsifying agent. In brief, lipid phase (liquid lipid and solid lipid) and water phase were heated in water bath until 75°C and 80°C. Then, the water phase was added into the lipid phase and stirred to obtain pre-emulsion. The pre-emulsion was reduced particle size by high pressure homogenizer with appropriate pressure (500 bar) for 8 cycles. Finally, NLCs formulations were cooled down to room temperature.

First of all, NLCs formulations were prepared using different amount of total lipid and fixed the ratio of solid lipid to liquid lipid at 3:1 with 5% Span[®] 80. During the experiment, the independent total lipid was varied at four levels (2.5, 5, 7.5 and 10%) and represented as A, B, C and D, respectively as shown in **Table 1**. All formulations (A-D) were observed physical appearance and characterize particle size, polydispersity index (PDI), and zeta potential by Zetasizer[®] (Zetasizer[®] ZS, Malvern Instruments Ltd., UK). Each formulation was diluted at a ratio of 1:100 with deionized water and then the diluted samples were measured in triplicate. (Nitthikan *et al.*, 2018). The formulation with nanosize range, narrow PDI and good zeta potential was selected for further study.

The second optimization, solid lipid to liquid lipid ratio which was an independent factor was varied at three levels as 2:1, 3:1 and 4:1 that represented as 1, 2 and 3. Then, the concentration of emulsifying agents (Span[®] 80 and Tween[®] 80) were varied with 2.5, 5, and 7 % w/w in the ratio of 1:1, represented as I, II and III (**Table 2**). All NLCs formulations were observed physical appearance and characterized particle size, PDI, and zeta potential. The stability of the best formulations were evaluated by heating cooling cycling test, which proceeded by keeping the formulations in refrigerator at 4±0.5°C for 48 h then moving to hot air oven at 45±0.5°C for 48 h (one cycle) for 6 cycle. The NLCs formulation with good stability was selected for loading WE.

Table 1: NLCs formulations composition

Formulation	Total lipid (g)	Ratio of solid lipid :liquid lipid (g:g)
A	2.5	3:1 (1.87:0.62)
B	5.0	3:1 (3.75:1.25)
C	7.5	3:1 (5.62:1.87)
D	10.0	3:1 (7.50:2.50)

Table 2: The composition of lipid ratio and percentage of surfactant used in NLCs formulations

Formulation	Ratio of solid lipid :liquid lipid(g:g)	%Mixed surfactants
D1-I	2:1 (6.67:3.33)	2.5
D1-II		5
D1-III		7
D2-I	3:1 (7.50:2.50)	2.5
D2-II		5
D2-III		7
D3-I	4:1 (8.00:2.00)	2.5
D3-II		5
D3-III		7

2.2 Development of watermelon extract-loaded NLCs

Fresh watermelon pulp was blended and freeze-dried. After that, it was extracted by Soxhlet's extraction with hexane: acetone: ethanol (2:1:1) (Haroon, 2014). Watermelon extract-loaded NLCs (WE-loaded NLCs) was prepared by adding the WE (1% w/w) into melting lipid phase of the selected NLCs formulation. Briefly, the lipid mixtures (D2-III and D3-III) were selected to load WE that consisted of solid lipid and liquid lipid with the ratio of 3:1 and 4:1 (% w/w), 10% (w/w) total lipid and 7% (w/w) surfactant as shown in **Table 3**. The pre-emulsion occurred after mixing the oil phase and water phase and then generated NLCs using the high pressure homogenizer. The WE-loaded NLCs was cooled down to room temperature. Physical appearance, particle size, PDI, zeta potential (by Zetasizer®) and percentage of entrapment efficiency were then evaluated.

Table 3: Compositions of watermelon extract-loaded NLCs

Composition	%w/w	
	D2-III	D3-III
Cocoa butter	7.5	8
Grape seed oil	2.5	2
Span® 80	3.5	3.5
Tween® 80	3.5	3.5
Watermelon extract	1	1
DI water	q.s .100	q.s .100

2.3 Determination of watermelon extract-loaded NLCs entrapment efficiency

The entrapment efficiency was evaluated by measuring the amount of free lycopene in formulation according to Liu and Wu, (2010) method with some modification. One milliliter of WE-loaded NLCs was carefully weighed and mixed with 4 mL of hexane as extracting solvent then vortex for 1 min and remain intact in hexane for 30 min. The sample was centrifuged at 3000 rpm/min for 3 min, supernatant was then separated and analyzed by high-performance liquid chromatography (HPLC) for free lycopene [W_{free}]. The amount of total lycopene in this formulation was determined by dispersed 1 mL of WE-loaded NLCs in hexane and ethanol with the ratio of 6:4. The mixture was sonicated for 20 min and centrifuged at 7000 rpm/min, 25 °C for 30 min. The solution was obtained because the NLCs were soluble in the mixture of hexane and ethanol. The sample was analyzed by HPLC and calculated as total compound [W_{total}]. The optimum mobile phase for HPLC consists of methanol: tetrahydrofuran: water at the ratio of 60:33:7 (v/v) (Okonogi and Riangjanapatee, 2015). Finally, the percentage of entrapment efficacy was calculated according to the following equation:

$$\% \text{ EE} = \frac{(W_{\text{total}} - W_{\text{free}})}{W_{\text{total}}} \times 100$$

Where [W_{total}] is the amount of total lycopene in the formulation and [W_{free}] is the amount of free lycopene in the formulation.

2.4 Statistical analysis

Statistical analysis was carried out using one-way analysis of variance (ANOVA) to evaluate significant difference followed by multiple comparisons (Tukey's test) between groups and paired t-test to evaluate significant difference before and after test at p values of 0.05.

3. Results and Discussion

3.1 Development and characterization of NLCs formulations

NLCs formulation is delivery system containing solid lipid, liquid lipid and emulsifying agent as main compounds. The NLCs formulation containing varied amount of total lipid (A-D) with fixed solid lipid and liquid lipid in the ratio of 3:1 and 5% Span[®] 80 were used in this studies. Cocoa butter and grape seed oil were used as solid lipid and liquid lipid. Following the preparation of pre-emulsion, particle size were reduced by high pressure homogenizer (HPH). Higher pressure and more cycles of the HPH provided higher force to break down the colloidal to nanoscale. In this experiment, the preparation of the NLCs formulations used 8 cycles of the HPH, because it could generated small particle size with a narrow PDI and was not significantly different from the result of using 10 cycles (data did not show). In this study, the pressure of homogenizer was 500 bar that is suitable to produce nanoparticles. NLCs formulations with varied total lipid was obtained. Then the particle size, PDI and zeta potential were analyzed. As in **Table 4**, the particle size of each formulation with different total lipid was in the range of 142.53±0.51 to 152.80±3.29 nm and narrow PDI (0.19±0.005 to 0.26±0.002) was obtained. The NLCs formulation with 10% (w/w) total lipid (formulation D) was selected for further study. Because formulation D contained the highest total lipid, it would entrapped higher amount of active compound compared to others. In addition, the particle size of formulation D was not significantly different when compared with other formulations and showed the narrowest PDI with good zeta potential (-39.30±1.73 mV).

For optimization, the solid lipid to liquid lipid ratio was varied at three levels (2:1, 3:1 and 4:1) and represented as 1, 2 and 3, respectively. In addition, concentration of Span[®] 80 and Tween[®] 80 were varied with 2.5, 5 and 7% w/w in the ratio of 1:1 and represented as I, II and III. Each factor was matched to determine the appropriate ratio of lipid and quantity of surfactant. The particle size, PDI,

and zeta potential were shown in **Table 5**. Increasing the ratio of solid lipid in formulation exhibited the increasing of particle size. Comparing of D1-III, D2-III and D3-III with similar concentration of surfactant, the D3-III showed the largest particle size. It might be due to oily droplets embedded in a solid lipid matrix. Therefore, the particle matrix contains imperfection that providing space to accommodate the drug molecules in amorphous clusters (Fang *et al.*, 2013).

Surfactant in formulation also affected the particle size of NLCs formulations. When increasing percentage of surfactant, particle size was decreased as shown in **Table 5**. The result demonstrated that 7% surfactant could generate the smallest particle size and low PDI. Suitable concentration of surfactant in formulation can generate the repulsive force among nanoparticles and reduce the interfacial tension as well as the surface energy of NLCs (McClements DJ. 2011). Therefore, it can help to break down lipid droplets to a smaller size.

D1-III, D2-III and D3-III showed the smallest particle size in D1, D2 and D3 group. So, their formulations were selected for stability test by heating cooling condition for 6 cycles. After testing, the physical appearance of D2-III and D3-III did not change and phase separation did not occur. However, D1-III showed phase separation. In addition, particle size, PDI and zeta potential of D2-III and D3-III did not change when compared with initial as shown in **Table 6**. Therefore, D2-III and D3-III were selected to load WE because they composed of appropriate total lipid, high solid lipid with small particle size and low PDI. The composition of lipid matrix, surfactant concentration and manufacturing parameters can affect the character of NLCs formulation that enhances a high loading capacity and drug release profile (Müller *et al.*, 2000).

Table 4: Mean particle size, PDI, and zeta potential of NLCs formulations with different total lipid

NLCs formulations	Mean particle size (nm)	PDI	Zeta potential (mV)
A	142.53±0.51 ^a	0.26±0.002 ^a	-25.80±0.53 ^a
B	145.80±1.65 ^{ac}	0.25±0.026 ^{ab}	-26.03±1.11 ^a
C	149.33±1.40 ^{bc}	0.22±0.013 ^{bc}	-21.13±1.42 ^b
D	152.80±3.29 ^b	0.19±0.005 ^c	-39.30±1.73 ^c

Values are mean ± S.D. from triplicate.

Different letters in the same column indicated significant differences (p<0.05).

Table 5: Mean particle size, PDI, and zeta potential of NLCs formulations (D) with different solid lipid to liquid lipid ratio and percentage of surfactant

Formulation	Ratio of solid lipid :liquid lipid(g:g)	% surfactant	Mean particle size (nm)	PDI	Zeta potential (mV)
D1-I	2:1	2.5	129.40 ± 1.01 ^a	0.146 ± 0.007 ^a	-29.37 ± 1.91 ^a
D1-II		5	120.03 ± 2.15 ^b	0.269 ± 0.017 ^b	-27.07 ± 4.01 ^a
D1-III		7	91.85 ± 0.62 ^c	0.111 ± 0.003 ^c	-25.10 ± 2.38 ^a
D2-I	3:1	2.5	135.97 ± 0.85 ^a	0.196 ± 0.010 ^a	-20.20 ± 0.17 ^a
D2-II		5	107.43 ± 0.93 ^b	0.148 ± 0.026 ^{ab}	-24.63 ± 3.33 ^a
D2-III		7	95.20 ± 1.15 ^c	0.115 ± 0.024 ^b	-20.47 ± 1.33 ^a
D3-I	4:1	2.5	141.17 ± 1.77 ^a	0.168 ± 0.003 ^a	-29.20 ± 1.77 ^a
D3-II		5	137.20 ± 0.66 ^b	0.267 ± 0.017 ^b	-23.93 ± 0.67 ^b
D3-III		7	106.80 ± 0.70 ^c	0.122 ± 0.004 ^c	-22.03 ± 1.76 ^b

Values are mean ± S.D. from triplicate.

Different letters in the same column indicated significant differences (p<0.05) in each group (2:1, 3:1 and 4:1).

Table 6: Mean particle size, PDI, and zeta potential of selected NLCs formulations before and after stability test [heating cooling (HC) for 6 cycles]

Formulation	Mean particle size (nm)		PDI		Zeta potential (mV)	
	Start	HC	Start	HC	Start	HC
D1-III	91.85 ± 0.62	NE	0.111 ± 0.003	NE	-25.10 ± 2.38	NE
D2-III	95.20 ± 1.15	97.27 ± 0.39	0.115 ± 0.024	0.113 ± 0.031	-20.47 ± 1.33	-20.53 ± 0.67
D3-III	106.80 ± 0.70	107.70 ± 1.06	0.122 ± 0.004	0.195 ± 0.023	-22.03 ± 1.76	-27.57 ± 1.29*

NE: not evaluated

Values are mean ± S.D. from triplicate.

* indicate significant differences (p<0.05) from paired t-test.

3.2 Development of WE-loaded NLCs

Both selected formulations (D2-III and D3-III) were loaded with 1% WE (w/w) (**Table 3**). The D2-III-WE showed significant higher particle size than that of D3-III-WE (167.37±3.76 vs. 113.23±1.76 nm) (p<0.05). However, PDI and zeta potential of WE-loaded NLCs were not significantly different when compared to each other as shown in **Table 7**. WE-loaded NLCs formulations gave larger particle size than the unloaded NLCs formulations because nanoparticle matrix was replenished by WE. However, they presented acceptance range of nanoparticle with narrow PDI value.

3.3 Entrapment efficiency of WE- loaded NLCs

Percentage of entrapment efficiency (%EE) was determined from above methodology and HPLC condition. D3-III-WE showed the highest %EE as 88.46±3.90 (**Table 7**). Entrapment efficacy depends on the amount of the lipid, the solubility of the compounds in lipid, and the concentration of the surfactant. Therefore, D3-III- WE with the highest total lipid and more solid lipid to liquid lipid ratio led to the higher encapsulation efficiency (Gambhire *et al.*, 2011; Singh *et al.*, 2016).

Table 7: Mean particle size, PDI, zeta potential and %entrapment efficiency of WE-loaded NLCs

Formulation	Mean particle size (nm)	PDI	Zeta potential (mV)	%Entrapment efficiency
D2-III-WE	167.37±3.76 ^a	0.119±0.040 ^a	-18.73±0.71 ^a	77.97±1.43 ^a
D3-III-WE	113.23±1.76 ^b	0.129±0.060 ^a	-20.23±0.76 ^a	88.46±3.90 ^b

Values are mean ± S.D. from triplicate.

Different letters in the same column indicated significant differences (p<0.05).

4. Conclusion

To develop appropriate NLCs system for loading watermelon extract, D3-III is the best NLCs formulation which showed small particle size, low PDI and good stability after heating-cooling condition. It composed of 10% (w/w) total lipid with cocoa butter and grape seed oil (4:1) and 7% of emulsifying agent. The D3-III-WE possessed small particle size, low PDI and high percentage of entrapment efficiency. In future study, stability testing and skin permeation should be conducted. Therefore, the D3-III-WE might be a promising formulation for further using in cosmetic products.

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Formulation of anti-wrinkle cream containing *Caesalpinia mimosoides* Lamk. extract

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Abstract

At present, cosmeceutical products containing natural extracts are increasingly popular all over the world. *Caesalpinia mimosoides* is a valuable indigenous plant mainly distributed in the north and north-eastern parts of Thailand. However, its potential for cosmeceutical applications has not been reported. This study aims to prepare the extracts, investigate the antioxidant activity and cytotoxicity *in vitro*, and subsequently, determine its potential development into anti-aging product. *Caesalpinia mimosoides* was extracted using 95% ethanol (CE) and partitioned with petroleum ether (PE), chloroform (CHF), and 50% aqueous ethanol (Aq-ETH), respectively. All the extracts were tested for antioxidant capacity using 1,1-diphenyl-2-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP), 2,2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), and lipid peroxidation inhibitory activity assays. Moreover, total phenolic and flavonoid contents were also determined. The most active extract was selected to be evaluated for cytotoxicity in human dermal fibroblast, chemical analysis by HPLC, and stability and was subsequently formulated into a cream. The results revealed that the Aq-ETH extract exhibited the highest antioxidant activities when compared with a standard by DPPH and ABTS assays, with IC₅₀ of 0.034±0.004 mg/mL (trolox; IC₅₀ = 0.089±0.002 mg/mL) and 0.003±0.002 mg/mL (trolox; IC₅₀ = 0.650±0.002 mg/mL), respectively. The Aq-ETH also showed high efficiency on lipid peroxidation inhibitory activity assay with IC₅₀ of 0.43±0.980 mg/mL (butylated hydroxyanisole [BHA]; IC₅₀ = 7.55±0.091 mg/mL) and FRAP assay with FRAP value of 1.73±0.099 mM FeSO₄/g (trolox; FRAP value = 3.33±0.064 mM FeSO₄/g). Moreover, the Aq-ETH extract showed the highest amount of phenolic and flavonoid contents and possessed great antioxidant activity with no cytotoxic effect; survival rates ranged from 98.76%-99.03%. The cream containing Aq-ETH extract was highly stable when stored under various conditions. Finally, a clinical study was performed in 30 healthy volunteers. The cream containing the Aq-ETH extract showed no skin irritation and exhibited a remarkable reduction in skin wrinkles and improvement in skin elasticity which were significantly apparent, after 30 days of application.

Keywords: *Caesalpinia mimosoides* Lamk., Antioxidant activity, Anti-wrinkle, cream

1. Introduction

Caesalpinia mimosoides Lamk. (*C.mimosoides* Lamk.) is a valuable indigenous plant with moderate antioxidant activity that has a high level of tannin and phenolic compounds (Lakshmi, 2015). *Caesalpinia mimosoides* is a prickly climbing shrub, with branches bearing small prickles and mainly distributed in the north and north-eastern parts of Thailand. In Thailand, this plant is usually known as a vegetable and is used to treat vertigo and dizziness (Deevisejkeaw, 2005). There is still no reported on cosmeceutical applications of this local plant. Therefore, the researchers are interested to study the benefits of this plant for cosmetic application. Consequently, the aims of this study were to prepare *C.mimosoides* extract and investigate the antioxidant activity and cytotoxicity *in vitro*. The study and data are used to develop *C.mimosoides* extract into cosmeceutical product, which has not been reported as anti-aging and skin whitening agent.

2. Materials and Methods

2.1 Preparation of plant extracts and fractionation

The fresh young shoots of *C. mimosoides* were purchased from the local market in Chiang Mai, Thailand, in January, 2019. The plant was authenticated by Herbarium, Faculty of Pharmacy, Chiang Mai university, Thailand, and was confirmed with a voucher specimen of Maxwell J.F (Herbarium No. 004289). The fresh samples were dried using hot air oven (Nakagawa *et al.*, 2019) at a temperature of 55°C for 24 hours, before ground into powder. The sample was completely extracted by Soxhlet apparatus using 95% ethanol as a solvent. The solvent containing extract was concentrated using a rotary evaporator at a temperature 40°C. Then, the crude extract was partitioned sequentially with petroleum ether, chloroform, and 50% aqueous ethanol to yield several partitions labeled as petroleum ether (PE), chloroform (CHF) and 50% aqueous ethanol (Aq-ETH), respectively.

2.2 Determination of antioxidant activities

2.2.1 Diphenyl-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH free radical scavenging activity was performed using a previously described method (Somdee *et al.*, 2016). First, the sample was dissolved in ethanol. Then, 20 µL of each sample was mixed with 180 µL of 0.2 mM DPPH solution in a microplate. The solution was incubated for 30 minutes in the dark at room temperature. The absorbance of the mixture was measured at 520 nm by a multimode detector (Netzel *et al.*, 2003). Trolox was used as a positive control.

2.2.2 Lipid peroxidation inhibitory activity test

Briefly, the linoleic acid was mixed with the sample and incubated at 37°C for 4 hours. Then, 50 µL of the mixture was mixed with 5 mL of 75% methanol, followed by 50 µL of 10% ammonium thiocyanide and 50 µL of 2mM ferric chloride. After the complete reaction, the absorbance was measured at 500 nm by UV-VIS spectrophotometer (Erian *et al.*, 2016). Butylated hydroxyanisole (BHA) was used as a positive control.

2.2.3 Ferric Reducing Antioxidant Potential (FRAP) assay

The FRAP solution was prepared by mixing acetate buffer, 2,4,6-Tripyridyl-S-triazine (TPTZ) solution and ferric chloride solution. Then, 20 µL of samples were mixed with 180 µL of FRAP solution. After the complete reaction, the absorbance was measured at 590 nm by using UV-VIS spectrophotometer (Somdee *et al.*, 2016). Trolox was used as a positive control. The results were expressed as ferrous equivalent in mM/gram of the extract (mM FeSO₄/g).

2.2.4 ABTS radical scavenging activity Assay

The ABTS radical scavenging activity of the samples was determined according to the method of an antioxidant to scavenge free radical as a measure of the ability to decolor the ABTS free radicals (Emad *et al.*, 2013). Briefly, the ABTS⁺ solution was produced by the reaction of 7mM ABTS solution in 2.45 mM potassium persulfate, and it was incubated for 16 hours in the dark at room temperature. After that, the sample was mixed with ABTS solution in a 96-well plate. The absorbance was measured at 736 nm by UV-VIS spectrophotometer (Re *et al.*, 1999).

2.3 Total Phenolic Content (TPC)

The TPC was determined by a method previously described as Erian *et al.* (2016) using Folin-Ciocalteu reagent. First, the sample was prepared at a concentration of 500 µg/mL in ethanol and gallic acid was used to determine standard curve. Then, 20 µL of the sample was mixed with 100 µL of 10%v/v Folin-Ciocalteu reagent and 80 µL of 7.5% w/v sodium carbonate, respectively. The mixture was incubated at room temperature for 30 minutes. The absorbance was measured at 765 nm by UV-VIS spectrophotometer (Bhatti *et al.*, 2010). The phenolic content of each partition was determined from the gallic acid calibration curve, and the results were recorded in mg of gallic acid equivalent per g of extract (mg GAE/g of extract).

2.4 Total Flavonoid Content (TFC)

The sample was prepared at a concentration of 500 µg/mL in ethanol. The 50 µL of sample was added to 96-well plate and quercetin was used as a standard. Then, the sample was mixed with 20 µL of deionized (DI) water, followed by 15 µL of 5% sodium nitrite solutions, 15 µL of 10% aluminium chloride, and 100 µL of sodium hydroxide, respectively. The absorbance was measured at 510 nm by UV-VIS spectrophotometer (Erian *et al.*, 2016; Bhatti *et al.*, 2010). The flavonoid content of each partition was determined from the quercetin calibration curve, and the results were recorded in mg of quercetin equivalent per g of extract (mg QE/g of extract).

2.5 Cytotoxic assay against human dermal fibroblast cells

The human dermal fibroblast cell line was obtained from the facial skin. The assay based on the measurement of cellular protein content was performed as described based on modification of Vichai *et al.* For the cytotoxicity assay, cells in triplicates were seeded in 96-well plates at 37°C, and 5% CO₂ in a humidified air for 24 hours. First, the test sample was dissolved in 10% dimethyl sulfoxide (DMSO) for the cell culture experiments. The test samples were sterilized by filtration and the test solution was diluted to the desired concentration with 10% DMSO in an uninoculated culture medium. The cells were then treated with various concentrations of test samples. Cytotoxicity was evaluated by sulforhodamine B colorimetric assay. The absorbance of the resulting solution was measured (at $\lambda = 510$ nm) by microplate reader (DTX-880 multimode detector, Beckman Coulter Inc., USA) equipped with multimode analysis software (Vichai *et al.*, 2016)

2.6 High-Performance Liquid Chromatography (HPLC)

The chromatographic fingerprint was performed on HPLC (Agilent G1311A) instrument equipped with a UV detector using an Inertsil reversed-phase C₁₈ column (5 µm, dimension, GL Sciences) and the following mobile phase: A: methanol and B: 5% acetonitrile in ultrapure water; pH-3.00 were adjusted by O-phosphoric acid with the ratio of 5 (A): 95 (B) with a flow rate 1.0 mL/min; and injection volume was 10 µL for the extract. Gallic acid was used as a reference standard. The chemical compositions were detected by a UV detector at 272 nm (Kardani *et al.*, 2013).

2.7 Formulation development

2.7.1 Development of cream

The cream containing *C. mimosoides* extract was developed as oil-in-water dispersion system by separately heating the aqueous phase containing hydrophilic surfactants at a temperature $75\pm 2^\circ\text{C}$ and the oily phase at a temperature $70\pm 2^\circ\text{C}$. Then, the oily phase was added to the aqueous phase under continuous stirring (Leelapornpisid, 1997). The suitable cream formulations with good characteristics and stability were selected for stability test.

Cream formulation was prepared by using the following list of ingredient:

Aqueous phase: Carbopol 940, Propylene glycol, Concentrated paraben, Polysorbate 80, and DI water.

Oily phase: Cetyl alcohol, Stearic acid, Cyclomethicone, Triethanolamine, Jojoba oil, Cetiol HE, Lanolin, White bee wax, Vitamin E acetate, Glyceryl monostearate, and Polysorbate 80.

2.7.2 Stability testing of the formulation

The physical and chemical properties of the cream containing *C. mimosoides* extract was evaluated after storage at various conditions including 4°C , 45°C , room temperature (light exposure) and room temperature (dark) for 3 months and also heating-cooling cycling (6 cycles; 4°C for 48 hours and 45°C for 48 hours). The antioxidant activity and HPLC analysis these samples were performed (Krasantisuk *et al.*, 2003).

2.7.3 Human skin primary irritation test

The research conducted on human subjects has been approved by Research Administrative, Academic Services and International Relations Affairs Faculty of Pharmacy Chiang Mai University (Approval No. 018/2561). The ethics committee is organized and operates according to GCPs and relevant international ethical guidelines, the applicable laws and regulations. Thirty healthy Thai were selected and written consent was obtained in each case. The age range for the research population that included males and females was 30–50 years (Shin *et al.*, 2017; Termogkolchai, 2012). The chamber patches stayed in place for 48 hours. Once the patches were removed, a reading was done after 1, 24, 48 and 72 hours and scored according to modified criteria proposed by the Draize scoring, Frosch and Kligman (Frosch *et al.*, 1979) and the Cosmetic, Toiletry, and Fragrance Association (CTFA) guidelines (Anita *et al.*, 1981).

2.7.4 Skin analysis for wrinkle reducing capacity and skin elasticity

The research conducted on human subjects has been approved by Research Administrative, Academic Services and International Relations Affairs Faculty of Pharmacy Chiang Mai University (Approval No. 018/2561). Thirty healthy Thai subjects were selected and written consent was obtained in each case. Healthy volunteers aged between 30–50 years, males and females with no congenital disease were selected. The *C. mimosoides* cream was applied to the lower left forearm and negative control was applied to the lower right forearm of the volunteers twice a day for 30 days. The skin was analyzed using the Visiometer® to observe the degree of wrinkling and roughness. Skin elasticity was measured by the Cutometer® (Termogkolchai, 2012).

2.8 Statistical analysis

Data are presented as the mean values $\pm$ standard deviation (SD) of three replicates for each parameter. The differences between the IC_{50} values were determined by the analysis of variance (ANOVA) and using the paired-sample t-test for assessment of skin analysis with the SPSS Statistics 17.0. version. Differences of $P < 0.05$ were considered significant.

3. Results and Discussion

3.1 Preparation of plant extracts and fractionation

The yield of 95% ethanol crude extract (CE) was 43.85%. Next, the CE was partitioned with different solvents in order obtained PE, CHF, and Aq-ETH, respectively. The result showed that the Aq-ETH fraction had the highest yield of 4.44%, followed by CHF and PE of 1.19% and 0.16%, respectively.

3.1.1 Antioxidant Activities

Table 1 Antioxidant activity of *C. mimosoides* extract

Sample	IC ₅₀ (mg/mL)			FRAP value (mM FeSO ₄ /g)
	DPPH	lipid peroxidation	ABTS	
CE	0.095±0.001*	1.27±0.179*	0.138±0.001*	0.99±0.030*
PE	0.119±0.001	0.21±2.066*	0.686±0.002*	2.88±0.063
CHF	0.041±0.007*	16.28±1.419*	0.066±0.001*	3.15±0.060
Aq-ETH	0.034±0.004*	0.43±0.980*	0.003±0.002*	1.73±0.099*
standard	0.089±0.002 (trolox)	7.55±0.091 (BHA)	0.650±0.002 (trolox)	3.33±0.064 (trolox)

Note; * is significantly different (P<0.05) when compared with standard (paired-samples T test), CE (crude extract), PE (petroleum ether), CHF (chloroform), Aq-ETH (50% aqueous ethanol)

Analysis of the free radical scavenging properties of the examined extracts revealed highest activities of the Aq-ETH partition. For example, in DPPH assay, the Aq-ETH showed the best activity with IC₅₀ value of 0.034±0.004 mg/mL, followed by CHF (0.041±0.007 mg/mL), CE (0.095±0.001 mg/mL) and PE (0.119±0.001 mg/mL), whereas, trolox used as positive control showed IC₅₀ value of 0.089±0.002 mg/mL. The IC₅₀ value of Aq-ETH showed significantly higher activity among all the extracts and when compared with the standard. The highest activity may attributed to more phenolic compounds in the extract that presented higher free radical scavenging ability.

For the lipid peroxidation inhibitory activity, the PE exhibited the best activity with IC₅₀ values of 0.21±2.066 mg/mL, followed by Aq-ETH (0.43±0.980 mg/mL), CE (1.27±0.179 mg/mL) and CHF (16.28±1.419 mg/mL), while BHA showed IC₅₀ value of 7.55±0.091 mg/mL. The IC₅₀ value of Aq-ETH, PE and CE were also lower than BHA. The PE was significantly different between fractionated extract and showed higher activity than BHA (P<0.05).

The ABTS assay was also used to measure the relative ability of the antioxidant to scavenge free radicals. The results revealed that the Aq-ETH showed the best activity with IC₅₀ of 0.003±0.002 mg/mL, followed by CHF (0.066±0.001 mg/mL), CE (0.138±0.001 mg/mL) and PE (0.686±0.002 mg/mL), while trolox showed IC₅₀ value of 0.650±0.002 mg/mL. The IC₅₀ value of Aq-ETH exhibited significantly higher ABTS scavenging activity (P<0.05) among all the extracts and also the standard. The results are corresponding to the DPPH assay.

For FRAP assay, the result showed that the CHF gave the highest antioxidant activity with a FRAP value of 3.15±0.06 mM FeSO₄/g, followed by PE, Aq-ETH, and CE at 2.88±0.063, 1.73±0.099 and 0.99±0.030 mM FeSO₄/g respectively. However, trolox demonstrated significantly higher antioxidant capacity (3.33±0.064 mM FeSO₄/g).

The Aq-ETH exhibited the highest antioxidant activities when tested by DPPH radical scavenging assay and ABTS assay. In addition, it also had high efficiency on lipid peroxidation inhibitory activity and FRAP assay. This may be due to the more polar active compound in the extract being able to scavenge free radical better than to inhibit the lipid peroxidation, which is corresponding to the previous study that result showed high polar solvent extract can exhibit good antioxidant activity when compared to other extracts (Lakshmi, 2015). The results of this study also showed that aerial

parts of *C.mimosoides* had strong radical scavenging and reducing capacities. The antioxidant activities of all fractions are summarized in table 1.

3.2 Total phenolic and flavonoid content

Table 2 Total phenolic and flavonoids content of *C.mimosoides* extracts.

Sample	TPC (mg GAE/g extract)	TFC (mg QE/g extract)
CE	827.75±0.02*	400.66±0.02*
CHF	773.88±1.01*	375.77±0.84*
PE	340.36±0.95*	190.52±0.76*
Aq-ETH	979.91±0.81*	478.08±1.50*

Note; * is significantly different ($P<0.05$) among the extract (ANOVA test), CE (crude extract), PE (petroleum ether), CHF (chloroform), Aq-ETH (50% aqueous ethanol), TPC (total phenolic content) TFC (total flavonoid content), GAE (gallic acid equivalent), QE (quercetin equivalent)

Phenolic and flavonoid compounds were found in *C. mimosoides*. The phenolic compounds are a large group of phytochemicals in plants that act as antioxidant. The results revealed that the highest content of phenolic compounds was found in the Aq-ETH (979.91±0.81 mg GAE/g extract) followed by CE, CHF and PE, respectively. The TPC was significantly different between the sample extracts ($P<0.05$). The Aq-ETH showed the highest amount of flavonoid content (478.08±1.50 mg QE/g extract), followed by CE, CHF and PE, respectively as shown in Table 3. The TFC was significantly different between sample extracts ($P<0.05$). Therefore, these compounds served as an antioxidant.

The Aq-ETH was found to contain the highest content of phenolic and flavonoid compounds, which are hydroxyl groups that can be dissolved in water or polar substances. This result was in accordance with the previous study (Aduang *et al.*, 2011). The results showed the highest content of phenolic and flavonoid compounds of Thai vegetables correlated with antioxidant activities.

3.3 High-Performance Liquid Chromatography (HPLC)

High-performance liquid chromatography (HPLC) analysis of gallic acid showed a retention time (t_R) of 9.90 minutes. The samples included CE, CHF, PE and Aq-ETH and reported retention time at 9.91, 9.91, 9.89 and 9.91 minutes, respectively. Therefore, the sample contained gallic acid due to the similarity of the retention time of standard and *C.mimosoides* as shown in Figure 1.

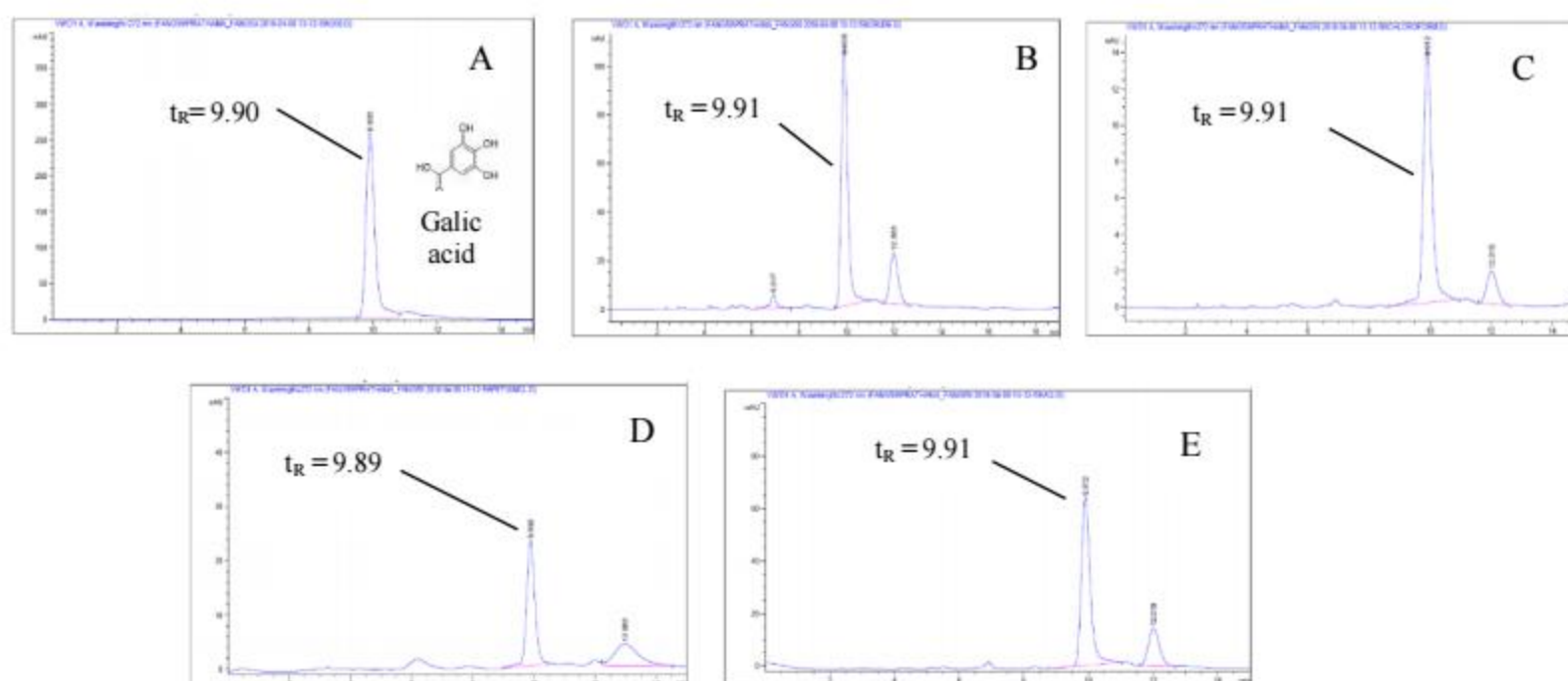


Figure 1. Chromatogram of gallic acid (A), CE (B), CHF (C), PE (D) and Aq-ETH (E)

3.4 Cytotoxicity test

The Aq-ETH showed no toxicity to human dermal fibroblasts at the concentration range of 0.0001-1 mg/mL, with survival rates ranging from 98.76%-99.03%. On the other hand, sodium lauryl sulfate as a positive control was toxic to human skin at 0.1 and 1 mg/ml, with survival rates of $32.28 \pm 4.35\%$ and $8.10 \pm 1.81\%$, respectively. The result of this study exhibited that the Aq-ETH is safe for cosmeceutical application.

3.5 Formulation development

3.5.1 Development of cream containing *C. mimosoides* extract

From the *in-vitro* antioxidant activity, Aq-ETH extract possesses great antioxidant activity, therefore it was selected to develop into the cream in the concentration of 0.3%, which is the lowest concentration that still provides good antioxidant activity. The result revealed that the cream has a yellowish color, fine texture, and low viscosity and is homogeneous. When it was applied to the skin, it spread easily, was not greasy, and highly moisturized the skin. It also provides the optimal pH for the skin (pH 5.5). In addition, it is highly stable when stored at various conditions for 3 months. After 3 months of stability testing, the product was stable but became liquefied when stored at 45 °C for 1 month.

3.5.2 Stability of formulation determined by DPPH free radical scavenging assay and HPLC

The DPPH free radical scavenging capability of the cream was determined after the stability test. When tested on day 0, the percentage of inhibition was $93.95 \pm 0.06\%$. The sample was stored at various conditions for 3 months including 4°C, 45°C, room temperature (light exposure), room temperature (dark) and heating-cooling for 6 cycles. After 3 months, the percentage of inhibition were $89.81 \pm 0.04\%$, $79.78 \pm 0.03\%$, $81.42 \pm 0.02\%$, $87.72 \pm 0.01\%$ and $91.87 \pm 0.03\%$, respectively. However, when determined with the HPLC assay, the results showed that gallic acid content of the cream was slightly reduced at 2 and 3 months. At 0 testing day, gallic acid content was 2.08 ± 0.02 ppm. After 3 months, storage at 4°C, 45°C, Room temperature (light exposure), room temperature (dark) and heating-cooling (6 cycles), the gallic acid content were 1.56 ± 0.04 , 1.46 ± 0.12 , 1.21 ± 0.32 , 1.19 ± 0.23 , and 1.21 ± 0.02 ppm, respectively. Storage at low temperature of 4°C showed the highest stability, followed by stored at room temperature (dark), 45°C, room temperature (light exposure) and after heating cooling. These results can be concluded that the cream was unstable when exposed light and high temperatures.

3.5.3 Human skin primary irritation test

The irritation test for the cream containing *C. mimosoides* extract was performed in 30 volunteers. The score of skin irritation was classified by the Draize scoring system and primary index irritation (PII) value. The cream of *C. mimosoides* extracts and untreated skin had PII values of 0, which were classified as non-irritation and 1% w/v of SLS had a PII value of 0.89, which was classified as slightly irritable. The result demonstrated that the cream was safe for cosmeceutical application.

3.5.4 Performance testing of the products from the *C. mimosoides* extract

The efficacy testing on the cream containing *C. mimosoides* extract was conducted on 30 volunteers within the age range 30-50 years composing of 15 male and 15 female. The skin measurements were wrinkles (surface, volume and roughness) using Visiometer® and skin elasticity using Cutometer®.

Skin wrinkles

The *C. mimosoides* cream showed that the most reduction of skin surface, skin roughness, and skin volume was after 30 days of application. The skin wrinkles were significantly reduced ($P<0.05$) on all skin wrinkle parameters (surface, volume, roughness) more than untreated skin and cream base. In this study, the skin wrinkles were improved after treatment with cream base when compare with untreated skin, but the improvement was not higher than the treatment with *C. mimosoides* cream.

Skin elasticity

The skin elasticity was significantly enhancing ($P<0.05$) more than untreated skin and cream base. In this study, the skin elasticity was improved after the treatment with cream base when compared with untreated skin, but it was not higher than the treatment with *C. mimosoides* cream. Finally, the results of satisfaction on cream containing *C. mimosoides* showed the high satisfactory response to the cream, appearance of the packaging, product characteristics, and product efficiency. Therefore, the product could be a promising anti-wrinkle cosmeceutical.

Table 3 Skin wrinkles and elasticity parameters before and after using cream containing *C. mimosoides* extract after 30 days.

Product		Skin wrinkles parameters			Skin elasticity parameters
		Surface	volume	roughness	
<i>C. mimosoides</i> cream	day 0	399.26±39.19	48.97±19.48	7.40±1.24	0.72±0.08
	day 30	376.20±41.01 ^a	42.51±14.23 ^a	6.57±1.07 ^a	0.79±0.06 ^a
Untreated skin	day 0	400.66±39.56	47.40±7.11	6.87±0.66	0.78±0.08
	day 30	399.95±39.11	48.21±11.91	6.92±0.71	0.79±0.08
Cream base	day 0	399.97±22.56	48.86±5.14	7.20±0.89	0.73±0.05
	day 30	390.67±36.87 ^{a, b}	46.88±7.02 ^{a, b}	6.77±1.02 ^{a, b}	0.75±0.06 ^{a, b}

Note; ^a is significantly different ($P<0.05$) when compared with day 0, ^b is significantly different ($P<0.05$) when compared with cream containing *C. mimosoides* extract

Table 4 % Efficacy of wrinkle reducing and skin elasticity enhancing after 30 days.

Product	% Efficacy of wrinkle reducing			Percentage of skin elasticity enhancing (%)
	Surface	volume	roughness	
<i>C. mimosoides</i> cream	-7.29±16.41	-9.45±19.98	-10.60±18.17	11.15±12.29
Untreated skin	-0.75±7.32*	0.67±4.55*	1.13±9.16*	0.67±4.75*
Cream base	-2.38±10.02*	-4.22±7.45*	-6.35±8.73*	2.67±5.22*

Note; * is significantly different ($p < 0.05$) when compared with *C. mimosoides* cream

4. Conclusion

The ethanolic extract (CE) and its fractionated extract from the shoot parts of *C. mimosoides* (PE, CHF, Aq-ETH) were investigated for their *in-vitro* antioxidant activity. The results showed that Aq-ETH possesses great antioxidant activity. This fraction has high stability and is non-toxic to human dermal fibroblasts. Moreover, the extract was found to contain high content of phenolic and flavonoid compounds. The cream containing 0.3% *C. mimosoides* extract (Aq-ETH) was successfully developed. It is highly stable when stored away from heat and light. The finished product showed no skin

irritation on humans and effective in skin wrinkles reducing capability and improving skin elasticity in 30 volunteers after 30 days of application.

5. Acknowledgements

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Encapsulation of *Dipterocarpus alatus* leaves extract by calcium alginate

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Abstract

Dipterocarpus alatus (*D. alatus*) is perennial plant that found in South-East Asia. Almost every part of *D. alatus* has been studied and the leaves show properties for cosmetic propose, i.e., inhibition of tyrosinase, inhibit growth of *Propionibacterium acne*, and anti-inflammatory. Moreover, *D. alatus* leaves extract consists of high content of phenolic compounds, especially, rutin and quercetin which related to high antioxidant activity by DPPH and ABTS assay and show nontoxicity effect to human skin cell determined by resazurin microplate assay (REMA). With these properties, *D. alatus* leaves extract has been used as an ingredient for some cosmetic products. However, the antioxidant ability and biological activities were previous reported to gradually lose overtime. Therefore, calcium alginate encapsulation of the extract was developed in this work in order to maintain its biological activities. The characterizations of alginate beads in terms of percentage of encapsulation efficiency of encapsulated form were studied. Alginate beads with different concentration of extracts (0.25, 0.50 and 0.75% w/v) and control alginate beads after drying showed the percentage of encapsulation efficiency was ranging from 22.56 ± 1.44 to $31.33 \pm 4.53\%$. To get the optimal method for encapsulation of *D. alatus* leaves extract, the Tween[®]20, sodium alginate and calcium chloride were used in various concentrations. The percentage of efficiency tends to increase when the concentration of Tween[®]20, sodium alginate and calcium chloride increase because Tween[®]20 can improve solubility of the extract and high concentration of sodium alginate and calcium chloride can increase porous of alginate bead therefore resulting in decrease diffusion of phenolic compounds from the beads.

Keywords: *Dipterocarpus alatus*; Phenolic compound; Antioxidant; Encapsulation; Alginate.

1. Introduction

Dipterocarpus alatus Roxb. Ex G. Don (*D. alatus*) is the large evergreen plant species belonging to the Dipterocarpaceae family found in evergreen and deciduous forest. *D. alatus* is native plant in South-East Asia (Orwa *et al.* 2009). Yang or Yang na is common name of *D. alatus* in Thailand and it was found in every region of the country. Every part of *D. alatus* including wood, leave, petal, and resin has been studied in biological activities and found that the ethanolic extract of *D. alatus* leaves has high content of phenolic compound (rutin and quercetin), high potential of anti-oxidation, anti-inflammatory, inhibition of tyrosinase enzyme and *Propionibacterium acne* (*P. acne*) bacteria, which these properties of leaves extract are proper to be applied in cosmetic more than the other parts (Nasok, 2015). However, we found that the biological activities *D. alatus* leaves extract was gradually decrease during storage. The degradation of phenolic compounds was due to exposure to environmental stress such as oxygen, moisture, temperature and light (Ozkan *et al.* 2019; Casanova *et al.* 2016).

Nowadays, the encapsulation is technology which able to improve stability and bioavailability of active compound in plant extracts (Aguirre Calvo *et al.*, 2017). Encapsulation can be as a method of entrapment of core material (i.e. enzyme, protein, essential oil, active compound) within another solid or liquid immiscible substance or wall material (Donsi *et al.*, 2011). The encapsulation of bioactive compound can be performed by several techniques and ionic gelation by extrusion or ionic extrusion method is a simple, efficient and low-cost encapsulation technique that does not require specific instrument, high temperature, organic solvents and it is suitable for encapsulating both hydrophobic and hydrophilic compound (Đorđević *et al.*, 2015). Sodium alginate is the wall material that most widely used in extrusion method because it has capability to undergo chain-chain association and form three dimensional gels in the presence of divalent cations such as Ca^{2+} and provide high encapsulation efficiency when used as wall material for phenolic compounds (Deladino *et al.*, 2008; Fang *et al.*, 2008).

The encapsulation of *Clitoria ternatea* petal flower extract (Pasukamonset *et al.*, 2016) and *stevia rebaudiana* Bertoni aqueous crude extracts (Arriola *et al.*, 2019) by ionic extrusion method have been reported. Thus, in the present research, we aimed to develop the encapsulation of *D. alatus* leaves extract by extrusion method using sodium alginate as a wall material and characterization of the alginate beads in term of percentage of encapsulation efficiency.

2. Materials and Methods

2.1 Materials

Folin-Ciocalteu's phenol reagent was obtained from Merck (Billerica Ma, USA). Alginic acid sodium salt or sodium alginate and gallic acid monohydrate were obtained from Sigma (St. Louis, MO, USA). Sodium carbonate anhydrous was purchased from Ajax Finechem (Taren Point, NSW, Australia). Calcium chloride dehydrate was obtained from QReC (New Zealand).

2.2 Preparation of *D. alatus* leaves extract

The fresh *D. alatus* leaves were collected from Khon Kaen University, Thailand and the ethanolic extract was done according to Nasok (2015) with slight modification. Briefly, the *D. alatus* leaves were washed and dried in hot air oven (Shel Lab, Cornelius, OR, USA) at 50°C for 24 h. The dried leaves were milled by blender and extracted with 95% ethanol. The extraction was performed using 100 g of dried leave powder with 400 ml of 95% ethanol, stirred by magnetic stirrer at room temperature for 24 h. The extract was filtered through Whatman #1 filter paper, evaporated by rotary evaporator (BUCHI, Flawil, Switzerland) and dried at 50°C for 24 h. The dried extract was stored at $4 \pm 2^\circ\text{C}$ until used.

2.3 Encapsulation of *D. alatus* leaves extract with common method

The encapsulation of leaves extract was performed according to Arriola *et al.* (2019) and Taofiq *et al.* (2018) with various extract concentration. Briefly 2 g of sodium alginate was added into 100 ml of 0.25, 0.50 and 0.75% (w/v) leaves extract solution. The mixtures were homogenized using magnetic stirrer until completely dissolution of alginate and left for 2 h to remove any air bubbles in the solution. The alginate solution was drawn into a 25 ml syringed attached with silicone tube that has a stainless steel needle (0.8×25 mm) for dropping of alginate solution. A peristaltic pump was applied to deliver 25 ml of alginate to 100 ml of 5% (w/v) calcium chloride solution at flow rate of 1.3 ml/min and distance between needle tip and surface of calcium chloride solution was set at 10 cm. The beads were maintained in calcium chloride solution to harden for 30 min and then they were filtered and washed with distilled water for three times. The obtained alginate beads were dried at 40°C for 24 h. then weighed and stored at $4 \pm 2^\circ\text{C}$.

2.4 Optimization of leaves extract encapsulation

The improvement of encapsulation of *D. alatus* leaves extract from common method was performed by using different concentration of Tween®20, sodium alginate and calcium chloride. Briefly, sodium alginate (1, 1.5 and 2 g) were mixed with 100 ml of 0.5% (w/v) extract in the present of 0, 5 and 10% (v/v) of Tween®20. The alginate solution was dropped into 100 ml of 1.5, 3 and 5% (w/v) calcium chloride solution with the same volume of alginate and calcium chloride solution, distance between solution, flow rate, and time of maintaining with optimal method (Section 2.3). The alginate beads were washed three times with distilled water and dried at 40°C for 24 h., then weighed and stored at 4 ± 2°C. This different concentrations of sodium alginate and calcium chloride solution were performed according to Pasukamonset *et al.* (2016).

2.5 Determination of total phenolic content (TPC) and Encapsulation efficiency (EE)

Alginate beads 0.1 g was dissolved in 5 ml of 5% (w/v) sodium citrate and shaken at 200 rpm for 1 h. (Arriola *et al.*, 2019). After fully dissolved, the TPC of the solution was determined according to Taga *et al.* (1984) with slight modifications. Briefly the solution 50 µl was added into 3 ml of distilled water, then 750 µl of 20% (w/v) sodium carbonate was added and vortexed. After 10 min, 250 µl of Folin-Ciocalteu's phenol reagent was added into the mixture, then mixed and left for 15 min. The 950 µl of distilled water was added to obtain 5 ml of final volume. The mixture was incubated at 50°C for 2 h. before the absorption at 765 nm was recorded on UV-Vis spectrometer (Molecular devices, SpectraMax M5, San Jose, CA, USA). A stock solution of 2 mg/ml gallic acid was used as standard (2.0 – 0.03 mg/ml).

The encapsulation efficiency (EE) of alginate beads was calculated using the following equation:

$$EE(\%) = \frac{TPC \text{ of encapsulated beads}}{TPC \text{ of initial extract}} \times 100$$

2.6 Statistical analysis

The results were expressed as mean ± standard deviation (SD) of three replicates. Data were analyzed by statistical software, SPSS version 26 for windows (SPSS Inc., Chicago, IL, USA) using One-way analysis of variance (ANOVA) followed by LSD's multiple range test were performed to determined differences of means among groups. Differences were considered to be significant when $P < 0.05$.

3. Results and Discussion

3.1 Optimization of leaves extract encapsulation

In this study, the different concentration of *D. alatus* leaves extract (0.25, 0.50 and 0.75% (w/v)) with the constant concentration of sodium alginate and calcium chloride were used to prepare alginate beads by extrusion method, then encapsulation efficiency was investigated. **Table 1** showed percentage of encapsulation efficiency of beads with different concentration of extract. The results showed that the encapsulation efficiency was ranging from 22.56 ± 1.44 to 31.33 ± 4.53%. The low value of encapsulation efficiency of extract may be due to an incomplete dissolved extract in distilled water cause the precipitation of insoluble part during homogenization with sodium alginate or removing bobbles. The beads with 0.25% (w/v) extract showed significant higher than other concentrations (0.50 and 0.75% extract). This behavior may occur from the different concentration between the beads and surrounding solution therefore resulting in diffusion of encapsulated compound to calcium chloride solution during gel forming through the porous hydrogel network of alginate beads (Aceval Arriola *et al.*, 2016). Thus, the high concentration of extract exhibited the higher level of diffusion than that of low concentration extract.

Table 1 The percentage of encapsulation efficiency of different concentration *D. alatus* leaves extract encapsulated in calcium alginate beads under different concentration of extract.

Concentration of extract (% w/v)	Encapsulation Efficiency (%)
0.25	31.33 ± 4.53 ^a
0.50	24.79 ± 1.40 ^b
0.75	22.56 ± 1.44 ^b

Data are expressed as mean ± standard deviation (SD) from triplicate.
Different letters in the same column indicate significant differences (P<0.05).

3.2 Optimization of leaves extract encapsulation

This experiment was designed to search for optimal condition for encapsulation of *D. alatus* leaves extract by extrusion technique. The different concentrations of Tween[®]20 (0 – 10% v/v), sodium alginate (1 – 2% w/v) and calcium chloride (1.5 – 5% w/v) were prepared several formulation of alginate beads. **Table 2** showed effects of Tween[®]20, sodium alginate and calcium chloride on encapsulation efficiency of alginate. The results showed that the encapsulation efficiency ranging from 16.95 ± 2.25 to 50.15 ± 4.52%. The encapsulation efficiency significantly increased when concentration of Tween[®]20 increased. For the alginate solution with Tween[®]20, the concentration of sodium alginate and calcium chloride had effect on increasing encapsulation efficiency of beads. Tween[®]20 can improve solubility of extract in alginate solution resulting in increasing of encapsulation efficiency. The high concentration of sodium alginate and calcium chloride result in rise of crosslinking extent on the surface of the beads, which decrease porous and diffusion of encapsulated compounds in alginate beads (Li *et al.*, 2019).

Table 2 The percentage of encapsulation efficiency of *D. alatus* leaves extract encapsulated in calcium alginate under different conditions.

Concentration of Tween [®] 20 (% v/v)	Concentration of sodium alginate (% w/v)	Concentration of calcium chloride (% w/v)	Encapsulation efficiency (%)
0	1.5	3	20.93 ± 1.6 ^e
0	1.5	5	16.95 ± 2.25 ^e
0	2	3	17.47 ± 1.52 ^e
0	2	5	21.08 ± 0.84 ^e
5	1.5	3	20.74 ± 0.43 ^e
5	1.5	5	29.50 ± 5.13 ^{c,d}
5	2	3	29.20 ± 1.22 ^{c,d}
5	2	5	33.62 ± 1.72 ^{b,c}
10	1.5	3	27.93 ± 1.43 ^d
10	1.5	5	33.28 ± 2.03 ^{b,c}
10	2	3	36.18 ± 1.01 ^b
10	2	5	50.15 ± 4.52 ^a

Data are expressed as mean ± standard deviation (SD) from triplicate.
Different letters in the same column indicate significant differences (P<0.05).

4. Conclusion

The present study demonstrates that the encapsulation by extrusion technique using sodium alginate has ability to encapsulate the *D. alatus* leaves extract as well as this ability can be improved by addition of surfactant and increase concentration of sodium alginate and calcium chloride. The further studies are required to increase encapsulation efficiency of encapsulated extract as well as investigation on more physiochemical, structure of the beads, stability and releasing of encapsulated compound.

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Antioxidant activities of Hom Nil rice (*Oryza sativa* L.) bran extract for cosmetic application

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Abstract

Hom Nil or black purple rice (*Oryza sativa* L.) is the pigmented rice that has been regarded as a health-promoting food due to an abundant amount of anthocyanins. The highest anthocyanin contents are typically found in the rice bran, which possess antimicrobial, anticancer, anti-inflammatory, and antioxidant properties. Therefore, Hom Nil rice bran extract has a great potential for use in cosmetic products. This study investigated the antioxidant activity of pigmented rice bran extracts with different solvents. Antioxidant activity was determined using the ferric reducing antioxidant power (FRAP) and ABTS radical scavenging activity assays. The content of total phenolic and their subtypes (flavonoid, and anthocyanin) were assessed by using the Folin-Ciocalteu assay, aluminium chloride colorimetric assay, and pH differential method. Hom Nil rice bran was extracted by maceration with four different solvents including 95% and 50% ethanol and 0.1 M citric acid in water and in 50% ethanol, respectively. The results revealed that 50% ethanolic rice bran extract (50BE) exhibited highest antioxidant activity in FRAP assay (0.96 ± 0.03 mM FeSO₄/g extract) and in ABTS assay (IC₅₀ value as 10.86 ± 0.50 mg/mL) as compared to other extracts. High correlations between the antioxidant activity of Hom Nil rice bran extract and quantity of phenolic, flavonoid, and anthocyanin indicated their contribution to the strong antioxidant activity. The highest total phenolic content of 44.41 mg GAE/g extract, total flavonoid content of 0.51 mg RE/g extract, and total anthocyanin content of 258.81 mg CGE/g extract were shown in the 50BE. In conclusion, the 50BE was the best Hom Nil rice bran extract with a promising antioxidant activity that is suitable for further use in cosmetic product.

Keywords: Anthocyanin; Hom Nil rice; Antioxidant activities; *Oryza sativa* L.; Rice bran extract

1. Introduction

Rice (*Oryza sativa* L.) is a plant in the family of grasses, Gramineae (Poaceae). It is one of the three main food crops of the world and the main diet of about half of the world's population. The global rice production has approximated at 650 million tones and the cultivation area is approximated 156 million hectares. Asia is the leader of rice production, which is about 90% of the world's production. People over 75% of the world have consumed rice, especially Asian. Therefore, rice is very important food of Asia (Tripathi *et al.*, 2011). Hom Nil rice is hybrid rice between Khao-Khaow-Dokmali 105 and Chinese black glutinous rice. Rice production process usually remains the valuable waste after milling such as husk, broken-milled rice and bran. The whole rice grain comprises the bran more than 10.5% by weight (Aguilar-Garcia *et al.*, 2007). It consists of full fat and defatted rice bran. The full fat rice bran compositions consist of moisture, protein, fat, fiber, ash and carbohydrate about 8.5%, 12.6%, 21.13%, 5.59%, 8.97%, and 43.12%, respectively. While the defatted rice bran presented moisture, protein, fat, fiber, ash and carbohydrate about 12.43%, 13.89%, 1.92%, 6.03%, 10.13% and 55.6% ,

respectively (Jiamyangyuen *et al.* , 2005) . It also contains high nutrients such as γ -oryzanol, tocotrienols, ferulic acid, and phenolic compounds (Wanna *et al.* , 2016). The pigmented rice (black purple rice) is the rich source of polyphenol compounds. The most abundant flavonoid compounds in the pigmented rice is anthocyanins (Abdel-Aal *et al.* , 2006; Yawadio *et al.* , 2007) The highest anthocyanin contents are typically found in the rice bran. Two types of anthocyanins which are frequency found in black rice bran are cyanidin-3-glucoside and peonidin-3-glucoside (Yodmanee *et al.* , 2011; Sompong *et al.* , 2011). They showed antimicrobial, anticancer, anti-inflammatory, and antioxidant properties (Heinonen *et al.* , 2016). The extract from black rice contained higher amount of anthocyanins than red and brown rice sources (Pengkumsri *et al.* , 2015). Black rice can be extracted by various methods including conventional solid–liquid extraction, supercritical fluid extraction and ultrasound assisted extraction. The solid–liquid extraction with organic solvent was used in this study due to it is the most popular and simple method. Therefore, the researchers had a plan to select the best Hom Nil rice bran extraction with different solvents and determine their antioxidant activity.

2. Materials and Methods

2.1 Materials

Hom Nil rice was obtained from Lampang province in 2018. Hom Nil rice bran was prepared by milling process of Homnil rice seed . 2,2-azino-bis(3-ethylbenzthiaoline-6-sulphonic acid) or ABTS, ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$), ferrous sulphate heptahydrate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$) and potassium persulfate were obtained from Sigma-Aldrich Inc., USA. 2,4,-6 tripyridyl-s-triazine (TPTZ) was obtained from Fluka, Switzerland. Kuromanin chloride (cyanidin-3-*O*-glucoside chloride), trolox, gallic acid and rutin were obtained from Sigma-Aldrich Inc., USA. Folin-Ciocalteu reagent, sodium carbonate, sodium nitrite, sodium hydroxide, sodium acetate and aluminium trichloride were obtained from Merck, Germany. Acetic acid, citric acid and hydrochloric acid were obtained from Labscan Ltd., Ireland.

2.2 Hom Nil rice bran extraction

Hom Nil rice bran was extracted by maceration with four different solvents including 95% and 50% ethanol and 0.1 M citric acid in water and in 50% ethanol using shaker. Then, the extract solution was filtrated through Whatman® filter paper No. 1. Finally, the filtrate was evaporated by rotary evaporator at 50°C. The crude extracts of Hom Nil rice bran were kept in amber bottle and stored at 4°C for further studies.

2.3 Antioxidant activity determination

2.3.1 Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power of each extract was determined by FRAP method with some modification (Skowrya *et al.* , 2014). The FRAP reagent consists of 300 mM acetate buffer (pH 3.6), 10 mM TPTZ in 40 mM hydrochloric acid in water and 20 mM ferric chloride in water with ratio of 10:1:1. The extract (20 μL) was mixed with 180 μL of FRAP reagent in 96-well plate. After 5 min of incubation in the dark, the absorbance was measured at 595 nm using microplate detector (Beckman Coulter® GmbH, Austria). The ferrous sulfate (FeSO_4) was used as the standard by this equation:

$$Y = 1.1032x + 0.2698$$

Where Y is the absorbance at 595 nm, X is concentration of FeSO_4 ($r^2 = 0.9981$). The results of ferric reducing antioxidant power were reported as the FRAP value and they were calculated following this equation:

$$\text{FRAP value (mM FeSO}_4 \text{ /g extract)} = \frac{[(A - B) - 0.2698]}{1.1032}$$

Where A is the absorbance of control and B is the absorbance of blank of sample.

2.3.2 ABTS radical scavenging activity (ABTS) assay

The ABTS radical scavenging activity of extract was determined by ABTS method with some modification (Skowrya *et al.* , 2014). The ABTS stock solution consists of 7 mM ABTS solution and

140 mM potassium persulfate solution. The mixing solution was incubated in the dark at room temperature for 16 h. The ABTS stock solution was diluted in water and the absorbance was measured at 0.5 nm using microplate detector. 3 μ L of each extract was mixed with 247 μ L of ABTS reagent in 96-well plate. After 6 min of incubation in the dark, the absorbance was measured at 734 nm using microplate detector. The percent inhibition was calculated following this equation:

$$\% \text{ inhibition} = \frac{(A_C - A_S)}{A_C} \times 100$$

Where A_C is the absorbance of control and A_S is the absorbance of the sample. The results were expressed as 50% inhibitory concentration (IC_{50}) as compared to trolox standard.

2.4 Total phenolic content determination

The total phenolic content was determined by Folin-Ciocalteu method with some modification (Ainsworth *et al.*, 2007). 0.5 mL of each extract (1 mg/mL) was mixed with 2 mL of Folin-Ciocalteu reagent and was neutralized with 4 mL of 7.5% sodium carbonate in water. After 30 min of incubation at room temperature, the absorbance was measured at 765 nm using UV-visible spectrophotometer. Gallic acid solution was prepared and plotted for the calibration curve. The results were expressed as milligram of gallic acid equivalent (GAE) per gram of extract.

2.5 Total flavonoid content determination

The total flavonoid content was determined by the colorimetric assay with some modification (Shoib *et al.*, 2015). 1 mL of each extract (1 mg/mL) was mixed with 4 mL of water and 0.3 mL of 5% sodium nitrite in water. After 5 min, 0.3 mL of 10% aluminium chloride in water was added. After 1 min, 2 mL of 1 M sodium hydroxide was added and water used to fill up to 10 mL. The absorbance was measured at 510 nm using UV-visible spectrophotometer. Rutin solution was prepared and plotted for the calibration curve. The results were expressed as milligram of rutin equivalent (RE) per gram of extract.

2.6 Total anthocyanin content determination

The amount of anthocyanins was determined by the pH differential method with some modification (Lee *et al.*, 2005). Each extract at concentration of 1 mg/mL was diluted by different pH of buffer solution. Potassium chloride buffer (pH 1.0) was added in the first test tube of sample and sodium acetate buffer (pH 4.5) was added in the second test tube of sample. These two buffers represent the conditions that anthocyanins show the highest and the least stability. After 15 min in the dark, the absorbance at wavelength of 520 and 700 nm of both test tubes were measured using the UV-visible spectrophotometer. Cyanidin-3-*O*-glucoside chloride solution was used to plot the calibration curve. The results were expressed as milligram of cyanidin-3-*O*-glucoside chloride equivalent (CGE) per gram of extract. The total anthocyanin content was calculated following this equation:

$$A_{\text{diff}} = (A_{520} - A_{700})_{\text{pH}1.0} - (A_{520} - A_{700})_{\text{pH}4.5}$$

2.7 Statistical analysis

All experiments were tested in triplicate. Statistical analysis was performed using One-way ANOVA, SPSS statistical analysis version 17.0.

3. Results and Discussion

3.1 Hom Nil rice bran extraction

Hom Nil rice bran was extracted by maceration with four different solvents including 95% and 50% ethanol and 0.1 M citric acid in water and in 50% ethanol. The results showed that Hom Nil rice bran extract from 0.1 M citric acid in water (CBE) exhibited the highest percentage yield of 94.20%, followed by extracts from 0.1 M citric acid in 50% ethanol (C50BE), 50% ethanol (50BE) and 95% ethanol (95BE), respectively that are shown in Table 1. From the results, CBE and C50BE are sticky liquid physical appearance which indicated that the water still retains in both extracts that revealed high

percentage yield. The results might be related to hydrolysis of glycosidic bond or breaking linkage between phenolic compounds and other constituents including sugar and protein. (Heinonen *et al.*, 2016; Benzie *et al.*, 1996.) It leads to increasing of percentage yield. In addition, small amount of acid in the extraction media could increase anthocyanins extraction (Heinonen *et al.*, 2016). Citric acid is the most appropriate acid for anthocyanins extraction and comparatively better than hydrochloric acid due to its less effect on anthocyanins structure (Heidari *et al.*, 2006; Devi *et al.*, 2012). In generally, ethanol is used to extract antioxidant compounds from plants and it shows better extraction power than water because it had lower viscosity, higher polarity and it generates faster diffusion into plant's cell. However, 50% ethanol has more extraction power than pure ethanol or pure water solvent because the combination of water and organic solvent may facilitate the chemical extraction of substances, which are soluble in water and/or organic solvent (Chatha *et al.*, 2006).

3.2 Determination of antioxidant activity, total phenolic, total flavonoid and total anthocyanin contents

The results of antioxidant activity determined by FRAP and ABTS assays, total phenolic, total flavonoid and total anthocyanin contents are shown in Table 2. The FRAP assay is used to measure the reducing ability of compound that donates the electron to the ferric ions ($[\text{Fe(III)} (\text{TPTZ})_2]^{3+}$) and changes to the ferrous ion ($[\text{Fe(II)} (\text{TPTZ})_2]^{2+}$). The results showed that 50BE exhibited the highest antioxidant activity of both assays, the FRAP assay was 0.96 ± 0.03 mM FeSO_4 / g extract which was significantly different ($p < 0.05$) from the other extracts. However, all extracts showed lower reducing power than that of trolox.

ABTS radical scavenging activity (ABTS) assay is used to measure the ability of the antioxidant compound to get rid of the ABTS^+ radical. The 50BE showed the highest IC_{50} value of ABTS assay as 10.86 ± 0.50 mg/mL which was significantly different ($p < 0.05$) from those under the series of $\text{C50BE} > 95\text{BE} > \text{CBE}$. However, all extracts presented lower antioxidant activity than trolox. In previous study, the antioxidant activity decreased when rice was extracted by higher concentration of citric acid in water. In addition, the ethanol extraction exhibited higher antioxidant activity than that extracted with acid that related to the results in this study (Halee *et al.*, 2018). Antioxidant activity of extract

Table 1 The percentage of yields and physical appearance of Hom Nil rice bran extracts

Extract	Extraction solvent	Yield (%)	Physical appearance	
95BE	95% ethanol	19.41	Purple solid extract	
50BE	50% ethanol	27.66	Purple sticky solid extract	
CBE	0.1 M citric acid in water	94.20	Purple sticky liquid extract	
C50BE	0.1 M citric acid in 50% ethanol	57.99	Purple sticky liquid extract	

Table 2 Antioxidant activity, total phenolic, total flavonoid and total anthocyanin contents of Hom Nil rice bran extracts

Sample or Standard	FRAP assay FRAP value (mM FeSO ₄ / g extract)	ABTS assay IC ₅₀ (mg/ mL)	Total phenolic content (mg GAE/ g extract)	Total flavonoid content (mg RE/ g extract)	Total anthocyanin content (mg CGE/ g extract)
95BE	0.69±0.05 ^c	29.75±5.64 ^b	24.85±0.06 ^b	0.28±0.00 ^b	210.48±9.60 ^b
50BE	0.96±0.03 ^b	10.86±0.50 ^d	44.41±0.00 ^a	0.51±0.00 ^a	258.81±9.60 ^a
CBE	0.28±0.01 ^d	51.26±12.47 ^a	21.28±0.00 ^b	0.12±0.00 ^c	ND
C50BE	0.28±0.04 ^d	18.29±1.91 ^c	21.96±0.06 ^b	0.13±0.00 ^c	130.36±4.41 ^c
Trolox	1.66±0.07 ^a	0.21±0.01 ^e	-	-	-

Where ND is not detectable. Values are given as mean ± S.D. from triplicate. Different letters in the same column indicate significant differences ($p < 0.05$).

decreases due to the free form of phenolic compounds are destroyed by the acid (Bridgers *et al.*, 2010; Ammar *et al.*, 2015).

The 50BE exhibited the highest total phenolic, total flavonoid and total anthocyanin contents as 44.41 ± 0.00 mg GAE/ g extract, 0.51 ± 0.00 mg RE/ g extract, and 258.81 ± 9.60 mg CGE/ g extract, respectively under values of which were significantly different ($p < 0.05$) from the other extracts.

From the results, it can be concluded that anthocyanins presented antioxidant properties by scavenging free radicals and reduce oxidative stress via donating protons to highly reactive radicals (Aniesrani Delfiya *et al.*, 2015). The high antioxidant activities related to high total phenolic, total flavonoid and total anthocyanin contents. The recent results of this study related to previous report, which revealed that dark purple grain had higher polyphenol and antioxidant activity (Yodmanee *et al.*, 2011). In contrast, the CBE could not detect the total anthocyanin because CBE is sticky liquid physical appearance which indicated that the water still retains in the extract that showed high sugar moiety and aglycone formation. In addition, water availability and hydrolytic reaction in the extract effect on anthocyanins degradation. This result is in agreement with the previous study which revealed that cyaniding-3-glucosides was degraded in weak acidified aqueous at various pH values between 1 and 4. The aglycone-sugar bond is the most unstable of glycosidic bonds at pH 2-4. Nevertheless, the glycosidic bonds are sensitive to hydrolysis at pH 1. (Adams, 1973). The anthocyanin degradation at various environmental conditions during production process and storage including high pH value and high temperature excess of 50°C (Heinonen *et al.*, 2016; Soldatkina *et al.*, 2017). In conclusion, the 50% ethanol was the solvent of choice for Hom Nil rice bran extraction that possessed the highest antioxidant activities which is suitable for further use in cosmetic product.

4. Conclusion

50% ethanol was appropriate solvent for Hom Nil rice bran extraction. It showed capability of extraction polar and semi-polar constituents from the rice bran. In addition, the 50BE was the best Hom Nil rice bran extract with good antioxidant activities that is suitable for further use in cosmetic product.

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Comparison of extraction methods of oil and protein from silkworm (*Bombyx mori* L.) pupae for application in cosmetic products

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Abstract

Silkworm pupae is considered as by-product left from silk thread preparation process. It is rich in oil and protein that could be useful for application in cosmetic product for skincare. The objectives of this research were to investigate the optimised method for extracting oil and protein from pupae of silkworm (*Bombyx mori* L. Var. Luang-Pairoj Thai hybrid). Three extraction methods were used including (1) soxhlet extraction followed by protein precipitation with ammonium sulfate, (2) maceration extraction followed by protein precipitation with ammonium sulfate, and (3) simultaneous oil and protein extraction by three-phase partitioning (TPP) technique. The percentage of oil extracted by soxhlet extraction, maceration extraction and three-phase partitioning were in the range of 36.25±1.36-37.34±3.07%, 11.64±1.17-12.54±1.23% and 7.32±0.14-8.24±0.98%, respectively, while the content of protein were equal to 1.77±0.15-1.87±0.15%, 1.15±0.12-1.22±0.10% and 2.56±0.29-8.40±0.87%. The comparison of different extraction methods, the soxhlet extraction method obtained the highest yield of silkworm pupae oil, followed by maceration extraction and three-phase partitioning (TPP) technique, respectively. All the three methods obtained different yields with no significant differences ($P \leq 0.05$). There were statistically significant differences ($P \leq 0.05$) for protein values. Three-phase partitioning method achieved the highest protein yield, followed by precipitation with ammonium sulfate of silkworm pupae oil extracted from soxhlet extraction. The least yield was from precipitation with ammonium sulfate of silkworm pupae oil extracted from maceration extraction. From this study, oil and protein extracted from silkworm pupae can be more analysed for chemical properties and used as a direction for further application in cosmetic products.

Keywords: Extraction Method; Silkworm Pupae; Oil; Protein.

1. Introduction

This research has a concept coming from the silk industry that having the by-product is silkworm pupae left from the silk thread preparation process. It is rich oil and protein that could be useful for application in cosmetic product for skincare. Silkworm (*Bombyx mori* L.) pupae var. Luang-Pairoj is the Thai hybrid's breeding in 2012 CE. from Japanese breed J108 crossed with the native Thai Nang-Lai breed (J108:bivoltine x Nanglai:multivoltine). Luang-Pairoj breed is suitable for farmers because of its simple raising, strength, good quality cocoons as one cocoon giving the silk for 700-800 metres. Those are the good reason for using in the industry. Proportion of Silkworm (*Bombyx mori* L.) pupae var. The Queen Sirikit Department of Sericulture in 2015. promoted Luang-Pairoj Thai hybrid in

various varieties by egg distribution to farmers for more than 50%, and this breed gives further advantages (The Queen Sirikit Department of Sericulture “Annual report 2015”, 2015 <https://qsds.go.th>). Currently, the farmers use most of the silkworm pupae for consumption. Moreover, there had various researches in pharmacy, food, cosmetics, livestock and food supplements. The oil is not only a part of the formation of the skin’s cell membrane but also protect the skin and reduce wrinkle and melanin pigment formation (Dhiraj *et al.*, 2005). Fat and protein extracted from natural insects could be used as optional ingredients in cosmetics. The researcher realised that the quantitative analysis of the nutrition information of silkworm pupae has very high in protein and good fat that are suitable for skin structure due to having the fatty acids are related to the sebum and fat of skin cells, that act as natural moisturising factors (NMFs). The objectives of this research were to investigate the optimised method for extracting oil and protein from pupae of silkworm (*Bombyx mori* L. Var. Luang-Pairoj Thai hybrid). Three extraction methods were used including (1) soxhlet extraction followed by protein precipitation with ammonium sulfate, (2) maceration extraction followed by protein precipitation with ammonium sulfate, and (3) simultaneous oil and protein extraction by three-phase partitioning (TPP) technique.

2. Material and Method

2.1 Material

Thai silkworm pupae (*Bombyx mori* L.) var. Luang-Pairoj (J108:bivotine x Nanglai:multivoltine), Thai hybrid’s breeding from The Queen Sirikit Department of Sericulture in Hua-Hin, Prachuapkhirikhan Province, Thailand were used in this study. The Thai silkworm pupae were collected from February to June in 2017 and 2018.

2.2 Preparation silkworm pupae

Thai silkworm pupae (*Bombyx mori* L.) var. Luang-Pairoj, Thai hybrid’s by-product from silk thread preparation process were washed with distilled water, air-dried and dried in hot air oven at 50°C until constant weight. After that, the dried pupae were ground to powder and stored at 4°C for further testing.

2.3 Soxhlet extraction followed by protein precipitation with ammonium sulfate

Soxhlet extraction (Doneanu *et al.*, 1997; Khobkol *et al.*, 2002) followed by protein extraction by precipitation with ammonium sulfate salt were studied. Samples of 100 grams of silkworm pupae powder were extracted by Soxhlet apparatus with 300 ml of petroleum ether solvent (Nipha and Arunyakorn, 1997; Doneanu *et al.*, 1997) for 3, 6, 12 and 18 hours. The extracted oil was filtered and evaporated with a rotary evaporator at a temperature of 40°C. The weights of oil were recorded to calculate the yield. The silkworm pupae powder was dried at 50°C for 6 hours and followed by protein extraction by precipitation with ammonium sulfate salt 30% w/v (Dawson *et al.*, 1969). The extracted silkworm pupae powder was blended with distilled water in the ratio of 1:3 w/v for 1 hour, filtered the residue and then the filtrate was allowed to precipitate protein by 30% (w/v) ammonium sulfate final concentration. During precipitation, the mixture was stirred slowly at 4°C for 30 min, and then centrifuged at 9000 rpm for 30 min to separate the protein. The protein was dried and recorded the weight obtained to calculate the yield.

2.4 Maceration extraction followed by protein precipitation with ammonium sulfate

The maceration method (Manosroi *et al.*, 2010) followed by protein extraction by precipitation with ammonium sulfate salt were studied. Samples of 100 g of silkworm pupae powder was shaken in 300 ml of petroleum ether solvent (Nipha *et al.*, 1997; Doneanu *et al.*, 1997) for 3 minutes with a shaker at 150 rpm at room temperature for 3, 6, 12 and 18 hours. The solution after oil extraction was filtered and evaporated with a rotary vacuum evaporator at a temperature of 40°C recorded the weight of the oil obtained to calculate the yield. The silkworm pupae powder was dried at 50°C for 6 hours and followed by protein extraction by precipitation with 30% w/v of ammonium sulfate salt (Dawson *et al.*, 1969). Silkworm pupae powder that has been extracted oil and then blended with distilled water in the ratio of 1:3 w/v for 1 hour, filtered the residue and then the filtrate was allowed to precipitate protein by 30% (w/v) ammonium sulfate final concentration. During precipitation, the mixture was

stired slowly at 4°C for 30 min, and then centrifuged at 9000 rpm for 30 min. to separate the protein. The protein was dried and recorded the weight obtained to calculate the yield.

2.5 Three-phase partitioning (TPP) technique

Three-phase partitioning (TPP) technique extracting oil and protein at the same time using TPP technique which consists of ammonium sulfate and t-butanol according to the modified method from Varakumar *et al.*, (2017). The silkworm pupae powder was blended with distilled water at the ratio 1:3 w/v for 5 min., then added ammonium sulfate salt, shaken until the salt dissolves, after that added t-butanol, stirred with a magnetic stirring rod or shook for 3, 6, 12 and 18 hours. The residue was filtered and then taken the clear solution to centrifuged at a speed of 7000 rpm for 30 minutes. The separated sample was shown in 3 layers. The top layer of t-butanol was used to evaporate the solvent with a rotary vacuum evaporator, recorded the weight of the oil obtained to calculate the yield. Separated the protein by filtering the middle layer between the bottom water and the top t-butanol. The protein was dried and recorded the weight obtained to calculate the yield.

$$\text{Oil Yield (\%)} = (\text{weight of extract (g)} / \text{weight of sample (g)}) \times 100.$$

$$\text{Protein Yield (\%)} = (\text{weight of extract (g)} / \text{weight of sample (g)}) \times 100.$$

2.6 Statistical analysis

The results are presented as the $\text{mean} \pm \text{SD}$ of three independent experiments. Analysis of variance (ANOVA) was used for analysis of the test results (least significant difference) at the significant level of P-value ≤ 0.05

3. Result and Discussion

3.1 Preparation silkworm pupae

Silkworm (*Bombyx mori* L.) Var. Luang-Pairoj Thai Hybrid was dried and ground to obtain dried powder which possessed $20.18 \pm 3.15\%$ dry weight. Kanika *et al.*, (2008) reported 23.6 and 37.8 g de-oiled pupae powder each was obtained from 100 g fresh male and female pupae in PM x CSR2 (Multivoltine x bivoltine) whereas 21.5 and 36.5 g in male and female pupae in CSR2 x CSR4 (bivoltine x bivoltine). Statistical analysis showed significant difference in de-oiled pupae powder recovery between the male and female pupae of both the hybrids. Female pupae yielded more pupae powder than male in both the hybrids.



Figure. 1 Physical appearance of silkworm (*Bombyx mori* L. Var. Luang-Pairoj Thai hybrid) pupae oil. A: Silkworm pupae B: Dried silkworm pupae C: Silkworm pupae powder

Table1 Yield of silkworm pupae oil and protein extract

Methods	Extraction time (h)	Oil yield (%w/w)	Protein yield (%w/w)
Soxhlet extraction	3	37.13±1.65 ^a	1.77±0.15 ^a
	6	36.84±2.20 ^a	1.87±0.15 ^a
	12	37.34±3.07 ^a	1.80±0.02 ^a
	18	36.25±1.36 ^a	1.82±0.09 ^a
Maceration extraction	3	12.54±1.23 ^a	1.22±0.10 ^a
	6	11.64±1.17 ^a	1.21±0.06 ^a
	12	12.31±1.39 ^a	1.15±0.12 ^a
	18	12.51±2.06 ^a	1.17±0.07 ^a
Three Phase Partitioning extraction	3	7.32±0.14 ^a	2.56±0.29 ^a
	6	7.67±0.29 ^a	3.75±1.00 ^b
	12	7.82±0.83 ^a	7.01±0.83 ^c
	18	8.24±0.98 ^a	8.40±0.87 ^c

⁽¹⁾ Calculated (mean ± SD) oil yield and protein yield of silkworm pupae extracts for three method and four extraction time. Values with different letters in the same column are significantly different ($P \leq 0.05$).

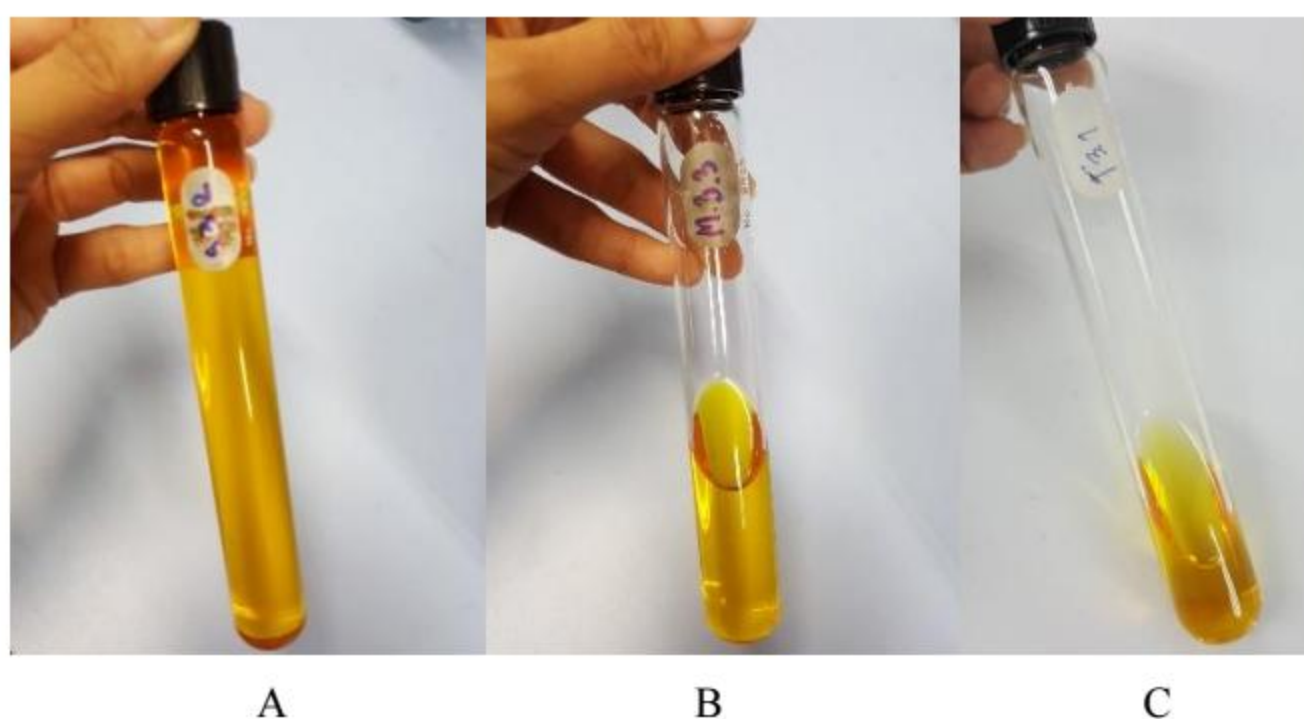


Figure 2 Physical appearance of silkworm (*Bombyx mori* L. Var. Luang-Pairoj Thai hybrid) pupae oil extracted by (A) Soxhlet extraction, (B) Maceration extraction and (C) Three-phase partitioning

3.2 Soxhlet extraction followed by protein precipitation with ammonium sulfate

The oils extracted from Thai silkworm pupae (*Bombyx mori* L.) var. Luang-Pairoj by Soxhlet extraction with petroleum ether for 3, 6, 12 and 18 hours were liquid at room temperature (Figure 2A). The yields of oil were 36.25 ± 1.36 - 37.34 ± 3.07 (%w/w) varying by time with no statistically significant differences. After the oil has been extracted for each period, continued by protein precipitation with Ammonium Sulfate. The results of protein extraction were 1.77 ± 0.15 - 1.87 ± 0.15 (%w/w) with no statistical differences, as shown in Table 1. From the study of Winitchai *et al.*, (2012) found that the percentage of the extracted oil from the five dried silkworm varieties, namely Keao-Sakol, Nang-Noi, Sri-Saket, Sumrong-Nang-Luang and Non-Luesi with petroleum ether were 24-29 percent of the total yield. Trivedy *et al.*, (2008) reported oil (removed by solvent soxhlet extraction process using chloroform: methanol = 2:1 (v/v)) and crude protein with other constituents were estimated as 40.02 and 59.98% in male pupae and 25.17 and 74.67% in female pupae of PM x CSR2 (multivoltine x bivoltine), whereas in CSR2 x CSR4 (bivoltine x bivoltine) these were 40.00 and 60.00% in male and 24.0 and 76.00% in female, respectively. It was found that in pupa powder oil is more in male than female of both the hybrids, whereas, crude protein was significantly more in female of both the hybrids as compared to male. Chatsuwana *et al.*, (2018) reported frozen silkworm pupae (*B. mori*) extraction was blended with cold water 4°C at a ratio of 1:4 w/v for 15 min using blender and stirred overnight at 4°C. The suspension was centrifuged at 12,500 g for 30 min at 4°C. After centrifugation, the sample was separated to 3 layers. Then, the middle layer, containing water soluble protein fraction was collected, freeze-dried and referred to as “water-soluble protein from *B. mori*: WSPB”. The yield of water-soluble protein extracted from silkworm pupae, *Bombyx mori* Linn. (WSPB) was 3.96% by wet weight basis.

3.3 Maceration extraction followed by protein precipitation with ammonium sulfate

The extracted oil from Thai silkworm pupae (*Bombyx mori* L.) var. Luang-Pairoj by maceration extraction with petroleum ether for 3, 6, 12 and 18 hours was transparent yellow liquid at the room temperature (Figure 2B). The yields were in the range of 11.64 ± 1.17 - 12.54 ± 1.23 (%w/w) varying by time with no statistical differences. Brought the extracted oil to extract protein by precipitation with ammonium sulfate, found the yield of protein in the range 1.15 ± 0.12 - 1.22 ± 0.10 (%w/w) with no statistical differences as shown in Table 1. From the study of Winitchai *et al.*, (2012) found that the percentage of the extracted oil from the 5 silkworm varieties both of dried and fresh, namely Keao-Sakol, Nang-Noi, Sri-Saket, Sumrong-Nang-Luang and Non-Luesi by Maceration with petroleum ether at 10°C for 5 and 7 days were 4-7 percent of the total yield with no statistical differences.

3.4 Three-phase partitioning (TPP) technique

The extracted oil from Thai silkworm pupae (*Bombyx mori* L.) var. Luang-Pairoj by three-phase partitioning (TPP) techniques with distilled water, ammonium sulfate and t-butanol for 3, 6, 12 and 18 hours was transparent yellow liquid at the room temperature (Figure 2C). The yields of oil were 7.32 ± 0.14 - 8.24 ± 0.98 (%w/w) varying by time with no statistical differences. The yields of protein extracted were statistical differences in the range of 2.56 ± 0.29 - 8.40 ± 0.87 (%w/w) and the yield increased by the time increased as shown in Table 1. The three-phase partitioning is the new technology of extraction that can extract fat and protein in the same time and do not denature the protein because the process is at the ambient and also get more protein (Xu and Wang, 2009). The extracted protein is concentrated and can apply for the mass production, therefore it is effective in economics. The extraction method is using the cells directly and is the cause of saving the time of sample preparation and saving the chemicals cost.

4. Conclusion

Comparing the oil extraction methods of Thai silkworm pupae (*Bombyx mori* L.) var. Luang-Pairoj found that the Soxhlet method at 3 hours gave the most cost-effective, but the three-phase partitioning gave the most protein yield, that increased with the time of extraction. The extraction of oil and protein from Thai silkworm pupae (*Bombyx mori* L.) var. Luang-Pairoj gave the effective and gained the possibility to be used as raw material in cosmetics, but it is necessary to analyse the chemical composition of the extracted oils and proteins in each method, including testing of various

properties and toxicity test in the cells before applying to develop further cosmetic products.

5. Acknowledgements

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Effect of high temperature stress on resveratrol and oxyresveratrol accumulation and related gene expression in mulberry callus

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Abstract

Resveratrol (Res) and oxyresveratrol (Oxyres) are the major stilbene phytoalexin that protects plant tissues from many environmental stresses. Res and Oxyres have many human-health benefits such as antioxidant, cancer-chemoprevention, cardioprotection as well as anti-aging activities. The previous study demonstrated that Res and Oxyres regulate mechanism of skin-whitening action by inhibition of tyrosinase activity and reduction in melanocytes proliferation. Moreover, these compounds can stimulate the fibroblast proliferation and promoting collagen type III. However, accumulation of Res and Oxyres in mulberry has not been well studied. Therefore, the aims of this study were to investigate the effect of high temperature on Res and Oxyres contents and determine the expression of *cinnamate-4-hydroxylase* (*C4H*), *4-coumarate: CoA ligase* (*4CL*) and *stilbene synthase* (*STS*) genes involved in Res biosynthesis in mulberry callus induced from root explant. Under high temperature stress condition, Res and Oxyres contents increased 2.67- and 3.49-fold, respectively when compared to normal growth condition. Gene expression analysis revealed that *C4H* expressions remain unchanged. In contrast, the expression of *4CL* and *STS* increased significantly after 6 h incubation at 40°C and showed the highest at 12 h when compared with untreated callus. These results suggested that high temperature stress can induce Res and Oxyres contents in mulberry callus. The study of Res and Oxyres accumulation including biological synthesis will bring benefits for quality improvement and industrial exploration of mulberry in the future.

Keywords: High temperature stress; Mulberry; Oxyresveratrol (Oxyres); Resveratrol (Res).

1. Introduction

Resveratrol (Res) and oxyresveratrol (Oxyres), the derivative of resveratrol and can be formed by 2-hydroxylation of resveratrol, are major hydroxystilbene phytoalexins. Res and Oxyres are produced by plants in response to stimulation from environmental stress, providing the plant resistance (Li, *et al.*, 2017). Res and Oxyres can be synthesized via the shikimate pathway, starting from phenylalanine or tyrosine. These compounds have been used as active ingredients and cosmeceuticals with powerful antioxidants and anti-inflammatory, involved in reducing the risk of many chronic disease (Riba, *et al.*, 2017). Res and Oxyres also has anti-aging property, involved in the inhibition of tyrosinase activity and reduction in melanocytes proliferation (Chatsumpun, *et al.*, 2011). Furthermore, Res can stimulate the fibroblast proliferation and promoting collagen type III, which is important for the development of skin (Lee, *et al.*, 2014). The biological potential mentioned above has resulted in Res and Oxyres being particularly interesting in the medical and cosmeceutical industry.

Mulberry (*Morus* spp.) is one of the economic plants grown widely in the Northeastern region of Thailand. Different tissues of mulberry contain high levels of vitamin and phenolic compounds (Yu, *et al.*, 2018). However, accumulation of Res and Oxyres in mulberry has not been well studied.

Therefore, the aims of this study were to investigate the effect of high temperature on Res and Oxyres accumulation in mulberry callus and determine the expression of genes involved in Res biosynthesis pathway.

2. Materials and methods

2.1 Plant materials and callus induction

Callus of mulberry plants cv. Kamphaeng Saen-MB-42-1 was induced from root tip *in vitro* and subcultured monthly on the Murashige and Skoog medium (MS medium) supplemented with 30 g/L sucrose, 8 g/L agar, 0.5 mg/L 6-benzylaminopurine (BAP) and 2,4-dichlorophenoxyacetic acid (2,4-D) in the same ratio. The pH of the plant growth media was adjusted to 5.7 ± 1 . The callus was cultured at $26 \pm 1^\circ\text{C}$ in darkness and 70-80 % of humidity.

2.2 High temperature stress treatments

The mulberry callus was cultured at $26 \pm 1^\circ\text{C}$ (Sahoo, *et al.*, 1997) in darkness for 45 days (Kamjad, unpublished). Then, the callus was selected for high temperature treatments (Figure 1A). After that, the callus was transferred to $40 \pm 1^\circ\text{C}$ and harvested at 0 (normal growth condition), 6, and 12 h (Figure 1B).

2.3 Res and Oxyres extraction from mulberry callus

Mulberry callus was dried in a vacuum freeze drier and ground into a fine powder using tissue lyser. The 2 mL of 80% methanol was added per 200 mg of powder. Sample along with solvent was sonicated on ice for 30 min (3 times) and then incubated at 4°C overnight. After that, the samples were centrifuged at 12,000 rpm for 10 min. The supernatant was evaporated with N_2 gas and the dried residue was dissolved in 200 μL of 80% ethanol. The sample was filtered through 0.2 μm syringe filter before HPLC analysis.

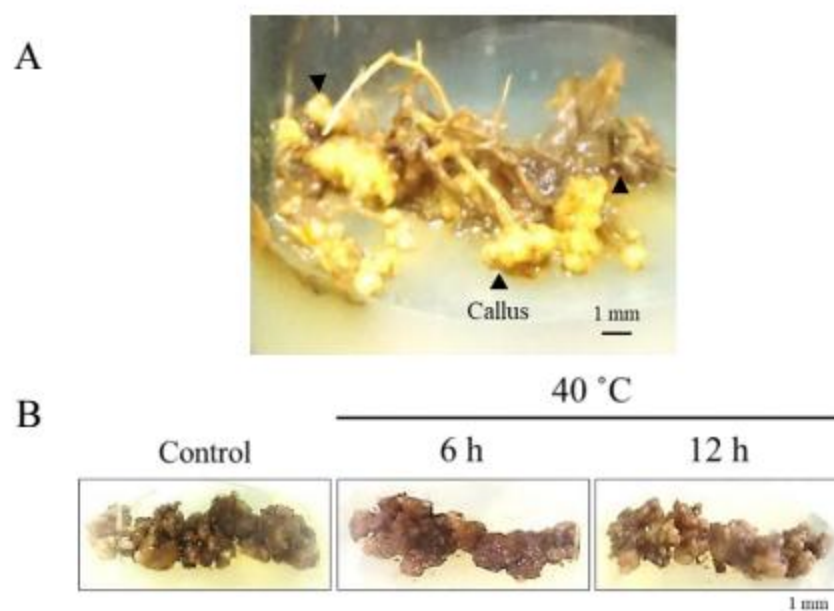


Figure 1 Callus was induced from root tip and grown for 30 days on the MS medium supplemented with 30 g/L sucrose, 8 g/L agar, 0.5 mg/L BAP and 2,4-D in the same ratio (A). For stress treatment, callus was transferred to $40 \pm 1^\circ\text{C}$ for 0 (normal growth condition), 6, and 12 h in darkness (B). Bar 1 mm.

2.4 Measurement of Res and Oxyres contents using HPLC

The HPLC analysis of Res and Oxyres contents in mulberry callus was performed using a Waters Alliance e2695 HPLC system (Waters, USA) with C18 reverse phase column (4.6 × 250 mm; particle size: 5 µm) (Thermo Scientific, USA). Solvent A consisted of 0.1% trifluoroacetic acid (TFA) in water, whereas solvent B was acetonitrile. The 20 µL of sample was injected into the column temperature of 25°C using a flow rate of 1 mL/min, and the detection occurred at 306 nm. Commercially available Res and Oxyres were used as standards (Sigma-Aldrich, USA, and Chengdu Biopurify Phytochemicals, China). Res and Oxyres were quantified by comparing retention times and peak areas with standard values.

2.5 RNA isolation and cDNA synthesis

Total RNA was isolated from 50 mg of callus tissue using TRI reagent (Molecular Research Center, USA). The quality and concentration of the RNA were determined using a NanoDrop One spectrophotometer (Thermo Scientific, USA). First-strand cDNA was synthesized from 1 µg total RNA using the RevertAid First Strand cDNA Synthesis kit (Thermo Scientific, USA). The cDNA samples were used as a template for RT-PCR.

2.6 Gene expression analysis by RT-PCR

The RT-PCR experiments were performed on a Thermo Electron Hybaid Px2 Thermal Cycler (Thermo Scientific, USA). The specific primers of Res biosynthesis genes (*C4H*, *4CL* and *STS*) and *Actin* as a housekeeping gene were designed from nucleotide sequences in the GenBank database (accessions number: KJ616396.1, KJ013409.1, KU596032.1 and KJ616403.1). The RT-PCR experiments were completed using the following program: 30 cycles of 95°C for 0.5 min 58°C for 1 min and 72°C for 1 min.

2.7 Statistical Analysis

Data were obtained from 2-3 biological replicates, and the results were expressed as means ± standard deviation (SD). The statistical analysis Two-way ANOVA was performed using GraphPad Prism 6 (GraphPad Software, Inc). The intensity of each band was analyzed using ImageJ (Wayne Rasband, National Institutes of Health, USA).

3. Results and Discussion

3.1 Effect of high temperature on Res and Oxyres accumulation

In this study, callus culture was used as a model system to determine Res and Oxyres accumulation in response to high temperature stress. The mulberry callus was subjected to high temperature at 40°C for 0-12 h. After that, Res and Oxyres were extracted from the callus and quantified using HPLC. The chromatograms of the standard are presented in Figure 2A-2B. The result showed that Res content increased significantly at 6 h (14.18 µg/g DW) and 12 h (19.44 µg/g DW), which is 1.96- and 2.67-fold, when compared to normal growth condition. Moreover, Oxyres content was 64.64 µg/g DW (2.47-fold) and 91.23 µg/g DW (3.49-fold) at 6 h and 12 h respectively, when compared to untreated condition (Figure 3A-3B). These results indicated that high temperature stress induces both Res and Oxyres accumulation in mulberry callus. From the previous study, Oxyres content was found to be highest in roots and stems of mulberry plants but the amount of Res was very low in both tissues (Zhou, *et al.*, 2013). The effect of high temperature stress on stilbene content was also investigated in *Gnetum parvifolium* stems. There was no change in stilbene content when the plants were exposed to high temperature over time, however significant fluctuations were observed in the roots (Deng, *et al.*, 2017). Another abiotic stress factor, UV irradiation was found to be effective for the induction of Res production at 48 h in *Vitis vinifera* L. callus culture (Keskin and Kunter, 2008). Similarly, Oxyres levels in mulberry leaves increased at 18 h after UV-C treatment (Li, *et al.*, 2016). These data suggested that abiotic stresses play important roles in Res and Oxyres production.

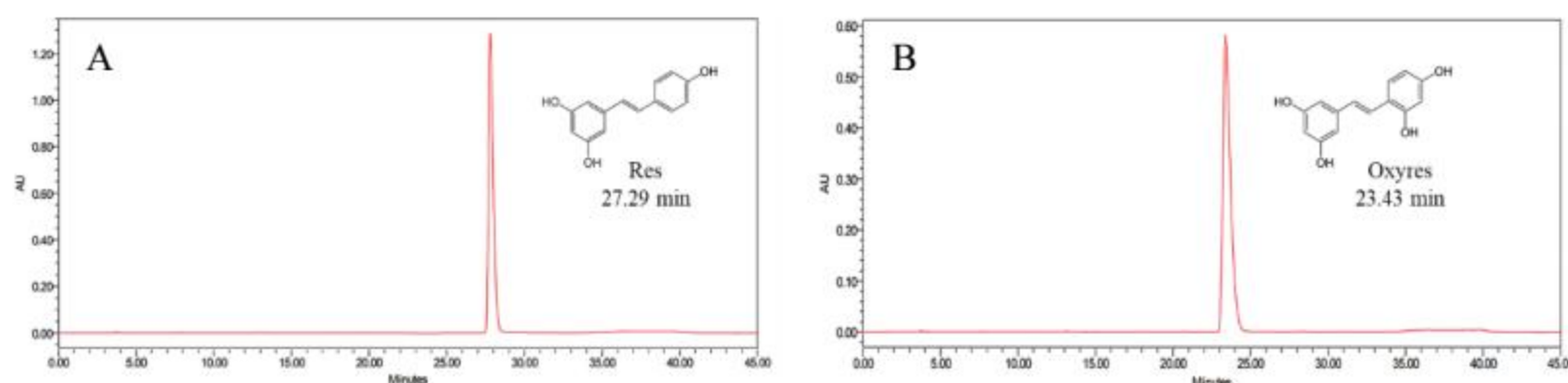


Figure 2 The chromatogram and structure of Res and Oxyres commercial standard (A-B). The retention time of Res and Oxyres are 27.29 and 23.43 min, respectively.

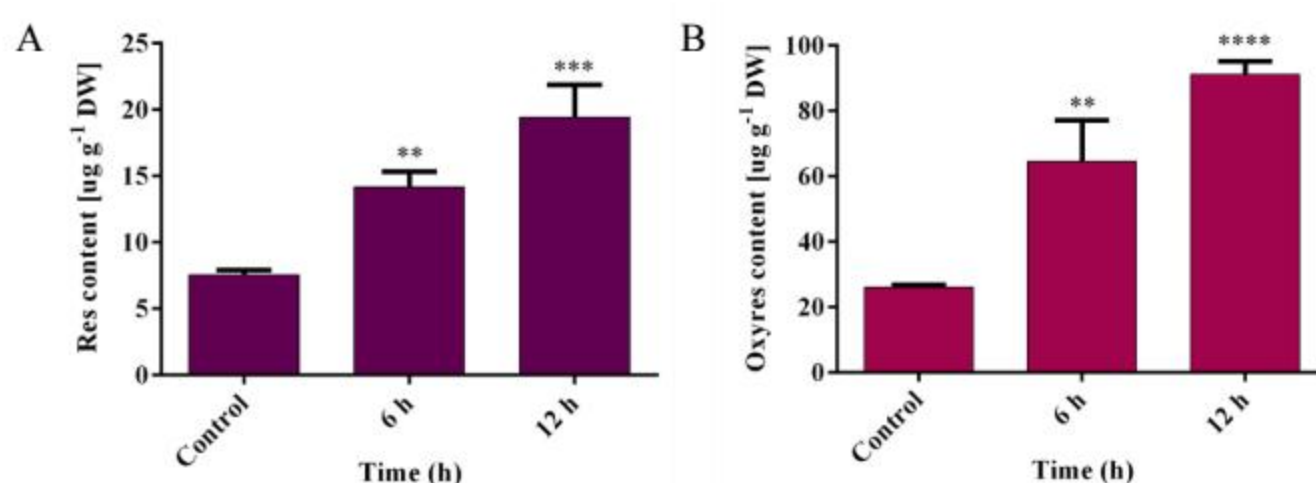


Figure 3 Contents of Res (A) and Oxy (B) in mulberry callus under stress conditions. X-axis represents the treatment time and Y-axis represents the content of resveratrol and oxyresveratrol ($\mu\text{g/g DW}$). Means $\pm$ SD, $n = 5$. **, *** and **** indicate significant difference at $P < 0.005$, $P < 0.0005$ and $P < 0.0001$, respectively, between the treated and normal growth conditions.

3.2 Expression patterns of genes involved in resveratrol biosynthesis pathway in response to high temperature stress

The expression of genes involved in resveratrol biosynthesis pathway, including *C4H*, *4CL* and *STS*, was investigated after high temperature exposure. For the upstream genes, no significant increase was detected in *C4H* expression. In contrast, the expression of *4CL* was up-regulated (1.55- and 1.61-fold at 6 h and 12 h, respectively) when compared to normal growth condition. Similarly, the downstream gene, *STS* expression level was 1.54- and 1.63-fold higher than that of the control after 6 h and 12 h, respectively (Figure 4). It was found that the expressions of *C4H*, *4CL*, and *STS* were significantly increased in *Gnetum parvifolium* in response to high temperature stress (Deng, *et al.*, 2017). In grapevine, *STS* expression was highly induced upon abiotic stress, especially wounding and UV-C exposure (Gatto, *et al.*, 2008). In addition, the UV-light and blue light irradiation stimulated *STS* transcript in berries (Ahn, *et al.*, 2015). Therefore, it can be concluded that the non-living environmental stress affects Res and Oxyres accumulation through the expression of Res biosynthesis genes.

4. Conclusion

The results herein indicate that high temperature stress affects the biosynthesis of Res and Oxyres in mulberry callus. Therefore, it can be concluded that this optimal stress condition has high potential to be used for enhancing specific stilbene compound production in mulberry.

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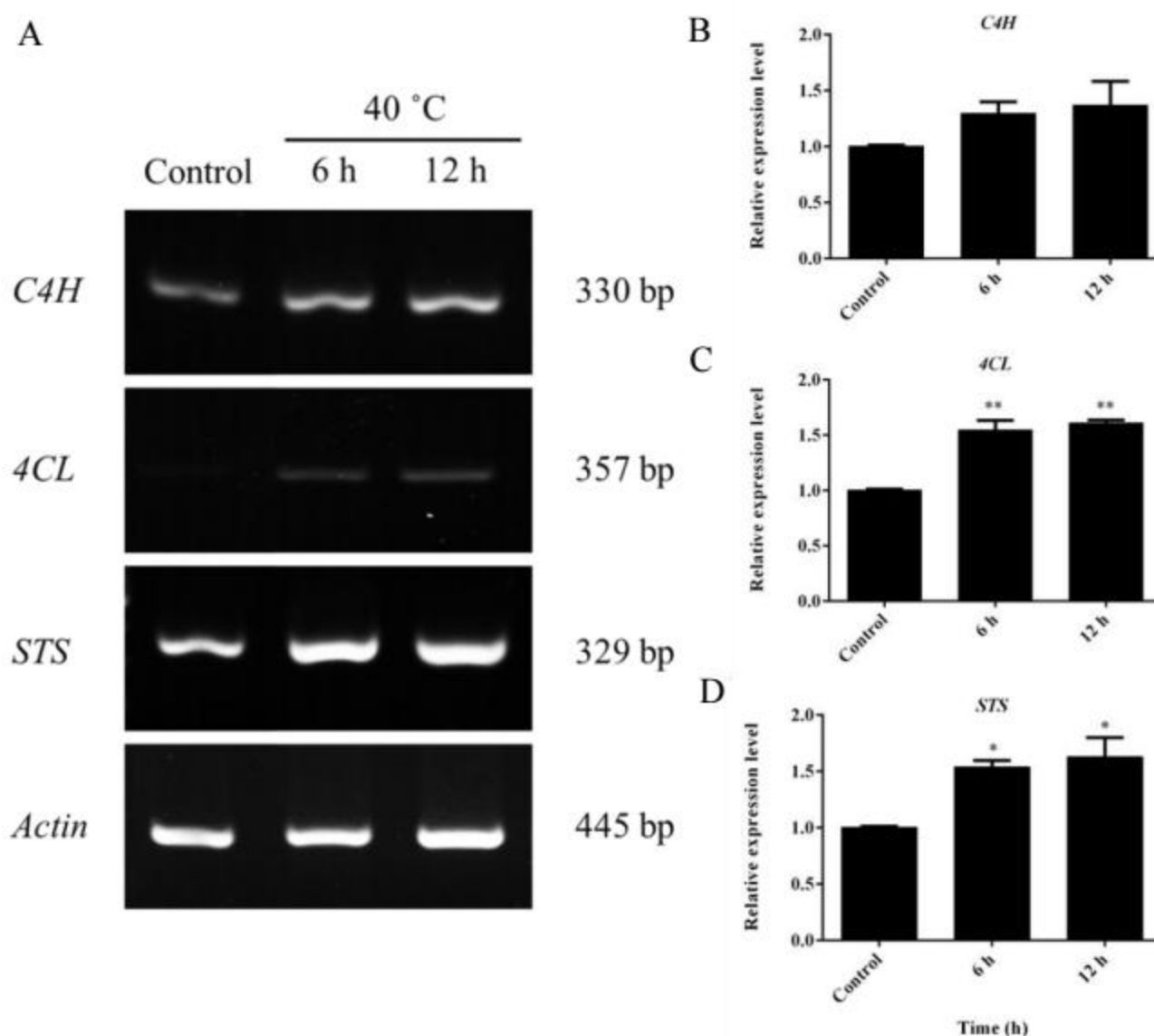


Figure 4 The expression of *C4H*, *4CL* and *STS* genes in mulberry callus after high temperature treatment (A). The PCR products were visualized on 1% agarose gel. *Actin* was used as an internal control. The relative expression pattern of Res biosynthesis genes compared with normal growth condition (B-D). Means $\pm$ SD, $n = 3$. * and ** indicate significant difference at $P < 0.05$ and $P < 0.005$, respectively, between the treated and normal growth conditions.

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Bioactive and antioxidant activity of *Centella asiatica* extract for hair cosmetic application

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Abstract

The objective of this study were to prepare *Centella asiatica* (CA) extract by using 2 methods: squeezing process and solvent extraction (DI water and ethanol) and to investigate the bioactive compounds (total phenolic: TPC, flavonoid: TFC and proanthocyanidin:TPAC contents) and antioxidant activity (DPPH, ABTS and FRAP activities) of these *Centella asiatica* extracts. Results showed that the ethanolic CA extract had the significantly highest TPC (262.5±1.1 mg GAE/g sample), TFC (100.7±0.6 mg QE/g sample) and TPAC (1236.0±0.0 mg ECE/ g sample) (p<0.05). In addition, the antioxidant capacity of these CA extracts tended to be greatest on ethanol extracts (DPPH 1157.6±26.8, ABTS 710.7±12.9 and FRAP 2648.8±12.0 mg TEAC /g sample) (p<0.05). The results suggested that *Centella asiatica* extract (especially ethanolic extraction) with high bioactive compounds (e.g. proanthocyanidin) and great antioxidant activity can be used as natural ingredient for hair care product.

Keywords: Antioxidant; *Centella asiatica* extract; Flavonoid; Hair care; Phenolic; Proanthocyanidin.

1. Introduction

Centella asiatica (common name Gotu kola) is one of Thai herbs that has been used for long time ago as health and beauty treatments in Thai traditional wisdom. The fresh leaves of *C. asiatica* is squeezed with clean water and then filtrated by cotton cloth as well as the extract is applied into hair and scalp for black shine hair and nourishing hair treatment. Moreover, the previous studies of this plant showed that *C. asiatica* extract by solvent extraction have high bioactive compounds (e.g. phenolic, flavonoid) and bioactivity (e.g. antioxidant activity) (Coldren, *et al.*, 2003; Kubola & Siriamornpun, 2011). These bioactive and its antioxidant activity can be used for skin and hair anti-aging treatment (Trüeb, Ralph M., 2006). However, the comparative study on bioactive and its antioxidant activity of *C. asiatica* extract from 2 methods; Thai traditional wisdom method and solvent extraction has little been investigated. Therefore, the objectives of this study were to prepare *Centella asiatica* (CA) extract by using 2 methods: squeezing process (following Thai traditional wisdom) and solvent extraction (DI water and ethanol) and to investigate the bioactive compounds (total phenolic: TPC, flavonoid: TFC and proanthocyanidin: TPAC contents) and antioxidant activity (DPPH, ABTS and FRAP activities) of these *Centella asiatica* extracts.

2. Materials and Methods

2.1 Chemicals and reagents

All chemicals and solvents were analytical grade, cosmetic ingredients were cosmetic grade. Acetone, dimethylsulfoxide (DMSO), ethanol (95%), ferric chloride, hydrochloric acid (37%),

methanol, sulphuric acid, vanillin was purchased from Merck (Darmstadt, Germany). Sodium carbonate (Na_2CO_3), aluminum chloride (AlCl_3), dibasic phosphate and monobasic phosphate. Potassium acetate (CH_3COOK) and potassium persulphate ($\text{K}_2\text{S}_2\text{O}_8$) were purchased from Ajax Finechem (Seven Hills, Australia). ABTS(2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid), DPPH(2,2-diphenyl-1-picrylhydrazyl), catechin, folin-ciocalteu, gallic acid, 2,4,6-tris(2-pyridyl)-1,3,5-triazine (TPTZ), quercetin, and trolox were purchased from Sigma (St. Louis, MO, USA).

2.2 Sample preparation

Gotu kola (*Centella asiatica*) were obtained from local market of Chiang Rai province and Bangkok, Thailand. The samples were cleaned and its root was removed. These CA were cut into small pieces and kept at 4°C until used.

2.3 Extraction of bioactive compounds

The clean fresh CA were extracted with 2 methods: squeezing process (following Thai traditional wisdom) and solvent extraction (DI water and ethanol). For squeezing process, CA was squeezed with clean water in ratio of sample: water, 1:2 w/w. And then filtrated by 2 layers of cotton cloth. The filtrate was lyophilized (Labconco, model 71061 6L, USA) and kept at -20°C until future used. In another method, CA was extracted using two different solvents; water and 95% ethanol, at a ratio 1:10 (w/v) using an incubator shaker (SHELLAB, model SSI3, USA) at 150 rpm for 6 h. The extracts were filtrated by Whatman filter paper No.1 and residue organic solvents (ethanol) were removed by rotary evaporator (EYELA, model n-1100, Japan) at 50°C. The extract samples were lyophilized (Labconco, model 71061 6L, USA) and kept at -20 °C until further used.

2.4 Determination of total phenolic content (TPC)

TPC was measured by according to the method of Negi *et al.*, (2003) with a slight modification. Firstly, 20 µl of CA extracts (1mg/ml) were mixed with 100 µl of 0.2 M Folin-Ciocalteu reagent for 1 min. Then, 80 µl of 7.5% (w/v) sodium carbonate was added into the reaction mixture. After incubation at room temperature for 30 min, the absorbance of mixture was measured at 765 nm by microplate reader (Biotek, Epoch, USA). Gallic acid was used as a reference standard, and the results were expressed as mg Gallic acid equivalents (GAE)/g extract.

2.5 Determination of total flavonoid content (TFC)

TFC was measured by slightly modified from Li *et al.*, (2006). Briefly, prepared 25 µl of sample, were mixed with 80 µl of 7.5% (w/v) Na_2CO_3 , 140 µl of DI water, 5 µl of 10% (w/v) AlCl_3 and 5 µl of 1M CH_3COOK . The mixtures were incubated in the dark at room temperature for 30 min. The absorbance was measured at 415 nm using a microplate reader (Biotek, USA). TFC was calculated using quercetin as standard and expressed as mg quercetin equivalents (QE)/g extract.

2.6 Determination of total proanthocyanidin content (TPAC)

TPAC was determined by the method modified from Zhenbin *et al.* (2011). Sample or catechin (standard)(20 µl) were added into 50 µl of 1% vanillin and 50 µl of 25% H_2SO_4 in methanol. The mixtures were incubated for 15 min in the dark at room temperature. The absorbance was measured at 500 nm by using a microplate reader. TPAC of samples were expressed as mg epicatechin equivalents (ECE)/g extract.

2.7 Determination of DPPH radical scavenging activity

A 190 µl of 0.1 mM DPPH in ethanol was added into 10 µl of sample (1 mg/ml). The reaction was performed in the dark at room temperature for 30 min and the absorbance was measured at 515 nm. Trolox was used as standard. Absorbance of samples were calculated to be % inhibition. % inhibition = $[(A \text{ control} - A \text{ sample}) / A \text{ control}] \times 100$ Where, A control = the absorbance of the control solution without antioxidant agent, A sample = the absorbance of sample to be tested. DPPH radical scavenging activity of samples were reported as mg trolox equivalent antioxidant capacity (TEAC)/g extract.

2.8 Determination of ABTS radical scavenging activity

The ABTS radical assay was adapted from Thaipong Italic (2006). Briefly, ABTS radical solution was prepared by mixing 7 mM ABTS with 2.45 mM $K_2S_2O_8$. Then, the ABTS working solution was prepared by mixing ABTS radical solution with 50 mM phosphate buffer (pH 7) at a ratio of 1:20 (v/v). A 10 μ l of samples was added into 190 μ l of the ABTS working solution. The reaction was performed in the dark at room temperature for 15 min and the absorbance was determined at 734 nm. Trolox was used as standard. Results were reported as mg Trolox equivalent antioxidant capacity (TEAC)/g extract.

2.9 Determination of ferric reducing antioxidant power (FRAP)

FRAP measurement of extract was adapted from Benzie and Stain (1996). FRAP solution was prepared by mixing 3 ml of 10 mM TPTZ (in 40 mM HCl), 3 ml of 3.2 mM $FeCl_3$ solution and 30 ml of 300 mM sodium acetate buffer (pH 3.6). A 10 μ l of sample was added into 190 μ l of FRAP solution. The reaction was performed in the dark at room temperature for 15 min and the absorbance was measured at 593 nm. Trolox was used as standard. FRAP was calculated as mg trolox equivalent antioxidant capacity (TEAC)/g extract.

2.10 Statistical analysis

All obtained data were expressed as mean $\pm$ standard deviation. The data were statistically analyzed by analysis of variance (ANOVA) and Tukey's HSD test for post-hoc analysis using IBM SPSS 21 for Windows. The comparison was considered at the significance level of $P < 0.05$.

3. Results and Discussion

3.1 Extraction yield

The extractable yield of extracts from *C. asiatica* by 2 methods; squeezing and solvent extraction were shown in Table 1. Ethanolic CA extract exhibited the highest yield (2.17%), while CA from squeezing method had lower yield (1.06%) than those extracted with solvent extraction.

3.2 Total phenol content (TPC)

TPC was significantly different between CA extracted from various methods and solvents ($P < 0.05$) (Table 2). The ethanolic CA extract had the significantly highest TPC (262.5 ± 10.8 mg GAE/g sample), whereas the lowest TPC was found in CA extract with squeezing process (113.3 ± 12.3 mg GAE/g sample) ($P < 0.05$).

3.3 Total flavonoid content (TFC)

TFC was significantly different between CA samples extracted from various methods and solvents ($P < 0.05$). The tendency of TFC was similar to their results of TPC that the greatest TFC was found in CA extract with ethanol solvent extraction (100.6 ± 0.5 mg QE/g sample) and the CA extract with squeezing method had lowest TFC (40.9 ± 3.6 mg QE/g sample).

3.4 Total proanthocyanidins content (TPAC)

Ethanolic CA extract showed the significantly highest TPAC (1236.0 ± 0.0 mg ECE/g sample) ($P < 0.05$). However, the lowest TPAC was obtained from CA extract with DI water extraction (232.0 ± 20.8 mg ECE/g sample).

3.5 Antioxidant activities

3.5.1 DPPH assay

Gotu kola extract had varying DPPH radical scavenging activity between each solvent ($p < 0.05$) as shown in Table 2. Ethanolic extract of CA exhibited the highest DPPH radical scavenging activity 1157.3 ± 26.7 mg TEAC/g sample ($P < 0.05$). The lowest DPPH was found in CA extract with squeezing process (503.1 ± 5.8 mg TEAC/g sample) ($P < 0.05$).

Table 1 Extractable yield and bioactive compounds (Total phenolic, Flavonoid and Proanthocyanidin contents) of *C. asiatica* extract from 2 methods Squeezing method and Solvent extraction

Extraction Method	Solvents	Extractable Yield (%)	Total Phenolic content (mg GAE/g sample)	Total Flavonoid content (mg QE/g sample)	Total Proanthocyanidin content (mg ECE/g sample)
Squeezing process	DI Water	1.06	113.3±12.3 ^c	40.9±3.6 ^b	352.0±24.0 ^b
Solvent extraction	DI Water	1.15	146.9±3.5 ^b	43.9±2.6 ^b	232.0±20.8 ^c
	EtOH	2.17	262.5±10.8 ^a	100.6±0.5 ^a	1236.0±0.0 ^a

Values are expressed as Mean ± S.D (n=5),

Different letters in the same column indicate significant difference at P < 0.05 (ANOVA, Tukey's HSD test).

3.5.2 ABTS assay

The tendency of ABTS result was similar to bioactive compound (TPC and TFC) results and DPPH activity result (P<0.05) as shown in Table 2. Gotu kola extract by ethanol extraction had highest ABTS capacity that was 2648.8±12.0 mg TEAC/g sample (P<0.05).

3.5.2 Ferric reducing antioxidant power (FRAP)

The results of FRAP activity from CA extracts by two methods was also shown in Table 2. The highest FRAP activity was obtained from CA ethanolic extraction (P<0.05) while, the squeezing CA extract had the lower FRAP than other extract (P<0.05).

Table 2 *In vitro* antioxidant activity (DPPH, ABTS and FRAP activities) of *C. asiatica* extract from 2 methods; squeezing method and solvent extraction

Extraction Method	Solvents	DPPH (mg TEAC/g sample)	ABTS (mg TEAC/g sample)	FRAP (mg TEAC/g sample)
Squeezing process	DI Water	503.1±5.8 ^c	1401.5±66.8 ^c	133.3±12.2 ^c
Solvent extraction	DI Water	577.3±16.0 ^b	1948.7±44.8 ^b	274.33±8.2 ^b
	EtOH	1157.3±26.7 ^a	2648.8±12.0 ^a	710.6±12.9 ^a

Values are expressed as Mean ± S.D (n=5),

Different letters in the same column indicate significant difference at P < 0.05 (ANOVA, Tukey's HSD test).

4. Conclusion

Centella asiatica leaves extract (especially ethanolic extraction) with high bioactive compounds (e.g. proanthocyanidin) and great antioxidant activity can be used as natural ingredient for hair care product. The comparison on bioactive compounds and its antioxidant activity between two methods squeezing process (following Thai traditional wisdom) and solvent extraction (DI water and ethanol) showed that the bioactive can be extracted by solvent extraction better than squeezing process. Moreover, the tendency of bioactive compounds (TPC, TFC and TPAC) was similar to their antioxidant activity. It suggested that these phenolic, flavonoid and proanthocyanidin may be act as antioxidant in these CA extract.

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Color cosmetic perception, utilization and purchasing behavior of female university students in Mae Fah Luang University, Chiang Rai, Thailand**Naphatsorn Ditthawutthikul^{*1,2}, Chakrit Sinsavetsakul¹, Waruttaya Sripattanakul^{1,2}**¹ *School of Cosmetic Science, Mae Fah Luang University, Chiang Rai, Thailand*² *Phytocosmetics and Cosmeceutical Research Unit, School of Cosmetic Science, Mae Fah Luang University, Chiang Rai, Thailand.***Corresponding author. E-mail: naphatsorn.kum@mfu.ac.th*

Abstract

Color cosmetics or makeup products are one of the important tools to improve physical appearance, attractiveness and personality of the people. In Thailand, color cosmetics are used since the women enter puberty and currently, there is no research indicated the perception, usage and purchasing behavior of color cosmetics in this people group. From this reason, this study was aimed to investigate the perception of color cosmetics, usage and purchasing behaviors of female university student whom currently studying in Mae Fah Luang University, Chiang Rai, Thailand. In this study, the questionnaire containing demographics data, perception of color cosmetics, usage and purchasing behavior was created and given to 183 female college students in Mae Fah Luang University, Thailand. The results revealed that the most prevalence skin type of the volunteers was a combination skin type. The most often used base and point makeup products among volunteers were loose powder and eyebrow decorating products (89.62 and 67.76%, respectively). Eyebrow decorating product was also chosen as the most important makeup product perceived in their opinion. Their favorite makeup style was dominantly inspired by Korean culture (47.22%). They perceived that the utilizations of color cosmetics can increase their self-confidence as they can conceal the impurities and camouflage the lesions on their face. From questionnaire, reviews and tutorials created by beauty blogger was the major factor that inspired the volunteer to purchase color cosmetics. The results from this survey provided better understanding in the utilization and purchasing behavior of color cosmetics among university students. This might be useful for the parents and cosmetic companies to understand the makeup behaviors and the utilization of these products.

Keywords: Loose powders; University students; Color cosmetics; Makeup products; Consumer behavior

1. Introduction

Personality traits and body image of the people play an important role in social interaction. People are judged each other from the physical appearances, such as facial makeup, hair dressing and clothing. The people with better physical appearances gain more attractiveness in society, and may be judged as a people of high rank status (Korichi and Tranchant, 2014). It was reported that people with higher attractiveness received higher opportunities in the society, such as higher salary and better job chance (Frieze *et al.*, 1991). For example, waitress in the restaurant who wore makeup earned more tips from male customers than waitress who do not as they think that she is more attractive (Guéguen and Jacob, 2011). From these reasons, decorative cosmetics are used to improve people attractiveness since an ancient period of time.

Skin is one of the major body components that affecting to physical appearance and attractiveness of the people. Color cosmetics can make people look younger and healthier. They also induce a state of self-confident and increase self-esteem of the people who wearing them. Furthermore, color cosmetics also improve the first impression of the people in social interaction (Dayan *et al.*, 2015; Nash *et al.*, 2016). It was also reported that female college students feel more self-confident and sociable when they are wearing color cosmetics (Cash and Cash, 1982). From these reasons, many color cosmetic products are employed by many people, including teenagers, to enhance the appearances, attractiveness and personality (Graham and Jouhar, 1981).

Color cosmetics or makeup products can be roughly classified into base makeup and point makeup products. Base makeup products are the products that designed to be used on the major area of the face. Examples of base makeup products are foundations, BB creams, makeup bases, and facial powders. Point makeup products are the products that designed to be used on the specific areas such as eyes and lip. Examples of point makeup products are lipsticks, eye shadows, eyeliners, mascaras and eyebrow products (Mitsui, 1997).

There was a survey in 2016 which indicated that 61% of US women use makeup products (Doyle, 2017). A previous survey in 2012 had indicated that the global market share of makeup products is accounted for 14% of all cosmetic categories (Maheswari, 2013). These suggested that makeup products are important cosmetic type. In Thailand, people start to care their skin and decorate themselves with cosmetics since their early teenage age. Usage of color cosmetics is also able to be found in primary school students. This may be because of the impact of social network, such as Facebook, Instagram, Twitter, etc. Teenagers, especially women, like to post their pictures online via these social platforms (Matthews, 2016). There was a report indicated that the women who spending time on facebook reported more awareness in their facial, hair and skin appearances than the women who do not (Fardouly *et al.*, 2015).

From afore mentioned, color cosmetics are important to people in a society. In addition, there is only limited literature about adolescent consumers' behavior on selecting and utilizing makeup. This study was aimed to evaluate the behavior of Thai adolescent consumers in the selecting and utilizing makeup. Their perception on makeup was also evaluated. The results of this study could be useful for parents, to understand their children and also for cosmetic industries to develop new products that related to the consumers' behavior.

2. Materials and Methods

2.1 Ethical considerations

A survey-based study is considered as an ethical exemption study by Ethical Committee at Mae Fah Luang University. From this reason, ethical consideration was exempted.

2.2 Participants

One hundred eighty three female university students were participated in this study. Inclusion criterion was female who used at least one makeup product on daily basis. Exclusion criteria were men, did had never used makeup products, or did not used makeup products on daily basis and unable to answer the questions in given questionnaire.

2.2 Intervention

A questionnaire composed of 24 questions regarding to demographics, skin types, perception and usage behaviors of base makeup and point makeup was developed. This questionnaire was given to recruit participants. They were then asked to complete a questionnaire. The study was conducted at Mae Fah Luang University, Chiang Rai, Thailand during, July – December, 2017.

2.3 Statistics

Data was collected and processed by using IBM SPSS version 17.0 (IBM Co., Armonk, NY, USA). For statistical analysis, Pearson's chi-square (χ^2)-test was used for the categorical data. The p-values < 0.05 was considered as a statistical significance.

3. Results and Discussions

All of 183 participants had completed the questionnaires. All participants were aged between 18 – 21 years old. Makeup products are outstanding than that of any other categories of cosmetic products. Their market share is increasing very fast from year to year (Donahue, 2016). The cosmetic industry targeted to younger customers in order to deliver beauty according to the demands of younger people (Aytuğ, 2015). It was surveyed that more than 64% of teenagers whom ages between 15 and 34 believed that staying active on social media was important (Mills, 2015). This may relate to the emerging ‘Selfie-beauty’ cosmetic trend that was previously mentioned elsewhere. Since the makeup products have been targeted on the adolescents and also teenagers, this research may be useful for any people in the cosmetic industries and also adolescents to understand the needs and makeup behavior of Thai University students, whom also are an important target consumer for cosmetic industries.

3.1 Demographics

Skin types, which were dry, normal, oily and combination, were included in the questionnaire. The distribution of each skin types is shown in Figure 1. The most prevalence skin type of these volunteers was combination skin (41.76%) and the least prevalence skin type was normal skin (10.99%). Combination skin is a skin type that the T-zone, area including forehead, nose and chin is oily and the cheek area is dry (LeQuesne, 2005). There was a report indicated that area in T-zone contains higher androgen receptor expression than other area. This can be explained by the effect of androgen hormones, testosterone and dihydrotestosterone that stimulate the production of sebum (Barraut *et al.*, 2015; Ju *et al.*, 2017). The skin type of the individual plays an essential role as one of the most important factor in selecting and utilizing cosmetic products. Different skin types required specific different cosmetic products (Mattocks, 2009).

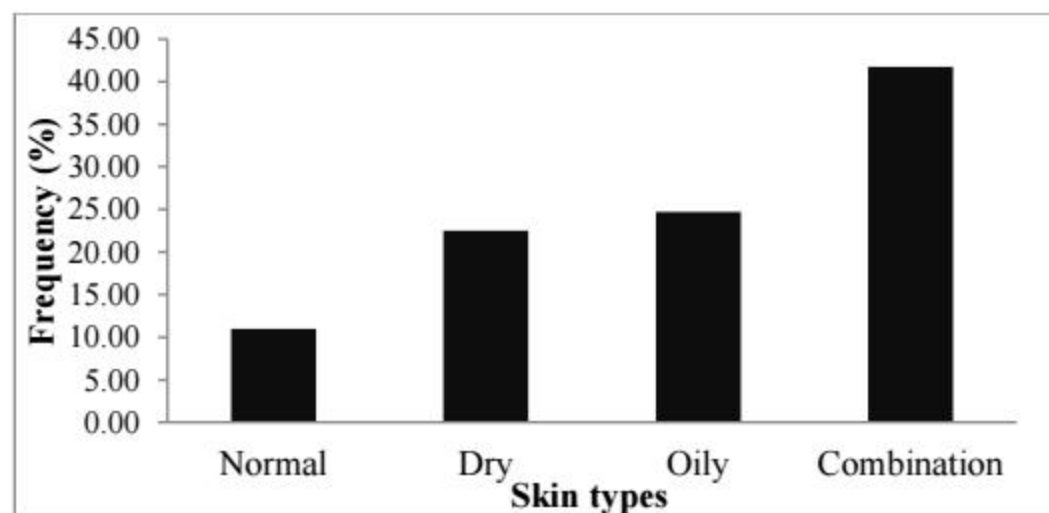


Figure 1 Skin types distribution of the participants included in this study.

3.2 Perception of color cosmetics and utilization behavior

In this part, we surveyed the perception of the participants toward utilization and purchasing makeup products. Six behavioral traits, attitudes and believes related to makeup products were rated by the volunteers. The score from 1 which is the least important to 5 which is the most important according to their own opinion was rated by the volunteers. These traits were listed as followed; (i) brand is the factor that can affect to the quality of the products; (ii) price is the factor that can affect to the quality of the products; (iii) celebrities can inspire you to utilize and purchase makeup products; (iv) beauty bloggers can inspire you to utilize and purchase makeup products; (v) makeup can conceal your impurities and (vi) makeup can give you confidences and increase self-esteem. The results are expressed in Figure 2. The results showed that 72.8% of the volunteers had selected that color cosmetics have high importance to the most importance tools that can improve their confidence. They also believed that cosmetics can conceal their imperfection, which 39.4% of them had selected in high importance to the most importance range. Color cosmetics purchased behavior of the volunteers was inspired by beauty bloggers, as 52.8% of them had selected in high importance to the most importance range. Volunteers perceived that the cosmetic brands had higher priority than the price which they chose high importance and the most importance percentage were 60.0% and 33.9%, respectively.

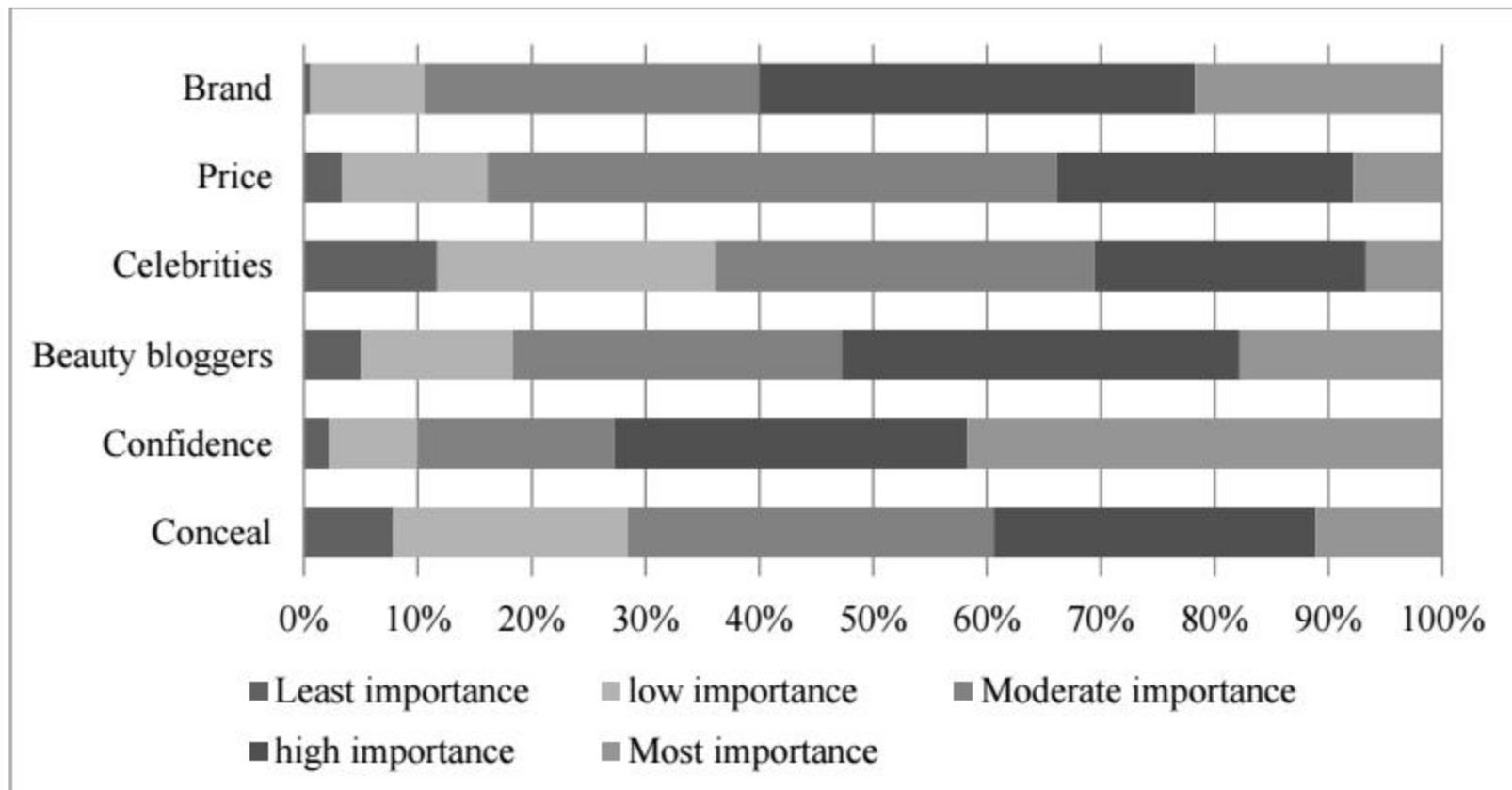


Figure 2 Behavior and perception of makeup products usage among participants. Behavioral traits, attitudes and believes regarding to makeup products. The scores from 1 to 5 from least important to most important were given by volunteers. Brand is stands for ‘brand is the factor that can affect to the quality of the products’. Price is stands for ‘price is the factor that can affect to the quality of the products’. Celebrity is stands for ‘celebrities can inspire you to utilize and purchase makeup products’. Beauty blogger is stands for ‘beauty bloggers can inspire you to utilize and purchase makeup products’. Concealing impurities is stands for ‘makeup can conceal your impurities’. Giving confidence is stands for ‘makeup can give you confidences’

The results were related to Cash and Cash (1982) whom indicated that makeup can increase the confidence of the person. They also perceived that brand and price of the color cosmetics are the major factors that can determine the quality of the products. This suggested that they thought that the brand has impact to the quality product. The company should create better brand image in order to target these adolescent customers. Pricing strategies is also important in order to target these consumers. Beauty bloggers, persons whom specialized in beauty and cosmetic products and write a review of their own opinions on Social Network Sites (SNSs) or blogs, can inspire them to purchase or utilize the makeup products. The effect from beauty bloggers is stronger than celebrity. Many beauty companies in Asia had hired beauty bloggers to advertise their products on various social network platforms. Cosmetic companies believed that this technique can increase the selling of their products. The results from this study suggested that the technique in hiring beauty bloggers might be useful for cosmetic companies, and this had stronger impact on adolescents target group than hiring the celebrities which cost more expensive.

3.3 Makeup inspiration

There are many makeup styles available in Thailand. They are inspired by many cultures. The most popular are Japanese, Korean, European and American-inspired makeup styles. From this reason, these four culture themes were included in this questionnaire. It was found that the most popular makeup styles for Thai University students is Korean-inspired makeup styles, in which nearly half (47.22%) of volunteers who answered the questionnaire had selected as their favorites makeup style, followed by European (24.44%) and American (18.89%). The least favorite makeup style among them was Japanese-inspired makeup style, which only 9.44% of them had chosen.

Korean-inspired beauty is quite popular in Thailand. Many of Thai people showed their preferences to the Korean soap operas, TV series, singers and celebrities. This may explain why nearly half of the volunteers selected Korean-inspired makeup style as their favorite. ‘K-beauty’ is a popular marketing term in cosmetic industry that was given elsewhere for a long time. K-beauty

indicates Korean beauty products. It was reported by Ziv (2016) that K-beauty had expanded their growth extensively since 2015. Many Korean beauty products are adopted by the American as their routine care products (Ziv, 2016). One of another reason that could explain why volunteers had selected Korean-inspired beauty as their favorite was the light and natural characteristics makeup look from this style (Kerry and Coco, 2015).

3.4 Base makeup products usage behavior

Base makeup products are applied on the major area of the facial skin to make flawless face. They are included with makeup base, makeup primer, foundation, concealer, powders, bronzers, contour products, and blushers. The makeup base is the product that applied over the face, after skincare process. The property of makeup base is to neutralize or camouflage the impurities and uneven pigmentation on face with the help of contrasting color. Contrasting colors are the colors that opposite together on the color wheel. For example, green makeup base is useful to cover redness because green is contrast to red (Le Quesne, 2005). It was found that 51.91% of these students used the makeup base as their routine makeup procedure. Among them, Thai adolescents showed more preferences toward the cream type makeup base (63.16%) followed by the mousse type (20.00%). Makeup primer was the next product surveyed. Only 29.51% of them used makeup primer in their routine. There was the significant relationship between multi-cultural beauty and the use of primers ($p < 0.005$). Half (54.55%) of the adolescents whom selected European-inspired makeup as their favorite use the makeup primer in their routine makeup procedure, while only 20.00% of Korean-inspired people used it. The further product surveyed was foundation. In this study, 76.50% of the volunteers used the foundation in their routine makeup regimen. Cream-type foundation was the most popular (64.29%) foundation among them. The second most favorite foundation type among them was liquid type (19.29%) and the least favorite foundation type was mousse type (7.89%). There was no relationship between the multi-cultural beauty and the routine use and type of foundation. Half of the volunteers (55.74%) used the concealer to conceal the imperfections on their face. There was a significant relationship between multi-cultural beauty and the use of concealers ($p < 0.005$). Two-third (77.28%) of the adolescents whom preferred European-inspired makeup style used the concealer, while only 29.41% of adolescents whom preferred Japanese-inspired makeup style used it in their routine. Among the volunteer who used concealer, cream type was the most favorite type, which accounted for 42.16%, followed by stick and liquid concealers which had equal frequencies of 25.49%. There was no relationship between the multi-cultural beauty and the favorite type of concealer. In this study, the female university students perceived that the loose powders are important in their makeup routine, as 89.62% of the volunteers always used them in their routine. Contrary to the loose powder, only 65.03% of the volunteers used the pressed powder in their routine. There were no relationship between the multi-cultural beauty and the use of loose powder and pressed powder. This suggested that loose powder was the vital base makeup product for every makeup styles. The last base makeup product surveyed in this study was a facial blusher. The most popular blusher color among the volunteers was pink (60.66%) followed by orange (25.68%) and brown (3.82%). The most favorite dosage form of the blusher was pressed powder (63.40%), followed by loose powder (19.59%) type. There was no relationship between the multi-cultural beauty and the use of blusher.

3.5 Point makeup products

Point makeup products are the products that used on the eye and lip area to create attractive makeup look. Examples of point makeup products are eye shadow, eyeliner, mascaras, eyebrow decorating products and lip products. It was reported by Mulhern *et al.* (2003) that eye makeup alone can draw the attractiveness better than foundation or lip makeup alone, but this was lower than full facial makeup. Five categories of point makeup products were included in this questionnaire. They were eye shadow, eyeliner, mascaras, eyebrow decorating products and lip products. For eye shadow, 67.75% of volunteers showed more preferences to the pressed powder eye shadow than any categories of eye shadow. The second and third most favorite eye shadow types in this survey were loose powder eye shadow (19.67%) and cream eye shadow (8.74%). In this study, 69.40% of the female adolescents used the eyeliner, and the most favorite color among them was the black color with the frequency of use at 88.98%. The most popular form of eyeliner in this study was the pen type which 65.36% of the volunteers had chosen as their favorite type. Pencil and gel-based eyeliners were the second most

popular eyeliner with the frequency around 23 – 27%. The next point makeup product surveyed was eyebrow decorating product. It was found that most of the participants used eyebrow decorating products. The results revealed that eyebrow pencil was the most popular product which 67.76% of the volunteers used them in their routine makeup regimen. Pressed powder eyebrow product was the second most popular product, with the frequency of 34.43% while cream eyebrow product was the least popular product (10.38%). Interestingly, there was no relationship between the multi-cultural beauty and the usage of eyeliner, mascara and eyebrow decorating products which suggesting the strong impact of eyebrow decorating products on all makeup styles. Lip decorating products were the last point makeup product surveyed. Several types of lip decorating products, including lipstick, lip gloss, lip cream and lip pencil were included in the questionnaire. The volunteers can answer more than one type of lip products. The results showed that 79.78% of all volunteers used the lipstick, and 45.36% of them used lip gloss. Lip cream and lip pencil were the least popular products, which only 25.14% and 10.38% of them used in their routine makeup, respectively. Pink colored-lipstick was the most favorite product in this study (46.44%), followed by orange (38.80%) and brown or nude tone (29.51%). Surprisingly, purple was the least favorite lip color with only frequency of 4.37%. We found the strong relationship between the multi-cultural beauty and the favorite lip color ($p < 0.005$). For Japanese-inspired makeup, 94.12% of the adolescents whom preferred Japanese-inspired makeup style exhibited disfavor against red-colored lip but 47.06% of them preferred pink-colored lip. This was contrary to European-inspired makeup, 38.64% of the adolescents whom preferred European-inspired makeup had chosen red-colored lip as their favorite, while 75% of them exhibited disfavor against pink-colored lip.

In the further part of questionnaire, volunteers were asked to give an example of the most important makeup product in their own opinion. The results were shown in Figure 3. From Figure 3, the most important makeup product in their opinion was eyebrow products, which 46.06% of all volunteers thought that it is the most important in their makeup routine. Eyeliner, makeup base and eye shadow were the least three important makeup products in their opinion.

Eyebrows are the frame for the eyes (Henshaw and Hanna, 2012). They also are an essential element of the face beauty and should be well decorated to provide attractiveness (Palma, 2006). It was mentioned by many Thai beauty companies that “eyebrows are the crown for the face”. Well-made eyebrow can make person younger (Mattocks, 2009). It was also previously mentioned by

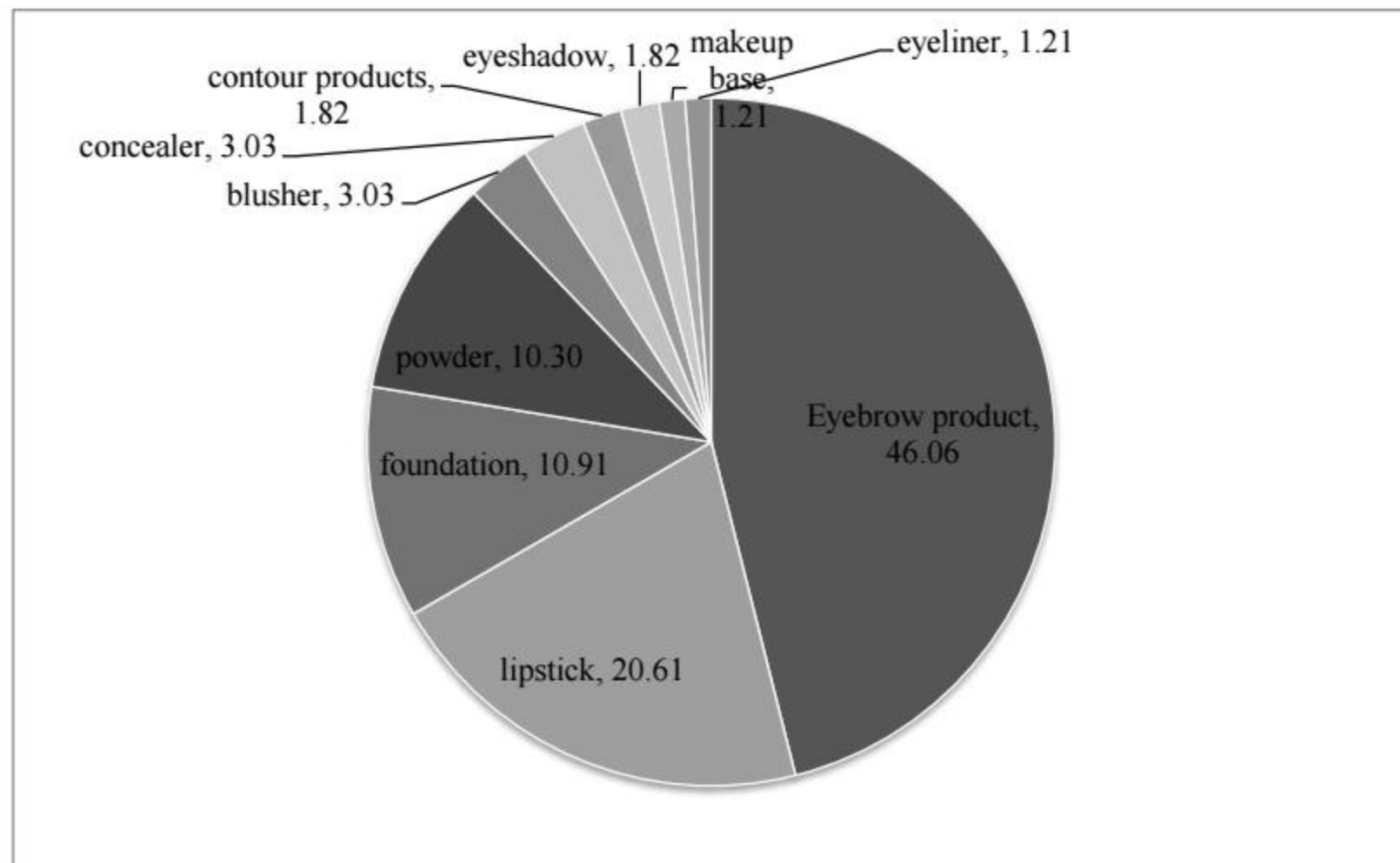


Figure 3 The most important makeup products in participants' opinion

LeQuesne (2005) that eyebrows are usually overlooked by many people. However, this is against to our study as almost 80% of female adolescents used eyebrow decorating products. This suggested that Thai adolescents strongly paid attention to their eyebrow. In order to achieve perfect brow shape, combination of several eye brow products is commonly used by many makeup artists. Normally, eyebrow pencil is used to draw the brow shape and pressed eyebrow powder is used to fill the shape (Mattocks, 2009). If people had their hair dyed, the brow mascara should be used to stain their brow color. The brow color should be lighter than the color of the person's hair (Mattocks, 2009).

5. Conclusion

In this study, questionnaire that consisted of demographics, skin types, base makeup and point makeup products and attitudes toward makeup products was given to 183 female adolescent University students whom wear makeup daily. The results found that the most common skin type of these adolescents was combination skin. Loose powder was the most common base makeup product among these adolescents, followed by foundation and pressed powder, while makeup primer was the least common base makeup product among them. The most important makeup product in their opinion was an eyebrow decorating product. Korea makeup style exhibited strong impact on these adolescents as it can inspire the selection and usage of some makeup products. They perceived that makeup can improve their confidence and beauty blogger can inspire them to utilize or purchase makeup products. Brand and price of the makeup products were also important to the decision in the usage and selection of these products. These data will help us understand the makeup products utilization of Thai adolescents and can be used by cosmetic companies, dermatologists and their parents to produce the makeup products according to their demands and understanding their makeup behaviors.

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Fanpage set up for providing health and beauty information

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Abstract

Many people are using Facebook fanpage for health and beauty purposes such as finding information, seeking some advices, sharing some experiences and professional counseling. However, there is no fanpage that establish according to the users demands. Therefore, this study was planned by surveying the requirements of online users. The collected data was then further used for Facebook fanpage creation. The aim of this fanpage was to provide the reliable information about health and beauty that achieve the online user requirements. Consumer behaviors on social media searching were surveyed via online questionnaire during October 2018 to January 2019. There were 900 participants completed the online questionnaire. Most participants were women (68.00%), aged between 20-29 years (54.00%). Most of them were bachelor's degree (43.56%). They used internet for finding health and beauty information once a week (30.56%). The most interest topic was food (60.00%) and most participants were required to receive knowledge from fanpage (81.00%). Facebook fanpage name "Counseling for your beauty" was constructed according to the online user requirement and distributed during February to April 2019. There were 229 users followed fanpage. Most of them preferred to participate in giveaway activities (45.12%) rather than reading post contents. The most preference type of content was picture (65.71%). In addition, the fanpage was evaluated via online satisfaction questionnaire. There were 500 evaluators assessed the fanpage. They were both fanpage followers and shared Facebook users. Most evaluators were women (68.20%) aged between 20-29 years (53.80%). They were known fanpage from their friends (31.60%). They assessed fanpage for reading new information about health and beauty (47.60%). In conclusion, the evaluators were satisfied in content, design and application of fanpage at very high level.

Keywords: Beauty; Behavior; Facebook; Fanpage; Health; Online.

1. Introduction

In a digital era, social media plays a front row of a communication channel. It is a virtual platform which helps people to make new connections, improve relationships, and exchange information. Social networking sites have become popular with the help of digital technologies (tablet, smartphone, notebook, etc.) and internet (Boyd and Ellison, 2007). The internet has always been a convenient and open source for educational material and information on many topics (Uittenhout, 2012). Online channels have become one of the most important sources for searching and exchanging health and beauty information, experiences, advice, support and opinions (Johnston and Petersen, 2015). Many people use the internet to find health and beauty information for themselves, family or friends. There is a lot of good quality health and beauty information online.

There are many social networking sites such as social network sites (e.g., Facebook and LinkedIn); wikis (e.g., Wikipedia); blogs (e.g., Blogger); micro-blogging services (e.g., Twitter); video-sharing services (e.g., YouTube); and photo-sharing services (e.g. Flickr) (Gok, 2016;

Greenhow *et al.*, 2017). Facebook is one of the most popular social networking sites. Facebook allows users to create their profiles and locate others to connect with in order to extend one's social network (Shachak *et al.*, 2017). The statistical data showed that there are more than 500 million people using Facebook and the majority of them are also the members of other social networking sites. Besides, approximately 250 million of these users visit Facebook site in each day at least one time (Boyd and Ellison, 2007). Facebook offers features to search and create an online social network of friends. In addition, Facebook can now be used for many different purposes. It may be used to create a fanpage for information and images sharing, videos and photo making, virtual gift sending, events calling and games section playing (Domingo *et al.*, 2017). Some people are using Facebook fanpage for health and beauty purposes such as finding information, seeking some advice, sharing some experiences and professional counseling (AlQarni *et al.*, 2016).

Due to Facebook fanpages are become one of the most popular places for people to search about health and beauty information, but there is no fanpage that establish according to the users demands. The information found in Facebook fanpages may be outdated or incorrect. Facebook fanpages owners may not keep their information up to date and may post information that is not based on any scientific data. Therefore, this study was planned by surveying the requirements of online users. Then, the collected data was used for Facebook fanpage creation. The aim of this fanpage was to provide the reliable information about health and beauty to achieve the online user requirements.

2. Materials and Methods

The scope of research was shown in Figure 1.

2.1 Survey for fanpage set up

The questionnaire was created by using Google forms and distributed online during October 2018 to January 2019. The population of this study was Thai people, who were using internet. The questionnaire composed of 2 parts:

Part 1: General information of participants such as sex, age, weight, height, marital status, educational level and health conditions.

Part 2: Behavior of participants such as topics of interest, data format preference, frequency of surfing the internet, requirement of page design, interaction with fanpage and suggestions.

2.2 Fanpage set up

Facebook fanpage name "Counseling for your beauty" (https://www.facebook.com/Counseling-for-your-beauty-รับปรึกษาความงามและพัฒนาบุคลิกภาพ-287554265258343/?modal=admin_todo_tour) was set up according to the results of online survey. The fanpage composed of articles, pictures and videos related to health and beauty. The first activity was like and share which performed by inviting Facebook users to like page and share content for fanpage promoting. The second activity composed of giveaways activities. The users were promoted to leave their comments and randomized for a free gift. The third activity composed of tagging activity. The objective of this activity was to add the post in the person's timeline and make the post more visible. The last activity composed of share knowledge without any activity to seek the number for handling user interests.

2.3 Data collection

Facebook page visiting number, interaction and comments were collected every 2 weeks during February to April, 2019.

2.4 Fanpage evaluation

The fanpage was evaluated by 500 Facebook users (both followers and shared user). The web link was shared and then the evaluators were asked to complete the online satisfaction questionnaire. The questionnaire composed of 2 parts:

Part 1: General information of evaluators such as sex, age, occupation, access channel and the purpose of page visiting.

Part 2: The satisfaction of Facebook Fanpage which composes of 3 parts as following:

- a) The satisfaction on content such as reliability, interest, conformity, clearness, consistency, update, knowledge and overall satisfaction.
- b) The satisfaction on design of fanpage such as easy to read, beautiful and clear meaning.
- c) The satisfaction on the application such as useful, adaptable, informative, on demand and overall satisfaction.

A five point Likert scale was used to evaluate the satisfaction on Facebook fanpage. The score of each factor was dependent on the evaluator satisfaction from the most satisfied to very unsatisfied. The mean was calculated and criteria for interpretation the satisfactions of Facebook fanpage were defined to 5 levels such as very high (4.21-5.00), high (3.41-4.20), medium (2.61-3.40), low (1.81-2.60) and very low (1.00-1.80), respectively (Wuttisin, *et al.*, 2016).

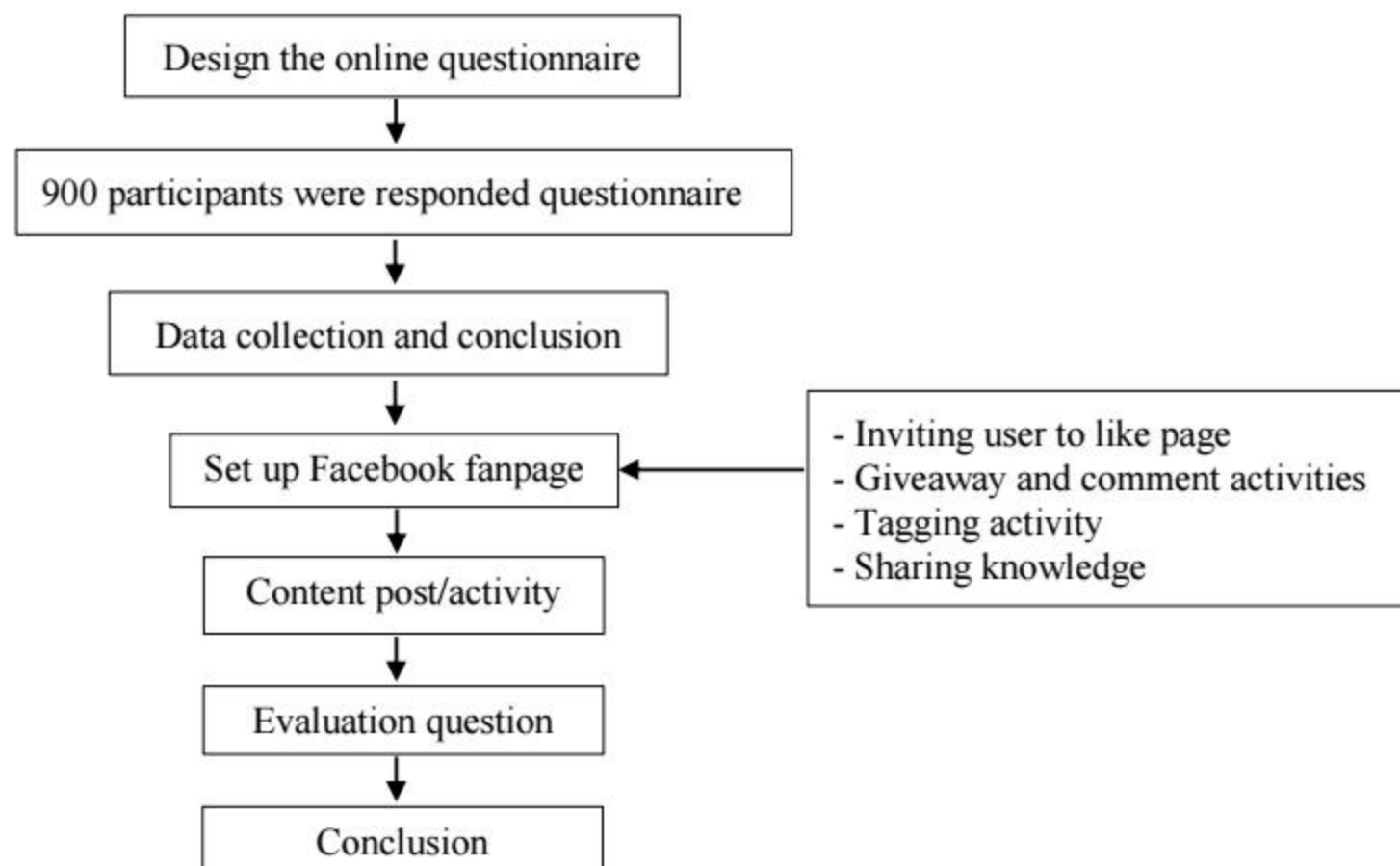


Figure 1 Scope of research

3. Results and Discussion

3.1 General information of participants

The demographic of participants are shown in Table 1. There were 900 participants responded to the online questionnaire. More than half of them were women (68.00%). Most participants aged between 20-29 years (54.00%). They were bachelor's degree (43.60%) and the most of them were single (83.50%).

The behaviors of participants about surfing the internet for finding health and beauty information are shown in Table 2. There were 30.60% of participants who often use internet for finding information once a week. The most interest topics were food (60.00%), exercise (54.00%), beauty (52.00%), health (36.00%), lifestyle (34.00%) and fashion (32.00%). The preference data format were video (49.00%) followed by article (28.00%) and picture (15.00%). Most of them usually surf the internet between 18.00-20.59 (45.00%) for 3-6 hours (35.80%). Most participants required to receive knowledge from fanpage (81.00%) and normally click like (73.00%) and shared content (55.00%) that they were interested.

Table 1 Demographic characteristics of participants.

Variables		Frequency (n=900)	Percentage (%)
Sex	Male	288	32.00
	Female	612	68.00
Age	10-19	198	22.00
	20-29	486	54.00
	30-39	99	11.00
	40-49	90	10.00
	>49	27	3.00
Weight	30-39	9	1.00
	40-49	180	20.00
	50-59	369	41.00
	60-69	198	22.00
	70-79	90	10.00
	>79	54	6.00
Height	140-149	9	1.00
	150-159	257	28.56
	160-169	416	46.22
	170-179	192	21.33
	>179	26	2.89
Education	Primary	37	4.11
	Secondary	363	40.33
	Diploma	48	5.33
	Bachelor's degree	392	43.56
	Master's degree	30	3.33
	Others	30	3.33
Status	Single	752	83.56
	Married	121	13.44
	Widow	9	1.00
	Divorce	10	1.11
	Separated	8	0.89
Chronic disease	No	756	84.00
	Migraine	39	4.33
	High blood fat	10	1.11
	Diabetes	8	0.89
	High blood pressure	8	0.89
	Heart disease	3	0.33
	Others	76	8.44

Table 2 Participant behaviors on internet surfing for finding health and beauty information.

Variables		Frequency (n=900)	Percentage (%)
Frequency	Every day	156	17.33
	Every second day	91	10.11
	2-3 days/time	237	26.33
	Once a week	275	30.56
	Others	141	15.67
Topic of interest*	Food	540	60.00
	Exercise	486	54.00
	Beauty	468	52.00
	Health	324	36.00
	Life style	306	34.00
	Fashion	288	32.00
	Others	90	10.00
Data format	Video	441	49.00
	Article	252	28.00
	Picture	135	15.00
	Poster	63	7.00
	Others	9	1.00
Time	06:00-08.59	27	3.00
	09.00-11.59	45	5.00
	12.00-14.59	45	5.00
	15.00-17.59	108	12.00
	18.00-20.59	405	45.00
	21.00-23.59	243	27.00
	00.00-05.59	27	3.00
Duration of using internet	1-2.59 hours	196	21.78
	3-6.59 hours	322	35.78
	7-9.59 hours	198	22.00
	>10 hours	184	20.44
Requirement of page*	Knowledge	729	81.00
	Review	513	57.00
	Giveaway	207	23.00
	Selling	198	22.00
	Others	9	1.00
Interaction with fanpage*	Like content	657	73.00
	Share content	495	55.00
	Join activity	171	19.00
	Question	135	15.00
	Comment	117	13.00
	Reaction with others	63	7.00
	Others	36	4.00

* The participants were answered more than one

3.2 Fanpage set up

Facebook fanpage name “Counseling for your beauty” was set up and distributed during February to April 2019. There were 229 users click like on this fanpage. Many topic contents were posted in the fanpage as shown in Appendix.

The contents in Facebook fanpage were classified into 6 groups as shown in Table 3. Most participants prefer giveaway activities (45.12%). The reason might be due to they want a free gift. On another hand, giveaway posts were created for bringing more people to the fanpage timeline and increasing fanpage like numbers (Nelson and Herndon, 2012). In addition, participants also preferred topic about food (16.93%), fashion (15.39%), health (10.77%) and beauty (6.72%). Table 4 indicates that the highest number of post accessing was found in picture contents (65.71%) the reason might be due to pictures take less time to consume. This finding was in accordance with the previous report that total views increased by 94 percent if a published article contained a relevant photograph or infographic when compared to articles without an image in the same category (Skyword Inc, 2018). Although, some social media analysis proposed that videos are get more reach than any other type of post further studies need to perform to get more reliable conclusions.

Table 3 List of topic contents in Facebook fanpage “Counseling for your beauty”.

Contents	Topics	Post reach	Mean	Percentage (%)
Giveaway activities	4	3,139	784.75	45.12
Food	2	589	294.50	16.93
Fashion	8	2,141	267.63	15.39
Health	12	2,249	187.42	10.77
Beauty	21	2,453	116.81	6.72
Exercise	3	265	88.33	5.08

Table 4 Frequency of post reach according to type of contents.

Type of content	Post	Post reach	Mean	Percentage (%)
Picture	16	6,927	432.94	65.71
Article	18	2,641	146.72	22.27
Video	16	1,268	79.25	12.03

3.3 Fanpage evaluation

The online satisfaction questionnaire (<https://forms.gle/Wk1cLtEv3HqCSPmZ7>) was posted on Facebook fanpage. The fanpage followers were asked to complete and share the satisfaction questionnaire. This activity was promoted by randomized a free gift for the evaluators. There were 500 participants evaluated the Facebook fanpage. The participants were both fanpage followers and shared Facebook users. Socio-demographic characteristics of evaluators were presented in Table 5. Most evaluators aged between 20 to 29 years, 31.80% were men and 68.20% were women. Half of them were students (45.20%). Many studies find that student spend a considerable portion of their daily life interacting through social media. Moreover, they are among the most prolific users of social network sites (Ahn, 2011). Most evaluators were known fanpage from their friends (31.60%). The previous study found that most adolescents receive information from friends because they need to be a part of society and need to be accepted from other people (Kongrach, 2011). The purposes of page visiting were reading new information (47.60%), participating in activities to get a reward (23.60%), searching information (19.40%) and consulting health and beauty problem (9.40%).

3.4 Evaluator’s satisfaction

The satisfaction of the evaluators on Facebook fanpage about content, design and application

were shown in Table 6. The overall satisfaction on content was at very high level (4.36 ± 0.61). The knowledge gained (4.35 ± 0.64), consistency (4.32 ± 0.62), clear (4.28 ± 0.62), conform (4.28 ± 0.66) and update (4.26 ± 0.66) content were also at very high level. In addition, the satisfactions of designs such as clear meaning (4.41 ± 0.63), beautiful (4.39 ± 0.66) and easy to read (4.25 ± 0.62) were at very high level. Furthermore, the overall satisfaction of application was as very high level. The evaluators also satisfied in adaptable (4.47 ± 0.63), on demand (4.46 ± 0.64), informative (4.42 ± 0.64), and usefulness (4.33 ± 0.62) of Facebook fanpage at very high level.

Table 5 General information of evaluators

Variables	Frequency (n=500)	Percentage (%)
Sex		
Male	159	31.80
Female	341	68.20
Age		
15-19	54	10.80
20-29	269	53.80
30-39	94	18.80
40-49	54	10.80
>49	29	5.80
Occupation		
Student	226	45.20
Teacher	51	10.20
Employee	77	15.40
Business owner	80	16.00
Government officer	56	11.20
Channel to know page		
Friends	158	31.60
Facebook timeline	119	23.80
Acquaintance	95	19.00
Workmate	60	12.00
Relative	59	11.80
Teacher	9	1.80
The purpose of page visiting		
Read new information	238	47.60
Participate in activities	118	23.60
Find information	97	19.40
Consultation	47	9.40

4. Conclusion

Facebook fanpage name “Counseling for your beauty” was set up according to the results of online survey. The fanpage composed of articles, pictures and videos related to health and beauty. Most participants prefer to participate in giveaway activities rather than reading post contents. The most preference type of content was picture. The Facebook fanpage was evaluated by evaluators. Most evaluators were known page from their friends. They were assessing Facebook fanpage for reading new information about health and beauty. In conclusion, the evaluators were satisfied in

content, design and application of Facebook fanpage at very high level.

Table 6 Evaluator's satisfaction

Factors	Mean $\pm$ SD	Satisfied level
Content		
Reliable	4.04 $\pm$ 0.67	High
Interesting	4.20 $\pm$ 0.61	High
Conform	4.28 $\pm$ 0.66	Very high
Clear	4.28 $\pm$ 0.62	Very high
Consistent	4.32 $\pm$ 0.62	Very high
Updated	4.26 $\pm$ 0.66	Very high
Knowledge gained	4.35 $\pm$ 0.64	Very high
Overall satisfaction	4.36 $\pm$ 0.61	Very high
Average	4.26 $\pm$ 0.64	Very high
Design		
Easy to read	4.25 $\pm$ 0.62	Very high
Beautiful	4.39 $\pm$ 0.66	Very high
Clear meaning	4.41 $\pm$ 0.63	Very high
Average	4.35 $\pm$ 0.64	Very high
Application		
Usefulness	4.33 $\pm$ 0.62	Very high
Adaptable	4.47 $\pm$ 0.63	Very high
Informative	4.42 $\pm$ 0.64	Very high
On demand	4.46 $\pm$ 0.64	Very high
Overall satisfaction	4.49 $\pm$ 0.60	Very high
Average	4.43 $\pm$ 0.63	Very high
Total satisfaction	4.35 $\pm$ 0.64	Very high

5. Acknowledgements

The authors wish to express our appreciation to people who participated in the survey.

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7. Appendix

Posting contents on Facebook fanpage

Date	Content	Type	Reach	Engagement			
				Post click ¹	Like	Comment	Share
20/2/2019	Yoga skin makeup trend	Picture	550	46	19	2	6
	Yoga skin makeup technique	Video	283	21	8	0	4
21/2/2019	What is your skin type?	Picture	400	25	11	1	5
22/2/2019	11 ways to reduce stretch marks	Article	365	34	9	0	8
23/2/2019	Mistake in skin care	Picture	216	15	5	0	3
24/2/2019	Why must to warm up?	Video	85	0	1	0	2
27/2/2019	Skincare routine	Picture	299	4	13	0	3
28/2/2019	Lazy girl style	Picture	172	15	7	0	2
1/3/2019	7 simple ways to make whitening teeth at home	Article	224	10	7	0	4
5/3/2019	How to solve dark mouth?	Picture	132	6	3	0	1
6/3/2019	Face washes along the hair	Video	196	3	5	0	3
7/3/2019	Pearl hair clip fashion	Picture	436	53	9	2	3
8/3/2019	Miss universe makeup	Video	51	0	1	0	3
9/3/2019	Lipstick for a healthy mouth	Picture	187	14	3	0	2
11/3/2019	How to treat acne on the back?	Video	50	2	2	0	3
12/3/2019	Giveaway Rojukiss mask	Picture	1,862	108	37	13	52
13/3/2019	Nail ideas	Picture	720	97	6	32	5
14/3/2019	Celery juice	Article	538	53	13	13	4
15/3/2019	How to treat Keratosis pilaris?	Video	42	1	1	0	3
19/3/2019	Announcement of winner (Rojukiss mask)	Article	93	11	6	2	1
20/3/2019	Perfume pulse points	Picture	130	7	5	0	1
21/3/2019	Medium hairstyles	Picture	216	13	5	0	2
22/3/2019	Yoga poses	Video	50	0	3	0	3
23/3/2019	Workout program	Picture	130	5	5	0	2
25/3/2019	Detox smoothie	Video	51	0	3	0	3
27/3/2019	Beauty tips from lemon	Article	98	1	1	0	2
29/3/2019	How to treat melasma?	Video	24	0	1	0	3
30/3/2019	Coconut oil	Article	103	2	2	0	2
1/4/2019	Sunscreen	Article	170	3	3	0	2
2/4/2019	Giveaway Biore sunscreen lotion	Picture	1,061	76	49	23	16
3/4/2019	How to treat acne scars?	Article	87	0	0	0	1
8/4/2019	Office women style	Picture	352	15	10	0	4
9/4/2019	Announcement of winner (Biore lotion)	Article	123	5	3	1	1
10/4/2019	Anti-aging skincare	Video	69	2	3	0	4
11/4/2019	How to get rid of vaginal odor?	Article	71	8	1	0	1
12/4/2019	Parisian makeup	Video	67	3	1	0	2
13/4/2019	How to check the expiry date of cosmetics?	Article	65	0	0	0	1
14/4/2019	Compare plankton essence products	Video	41	0	2	0	4
15/4/2019	Beauty products store in fridge	Article	56	0	0	0	3
16/4/2019	How to read cosmetic label?	Video	54	2	1	0	3
17/4/2019	Skin care techniques from Victoria's secret	Video	55	0	2	0	2
18/4/2019	Coachella dress up ideas	Picture	64	0	1	0	2
20/4/2019	How to select contact lens to match with skin color?	Article	75	2	2	0	2
21/4/2019	Rihanna makeup	Video	67	0	2	0	3
25/4/2019	Review La Roche Posey sunscreen	Article	50	1	2	0	6
26/4/2019	Student makeup	Video	83	0	5	0	1
27/4/2019	5 trends fashion 2019	Article	106	0	4	0	2
28/4/2019	Cosmetics in Japan	Article	176	2	4	0	2
29/4/2019	How to change personality?	Article	128	1	3	0	3
30/4/2019	Lose weight tips	Article	113	4	5	0	1

¹ The number of users who clicked to read the contents.

The Efficacy of 0.025% Licochalcone A cream in treatment of moderate to severe acne vulgaris

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Abstract

Acne is one of the most common disorders which are more than 90 percent of world population is affected by acne at some period in their life. Nowadays, herbs, especially Licochalcone A are very popular to cure inflammatory skin lesion. Therefore, researcher is interested to study more about Licochalcone A so that it will be the other alternative treatment to cure inflammatory acne, and can reduce using steroid to patient. Objectives: To examine the efficient of using Licochalcone A to treat moderate to severe inflammatory acne vulgaris. Methods: The study design is a Clinical trials by using Double-blind Randomized controlled trials with 30 patients and separate them into 2 groups; an Experimental group with Licochalcone A treatment and Controlled group with Cream base. Results: Acne severity at 0, 4th, 8th, and 12th week, there is no different in Experimental group with Licochalcone A and Controlled group with Cream base in statistically. As a result of Experimental group with Licochalcone A, 13 of 30 patients have a better result at 4th rating at 4th week, 16 patients at 8th week, and 13 patients at 12th week. In addition, a better result at 5th rating, there are 3 patients have a 5th scores at 4th week, 3 patients at 8th week, and increasing to be 9 patients at 12th week. In summary, the results show that Experimental group with Licochalcone A have an efficient in cure and treat non-inflammatory acne, Closed Comedones and inflammatory acne called Papules and Nodules until period of experiment at 12th week, although there is some Scaling side effect which is evaluated by dermatologist. However, both dermatologist and all 30 patients have higher satisfaction compared with at the beginning of the experiment. Therefore, Licochalcone A is the other alternative treatment of moderate to severe inflammatory acnes.

Keywords: Licochalcone A; Acne; Inflammatory Acne; Moderate Acne Vulgaris; Severe Acne Vulgaris.

1. Introduction

Acne is one of the most common disorders that has been treated by dermatologists and other health service providers. More than 90% of world population is troubled by the effects of acne at a certain period in their lives. Although acne is considered as skin disease, it could lead to emotional and mental condition. There are various researches indicated that acne can affect the quality of life, self-confident, and negative emotional expression (Yazici, 2004).

Acne is a common chronic condition of the sebaceous gland, which results in inflammation process that demonstrates itself in many forms of inflammatory acne (papules, pustules, nodules, cysts), close comedones (blackheads, white heads), and post-inflammatory scars on skin (EffeMarket, 2017, pp. 111). The cause of acne is composed of many reasons, such as hormonal changes during puberty and teenage years, and will become more severe by genetic influence and other factors.

Acne inflammatory process is caused by the mechanism of immune system when detected bacteria *P. acne*, which triggered the releasing of inflammation-inducing chemicals (Lymphocytes, Neutrophils, and Macrophages). These mechanism then caused damage to sebaceous gland, which leads to leakage of fluid within the gland (bacteria, fatty acid, fat) into surrounding tissues. Moreover, it was discovered that Neutrophils trigger Reactive Oxygen Species (ROS) reaction that affect the inflammation of acne by destroying sebaceous gland's membrane, which then result in the leakage of the gland that will cause inflammation afterward (Lizelle Fox et al, 2016).

Current treatment for acne can be divided into 3 methods (Noppakun et al, pp. 57-80).

1. Topical treatment, using topical application to dissolve acne and reduce inflammatory (Adapalene, topical isotretinoin, benzyl peroxide, topical antibiotics)
2. Oral antibiotics, (tetracycline, doxycycline, erythromycin, sulfamethoxazole trimethoprim, dapsone), sebaceous glands reduction, anti-inflammation medication (isotretinoin), and hormonal medication
3. Alternative treatment – Intralesional Steroid, Systemic Steroid, Laser and light therapy

As the main treatment using topical and oral medication cannot yield satisfying treatment results within limited period of time, other alternatives treatments were invented, especially the application of steroid medication in order to abruptly end the inflammation of acne and causing less scarring as much as possible (Wattanakrai, #10)

Licochalcone A is an ingredient extracted from Licorice roots (*Glycyrrhiza inflata*) that has anti-inflammatory property.

Licochalcone A or Retrochalcone has component as reverse structure of Chalcone, which found to have potential as various anti-inflammatory against inflammatory mediator within the body as following list (Kolbe, 2006 #14)

1. Reduce Oxidative Burst of Granulocytes
2. Inhibit the production of Prostaglandin E2 from Keratinocytes and Dermal Fibroblast (Prostaglandins)
3. Inhibit the releasing of LTB4 from Granulocytes (Leukotrienes)
4. Inhibit the releasing of IL-6/TNF-alpha from Immature Dendritic Cells (Interleukins/ Tumor Necrosis factor)
5. Inhibit T Cells Proliferation
6. Inhibit the production of Cytokine
7. Inhibit Phorbol Ester-Driven Inflammatory Responses

From the above properties of Licochalcone A as anti-inflammatory or inhibitor against many inflammatory mediator found in acne inflammation process, the authors anticipate that Licochalcone A could be an effective treatment for acne vulgaris as it has never been studied with moderate to severe acne before.

2. Materials and Methods

This research is a clinical trial performed in volunteers, experimental and control groups, which 0.025% Licochalcone A cream and Cream base treatment was assigned randomly to each individual unknown by both researchers and volunteers (Double-blind Randomized controlled trial). The experiment began by dividing half of the face of 30 male and female subjects age 14 to 40 years old, who were diagnosed with facial acne vulgaris and have been assessed by dermatologists with moderate to severe acne vulgaris, all subjects gave consent to participate in the experiment and follow-up at Mae Fha Luang University Hospital, Bangkok. Researchers performed lesion counting on both sides of the face, categorized by non-inflammatory and inflammatory acne, recorded the numbers. The assessment of acne severity was according to Clinical Practice Guideline for Acne established by Dermatological Society of Thailand. Sebum level was measured on both sides of the face with sebum meter, then took photographs of subjects from the front, left side, and right side for further comparison in follow-ups using digital camera. Subjects were instructed to apply cream daily by using 1.25 FTU each cream per their faces side in the morning and before bedtime which is

assigned randomly on either left or right side of the face. In addition, all participants in this experiment had received detailed information on the cause of acne, factors that triggered acne, instruction for self-care, face cleaning routine, and avoiding acne inducing behaviour. After washing their faces daily for 12 weeks, volunteers will rate satisfaction scores of treatment results from 1 to 5 (low to high).

3. Results and Discussion

Majority of the subjects are female (21 individuals), which constitutes as 70% of all participants, and male of 30% (9 individuals) respectively. Average age is 21.3 ± 3.88 years, with the lowest at 15 years old and eldest at 34 years old (Table 1).

Amount of non-inflammatory and inflammatory acne between each side of the face that received Licochalcone A and cream base before treatment, was followed up at 4th, 8th, and 12th week. The results found that the amount of non-inflammatory acne (closed comedones) between both sides displayed no statistical differences in every follow-up period. But comparison made during follow-up found that non-inflammatory acne (closed comedones) on side that received Licochalcone A showed statistically significant reduction at 0.05 ($p < 0.001$), while the side that received cream base showed no statistical change ($p = 0.259$) at 12th week (Table 2).

Amount of Papule-type inflammatory acne between both sides have no statistical differences in almost every follow-up period, except on 12th week that amount of papule acnes on the side that received Licochalcone have reduced significantly more than cream base side, with statistically significant at 0.05 ($p < 0.001$). However, comparison made during follow-up found that amount of inflammatory papule acnes on both sides of the face, received Licochalcone A and cream base, have reduced with statistically significant at 0.05 ($p < 0.001$ and $p = 0.002$ respectively), but the side that received Licochalcone A demonstrated higher reduction rate than cream base.

Amount of Pustule-type inflammatory acne between each side of the face displayed no statistical differences in every follow-up period. Comparison made during follow-up found that inflammatory pustule acne on sides that received Licochalcone A and cream base showed no statistical change.

Amount of Nodule-type inflammatory acne between both sides have no statistical differences in almost every follow-up period. However, comparison made during follow-up found that amount of inflammatory nodule acnes on both sides of the face, received Licochalcone A and cream base, have reduced with statistically significant at 0.05 ($p < 0.001$ and $p = 0.001$ respectively), but the side that received Licochalcone A demonstrated higher reduction rate than cream base.

Amount of Cyst-type inflammatory acne between each side of the face displayed no statistical differences in every follow-up period. Comparison made during follow-up found that inflammatory cyst acne on sides that received Licochalcone A and cream base showed no statistical change.

Table 1 Demographic data of participants

Variables	Frequency (%)
Sex	
Male	9 (30.0)
Female	21 (70.0)
Age	
15 – 20 years	13 (43.3)
21 – 30 years	16 (53.3)
Over 30 years	1 (3.4)
mean $\pm$ SD	21.3 ± 3.88
Min - Max	15 - 34

Table 2 Amount of inflammatory and non-inflammatory acne between sides that received Licochalcone A and cream base before treatment, follow-up on 4th, 8th, and 12th week

Variables	Licochalcone A	Cream Base	p-value ^a
Non-inflammatory acne			
Closed Comedones			
Before treatment	11.17±10.35	9.00±6.60	0.521
Follow-up on 4 th week	9.04±5.85	8.04±6.50	0.411
Follow-up on 8 th week	7.74±6.55	6.84±4.99	0.868
Follow-up on 12 th week	6.91±5.77	7.08±4.26	0.468
p-value ^b	<0.001*	0.259	
Inflammatory acne			
Papules			
Before treatment	13.87±6.88	11.53±4.33	0.076
Follow-up on 4 th week	9.70±5.19	10.27±4.66	0.467
Follow-up on 8 th week	7.77±4.59	9.67±6.73	0.440
Follow-up on 12 th week	5.17±4.16	9.00±5.19	<0.001*
p-value ^b	<0.001*	0.002*	
Pustules			
Before treatment	2.43±1.13	2.00±1.73	0.663
Follow-up on 4 th week	3.42±3.50	1.83±2.04	0.120
Follow-up on 8 th week	1.67±1.21	4.50±2.12	0.076
Follow-up on 12 th week	2.00±1.73	3.00±0.00	0.223
p-value ^b	0.126	0.244	
Nodules			
Before treatment	1.81±0.96	2.37±1.81	0.302
Follow-up on 4 th week	1.80±1.15	1.91±1.08	0.547
Follow-up on 8 th week	1.71±0.92	1.94±0.83	0.298
Follow-up on 12 th week	1.46±0.97	1.43±0.64	0.651
p-value ^b	<0.001*	0.001*	
Cysts			
Before treatment	2.75±3.50	3.67±1.53	0.271
Follow-up on 4 th week	1.50±0.71	2.17±1.17	0.483
Follow-up on 8 th week	2.33±1.53	3.00±1.00	0.500
Follow-up on 12 th week	1.50±0.70	2.33±1.53	0.543
p-value ^b	0.822	0.714	

Statistical analysis assessment using Mann-Whitney U test ^(a) and Friedman test ^(b)

* Statistically significant at < 0.05

The changes in the amount of inflammatory and non-inflammatory acne between sides that received Licochalcone A and cream base during follow-up on 4th, 8th, 12th week were compares with before treatment (Table 3). The research data showed that the reduction of papule-type inflammatory acne on the Licochalcone A side was higher than bream base side during 4th, 8th, and 12th week with statistically significant at 0.05.

Average facial sebum level on sides that received Licochalcone A and cream base measured before treatment and during follow-up on 4th, 8th, 12th week, were evaluated (Figure 1). The research found that average sebum level on both sides showed no statistical differences in every follow-up period. Also, comparison made during follow-up found that sebum level on sides that received Licochalcone A and cream base showed no statistical change.

Satisfaction in treatment process, in terms of physicians and participants, between sides that received Licochalcone A and cream base, was assessed (Table 4). The result found that satisfaction rate for physicians in side that received Licochalcone A was different from side that received cream

Table 3 Data of changing amount of inflammatory acne and non-inflammatory acne between sides that received Licochalcone A and cream base during follow-up period on 4th, 8th, and 12th week, compare to amount measured before treatment

Compare to before treatment	Licochalcone A	Cream base	df	p-value
Non-inflammatory acne				
Closed Comedones				
Follow-up on 4 th week	-2.33±1.40	-1.17±0.87	42	0.478
Follow-up on 8 th week	-3.67±1.28	-2.21±0.75	43	0.317
Follow-up on 12 th week	-4.57±1.41	-2.13±0.82	42	0.133
Inflammatory acne				
Papules				
Follow-up on 4 th week	-4.17±0.91	-1.27±0.64	58	0.011*
Follow-up on 8 th week	-6.10±1.17	-1.87±0.75	58	0.003*
Follow-up on 12 th week	-8.70±1.19	-2.53±0.86	58	<0.001*
Pustules				
Follow-up on 4 th week	-1.00±0.44	-1.00±0.00	4	1.000
Follow-up on 8 th week	1.60±1.47	1.00±1.00	5	0.821
Follow-up on 12 th week	-0.80±0.58	-1.00±0.00	4	0.895
Nodules				
Follow-up on 4 th week	-0.22±0.19	-0.70±0.39	39	0.323
Follow-up on 8 th week	-0.38±0.31	-1.06±0.50	31	0.265
Follow-up on 12 th week	-1.08±0.34	-1.79±0.62	24	0.353
Cysts				
Follow-up on 4 th week	-3.00±4.00	-1.67±1.33	3	0.797
Follow-up on 8 th week	-3.00±4.00	-0.67±0.67	3	0.663
Follow-up on 12 th week	-3.00±4.00	-1.33±1.33	3	0.750

Statistical analysis assessment using Independent t-test

base during follow-up on 8th and 12th week with statistically significant at 0.005 ($p < 0.001$ and $p < 0.001$ respectively), while for participants satisfaction rate in side that received Licochalcone A was different from side that received cream base during follow-up on 8th and 12th week with statistically significant at 0.005 ($p = 0.044$ and $p = 0.035$ respectively).

4. Conclusion

From the research results, it is showed that Licochalcone A has efficacy in acne treatment in non-inflammatory acne (Closed Comedones) and inflammatory acne, Papules and Nodules. Physicians and participants have increasing satisfaction rate in treatment up until 12th week when comparing to the early period of treatment for moderate to severe acne vulgaris on face. Therefore, Licochalcone A can be considered as an option in treatment for patients with moderate to severe acne vulgaris on face.

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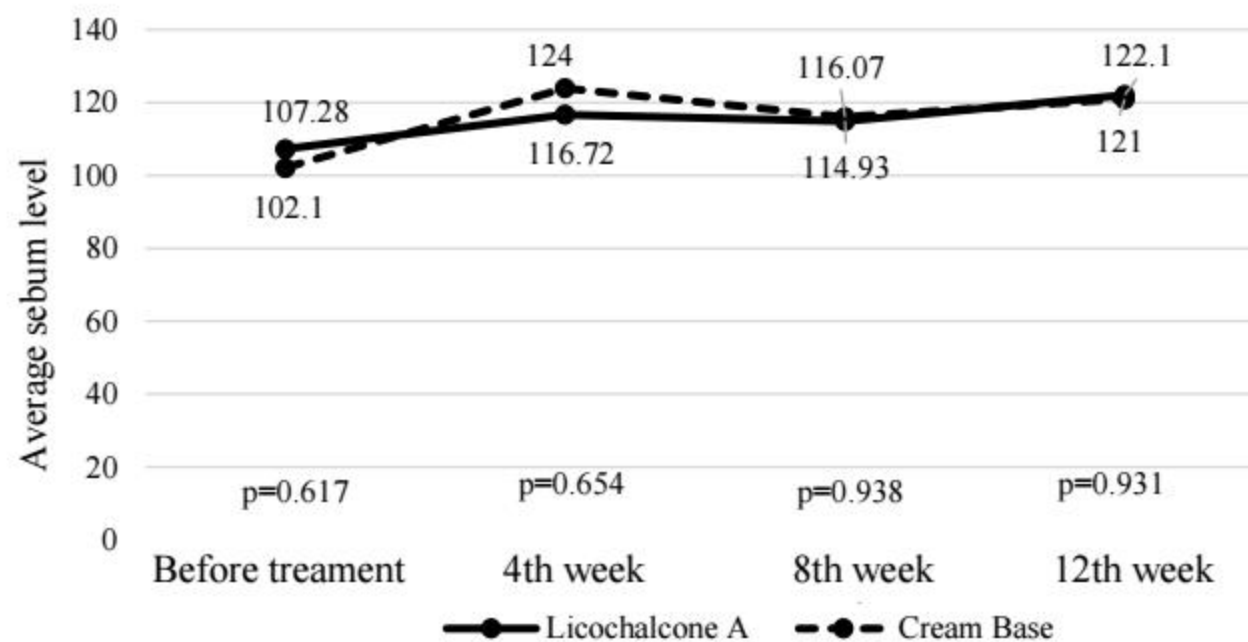


Figure 1 Average sebum level on face between sides that received Licochalcone A and cream base

Table 4 Satisfaction level in treatment, for physicians and participants, between sides that received Licochalcone A and cream base

	Licochalcone A	Cream base	p-value
Satisfaction level in treatment			
Physicians, median (IQR)			
Follow-up on 4 th week	3 (2, 3)	3 (2, 3)	0.217
Follow-up on 8 th week	3 (3, 4)	3 (3, 3)	<0.001*
Follow-up on 12 th week	4 (3, 4)	3 (3, 3)	<0.001*
Satisfaction level in treatment			
Participant, median (IQR)			
Follow-up on 4 th week	4 (3, 4)	4 (3, 4)	0.865
Follow-up on 8 th week	4 (4, 4)	4 (3, 4)	0.044*
Follow-up on 12 th week	4 (4, 5)	4 (3, 4)	0.035*

Statistical analysis assessment using Mann-Whitney U test

* Have statistically significant at 0.05

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Fatty acid contents and antioxidant activity evaluation of Pra (*Elateriospermum tapos*) seed oil obtained from different extraction techniques

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Abstract

Pra (*Elateriospermum tapos*) belongs to family Euphorbiaceae, is abundant in Southern of Thailand. The seeds are normally consumed as a snack in Southern part of Thailand. Its seeds contain abundant amount of fixed oil, which could be applied in cosmetic, pharmaceutical and food industries. The aim of this work was to investigate the effects of extraction techniques including soxhlet extraction, maceration and cold press technique on the chemical constituents and antioxidant activities of pra seed. Unsaturated fatty acid contents were determined by Gas chromatography with flame ionization detector (GC-FID) and antioxidant activity of obtained extracts were evaluated by DPPH assay. The results revealed that pra seed oil obtained by Soxhlet extraction showed the highest unsaturated content was linoleic acid (35.97 ± 0.14 %), followed by oleic acid (32.75 ± 0.06 %) and linolenic acid (14.08 ± 0.03 %). The oil derived from cold press extraction exhibited the highest DPPH radical scavenging potential (IC_{50} ; 12.69 ± 2.42 mg/ml), followed by soxhlet continuous extraction (IC_{50} ; 19.55 ± 2.14 mg/ml) and maceration technique presented the lowest potential for antioxidant (IC_{50} ; 43.09 ± 2.81 mg/ml). This suggested that pra seed oil prepared by Soxhlet extraction should be recommended to apply in cosmetic, pharmaceutical and food industries.

Keywords: Antioxidant; Euphorbiaceae; Extraction method; Omega; Pra; *Elateriospermum tapos*

1. Introduction

Pra (*Elateriospermum tapos*) is an edible nut that has been popular in Southern of Thailand. It is a native plant from Malay Peninsula and Indonesia Island. In Thailand, pra are found in the Kao-nan national park, Nakhon Si Thammarat. Fruits or nuts are obtained around August to October in every year. There are 3-4 seeds are enclosed within the nuts. When the fruits are mature, fruit are dehiscent and scatters seeds (Jantarit *et al.*, 2009; Yong and Salimon, 2006). People always harvest these seeds for consumption.

However, Seeds of pra contain many compounds which make human feel not well, especially cyanogenic glycosides such as amygdalin. Plants are mostly secreted these compounds from the

interference by herbivore. Therefore, cyanogenic glycosides can also affect on human health. Nonetheless, these glycosides can be eliminated by heating or fermenting (Ngamriabsakul and Kommen, 2009).

Oil are extracted from seeds. It has pale yellow color and odorless. Oil from seed has potential to emerge as a major source of vegetable oil and can be produced for oil lamp (Sam and Welzen, 2004). Many researches have been found that Pra oil consists of squalene, vitamin E and L beta-sitosterol. All mentioned compounds, have beneficial effect to body such as improving the skin body, anti-aging or anti-oxidant activity. Moreover, this seed obtains good nutritional because of the component of fatty acids. Fatty acids which have been found in pra seed oil include linolenic acid, linoleic acid and oleic acid (Choonhahirun, 2010; Hamidah *et al.*, 2011; Yong and Salimon, 2006). The information about it, exhibits the high content of linolenic acid of pra oil is higher than Palm oil and soybean oil.

In addition, researchers attempted to extract linolenic acid (omega-3) from pra seed oil by using Soxhlet extraction method by 3 different solvents (hexane, hexane: methanol and ethanol: methanol). The results were found that linolenic acid (omega-3) amount in 3 solvents were not significant differences. It was about 1.15, 1.41, 1.32 mg/g oil, respectively (Tan *et al.*, 2014). Moreover, oils extracted from maceration procedure showed the present of oleic acid (34.55%), linoleic acid (31.76%) and α -linolenic acid (16.10%) (Yong and Salimon, 2006). It could be implied that extraction methods play an important role in fatty acid contents.

Therefore, the objectives of this study were attempted to compare the extraction methods and solvent to provide oil from pra-seed that have nutritional values, high antioxidant activity to produce the oil that showed the potential to emerge as a major source of vegetable oil.

2. Material and Method

2.1 Chemical

Hexane, petroleum ether, potassium hydroxide, potassium iodide, sodium thiosulfate, and Wijs solution were purchased from Sigma-Aldrich Chemical Corporation (St. Louis, MO, USA). All other analytical reagents were obtained from Merck Chemical Company (Darmstadt, Germany). The titrations were performed by using an auto titrator Titrino plus 848 (Metrohm, Herisau, Switzerland).

2.2 Seed material

Pra seeds were purchased from Hua It Market, Nakhon Si Thammarat Province. The seeds were washed thoroughly and dried in a hot air oven at 55°C for 5 days. The dried sample (removing crusts) was powdered and passed through a 40 mesh, it was stored in the refrigerator for further use.



Figure 1 Physical appearance of pra seeds (*Elateriospermum tapos*). A: pra seed B: pra seed without peel C: pra seed fine powder

2.3 Oil Extraction

2.3.1 Soxhlet extraction (Tan *et al.*, 2013)

Pra seed powder was extracted by using soxhlet extractor operated at 55-60°C for 6 hours. Solvents such as hexane and petroleum ether were used. After the extraction period, the solvent was evaporated with a rotary evaporator (Buchi, Model B 720, Switzerland) then stored at -5°C for further analysis.

2.3.1 Macerations extraction (Yong and Salimon, 2006)

Pra seed powder extracted with solvents such as petroleum ether and hexane in a shaking incubator (BIOSAN Model ES-20) (55°C) at constant stirring rate 175 rpm. It was left for 6 hours and solids were filtered. The solvent was evaporated with a rotary evaporator (Buchi, Model B 720, Switzerland) and stored the extract in a tightly closed container at -5°C.

2.3.3 Cold press

Pra seed powder was extracted with manual hydraulic pressure extraction (Specac Ltd, Uk) at 15 metric ton

2.4 Fatty acid composition

Methanol: sulfuric acid: chloroform (17:3:20 v/v/v), 1 mL of distilled water were added to pra seed oil (50 mg) and mixed. The fatty acid compositions of the FAMES from pra seed oil were evaluated using an Agilent gas chromatography equipped with a silica column (HP-INNOWax 19091N-113, 30 m × 0.32 mm × 0.25 µm film of polyethylene glycol), a flame ionization detector and split injection port. The initial oven temperature was 200°C, subsequently increased to 240°C at 10 °C/min. The injector temperature was set at 250°C. Nitrogen was used as the carrier gas at a flow rate of 1.5 ml/min. The standards of fatty acids that were used in this study including caprylic acid, capric acid, lauric acid, myristic acid, palmitic acid, stearic acid, oleic acid (omega-9), linoleic acid (omega-6) and linolenic acid (omega-3).

2.5 Antioxidant assay

2.5.1 Assay of DPPH radical-scavenging activity (adopted from Bennett *et al.*, 2008)

The DPPH assay was used to determine the free radical scavenging activity. Oil sample (1 mL) was added to 3 mL of 0.1 mmol of DPPH solution then kept in a dark place for 90 min and measured the absorbance of the resulting solution at 517 nm, using Trolox as standard and inhibitory concentration (IC₅₀) was calculated.

2.5.2 Assay of lipid oxidation through ferric thiocyanate assay (FTC assay) (adopted from Sen Gupta & Ghosh, 2013)

Linoleic acid was weighed approximately 0.28 g and then mixed with 0.2 mL of PBS pH 7.0. 400 mg of pra seed oil was added to linoleic acid solution (5.0 mL) and PBS (4.6 mL) then incubated in a heat block (AccuBlock™ Digital Dry Bath, Labnet International, Inc., New Jersey, USA) at 40°C in dark place. Mixed 4.3 mL of 75% (v/v) ethanol and 0.2 mL of 30% (w/v) NH₄SCN solution with the sample (sampling at 1, 2, 3, 4, 5, 6 and 7 days) and left for 3 min before measuring the absorbance at 500 nm using Trolox as standard.

3. Result and Discussion

3.1 Fatty acid composition

The results expressed that all three extraction methods (soxhlet, maceration and cold press) of dry pra seed had no different in chemical properties ($p < 0.05$), the highest linoleic acid (35.35-36.75%), followed by oleic acid (32.09-32.79%), linolenic acid (13.61-14.44%) and palmitic acid (12.72-13.97%) as shown in Table 1, respectively. Comparing various solvents using in soxhlet method of omega 3 in pra

seed oil, the omega 3 concentration is demonstrate as follows by hexane:methanol (90:10) (1.41 g ω -3/g oil), ethanol:methanol (70:30) (1.32 g ω -3/g oil) hexane (1.15 g ω -3/g oil) (Tan *et al.*, 2014). According to the results, oil derived from Pra seed exhibited the highest unsaturated fatty acid content that can be used as the alternative choices to produce the natural oil.

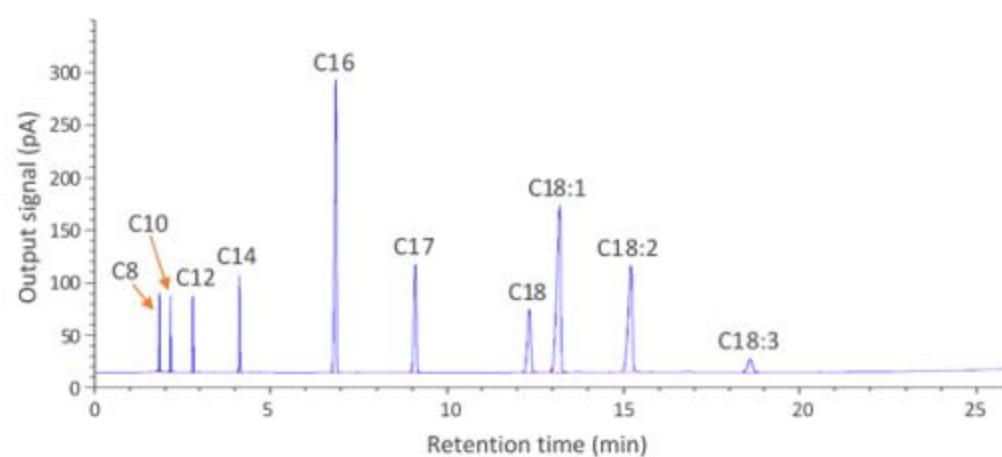


Figure 2 GC chromatogram of standard FAMES. (C8, methyl caprylate; C10, methyl capricate; C12, methyl laurate; C14, methyl myristate; C16, methyl palmitate; C17, methyl heptadecanoate; C18, methyl stearate; C18:1, methyl oleate; C18:2, methyl linoleate; C18:3, methyl linolenate).

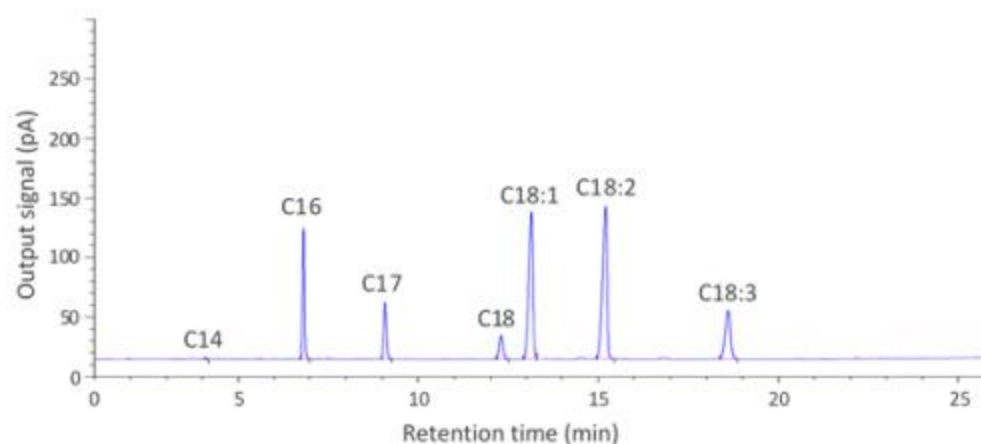


Figure 3 GC chromatogram of FAMES in the pra seed oil sample (C14, methyl myristate; C16, methyl palmitate; C17, methyl heptadecanoate; C18, methyl stearate; C18:1, methyl oleate; C18:2, methyl linoleate; C18:3, methyl linolenate).

Table 1 Fatty acids composition of pra seed oil extracted by GC-FID

Fatty acid	Soxhlet		Maceration		Cold press
	Hexane	Petroleum ether	Hexane	Petroleum ether	
Luaric acid	0.09±0.01 ^b	ND	0.07±0.02 ^b	0.03±0.01 ^a	ND
Myristic acid	0.10±0.02 ^{ab}	0.07±0.01 ^a	0.12±0.05 ^{ab}	0.08±0.03 ^{ab}	0.04 ± 0.01 ^b
Palmitic acid	13.97±0.49 ^b	12.72±0.02 ^a	13.22±0.45 ^{ab}	13.93±0.15 ^a	12.82 ± 0.02 ^{ab}
Stearic acid	5.03±0.23 ^b	5.79±0.02 ^c	5.14±0.23 ^{bc}	5.45±0.16 ^c	5.2 ± 0.03 ^a
Oleic acid	32.79±0.52 ^b	32.75±0.06 ^a	32.09±0.16 ^a	32.30±0.03 ^a	31.24 ± 0.01 ^a
Linoleic acid	35.35±0.64 ^a	35.97±0.04 ^{ab}	35.01±0.43 ^{ab}	35.34±0.24 ^{ab}	36.75 ± 0.10 ^b
Linolenic acid	14.29±0.53 ^a	14.08±0.03 ^b	14.44±0.17 ^b	14.35±0.11 ^b	13.61 ± 0.06 ^b
Total Omega	82.43±1.68	82.81±0.13	81.55±0.75	81.98±0.38	81.61 ± 0.16

The data are the means of three independent experiments ± standard deviations (n = 3).

^{a, b, c}Values in the same row with different superscript letters differ significantly (P < 0.05). ND: not determined

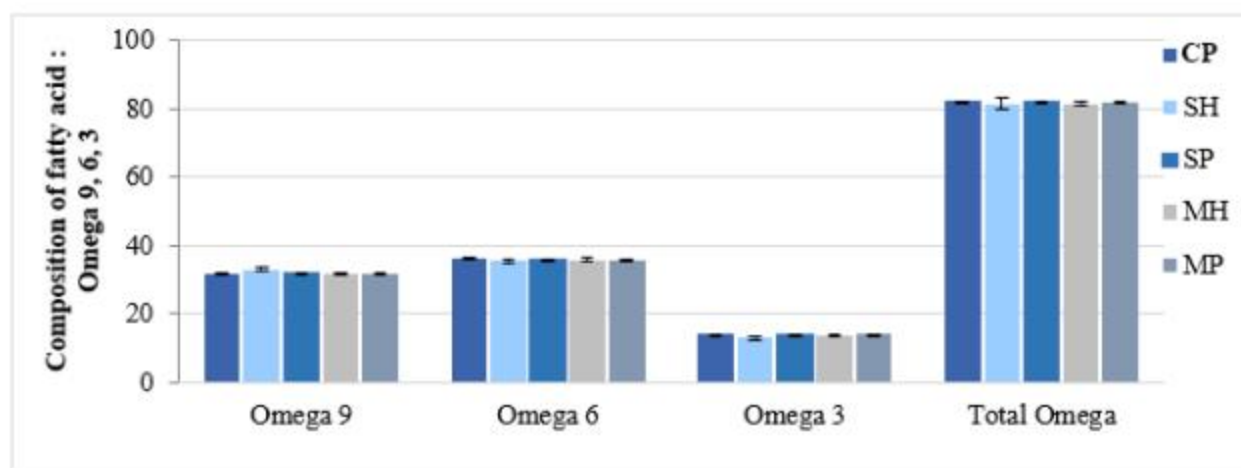


Figure 4 The total composition of fatty acid: Omega 3, 6, 9 (CP: Cold press, SH: Soxhlet - Hexane, SP: Soxhlet - Petroleum ether, MH: Maceration - Hexane and MP: Maceration - Petroleum ether)

3.2 Antioxidant assay

Antioxidant activity of oil derived from cold press extraction exhibited the lowest IC_{50} value that has a potential for antioxidant, followed by soxhlet continuous extraction method. Maceration technique presented the lowest potential for antioxidant. Scavenging activities of the DPPH radicals (IC_{50}) were shown in Table 2, the results revealed that pra seed oil extracted by using petroleum ether had the most anti-oxidant activity (19.55 mg/mL) when comparing to using hexane. This result could be explained that polyunsaturated fatty acids, especially omega-3, omega-6 and omega-9 reduce the oxidative stress and increase the antioxidant markers.

Table 2 Comparison of antioxidant activities of pra seed oil.

Methods	Solvent	Scavenging of DPPH, IC_{50} (mg/mL)	Inhibition of linoleic acid oxidation (%)
Soxhlet	Hexane	77.33 ± 1.50^c	28.76 ± 2.07^a
	Petroleum ether	19.55 ± 2.14^b	43.27 ± 1.87^b
Maceration	Hexane	46.72 ± 2.36^d	45.84 ± 2.38^c
	Petroleum ether	43.09 ± 2.81^c	49.89 ± 1.83^d
Cold Press		12.69 ± 2.42^a	57.83 ± 2.42^c
Trolox		5.48 ± 2.59	60.95 ± 1.36

The data are the means of three independent experiments $\pm$ standard deviations (n = 3).

^{a, b, c}Values in the same row with different superscript letters differ significantly ($P < 0.05$).

4. Conclusion

The results of physicochemical properties and fatty acid composition demonstrated that pra seed oil can be used to develop drugs, supplements, and cosmetics and to reduce oil imports which will improve the economic potential of the country. Oil from pra-seed derived from all extraction methods have nutritional values, high antioxidant activity. For these information, pra seeds showed the potential to emerge as a major source of vegetable oil.

5. Acknowledgements

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Preparation of Colorant from Teak Leave for Cosmetic Utilization

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Abstract

This research investigated the feasibility of teak (*Tectona glandis*) leave utilization as cosmetic colorant. The color extracts from young and mature of the teak leaves were prepared by using 50% ethanol and 95% ethanol in water. Young teak leave extract from 50% ethanol provided the most bright orange-red color with $L^* = 18.85 \pm 0.66$, $a^* = 9.91 \pm 0.27$ and $b^* = 2.93 \pm 0.09$. Pigment from 50% ethanolic young teak leave extract was prepared by using aluminum chloride, aluminum oxide, potassium aluminum sulfate and magnesium aluminum silicate yielding 2.87%, 90.96%, 4.19% and 84.27% w/w, respectively. The pigment prepared from the potassium aluminum sulfate was selected for study the effects of salt amount, extract to salt ratio, reaction time, temperature, and pH during pigment preparation. The 5.00% of salt in the reaction gave the highest yield, despite the 2.62% provided the higher chroma value of the pigment. Suitable ratio of the color extract solution to the salt solution was 2:1 v/v. Pigment preparation reaction at 60 min, pH 3-4 and 95°C was the optimal condition. The prepared teak leave pigment was bleeding stable in DI water with ΔE 0.47 ± 0.08 . After 6 cycles of heating-cooling acceleration test, the pigment also exhibited its color stability with ΔE 0.48 ± 0.04 . Results from this study showed that the teak leave could be considered as a source for cosmetic pigment preparation.

Keywords: Teak leaf; Teak young leaves; Color; Lake

1. Introduction

Color additives are another important component of cosmetic products. Because it makes the product more colorful, attractive to use, and can be used for beauty as well as to conceal unwanted appearance. Most cosmetic coloring agents are synthetic dyes due to their good stability and cheap price. However, some of them are high risk on consumer health, which have been reported some of them contain heavy metal contamination such as chromium, cadmium, mercury, lead and arsenic etc. (Dumas, 2012). Nowadays the natural pigment has more interest because it is believed to be safer resulting in an increase demand of natural colors in cosmetics. *Tectona grandis* (Teak) belongs to Verbenaceae family, is well known for containing orange-red dye that has been using for dyeing silk. The young teak leaves produce a yellowish brown-reddish dye (Saxena, 1989). The chemical composition of the teak leaves colorant is mainly anthraquinoid (Vankar, 2000). There has been reported to use color extract from teak leaves in lipstick but the color stability was low which it becoming turn to brown shade (Setyawaty and ratama, 2018). The author explained that the use of ethanol as solvent provided the extract with high anthocyanin and tannin contents resulting in color change in lipstick (Setyawaty and ratama, 2018). This indicated that direct use of the teak leave extract in the cosmetic formula might suffer from color instability. Therefore, this research was aimed to prepare more stable teak leave color extract in the form of lake by using aluminum salts. Types and amount of salts, temperature, time, pH and ratio of color extract to salt solution were also investigated.

2. Materials and Methods

2.1 Chemicals and Reagents

Aluminum chloride was obtained from Sigma-Aldrich, USA. Aluminum oxide was purchased from KemAus, Austria. Potassium aluminum sulfate dodecahydrate was provided by Ajax Finechem, Austria. Magnesium aluminium silicate was obtained from Vanderbilt Minerals, USA. 1,3-Butylene glycol was purchased from KH Neochem, Japan. Castor oil was bought from Thai castor oil industries, Thailand. Hydrochloric acid was obtained from J.T. Baker, USA. Sodium hydroxide was bought from Carlo Erba, Italy. Young and mature teak leaves were obtained from NongmuangKhai district, Phrae, Thailand during March 2019.

2.2 Preparation of the Color Extract from Teak Leaves

The young teak leaves and mature teak leaves were chopped to small pieces and dried in oven-dried at 50°C until their weight were constant. The dried teak leaves were then pulverized into powder. Each extraction was performed by mixing the sample powder with solvent at the ratio of sample to solvent of 1:15 w/v. The solvent system used were the mixture between 95% ethanol and DI water varying at 0:100, 50:50 and 100:50 by volume. After shaking for 2 h, the mixture was filtered by vacuum filtration for removal of solid suspensions. The filtrate obtained was color extract which was then measured its color by using Spectrophotometer CM-700d.

2.3 Preparation of Lake from Teak Leaf

Preparation of lake from the teak leaf extract was modified from previous study (Kongnoraset, 2009). Briefly, 0.50 g aluminum salt was dissolved with 10.00 mL DI water and then added into 10.00 mL of the teak leaf color extract. The mixture was heated to 95°C for 60 min. The mixture was cooled to room temperature. Precipitate was filtered through Whatman filter paper No.1 and dried at 45°C for 20 h. Yield of lake was calculated from dried lake precipitate compared with the weight of the aluminum salt used. Types of aluminum salts influence the lake preparation was investigated by using potassium aluminum sulfate ($\text{KAl}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}$), aluminum chloride (AlCl_3), aluminum oxide (Al_2O_3) and Magnesium Aluminium Silicate. Reaction time and temperature were also studied at 15, 30, and 60 min and 45, 70, and 95°C, respectively. After selection for the aluminum salt that provided the most proper properties of the lake, the ratio of color extract and salt solution of 1:1, 1:2 and 2:1 and the pH of reaction ranging between 2-8 were determined for their optimum.

2.4 Investigation of Bleeding Property for Teak Leaf Lake

Bleeding test of the teak leaf lake was performed (American society for testing and materials international, 2002) by mixing 0.01 g of lake with 10 mL of each of toluene, DI water, castor oil, 1,3-butylene glycol and 95% ethanol. The mixtures were closed with parafilm and shaken for 10 sec and then let stood for 15 min. The mixtures were re-shaken for 10 sec and let stood for 45 min. Then, the samples were filtered through a filter paper using glass funnel. The filtrates were collected to measure the color and the result of bleeding was expressed as color change (ΔE) from solvents without the lake. Bleeding was evaluated as ΔE less than 1 (no perceptible color, no bleeding), ΔE equal or more than 1 (perceptible color, bleeding).

2.5 Determination of Teak Leaf Lake Stability

The prepared teak lake stability was determined by storage the lake in accelerated condition using heating-cooling cycle at 4°C for 24 h and at 45°C for 24 h which was accounted as 1 cycle. The experiment was done for 6 cycles (Mamah et.al, 2017). The lake pigment was evaluated for its color and appearance before and after storage.

3. Results and Discussion

3.1 Preparation of the Color Extract from Teak Leaves

The Young and mature teak leaves were chopped and dried at 50°C to obtain the teak leaf

powder as shown in Figure 1.



Figure 1. Teak leaves powder (A) young leave and (B) mature leave.

Extraction of the color extract of young and mature teak leaves by different ratio of 95% Ethanol: H₂O (0:100, 50:50 and 100:0 by volume) gave the result shown in Figure 2. The ratio of 0:100 of both ages teak leaf extracts exhibited yellow-orange in which the young leaf extract showed lighter than that of the mature teak leaf. The ratio of 50:50 gave red-orange shade in the young leaf while the mature leaf provided darker red shade. The ratio of 100:0 showed red-brown shade in both young and mature leaf. Simatupang and Yamamoto (1999) reported that young teak leaves give a water soluble red dye on wounding underside. The dye is located in gland at the bottom of the leaf between the fine hairs. It is easily soluble in water and polar solvent. In mature leave the glands contain non colored liquid. The compound is apparently in reduced form as a water extract is only yellow and not red.

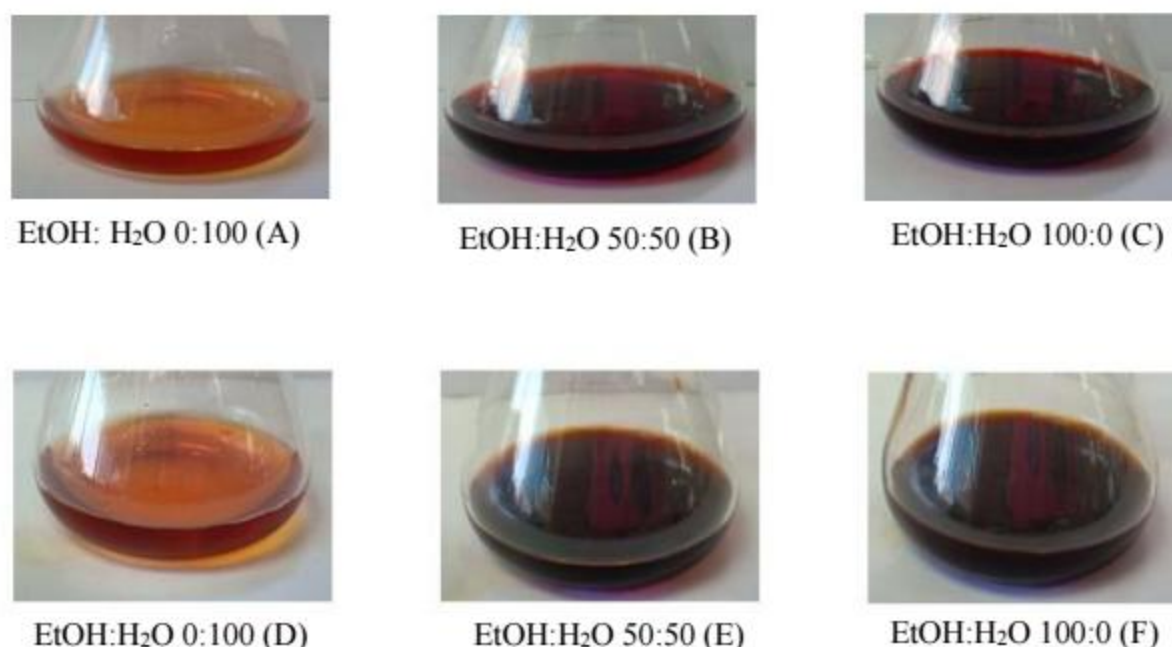


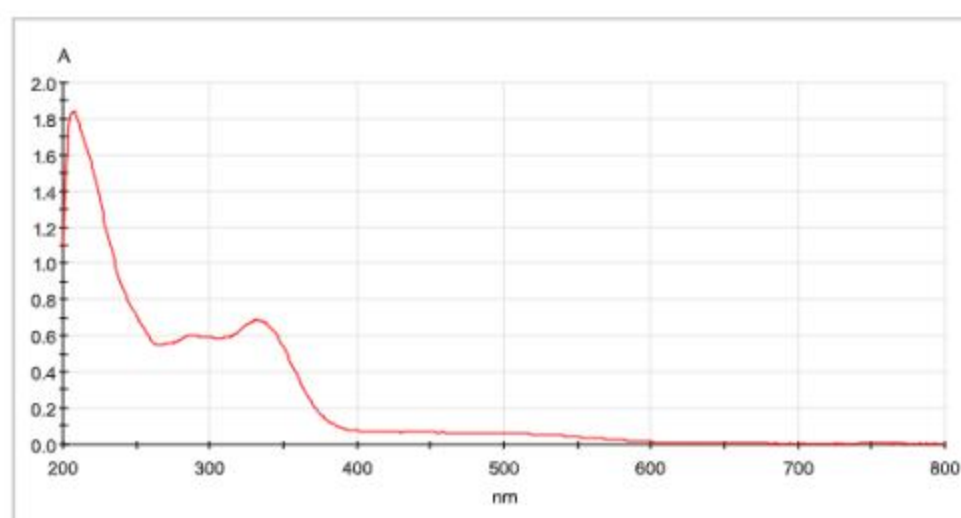
Figure 2. Color of the teak leave extract (A) young leave and (B) mature leave.

The pH and color measurement of the teak leaf extracts are aligned in Table 1. The mature leave extract showed slightly higher pH than the young leave extract. The 50:50 solvent showed high intense red shade in the young leaf which possessed higher positive a^* and b^* than those of the mature leave extract. Based on the appearance and color measurement, the young teak leaves extracted with 95% ethanol and water at 50:50 ratio was selected for further studied of lake preparation.

Table 1 Color value of the color extract of young and mature teak leaves from different ratios of 95% ethanol:H₂O (0:100, 50:50 and 100:0).

Sample	Ratio of 95% EtOH:H ₂ O	pH	L*	a*	b*	C*	h
Young teak leaves	0:100	5.45±0.04	39.10±0.10	11.06±0.03	25.09±0.15	27.43±0.13	66.21±0.16
	50:50	5.82±0.03	18.85±0.66	9.91±0.27	2.93±0.09	10.34±0.29	16.45±0.15
	100:0	5.72±0.02	18.04±0.20	3.83±0.09	0.77±0.04	3.90±0.10	11.38±0.36
mature teak leaves	0:100	6.71±0.03	32.92±0.02	14.90±0.25	25.33±0.81	29.39±0.80	59.52±0.41
	50:50	6.54±0.02	17.67±0.02	1.55±0.11	0.06±0.05	1.55±0.12	2.76±0.46
	100:0	6.39±0.03	16.70±0.20	1.10±0.15	-0.18±0.06	1.12±0.16	350.77±3.17

UV-vis absorbance of the young teak leave color extract from 50:50 ratio of 95% ethanol:H₂O 50:50 was measured and the result is depicted in Figure 3. The extract showed maximum absorption at 250 nm and 340 nm which were similar to anthraquinone absorption (Rodrigues et. al., 2006; Thongthao et. al., 2015)

**Figure 3.** UV-vis absorption spectrum of 50% ethanol young teak leave extract.

3.2 Preparation of Lake Pigment

3.2.1 Effect of Metal Salt

Aluminum chloride, aluminum oxide, potassium aluminum sulfate and magnesium aluminum silicate were used in this study. The precipitates of aluminum chloride was black color, aluminum oxide and potassium aluminum sulfate were dark-violet color with brilliant and fine particle, while the magnesium aluminum silicate gave dark-gray color (data not shown). Aluminum oxide and magnesium aluminum silicate gave 90.96 and 84.27% yields, respectively. Although they showed substantially high yield but their appearance and color were less preferable for further application in make-up cosmetics. Potassium aluminum sulfate yielded 4.19%, whereas the aluminum chloride gave the lowest yield of 2.87%. Acid-base of the mixed solution may affect the amount of lake precipitate. Acidic solution of aluminum salt caused lower yield of lake, while basic solution provided remarkable higher yield. However, even lower lake yield obtained, potassium aluminum sulfate exhibited acceptable appearance and color than other metal salts, it was selected for further investigation.

3.2.2 Effect of Reaction time

The effect of reaction time on teak lake preparation was studied at 15, 30, and 60 min with a constant temperature of 95°C. Aluminum potassium sulfate was used to prepare the lake. It was found that when reaction time was increased from 15 to 30 and 60 min, the lake yields were also raised to 2.33, 4.26 and 6.28% w/w, respectively.

3.2.3 Effect of Temperature

The effect of temperature on the teak lake preparation was also studied at 45, 70, and 95°C with a constant reaction time at 60 min. Higher temperature resulted in higher yield. At 45°C of reaction, the lowest yield of 0.96% was obtained. When the temperature was increased to 70°C and 95°C, the yields increased to 2.26% and 4.09%, respectively.

3.2.4 Effect of Metal salt Concentration

In this study, varying concentrations of salt between 0.25-10% decreased the lake yield from 4.92% to 3.42% with slightly decreased of pH from 3.64 to 3.42 (Table 2). This might be explained that the acidic condition was not proper to prepare the lake from teak leave extract. This result was agreed with the result of section 3.2.1 in which acidic salt provided lower yield than those of basic salt.

Table 2 Effect of metal salt concentration on preparation of lake from teak leave extract

Amount of $\text{KAl}(\text{SO}_4)_2 \cdot 12\text{H}_2\text{O}(\text{g})$	Concentration (%)	pH of mixture solution	Yield (%)
0.25	2.5	3.64	4.92
0.50	5	3.54	3.75
0.75	7.5	3.47	3.67
1.00	10	3.42	3.10

3.2.5 Effect of pH

The effect of pH on preparing the lake from teak leave was studied by adding 0.5% HCl and 0.5% NaOH in the mixture solution to give the final pH ranging between 2-8. When the pH was altered from acidic region to basic side, the color was changed from orange-red to dark violet. Moreover, when the pH was 5 the black precipitate was observed. It was found that that the suitable pH for lake preparation were between 3-4 due to more preferable dark violet precipitate appearance. The yield of 2.47 and 3.76% were obtained from the pH 3 and 4 condition, respective, while the pH 5-8 gave dark black precipitates.

3.2.6 Effect of Color Extract Solution to Metal Salt Solution Ratio

The effect of ratio of teak color extract solution to metal salt solution to the lake yield was studied by using the ratios of 1:1, 1:2 and 2:1 v/v. Table 3 shows the 2:1 ratio exhibited the highest yield of 16.38% with dark violet precipitates. Lower yields of 9.40 and 9.77% were observed from the 1:1 and 1:2 ratios, respectively.

For lake preparation reaction, the metal ion can act as acceptors to electron donors to form co – ordinate bond with the dye molecule, which is insoluble in water (Kongkachuichay *et al.*, 2002). The result implied that higher quantity of the color extract molecule determined the increase of the lake yield rather than amount of the salt. The color extract should be also high water soluble which could render the the precipitation of the lake. Therefore, the 50% ethanol used in this study was an appropriate solvent to extract the color compound containing high amount of water soluble dye anphtoquinone from the teak leave (Vankar, 2000).

Table 3 Effect of teak color extract solution to metal salt solution ratio on lake preparation.

Ratio of color extract solution to metal salt solution (mL)	Volume of color extract solution (mL)	Volume of $KAl(SO_4)_2 \cdot 12H_2O$ (mL)	Yield (%)
1:1	10	10	9.40
1:2	10	20	9.77
2:1	20	10	16.38

2.4 Bleeding Test of Lake from Teak Leave

Results of bleeding test is show in Table 4. The prepared lake was bleeding resistance against DI water providing ΔE of 0.47 ± 0.08 with could not be observed color change by naked eyes. Bleeding was observed when mixing the lake with 1,3-butylene glycol, castor oil, and 95% ethanol exhibiting the ΔE of 7.18 ± 0.07 , 4.70 ± 0.09 , 11.68 ± 0.08 , and 5.56 ± 0.34 , respectively.

Table 4 Bleeding test of lake from teak leave extract

Solvents	B leeding	ΔE	Solvent	Solvents +lake	Filtrate
DI	No bleed	0.47 ± 0.08			
1,3butylene glycol	bleed	7.18 ± 0.07			
Castor oil	bleed	4.70 ± 0.09			
Ethanol	bleed	11.68 ± 0.08			
Toluene	bleed	5.56 ± 0.34			

2.5 Stability Test of Lake from Teak Leave Extract

Stability of the prepared lake was investigated. The result shown in Table 5. The L^* , a^* and b^* values were quite similar at before and after cycle test. The L^* value was slightly increased indicated slightly increased lightness of the lake. The ΔE value was 0.48 ± 0.0 indicating high stability of the lake (Wrolstad et. al, 2005).

Table 5 Color measurement of lake from teak leave extract at before and after 6 cycles acceleration test.

Color unit	Color of teak leave lake before and after acceleration test	
	Before	After 6 cycles
L^*	39.38±0.09	39.75±0.08
a^*	3.10±0.10	3.36±0.06
b^*	-1.56±0.13	-1.47±0.06
C^*	3.48±0.14	3.67±0.06
ΔE	0.48±0.04	

The color ($L^*=0$: black, $L^*=100$: white), a^* represents the green-red axis (a^* negative: green, a^* positive: red), b^* represents the blue-yellow axis (b^* negative: blue, b^* positive: yellow)

4. Conclusion

In this study, the color extraction from young teak leaves was performed by a combination a solvent between 95% ethanol and DI water at ratio of 50:50 v/v. The suitable condition for preparation of lake from young teak leave color extract by using potassium aluminum sulfate were the extract to metal salt ratio of 2:1 by volume, reaction pH was around 3-4 at 95°C for 60 min. The lake was no bleed in with DI water, while bleeding was observed in 1,3- butylene glycol, castor oil, 95% ethanol and toluene. The teak leave lake was stable under accelerated storage test of heating-cooling for 6 cycles providing ΔE of 0.48±0.04.

5. Acknowledgements

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Variation of extraction conditions on antioxidant activities and phenolic constituents of rambutan (*Nephelium lappaceum* L.) rind

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Abstract

Rambutan (*Nephelium lappaceum* L.) is well known tropical fruit distributed in Southeast Asia. Its rind has been regarded as a source phenolic compounds which exhibit many biological properties including antioxidant properties. This study was aimed to vary extraction conditions including ethanol concentration, shaking speed and extraction time for rambutan rind extraction by single factor experiment using the one factor at a time design (OFAT) method. The extracts were determined on DPPH and ABTS radical scavenging activities and quantified for the phenolic contents. Geraniin, corilagin and ellagic acid were quantified by HPLC. The results revealed that all extraction conditions especially ethanol concentration influenced antioxidant activities and phenolic contents of the rambutan extract. Increasing in ethanol concentration obviously increased phenolic content and antioxidant activities of the extract ($P < 0.05$). The extraction that performed by using 65% (v/v) ethanol, shaking at 200 rpm for 12 h provided the extract with the highest phenolic compounds (159.59 ± 2.81 mg GAE/g sample) and exhibited strongly DPPH (255.75 ± 7.47 mg TE/g sample) and ABTS (573.12 ± 24.48) radical scavenging properties ($P < 0.05$). Geraniin was the major phenolic compound of rambutan rind representing 75.94 ± 0.95 mg/g sample compared with corilagin and ellagic acid ($P < 0.05$) at the appropriate condition. In conclusion, varying extraction conditions could enhance extraction's efficiency of *Nephelium lappaceum* L. rind. Therefore, the extraction condition of phenolic compounds from *Nephelium lappaceum* L. rind should be further optimized.

Keywords: Antioxidant activities; Extraction conditions; *Nephelium lappaceum* L.; Phenolics.

1. Introduction

There has been considerable interest in finding natural antioxidants from plant materials that exhibit high redox potential. Recently, waste materials are considered to investigate their feasible in value-added through reutilization as biological active substances. Rambutan (*Nephelium lappaceum* Linn.) is a tropical fruit in South-East Asia and its production generate vast amount of waste including seeds and rind. Rambutan rind is potential source of naturally-occurring antioxidants especially phenolic compounds such as corilagin, ellagic acid and its major constituent is geraniin (Thitilertdecha *et al.*, 2010). These phytochemical compounds exhibit various biological properties. Geraniin possess high antioxidant activity with low pro-oxidant (Palanisamy *et al.*, 2008). It has also been credited with a range of bioactive properties related to cosmetic applications; stimulating cell proliferation, strengthening skin barrier and enhancing extracellular matrix proteins formation through the stimulation of dermal fibroblasts and keratinocytes (Agyare *et al.*, 2011). Ellagic acid slows down the melanogenesis process (Ortiz-Ruiz *et al.*, 2016) while corilagin has been revealed to possess

antioxidant and anti-inflammatory properties (Kinoshita *et al.*, 2007; Zhao *et al.*, 2008; Thitilertdecha *et al.*, 2010).

Various conventional and advanced extraction techniques are used to extract phenolic compounds from plants such as ultrasound assisted extraction, microwave assisted extraction, pressurized liquid extraction because of their low requirement in extraction time and less solvent consumption compared with then conventional extraction techniques. However, limitations in extraction scale as well as equipment cost of those advanced extraction should be considered in an industrial scale. Many factors, such as solvent concentration, extraction temperature, pH and extraction time, can influence and increase the extraction's efficiency in term of quality and quantity of bioactive substances (Samuagam *et al.*, 2013; Kamaludin *et al.*, 2016). The optimal extraction condition that provides the extract enrich in bioactive substances is important to improve the extract's quality. Many experimental designs, such as single factor experimental design, completely randomized design, response surface design, etc. are used to optimize the extraction's efficiency to recover the maximum yield of bioactive substances (Mokhtarpour *et al.*, 2014; Kamaludin *et al.*, 2016). Therefore, this study was aimed to screen the extraction conditions of *N. lappaceum* L. rind to acquire the extract possessing high amount of phenolic compounds and antioxidant properties by using single factor experimental design. The phenolic constituents in the rambutan rind extract (geraniin, corilagin and ellagic acid) were also evaluated and quantified by HPLC.

2. Materials and Methods

2.1 Materials and chemicals

Rambutan fruits (Rongrien cultivar) were collected from Chanthaburi province, Thailand on July, 2015. Rambutan rinds were dried with hot air dryer at 50°C and pulverized by sieving machine (500 meshes) to obtain sample powder for this study.

All chemicals in this study were analytical grade. Folin-Ciocalteu's reagent was purchased from Carlo Erba (Val-de-Reuil, France). Acetonitrile (HPLC grade) (RCI Labscan, Bangkok, Thailand) and formic acid (analytical grade) were obtained from MERCK, Darmstadt, Germany. The water used for HPLC analysis was obtained by water purification system (Millipore® Milli Q (Bedford, MA, USA)). Ellagic acid, gallic acid, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) and trolox were purchased from Sigma-Aldrich (St. Louis, MO, USA). Corilagin and geraniin were isolated and purified according to the method described by Thitilertdecha *et al.* (2010).

2.2 *Nephelium lappaceum* L. rind extraction

The experiment for the extraction of *N. lappaceum* rind was designed by using single factor design previously described by Kamaludin *et al.* (2016) with some modifications. The extraction was performed by using maceration technique with the observation of three consecutive factors including ethanol concentration, shaking speed and extraction time, respectively. The highest phenolic content of each extraction factor was used to study for further extraction factor.

The sample (5 g) was extracted, with an LS ratio of 1:10, by using various ethanol concentrations (5, 35, 65 and 95% ethanol (v/v)) for 12 h at the shaking speed of 150 rpm. The best solvent concentration was then used to study influence of shaking speed on the phenolic content. The sample (5 g) was extracted by using the best solvent concentration for 12 h at various shaking speeds (0, 50, 100, 150, and 200 rpm). The best shaking speed was chosen based on the highest phenolic content. Finally, the influence of extraction time on extraction was determined by using the optimum solvent and shaking speed selected from previous steps. The sample was extracted various extraction times (0, 6, 12, 18 and 24 h). The best extraction time was selected based on the highest phenolic content.

2.3 Determination of phenolic compounds

2.3.1 Determination of phenolic contents

Extractable phenolic content (EPC) was determined according to the method described by Waterman and Mole (1994). Folin-Ciocalteu reagent (0.5 mL) was mixed with 0.1 mL of the sample solution. Three minutes later, 200 g/L sodium carbonate (1.5 mL) was added and allowed to stand for

1 h at ambient temperature. The absorbance was measured at 760 nm by using UV-Vis Spectrophotometer (Metertech SP-880). Gallic acid was used as reference compound and result was expressed as gallic acid equivalent.

2.3.2 Identification and quantification of phenolic compound by HPLC

The quantification of phenolic compounds was employed according to the method of Fecka and Turek (2008). A HPLC was performed in an Agilent 1100 UV-VIS high-performance liquid chromatography (Agilent, Germany). The samples were separated on a Zorbax SB-C18 column (5 μ m, 4.6 $\times$ 150 mm; Agilent, USA). The eluent system was a combination of solvent A (0.2% formic acid in acetonitrile) and solvent B (0.2% formic acid in water). The gradient of mobile phase was 10%A to 40% from 0 to 25 min, and then increased up to 70% within 5 min and finally maintained at 70% after 30 min. The injection volume was 20 μ L and the chromatogram was detected at 280 nm. Internal standard and linearity of standard calibration curve by plotting the area under peak versus the concentration (mg/mL) were used to quantify the amount of phenolic compounds.

2.4 Determination of antioxidant activities

2.4.1 DPPH radical scavenging assay

The DPPH radical scavenging activity was determined according to the method of Gülçin *et al.* 2003. Sample (1 mL) was mixed with 3 mL of DPPH solution (0.1 mM). The reaction mixture was shaken and incubated in the dark place for 30 min, at ambient temperature. The absorbance was read at 517 nm by using UV-Vis Spectrophotometer (Metertech SP-880). Trolox was used as reference compound. The DPPH radical scavenging activity was expressed as trolox equivalent (mg TEAC). The ability to scavenge the DPPH radical scavenging was calculated by following equation:

$$\% \text{DPPH radical scavenging activity} = [(A - A_1)/A_0] \times 100 \quad (1)$$

Where A_0 is the absorbance at 517 nm of the control and A_1 is the absorbance at 517 nm of the mixture containing standard or extract

2.4.2 ABTS⁺ radical scavenging assay

The ABTS⁺ scavenging activity was performed according to the method of Re *et al.* (1998) with some modifications. The stock solution of ABTS cation chromophore was prepared by the reaction between 7 mM ABTS solution (100 mL) and 2.45 mM potassium persulfate (final concentration) (100 mL) in the dark place at ambient temperature for 16 h. The ABTS⁺ solution was diluted with phosphate buffer (50 mM, pH 7.4) to an absorbance of 0.70 ± 0.02 at 734 nm. An aliquot of each extract (100 μ L) was added to 3 mL ABTS⁺ solution and the resulting mixture was then incubated for 30 min at ambient temperature prior to being measured the absorbance at 734 nm. Trolox was used as reference compound and the ABTS⁺ scavenging activity was expressed as trolox equivalent.

2.5 Statistical analysis

The experimental designs were generated and their results were analyzed using IBM SPSS Statistics software (version 24). One-Way Analysis of Variance (ANOVA) and Duncan multiple range test were used for determining significant difference at $P < 0.05$.

3. Results and Discussion

The single factor experiment using the one factor at a time (OFAT) was used to find out the appropriate condition for the extraction of *N. lappaceum* rind based on solvent maceration extraction technique. The ethanol concentrations, shaking speed and extraction time were factors that conducted in this study.

3.1 Determination of phenolic content and phenolic constituents

The effect of ethanol concentration on the phenolic contents is shown in Table 1. The results showed that the phenolic contents in the extracts increased in accordance with an increasing ethanol concentration. The highest phenolic content was found in the 65% ethanolic extract ($P < 0.05$). However, lower phenolic content was observed in the 95% ethanolic extract. Therefore, 65% (v/v)

Table 1 The phenolic content, antioxidant, and tyrosinase inhibition activity of single factor experiment using the one factor at a time (OFAT) for extraction condition.

Extraction factors	Total Phenolic content (mg GAE/g sample)	DPPH radical scavenging activity (mg TEAC/g sample)	ABTS radical scavenging activity (mg TEAC/g sample)
Ethanol concentration (v/v)			
5%	37.70±0.71 ^d	99.36±0.47 ^d	120.98±4.41 ^c
35%	97.77±2.44 ^b	111.32±1.24 ^c	366.83±23.18 ^b
65%	130.14±1.48 ^a	355.19±2.80 ^a	476.62±17.41 ^a
95%	92.12±1.38 ^c	325.38±0.46 ^b	478.40±15.56 ^a
Shaking speed (rpm)			
0	108.24±7.78 ^c	321.64±3.80 ^b	426.53±8.71 ^d
50	146.86±7.31 ^b	316.28±0.95 ^b	561.24±11.48 ^a
100	135.61±3.53 ^c	316.57±8.21 ^b	520.32±20.44 ^b
150	118.73±0.90 ^d	357.82±3.24 ^a	484.12±20.68 ^c
200	159.59±2.81 ^a	322.10±9.72 ^b	548.30±20.60 ^{ab}
Extraction time (hour)			
0	84.40±4.07 ^c	239.20±2.90 ^d	358.02±1.88 ^c
6	134.88±4.73 ^b	305.09±5.87 ^c	566.20±24.47 ^b
12	158.99±2.42 ^a	355.75±7.47 ^a	573.12±24.48 ^b
18	157.56±5.40 ^a	364.86±2.80 ^a	639.50±21.16 ^a
24	153.84±1.81 ^a	342.43±5.29 ^b	656.50±8.89 ^a

Values are given as mean ± S.D. from triplicate.

Different letters in the same column and extraction factor indicate significant differences (P<0.05).

was then used to find out an appropriate shaking speed. Tendency of the phenolic contents of the extract increased when shaking speed ranged 0 to 50 rpm. In contrast, shaking speed ranging from 50 to 150 rpm influenced to decrease the phenolic content of the extracts. The highest phenolic compound was observed in the extract that was shaken at 200 rpm (P<0.05). Therefore, shaking speed at 200 rpm was selected to study the effect of extraction time. As the data shows in Table 1, the phenolic contents of the extracts increased with an increasing extraction time and reached the maximum when the extraction time was 12 h and longer (P<0.05). Thus, the suitable time to extract phenolic compounds of *N. lappaceum* rind was 12 h.

Hayouni *et al.* (2007) and Spigno *et al.* (2007) revealed that molecules of phenolic compounds had high polarity, which similar to water and alcohol. However, the mixture of alcohol and water had more efficient to extract phenolic compounds when compared to alcohol or water alone (Zhang *et al.*, 2007; Chew *et al.*, 2011). Muhamad *et al.* (2014) found that solute moves from inside the solid to the surface through diffusion or capillary action, and one at the surface, it is limited by convective mass transfer. Higher agitation rate can results higher mass transfer, which influence to increase the extraction yields. However, prolonged extraction time might induce decomposition of phenolic contents by endogenous factor (e.g., enzyme in plant tissue), or exogenous factors (e.g., temperature, light and oxygen exposure), which lead the occurrence of phenolic oxidation (Nacz & Shahidi, 2004; Kuljarachanan *et al.*, 2009; Thabit *et al.*, 2014).

3.2 Determination of antioxidant activities

The effect of ethanol concentration on the antioxidant activities is shown in Table 1. The antioxidant activities of the extracts increased in accordance with an increasing ethanol concentration. However, lower antioxidant activities were observed in the 95% ethanolic extract. The highest antioxidant activities was observed in the 65% ethanolic extract while the 5% ethanolic extract exhibited the lowest antioxidant activities (P<0.05). These results were in agreement with the total phenolic content. The influence of shaking speed was then observed. It was found that all shaking speeds, in this study, gave the extracts possessing potential antioxidant properties. In the consideration

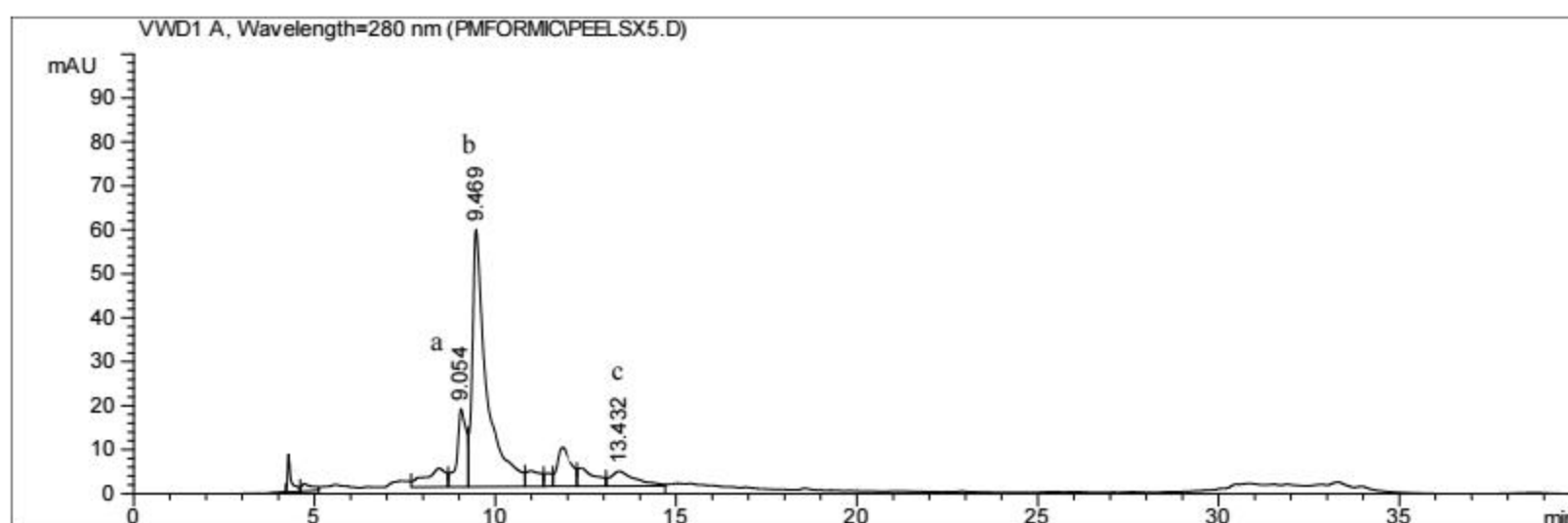


Figure 1 HPLC chromatogram of *Nephelium lappaceum* rind extract. a; corilagin, b; geraniin, and c; ellagic acid detected at 280 nm.

of the extraction time, the antioxidant properties of the extracts increased in accordance with an increasing extraction time ($P < 0.05$) and the highest antioxidant activities of the extracts was observed when the extraction time was conducted for 18 h.

From overall results, the condition for *Nephelium lappaceum* rind extraction in this study was 65% ethanol, shaking speed of 200 rpm and extraction time of 12 h. this condition provided the extract. This study was preliminary screening for suitable extraction condition. However, further study to optimize the best condition is still be required to obtain the extract with the highest recovery yield of phenolic compounds accompanying with strong antioxidant properties. Different phenolic compounds not only vary in solubility against solvent in terms of polarity but also antioxidant potential (Thitilertdecha *et al.*, 2010). This might be the reason that, in this study, similar antioxidants activities were observed with different phenolic content.

3.3 Determination of *N. lappaceum* phenolic constituents

The phenolic constituents of *N. lappaceum* rind were also identified and quantified by HPLC. The authentic compounds, geraniin, corilagin and, ellagic acid were used as reference standard for the

Table 2 Phenolic constituents analyzed by HPLC of the extracts from single factor experiment using the one factor at a time (OFAT) of extraction conditions

Extraction factors	Geraniin (mg/g sample)	Corilagin (mg/g sample)	Ellagic acid (mg/g sample)
Ethanol concentration (v/v)			
5%	6.58±0.29 ^d	N.D.	N.D.
35%	38.23±1.76 ^c	4.60±0.38 ^b	N.D.
65%	66.37±0.80 ^b	5.38±0.19 ^{ab}	N.D.
95%	72.41±0.17 ^a	6.11±0.17 ^a	2.29±0.06
Shaking speed (rpm)			
0	59.44±1.92 ^d	7.84±0.01 ^c	2.86±0.06 ^c
50	67.93±0.86 ^c	9.07±0.47 ^b	3.10±0.02 ^{ab}
100	81.24±0.03 ^a	10.23±0.45 ^a	3.60±0.19 ^a
150	66.37±0.80 ^c	5.52±0.19 ^d	3.06±0.13 ^{ab}
200	75.94±0.95 ^b	10.15±0.24 ^a	3.62±0.01 ^a
Extraction time (hour)			
0	43.94±0.01 ^d	5.05±0.23 ^c	2.33±0.55 ^b
6	67.29±0.07 ^b	8.37±0.18 ^b	3.03±0.07 ^{ab}
12	75.94±0.95 ^a	10.15±0.24 ^a	3.62±0.01 ^a
18	68.72±0.93 ^b	8.68±0.40 ^b	3.34±0.17 ^a
24	64.53±0.20 ^c	6.67±1.43 ^{bc}	3.35±0.05 ^a

Values are given as mean ± S.D. from triplicate.

Different letters in the same column and extraction factor indicate significant differences ($P < 0.05$).

identification and quantification of geraniin, corilagin and ellagic acid content in the *N. lappaceum* extracts. The quantification of the phenolic compound in the extract was conducted by using standard calibration curve. The HPLC chromatogram of the sample extract is illustrated in Figure 1. The comparative analysis of the extracts with reference phenolics allowed the characterization of three peaks in the chromatogram; corilagin (t_R 9.054 min), geraniin (t_R 9.469 min) and ellagic acid (t_R 13.432 min). The amounts of three identified compounds in the *N. lappaceum* extracts are shown in Table 2. It was obvious that geraniin represented the major phenolic compound in rambutan rind. From the results, increasing ethanol concentration significantly increased the recovery yield of geraniin, corilagin and ellagic acid ($P < 0.05$) and the 95% ethanolic extract possessed the highest amount of three phenolic compounds ($P < 0.05$). The influence of shaking speed on the amount of geraniin was similar to those of phenolic content. The amount of geraniin increased in accordance with an increasing shaking speed from 0 to 100 rpm. Decreasing in the amount of geraniin was observed when shaking speed at 150 rpm was used, however, the amount of geraniin increased when the shaking speed was 200 rpm. The highest amount of geraniin was observed when shaking speed at 100 rpm was used. In the consideration of extraction time, all identified compounds proportionally increased with extraction time ranged 0 to 12 h. The suitable extraction time was 12 h because of the amount of geraniin and corilagin decreased when extraction time was longer than 12 h. Prolonged extraction time may lead the decomposition and oxidation process of phenolic compound from external factors such as temperature, light and oxygen (Naczek and Shahidi, 2004). Geraniin, when exposed to acids/bases, boiling water, or aqueous condition in long time, will be possibly hydrolyzed to yield corilagin, gallic acid and hexahydroxydiphenic acid to which the latter have a high tendency spontaneously undergo lactonization to afford ellagic acid (Luger *et al.*, 1998; Ito *et al.*, 2008). Rambutan rind not only contains geraniin, corilagin and ellagic acid but also other phenolic compounds such as phenolic acids, flavonoids (Hernández *et al.*, 2017). This might be the reason that phenolic content, when extraction time was longer than 12 h, did not decrease (Table 1). In overall results, the extraction condition which was suitable to extract bioactive phenolic compounds from *N. lappaceum* rind was the extract obtained by using 65% ethanol, shaken at 200 rpm for 12 h.

4. Conclusion

The extraction condition of *Nephelium lappaceum* rind, based on maceration extraction, was screened by using single factor experiment using the one factor at a time (OFAT) to obtain the extract possessing high amount of phenolic content and antioxidant properties. Three factors including ethanol concentration, shaking speed and extraction time were conducted in this study. Ethanol concentration and extraction time significantly influence phenolic content and antioxidant properties of the extracts of *N. lappaceum* rind. Geraniin, identified and quantified by HPLC, is major phenolic compound of *N. lappaceum* rind. The suitable extraction condition to extract *N. lappaceum* phenolic compound is 65% ethanol, shaking speed at 200 rpm, 12 h. Nevertheless, the optimization of extraction condition still is unraveled due to interaction among extraction factors which could not be archived by OFAT. Therefore, response surface methodology (RSM), an effective statistical procedure using a minimum set of experiments for determining coefficients of mathematical model for condition optimization, should be conducted in further study.

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Extraction and characterization of banana (*Musa acuminata*) starch for cosmetic application

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Abstract

Starch is a natural polysaccharide derivative from plant such as cereals, tubers, and some unripe fruits like banana. The natural starch is widely used in food and cosmetic industry for the reasons of its low cost, biodegradability, renewability and easy availability. Starch plays a role in the cosmetic industry and normally it is used delivery system, anti-irritant agent, gelling agent, thickener, binder, stabilizer or texture modifier. In the present work, banana starch was extracted from unripe banana (*Musa acuminata*) fruits using 1%w/v of sodium sulfite. The starch yield was 16.88% based on fresh-peeled banana. The scanning electron microscopy (SEM) analysis showed that the starch granules were oval and elongated shape with smooth surface. The color of banana starch was light yellow with L*, a*, and b* values of 80.58 ± 0.01 , 3.61 ± 0.04 , and 11.76 ± 0.17 , respectively. The amylose content of the starch was equal to 22.96%. Study of x-ray diffraction (XRD) showed that the starch had B type crystalline structure. The gelatinization of banana starch was determined by using differential scanning calorimetry (DSC) and the result showed that the peak temperature (Tp) of gelatinization was 77.97 ± 0.18 °C and the enthalpy change (ΔH) was -3.07 ± 1.86 Jg⁻¹. The obtained properties could be therefore useful for further utilization of banana starch in foods and cosmetics.

Keywords: *Musa acuminata*; Banana starch; Morphology; Amylose; Gelatinization.

1. Introduction

Bananas are tropical and sub-tropical fruits that belong to the genus *Musa* of the family *Musaceae*. The origin of bananas is mostly from Southeast Asia, Africa, India and Latin America (de la Torre-Gutiérrez et al., 2008; Carmona-Garcia et al., 2009). Bananas are monocotyledon herbaceous plants that divided into two species which are *Musa acuminata* (AA) and *Musa balbisiana* (BB), and most banana cultivars are hybrids of these two species. In Thailand and Southeast Asia countries, there are more than 50 varieties of banana such as Kluai Namwa (ABB group), Kluai Hom (AAA group), Kluai Khai (AA group) and Kluai Lep Meu Nang (AA group). Temperature and moisture are the most important factors to cultivate the banana. The optimum temperature for cultivate banana under warm to hot condition is around 25-30 °C and lack of water can reduce the fruit number and size of banana. In general, the bananas with a high proportion of A produce sweet fruit, whereas those with a high proportion of B produce starchy fruit. Banana cultivars with B genes tend to be more drought tolerant than cultivars of A genes (Valmayor et al., 2000).

Starch is a natural polysaccharide derivative from crops especially in tropical plants. Cereals (corn, sorghum, wheat, rice and barley) have the starch content about 60-80%, tubers (cassava, sweet potato, potato, taro and yams) contain about 60-90% and some unripe fruits (banana and mango) contain about 70% (Moorthy, 2004; de la Torre-Gutiérrez et al., 2008). Starch is used in a variety of

industries, such as paper industries (improvement of mechanical strength, faster drainage, reduction of waste water pollution) (Nachtergaele, 1989), textile industries (improves tensile strength and reduce elongation at break) (Meshram et al., 2009), food industries (production of glucose and fructose syrups) (Bello-Pérez et al., 2006), pharmaceutical industries (pharmaceutical excipients and controlled-release) (Zografi & Kontny, 1986), and cosmetic industries (delivery system) (Lenaerts et al., 1998). There have been reported that banana starches have potential to be used in various industries especially food industries but in cosmetic application it has a few researches about it. Thus, the objective of this research is to extract the starch from Kluai Hom Khieo (*Musa acuminata*) starch. The basic properties of banana starch such as morphology, amylose content, crystalline structure and thermal properties were studied. The results obtained from the study will be useful in applying banana powder for future use in cosmetics and other industry.

2. Materials and Methods

2.1 Materials

Unripe banana (*Musa acuminata* 'Kluai Hom Khieo') used was at 1st state of mature that obtained from Chiang Rai, Thailand. The chemicals used for extraction is sodium sulfite from Loba Chemie Pvt. Ltd. Iodine (I₂) from J.T. Baker and amylose and amylopectin from Sigma-Aldrich.

2.2 Methods

2.2.1 Starch extraction

The method that used to extract the banana starch was slight modified version of Bello-Pérez et al (1999). Unripe banana (*Musa acuminata*) was peeled and cut into small pieces then immersed in 1%w/v of sodium sulfite solution (pH 4.5) in a ratio of 1:2 w/v. The sample was blended in blender at maximum speed for 10 min. The suspension was filtrated with 200 meshes sieving and white cloth to remove pulp. The sample after being sieved was centrifuged at 8000 rpm for 5 min to precipitate the starch. The precipitated starch was dried at 40 °C overnight in hot air oven. The dried banana starch was ground by mortar and pestle then sieved at 125 µm mesh. The yield of banana starch was calculated as the following equation.

$$\text{The percentage of yield} = \frac{\text{weight of dry starch}}{\text{initial weight of peeled unripe banana fruit}} \times 100$$

2.2.2 Scanning electron microscopy (SEM)

The morphology of banana starch was examined by using a scanning electron microscopy (Leo 1450, VP LEO, USA) with SEI detector. The starch sample was placed on double-side adhesive carbon tape then covered with gold layer at 100x, 500x, 1000x magnification.

2.3 Amylose content

The amylose content was followed the method of Kaufman et al., (2014). 5 mg of starch sample or standards were weighed into microcentrifuge tube then added 1 ml of 90% DMSO in distilled water and heated at 95 °C for 60 min and shaken every 10 min with vortex. The starch sample was cooled the for 5 min then 100 µl of each sample was added into a 96-well plate. The standard curve was prepared using both of amylose and amylopectin by varies the ratios (Table 1) were placed into the 96-well plates. The 100 µl of 90% DMSO in distilled water with 3.04 g/L of iodine was added into each well then shaken the plate for 2 min. 2 µl of each well was transferred to the empty plate and added 180 µl of distilled water into each plate then shaken for 2 min before analyzed for absorbance at 620 nm. 100 µl of 90% DMSO with 100 µl of DMSO with 3.04 g/L iodine were used for control blank. The amylose content was calculated as the following equation.

$$\text{Amylose content} = \frac{\text{Absorbance at 620 nm}}{\text{Slope of regression}} \times 100$$

Table 1 Standard curve preparation

Amylose content (%)	Amount of 5 mg/ml amylose solution (μ l)	Amount of 5 mg/ml amylopectin solution (μ l)
0	0	100
5	5	95
10	10	90
15	15	85
20	20	80
25	25	75
30	30	70
50	50	50
75	75	25
100	100	0

2.2.4 X-Ray Diffractometer (XRD)

The method that used to measure the intensities of banana starch was slight modified version of Chávez-Salazar et al (2017). It measured by the x-ray diffractometer (X'Pert PRO diffractometer, Malvern Panalytical, UK) using Cu K α radiation ($\lambda = 1.542$) in the 3-40° 2 θ range with 0.01° step size and measuring time of 10.0s per point.

2.2.5 Thermal properties

The thermal properties of banana starch were analyzed by using differential scanning calorimetry (DSC 822e, Mettler Toledo, Switzerland) and slight modified by Carmona-Garcia et al (2009). 7 mg of starch sample were weighed into a 40 μ L of aluminum pan then added 20 μ L of distilled water and sealed tightly. The suspension was allowed to stand at room temperature for 1 h before analysis. The sample was subjected to the range of temperature from 40-90 °C and a heating range of 10 °C/min under N₂ atmosphere. The empty aluminum pan was used as a reference.

3. Results and Discussion

3.1 Extraction of banana starch

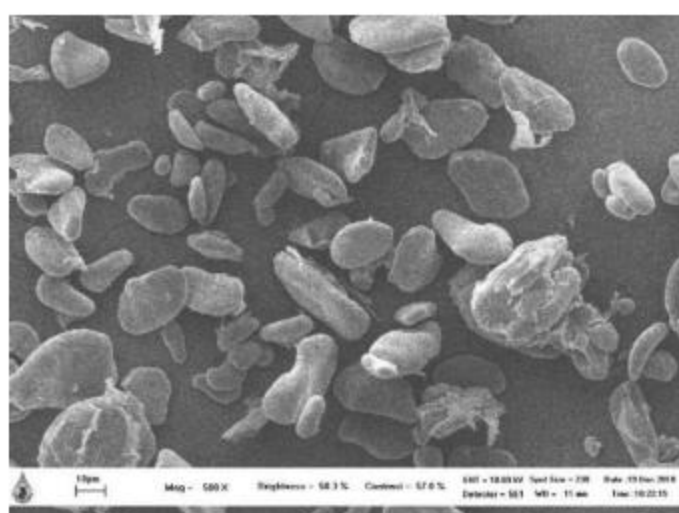
The percentage of banana starch yield that obtained from extracted with 1% sodium sulfite was 16.88% and the appearance was light yellow powder (Figure 1). The L*, a*, and b* were 80.58 ± 0.01 , 3.61 ± 0.04 , and 11.76 ± 0.17 , respectively. The banana starch powder had fine texture with light banana odor.

3.2 Morphology of starch granules

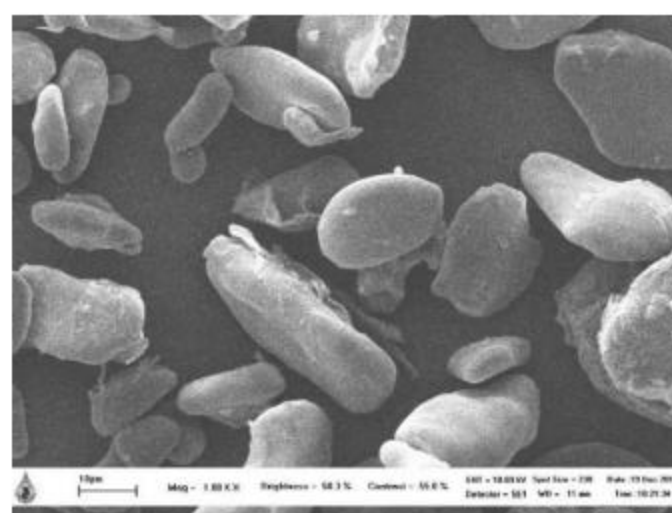
The starch granules had various size. The large size was elongated and oval shape and the narrow size was round. The appearance of granules showed that the surface of them were smooth and dense (Figure 2). Generally, the unripe banana starches had dense granules with elongated shape and spheroid forms (Kayisu & Hood, 1981). Rice starch granules has 3-5 μ m and polygonal with angular affect to caking and stickiness of cosmetic powder formulation (Hoover et al., 1996). Corn starch granules has polygonal with angular like rice starch granules but larger size (13-15 μ m) than rice starch granules. It affects silky feeling (Ellis et al., 1998). The banana starch from this work gave smooth and soft feeling when apply on skin. It didn't give silky like corn starch



Figure 1 The appearance of banana starch



(a)



(b)

Figure 2 The morphology of banana starch granules obtained from scanning electron microscopy (SEM); (a) 500x and (b) 1000x

and stickiness like rice starch. From the previous report showed that the size, shape and surface of starch granules effect the properties of starch.

3.3 Amylose content

The starch granules were extracted from *Musa acuminata* and the amylose content of banana starch was 22.96%. The amylose content that obtained from *Musa acuminata* is in the range of most other starches which are 20 to 27% (Moorthy., 2004). From the previous report, the amylose content of banana (*Musa spp.* 'Valery') was 16% by Kayisu & Hood (1981), *Musa acuminata* 'Cavendish' and *Musa spp.* 'Prata' were in the range of 25-29% by de Barros Mesquita et al. (2016) and *Musa acuminata* 'Cavendish', *Musa acuminata* 'Red dacca' and *Musa spp.* 'Valery' were in the range of 19-27% by Utrilla-Coello et al. (2014). The amylose content effect the properties of starch such as the thermal properties that the gelatinization peak and gelatinization enthalpy increase when the amylose content decreases (Geera et al., 2006). The different of amylose content depend on the variety and the state of maturity of plant (Chávez-Salazar et al., 2017).

3.4 X-ray diffractometer (XRD)

The X-ray diffractometer pattern of banana starch presented in Figure 3. The A-type crystalline pattern showed the strong peaks at 2θ about 15 and 23°, the double peaks about 17 and 18°, and weak peak about 20°. The B-type crystalline pattern showed the strong peaks at 2θ about 17°, the medium peaks about 5.6, 14.4, 22, and 24°, and weak peak about 19.5° (Jiranuntakul et al., 2011). For the starch sample showed B-type crystalline pattern with the strong peaks observed at 2θ about 17° and the several medium peaks at 2θ about 5.7, 15.3, 20.2, and 23.5°. The XRD pattern affected by amylose content because the amylose is higher in A-type than B-type starch (Cornejo-Ramírez et al.,

2018). The B-type starch had high proportion of amylopectin that effect water absorption and gel clarity from starch (de la Torre-Gutiérrez et al., 2008).

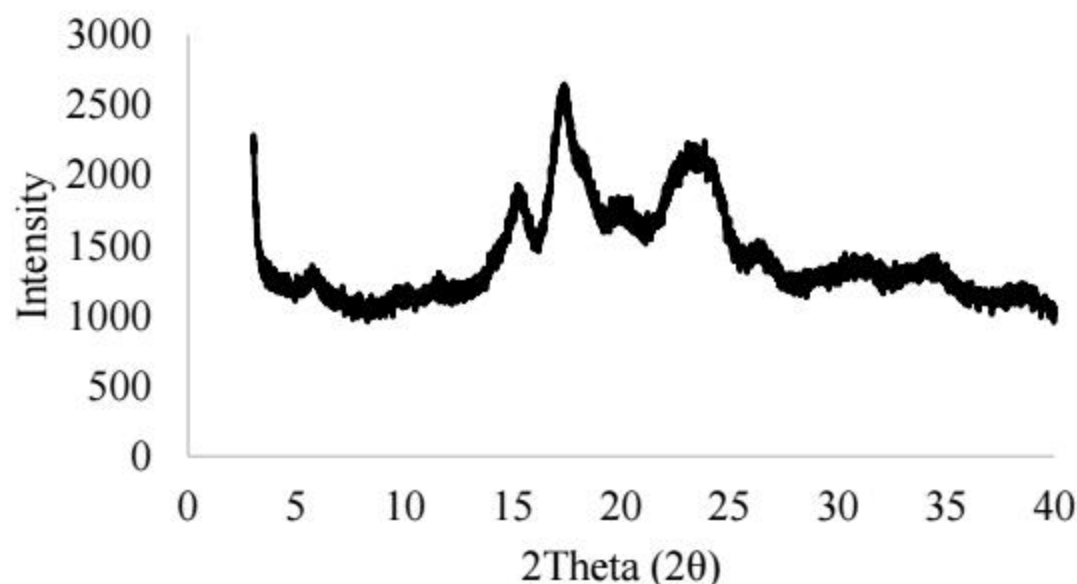


Figure 3 X-ray diffraction pattern of banana starch.

3.5 Thermal properties

The thermal properties of banana starch were determined by DSC. The banana starch has an onset gelatinization temperature (T_o) at 75.17 °C, a peak gelatinization temperature (T_p) at 78.57 °C, and an endpoint gelatinization temperature (T_e) at 86.52 °C with gelatinization enthalpy (ΔH) of 2.33 J/g (Figure 4). The gelatinization of starch depended on the amylose content that the gelatinization temperature increased with the amount of amylose content decreased because higher amylose content has less crystalline and more amorphous region so use lower energy to melted the crystalline regions (Sasaki et al., 2000).

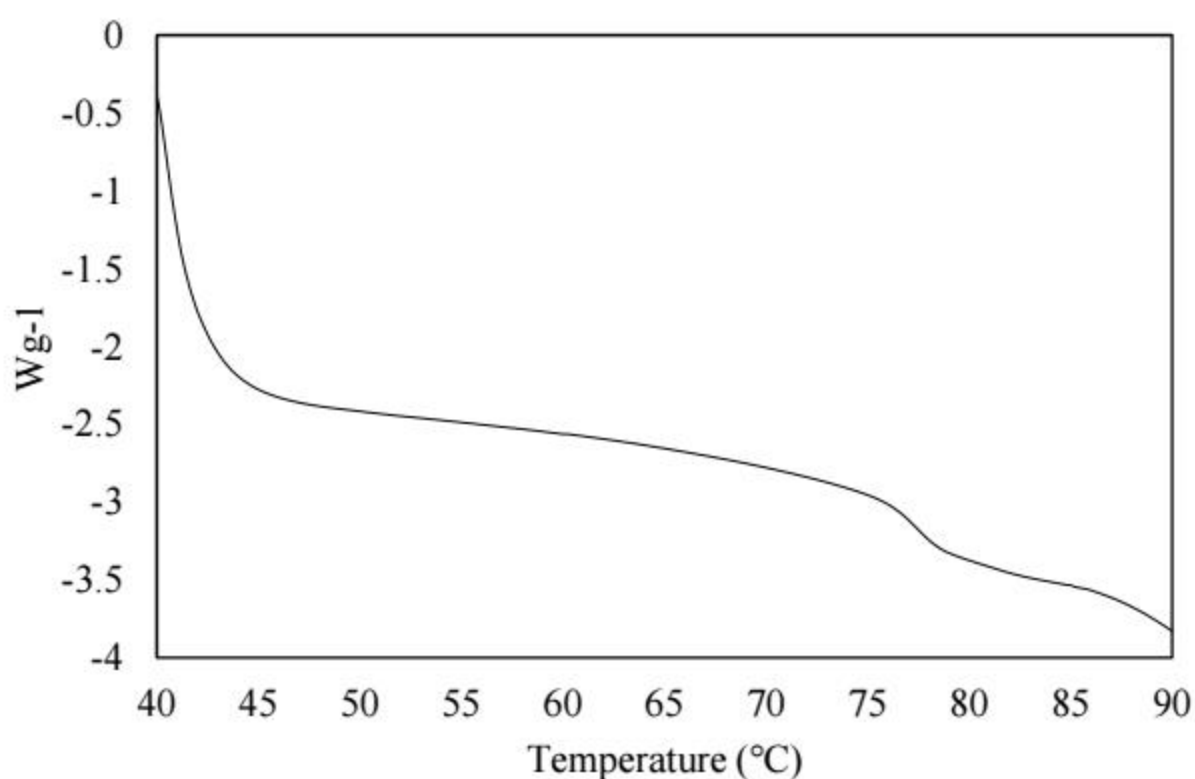


Figure 4 Differential scanning calorimetry (DSC) curve of gelatinization of banana starch.

4. Conclusion

The starch was successful extracted from *Musa acuminata*. It can be new source of natural starch for uses as an ingredient in cosmetic, food and pharmaceutical industries. However, the other properties such as moisture content, flowability, swelling and solubility are suggested to study which will be useful information for further use in the industry.

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Cosmetic emulsion of *Coix lachrymal* extract

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Abstract

In this study, *Coix lachrymal jobi* Linn. (job's tears) water extract was prepared by simple and easy method for being used as water portion in preparation of cosmetic emulsion. Antioxidant and anti-tyrosinase activities of the extract were studied. The suitable substance to preserve the extract against growth of microbes was investigated using BF-indicator dip T microbial test kits. The extract was prepared by immersion of the job's tears powder in water at ratio of 1:4 (w/v) and shaken by digital shaker for 2 hours. The job's tears water extract was turbid, white-yellow with pH value of 6.65 ± 0.37 . The use of 1,2-Hexanediol with caprylyl glycol at ratio of 1:1 total 2% can inhibit the growth of microbes in job's tears water extract. The extract exhibited antioxidant ability with IC_{50} of 94.73 mg/ml determined by DPPH assay which is lower than ascorbic acid (IC_{50} 0.035 mg/ml). Moreover, the extract was efficient in inhibition of tyrosinase with IC_{50} of 212.32 mg/ml which was lower than kojic acid (IC_{50} 0.027 mg/ml). The emulgel formulation was prepared using 91.80% of job's tears water extract. The product was white-yellow with pH value of 5.34 and the product was physically stable at 45°C and ambient temperatures after being tested for 1 month. Additionally, the emulgel formulation possessed antioxidant and anti-tyrosinase activities with IC_{50} of 133.67 mg/ml and 57.92 mg/ml, respectively, and still exhibited both activities over the stability test period. The results suggest that job's tears water extract, which prepared by simple and environmental sustainability method, can function as both carrier and anti-aging and skin lightening active in emulsion preparation. Product efficacy in human volunteers is advised for further study.

Keywords: Antioxidant; Cosmetic emulsion; Inhibition of tyrosinase; Job's tears water extract; Stability

1. Introduction

Coix (*Coix lachryma-jobi* L.), commonly known as adlay or Job's tears, is a minor cereal. *Coix* belongs to the grass family Poaceae and is a close relative of mize (Corke, Huang, Li, Wringley and Seetharama, 2016). *Coix* seeds contain a range of bioactive components including polysaccharides, proteins, lipids, polyphenols, etc. The chemical constituents of *coix* contribute to a range of medical and nutritional benefits. They are claimed for health benefits including anti-oxidation, anti-cancer, anti-inflammation, anti-allergy, enhancing immunological activity (Zhu, 2017). The study found that the Job's tears can use in cosmetic products. It stimulates blood circulation in the skin, promotes hair growth and anti-inflammation of the skin and makes the skin look healthier (Chaichantippayuth, Hantrakul, Poonsuk, Pangpreecha and Pethsom, 1982). Job's tears extract has been reported that has DPPH radical scavenging property (SC_{50} 0.31 $\pm$ 0.07 mg/ml) and job's tears extract has inhibit tyrosinase property (IC_{50} 0.28mg/ml). Job's tears extract has no cytotoxic effect on normal human

skin fibroblast (Manosroi, Khonsitsunti Wong, Mamosroi, 2014). The coix seed is an important food and the seeds are used in soups, porridges, and drinks, and are also brewed and fermented for tea, beer and other alcoholic beverages (Corke et al. 2016). Various medical effects of coix seeds in Traditional Chinese Medicine include removing heat, inducing diuresis, helping the drainage of pus, stimulating the function of lung and spleen and treating arthritis and diarrhea (Zhu, 2017). The objective of this study was to prepare job's tears water extract by simple and easy method. The substance to preserve to the extract against growth of microbes was investigated. The biological activities of Job's tears water extract including antioxidant activity by DPPH free radical scavenging, anti-tyrosinase were studied. The results from this study can be applied for the further development of cosmetic ingredient.

2. Materials and Methods

2.1 Materials

Coix lachrymal jobi L. (job's tears) seed of Khaothong® brand were purchased from Thai Food Industry (Bangkok, Thailand). Ascorbic acid was purchased from Fluka, Switzerland. 2,2-diphenyl-1-picrylhydrazyl (DPPH), kojic acid, L-DOPA, and tyrosinase enzyme were purchased from Sigma-aldrich, USA, 1,2-Hexanediol, Caprylyl glycol, solvents and other chemicals used were cosmetic grade.

2.2 Preparation of job's tears water extract

The job's tears water extract was prepared by water extraction method. Briefly, job's tears seed was ground and pass through a 40 mesh sieve. Job's tears powder was mixed with distilled water at the ratio of 1:4 w/v and was shaken by digital shaker for 2 hours and filtered through white cloths. Next, the filtrate was centrifuged at 6,000 rpm for 10 minutes by Compact Centrifuge Z206A.

2.3 Preservation of job's tears water extract

Preservatives are substances added to job's tears water extract to prevent microbiological growth. The antimicrobial activity was investigated by using BF-indicator dip T-microbial test kits. Each dip slide contains two agar surfaces, one for testing bacteria count (light yellow agar) and the other for yeast and mold (brown agar) count. The slide was incubated at 27-30°C for 3 days. The chemical used as preservatives are shown in Table 1

Table 1 List of preservative and amounts used to preserve the extract

Ingredients	%w/w		
	F1	F2	F3
Job's tears water extract	99	95	98
Phenoxyethanol	1	-	-
Pentylene glycol	-	5	-
1,2-Hexanediol	-	-	1
Caprylyl glycol	-	-	1
Total	100	100	100

2.4 Antioxidant activity

Determination of antioxidant activity was based on Williams, Cuvelier and Berset (1995) with some modification. Antioxidant activity of Job's tears water extract was measured by using DPPH radical scavenging method, and vitamin C was used as standard. Briefly, 100-500 µl of five serial concentrations of the job's tears water extract was added to 1000 µl of DPPH solution and ethanol mixing in the tube for total 2000 µl and then incubated at room temperature and dark area for 30

minutes and absorbance was measured at 515 nm. The result was compared with ascorbic acid. Antioxidant activity of job's tears water extract was calculated using the following equation:

$$\% \text{ Radical scavenging} = [(A_{\text{con}} - A_{\text{sam}}) / A_{\text{con}}] * 100$$

Where A_{con} was the absorbance of the control and A_{sam} was the absorbance of the job's tears water extract. The result was described as IC_{50} value. The IC_{50} value was calculated as the concentration of sample (mg/ml) between the percentages of scavenging 50% of the DPPH in the reaction mixture.

2.5 Tyrosinase inhibition activity

Determination of tyrosinase inhibition activity was based on method reported by Sari et al (1995) with some modification. Standard of tyrosinase inhibition was kojic acid. 20 μ l of tyrosinase and 50-200 μ l of five serial concentrations of the job's tears water extract was added into the tube containing buffer solution for total 600 μ l (50mM, pH 6.8) incubated at 37°C for 3 minutes and then L-Dopa (25mM) was added into tube and incubated at 37°C for 6 minutes and absorbance was measured at 475 nm. The result was compared with kojic acid. Tyrosinase activity of job's tears water extract was calculated using the following equation:

$$\% \text{ Inhibition} = [(A_{\text{con}} - A_{\text{sam}}) / A_{\text{con}}] * 100$$

Where A_{con} was the absorbance of the control and A_{sam} was the absorbance of the job's tears water extract. The result was described as IC_{50} value. The IC_{50} value was calculated as the concentration of sample (mg/ml) between the percentages of scavenging 50% of the DPPH in the reaction mixture.

2.6 Development of product containing job's tears water extract

The job's tears water extract was used as water portion in formulation of emulgel product. The pH value, viscosity and appearance of emulsion were evaluated. The microbial contamination of product was determined by BF-indicator dip T-microbial test kits.

2.7 Stability test of emulgel

The product was loaded in the glass bottles with net weight 100 g for 4 bottles. The product was kept at ambient temperature, 45, 4°C for 1 month. In addition, the cycling test was performed by keeping the product in the refrigerator temperature at 4°C for 24 hours and 45°C for 24 hours per one cycle. The test was performed for six cycles Charoenjittichai (2016).

2.8 Biological activity of emulgel

Determination of antioxidant activity and tyrosinase inhibition activity of products was based on Prommakool (2015) with some modification. 10 g of the product was mixed with 10 ml of ethanol and was extracted by ultrasonic for 30 minute. Next, the product was centrifuge at 10,000 rpm for 5 minutes by Micro Centrifuge IEC 5590 micro-centrifuge HP, USA. Analyze and the result was described as IC_{50} value. The product was kept at 45°C for 1 month.

3. Results and Discussion

3.1 Preparation of job's tears water extract

The job's tears seed (Figure 1) was ground and passed through a 40 mesh sieve and the powder was mixed with distilled water at the ratio of 1:4 w/v. The job's tears water extract was turbid, characteristics odor and white- yellow color (Figure 2) with pH value of 6.65 ± 0.37 and viscosity value of 14.01 ± 0.01 cP. The job's tears water extract was stored at 10°C for further use.



Figure 1 Appearance of job's tears seed



Figure 2 Appearance of job's tears water extract

3.2 Preservation of job's tears water extract

The job's tears water extract had a composition of carbohydrate which makes microorganisms growth. The water extract was mixed with 1% of phenoxyethanol (F1). According to the Ministry of Health announced and EU Regulation, the maximum content allowed to be used as an ingredient in cosmetics. Yeast and mold was found at $<10^2$ CFU/ml (brown agar) and bacterial was 10^3 CFU/ml (light yellow agar). The color, odor of extract changed from white-yellow to yellow after the formulation stored for 3 days. Next, the water extract was mixed with 5% of pentylene glycol (F2). It's humectant and preservative booster. The test kits showed the presence of yeast, mold 10^2 CFU/ml and bacterial 10^4 CFU/ml. Finally, mixture 2% of 1,2-Hexanediol, caprylyl glycol, microbe was not found in the extract. The results showed that 1,2-Hexanediol, caprylyl glycol used in job's tears water extract can inhibit of microorganisms growth (Table 2).

Table 2 The microbial contamination of job's tears water extract

Formula	Substance	Bacterials (CFU/ml)	Yeasts/Mold (CFU/ml)
F1	Phenoxyethanol	10^3	$<10^2$
F2	Pentylene glycol	10^2	10^4
F3	1,2-Hexanediol,caprylyl glycol	-	-

3.3 Antioxidant activity

DPPH activity was measured by using Genesys 10s UV-Vis spectrophotometer at a wavelength of 515 nm. IC_{50} value of job's tears water extract was 94.73 mg/ml which was lower than standard (ascorbic acid) that showed IC_{50} value 0.035 mg/ml. Because the ratio of water was too high compared to the job's tears powder in preparation, therefore less concentrated. It has been reported that the coix crude extract showed antioxidant property very close to that of vitamin C, vitamin E and butylated hydroxyanisole (BHA) at same concentration Huang, Hsieh, Niu and Chang (2014).

3.4 Tyrosinase inhibition activity

Tyrosinase inhibitor activity was measured by using Genesys 10s UV-vis spectrophotometer at a wavelength of 475 nm. The extract showed the presence of tyrosinase inhibitory activity with IC_{50} value of 212.32 mg/ml. When compared with IC_{50} value of kojic acid (0.027 mg/ml), the activity of job's tears water extract was lower than positive control. It has been reported that the 125 mg/ml of

adlay extract showed tyrosinase activity at $46.68 \pm 3.52\%$ which is close to kojic acid (0.028 mg/ml) that showed tyrosinase activity of $45.67 \pm 4.21\%$ Huang et al (2014).

3.5 Development of product containing job's tears water extract

The emulsion was prepared using 91.80% of job's tears water extract compared with emulsion base which use of DI water. 5% of thickener (simulgel INS 100) was used in the preparation (Table 3). The product containing extract was white-yellow cream gel (Figure 3 a) with pH 5.34 and viscosity value of 2,203 cP. The emulsion base was white cream gel (Figure 3 b) with pH 4.51 ± 0.01 and viscosity value of 3,940 cP. The extract has a neutral of pH value that may cause pH value of emulsion increased. The viscosity of product containing extract decreased because the job's tears water extract contain organic compounds, thus the conductivity of the water was low. United States Environmental Protection Agency (EPA) (2014).

3.6 Stability test of emulgel

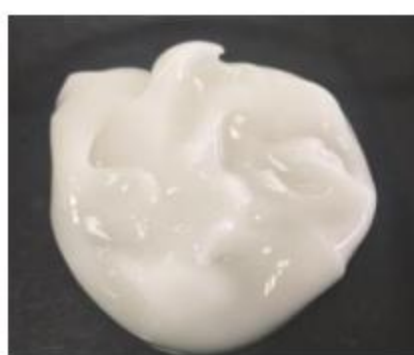
The product was physically stable at ambient temperature, 45 and 4°C after being tested for 1 month but the color is yellow, dark yellow, and white-yellow (Figure 4a, 4c, 4e), respectively. The pH of product containing extract did not change but the viscosity increase at low temperature (4°C). The emulsion base color did not change (Figure 4b, 4d, 4f) with pH value and viscosity value were relatively stable (Table 4). The results showed that the product was stable and the temperature may affect thickening agent that cause the viscosity changed.

3.7 Biological activities of emulgel

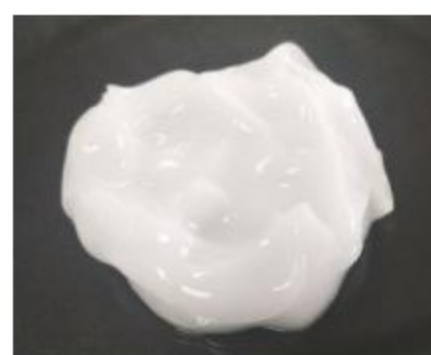
The job's tears emulgel after instant preparation showed antioxidant and antityrosinase activities with IC_{50} of 133.67 mg/ml and 57.92 mg/ml. The product was kept at 45°C for 1 month and that showed IC_{50} of 130.70 mg/ml and 56.12 mg/ml, respectively. The results indicated that emulgel still exhibited both antioxidant and antityrosinase activity after the stability test for 1 month.

Table 3 List of raw materials in emulsion formulation

Raw materials	Function	%w/w	
		Job's tear emulgel	Base
Job's tears water extract	Extract	91.80	-
Water	Solvent	-	91.80
EDTA	Chelating agent	0.10	0.10
Xanthan gum	Thickener	0.10	0.10
Simulgel INS 100	Thickener	5.00	5.00
1,2-Hexanediol	Preservative	1.00	1.00
Caprylyl glycol	Preservative	1.00	1.00
Caprylic/capric triglyceride	Emollient	1.00	1.00
Total		100.00	100.00



(a) Job's tears emulgel



(b) Base of cream gel

Figure 3 Appearance of (a) Job's tears emulgel and (b) Base of emulgel



(a) Job's tears cream gel (ambient temperature)



(b) Base of cream gel (ambient temperature)



(c) Job's tears cream gel (45°C)



(d) Base of cream gel (45°C)



(e) Job's tears cream gel (4°C)



(f) Base of cream gel (4°C)

Figure 4 The color of cream gel after stored at ambient temperature, 45 and 4°C for 1 month

Table 4 Properties of cream gel after stability test for 1 month

Property	Job's tears cream gel				Base of cream gel			
	Initial	1 month			Initial	1 month		
		RT	45	4		RT	45	4
Appearance	Cream gel, white-yellow	++	++	+	Cream gel, white	-	-	-
Odor	specific	++	+++	+	No odor	-	-	-
pH	5.34	5.33	5.32	5.32	4.51	4.70	4.84	4.59
Viscosity	2,265	1,945	1,976	2,149	3,940	3,799	3,563	3,726

Note: - is mean appearance or odor is not change., + is mean slightly of color appearance, ++ is mean very deep of appearance

Table 5 Biological activity of cream gel after stability test at 45°C for 1 month

Biological activity	Innitial activity (IC ₅₀ mg/ml)	After stability test (IC ₅₀ mg/ml)
Antioxidant	133.67	130.70
Antityrosinase	57.92	56.12

4. Conclusion

In this study, *Coix lachrymal* jobi Linn. (job's tears) water extract was prepared by simple, easy and environmental sustainability method. The job's tears water extract was turbid, characteristic odor and white- yellow color with pH value of 6.65±0.37. The use of 1, 2-hexanediol with caprylyl glycol at ratio of 1:1 total 2% can inhibit the growth of microbes in the extract. The extract exhibited antioxidant ability with IC₅₀ of 94.73 mg/ml. Moreover, the extract is efficient in inhibition of tyrosinase with IC₅₀ of 212.32 mg/ml. The cream gel emulsion was prepared using 91.80% of job's tears water extract. The product was white-yellow cream gel with pH 5.34 and the product was physically stable at 45°C and ambient temperatures after being tested for 1 month and still exhibited both antioxidant and antityrosinase activities over the stability test period.

5. Acknowledgements

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Utilization of parchment coffee bean for value-added active compounds of cosmetic

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Abstract

The aims of this study were to study on utilization of parchment coffee bean (PCB) (as waste from coffee bean process) for value-added active compound of cosmetic and to develop the deep facial moisturizing serum and mild cleansing foam PCB extract. The PCB was obtained from coffee (*Coffea arabica* L.) production process in Chiang Rai province and the active compound was extracted by using four solvents (hot water at 80°C, ethanol, methanol and ethyl acetate) with shaking method at 200 rpm for 10 hours. Total phenolic content (TPC), total flavonoid content (TFC), total tannin content (TTC) and antioxidant activities of PCB extracts were determined. The results showed that the ethanol extract of PCB (PCBE) had the highest bioactive compounds (TPC 117.17 mg GAE/mg extract, TFC 0.094 mg QE/mg extract and TTC 0.539 mg GE/mg extract, respectively, $P < 0.05$) and also showed the most potent antioxidant activities (DPPH 1.198 ± 0.040 , ABTS 9.886 ± 0.208 , and FRAP 2.117 ± 0.026 mg TEAC/mg extract, respectively, $P < 0.05$) without irritation to human skin (close patch test). Moreover, the deep facial moisturizing serum and mild cleansing foam with PCBE extract could increase the skin moisture of volunteers after used 3 days used ($583.0 \pm 18.5\%$, $n = 7$, $P < 0.05$). In conclusion, PCB (agricultural waste) could be used as cosmetic ingredient with antioxidant and moisture retention properties as utilization of waste from coffee production and industry.

Keywords: Antioxidant; Bioactive; Deep moisturizing serum; Mild cleansing foam; Parchment of coffee bean; Waste utilization.

1. Introduction

Nowadays, coffee is widely consumed beverage in worldwide and is also one of the important economic plants in Northern Thailand. Two species of coffee including *Coffea arabica* L., known as Arabica coffee, and *Coffea canephora* or Robusta coffee are economically important for coffee production. Arabica coffee has preferable cultivated for roasted coffee bean, while Robusta has cultivated for instant coffee. Chiang Rai province has a proper environment for cultivated Arabica coffee which can be produced approximately 8,000-9,000 tons in 2015. The tendency of coffee production has increased for consumers demand. However, there are various wastes from coffee production which has approximately 45% of coffee berry such as coffee pulp, husk and parchment of coffee bean. Parchment of coffee bean (PCB) is one of the main by-products in roasting process and has no commercial value regarded as solid waste. Previous study on bioactive and bioactivity of coffee

showed that green coffee bean had antioxidant activity by inhibit superoxide anion scavenging activity and also inhibit tyrosinase activity (Iwai *et al.*, 2004). However, there are few studies demonstrated that PCB has bioactive compounds and antioxidant activity (Madhava *et al.*, 2008). Therefore, the aims of this study were to study on utilization of parchment coffee bean (PCB) (as waste from coffee bean process) for value-added active compound of cosmetic and to develop the facial deep moisturizing serum and mild cleansing foam PCB extract.

2. Materials and Methods

2.1 Materials

All solvents and chemicals were analytical grade. Ethanol (95%), acetone, hexane, methanol (95%), Dimethyl sulfoxide (DMSO), iodine, potassium iodine, sodium carbonate, sodium citrate dehydrate, copper sulfate pentahydrate, vanillin, sulphuric acid, ferric chloride, gelatin, sodium hydroxide, hydrochloric acid (37%), vanillin were purchased from Merck. Sodium carbonate, aluminum chloride, dibasic phosphate and monobasic phosphate were purchased from Univar. Potassium acetate and Potassium persulphate were purchased from Unilab. Gallotannin was purchased from Himedia. Folin-Ciocalteu, 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), Gallic acid, Quercetin and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma.

2.2 Plant preparation and extraction

Parchment coffee bean obtained from coffee production in Chiang Rai province. Samples were dried at 50°C in hot air oven for 24 hours and blended to powder. Then the samples were kept in auto desiccator at room temperature until used.

Then, the extract was prepared by serial extraction using 6 different solvents including hexane, dichloromethane, ethyl acetate, 95% methanol, ethanol and hot water 80°C deionized water (DI) at ratio sample: solvent (1:20 w/w) with shaking extraction 200 rpm at room temperature (RT) for 10 hours. The extracts were freeze-dried and then calculated for % yield of dry basis and then stored at -20°C prior use.

2.3 Determination of bioactive compound

2.3.1 Total phenolic content (TPC)

PCB extracts (1mg/ml), 20 µl were reacted with 100 µl of 0.2 M Folin-Ciocalteu reagent for 1 min. Then, 80 µl of 7.5% (w/v) sodium carbonate was added into the reaction mixture. After incubation at room temperature (RT) for 30 min, the absorbance of extracts was measured at 765 nm by microplate reader (Singleton & Rossi, 1965). Gallic acid was used as a reference standard, and the results were expressed as mg gallic acid equivalents (GAE)/g extract.

2.3.2 Total flavonoid content (TFC)

The 25 µl (1mg/ml) of PCB extracts were mixed with 75 µl of 95% ethanol, 5 µl of 10% aluminum chloride, 5 µl of 1 M potassium acetate and 140 µl of DI water. The mixture was incubated at RT for 30 minutes and then measured the absorption at 415 nm by using microplate reader (Chang *et al.*, 2002). Extractable flavonoid content was reported as mg of quercetin (QE)/g extract.

2.3.3 Total Tannin content (TTC)

Total tannin content were determined by Folin-Ciocalteu method (Singleton & Rossi, 1965). About 20 µl of samples were mixed with Folin-Ciocalteu. Then the mixture was added with 7.5% sodium carbonate (%w/v). The mixture was shaken well and kept at room temperature for 30 min. A set of reference standard solutions of Gallotannin was prepared. Absorbance were measured against

the blank at 765 nm by microplate reader. The tannin content was expressed in terms of mg of Gallotannin Equivalent (mg GE/g sample).

2.4 Determination of antioxidant activity

2.4.1 DPPH radical scavenging activity

The 10 μ l (1mg/ml) of PCB extracts were mixed with 190 μ l of DPPH reagent. The mixture was vortexed and then left to stand in dark place for 30 min at RT. The absorbance was measured at 515 nm by using microplate reader (Prior wu & Schaich, 2005). Trolox was used as a reference standard, and the results were expressed as g Trolox equivalents (TE)/g extract.

2.4.2 ABTS Radical Scavenging activity

The mixture of ABTS radical cation was prepared by adding 7mM ABTS with 2.45mM potassium persulphate and then the mixture was left to stand overnight in the dark at RT. The ABTS radical cation solution was diluted with 50 mM phosphate buffer in a ratio of 1:20 (v/v). For analysis, 10 μ l (1mg/ml) of PCB extracts were mixed with 190 μ l of ABTS radical cation solution. The mixture was vortexed and then left to stand in dark place for 15 min at RT. The absorbance was measured at 734 nm by using microplate reader (Biotek, USA). Trolox was used as a reference standard, and the results were expressed as g TE/g extract (Thaipong *et al.*, 2006).

2.4.3 Ferric Reducing Antioxidant Power (FRAP) assay

FRAP solution was prepared freshly by mixing 1.0 ml of 2,4,6-TPTZ solution in 40 mM hydrochloric acid with 1.0 ml of 20mM ferric chloride and 10 ml of 0.3M acetate buffer (pH3.6). For analysis, 10 μ l (1mg/ml) of each extracts was mixed with 190 μ l of FRAP solution. The mixture was vortexed and then left to stand in dark place for 15 min at RT. The absorbance was measured at 593 nm by using microplate reader (Biotek, USA). Trolox was used as a reference standard, and the results were expressed as g TE/g extract (Thaipong *et al.*, 2006).

2.5 Irritation test of PCB extract

The irritation test was performed on seven volunteers with age between 20 and 40 years on human skin using close patch test. All volunteers were not suspected of having any allergic contact. The ethanol PCB extract (1 mg/ml) and distilled water (negative control), were applied on Finn chambers to a 3x6 cm² area of skin on forearm. The test materials were removed after 24 h and the treated sites were examined (time 0) and after 30 min (time 30). Five types of reactions were classified according to the International Contact Dermatitis Research Group (ICDRG) scoring system. The five reactions include negative reaction (-), possible reaction ($\pm$, faint erythema only), weak positive reaction (+, erythema, infiltration, and possible papules), strong positive reaction (++ , erythema, infiltration, papules or vesicles), and extreme positive reaction (+++, intense erythema, infiltration, and coalescing vesicles).

2.6 Cosmetics Formulation

2.6.1 Development of base formula for deep facial serum and mild facial cleansing foam

Base formula for deep facial serum was developed into five formula depending on amount (1-5% in formula) of thicker agent (Polyacrylamide and C13-14 Isoparaffin and Laureth-7). These five base serums were stability tested by centrifuge test and heating-cooling test.

For mild facial cleansing foam, the base formula was developed into three formulas depend on percentage of surfactants (10-30% of disodium laureth sulfosuccinate, 5-15% of cocamidopropyl betaine and 5-15% of cocamide DEA). This mild facial cleansing foam was characterized by mild cleansing for facial without SLES, paraben and Silicone. These three bases of facial foam were stability tested by centrifuge test and heating-cooling test.

2.6.2 Development of deep facial serum and mild facial cleansing foam containing PCB extract

The best of base for deep facial serum and mild facial cleansing foam were developed to be cosmetic product with PCB extract (1, 2.5, 5% for serum and 1, 3, 5% for cleaning foam in formula at concentration 1 mg/ml). These serum and cleansing foam products were stability tested by centrifuge test and heating-cooling test.

2.7 Efficacy testing of deep facial serum and mild facial cleansing foam containing PCB extract

Skin hydration and elasticity were determined by using skin analyzer (Dermalab Combo, Cortex technology, Denmark). The baseline reading of skin hydration level and elasticity were measured and were taken in a sitting position with the forearm lying on a table. The products were applied to the test site which is the left forearm of the volunteers (n=7). They needed to apply mild cleansing foam before apply deep facial serum to the test side daily, in the morning or in the evening. The skin hydration value was measured and recorded on day 0, 3, 7 and 14.

2.8 Ethical consideration

This study was performed following the ethical guidelines of the Declaration of Helsinki, after prior permission from Institutional Ethical Committee of Mae Fah Luang University (Certification document no. 065- 2561). Efficacy evaluation on human volunteers was carried out for 2 weeks. Seven subjects were enrolled in the study. Skin hydration and skin elasticity were determined by using Derma Combo Lab, respectively.

2.9 Statistical analysis

All obtained data were carried out by the one-way analysis of variance (one-way ANOVA) and Tukey's post hoc test in SPSS (IBM SPSS, USA) at least in triplicate and expressed as mean $\pm$ SD and statistically analyzed by using statistical analysis software at the significance level $P < 0.05$.

3. Results and Discussion

The extractable yield of parchment coffee bean (PCB) extracts ranged from 0.12% to 0.63% dry basis that the highest yield was found in the hot water (80°C) extract of PCB (0.63% dry basis), whereas the yield of hexane and dichloromethane extract were not determined (Table 1). PCB extract may not contain low polarity substances; therefore low polarity solvent could not extract any substance from PCBE.

The results showed that the ethanol extract of PCB (PCBE) had significantly the highest phenolic contents (1.117 ± 0.014 mg GAE/mg extract, $P < 0.05$) following by hot water and methanol extract (0.750 ± 0.015 and 0.701 ± 0.008 mg GAE/mg extract, $P < 0.05$). According to the TPC, the ethanol extract of PCB showed the highest total flavonoid content (0.094 ± 0.004 mg QE/mg extract, $P < 0.05$), which significantly higher than the methanol extract of PCB (0.063 ± 0.003 mg QE/mg extract). Furthermore, the ethanol extract also had the highest total tannin content (0.539 ± 0.015 mg GE/mg extract, $P < 0.05$), while the ethyl acetate extract had the significant lowest TPC, TFC and TTC ($P < 0.05$, table 1).

Table 1 Extractable yield and bioactive compounds (Total phenolic: TPC, Total flavonoid: TFC and Total tannin contents: TTC) of parchment of coffee bean (PCB) using different solvents.

Solvent	Yield of Dry Basis (%)	Total Phenolic Content (mg GAE/mg extract)	Total Flavonoid Content (mg QE/mg extract)	Total Tannin Content (mg GE/mg extract)
Hot water (80°C)	0.63	0.750 ± 0.015 ^b	0.018 ± 0.001 ^c	0.364 ± 0.013 ^b
Methanol	0.13	0.701 ± 0.008 ^c	0.063 ± 0.003 ^b	0.343 ± 0.007 ^c
Ethanol	0.32	1.117 ± 0.014 ^a	0.094 ± 0.004 ^a	0.539 ± 0.015 ^a
Ethyl Acetate	0.12	0.164 ± 0.007 ^d	0.019 ± 0.005 ^c	0.070 ± 0.007 ^d

Mean ± S.D (n=5) Different superscript letter in a column indicated the statistically different between samples (ANOVA, Turkey test, P<0.05); GAE= Gallic acid equivalent, QE= Quercetin equivalent, GE=Gallotannin equivalent

The study of antioxidant activities assay using DPPH, FRAP and ABTS assay showed that the ethanol extract of PCB had the significant highest antioxidant activities (P<0.05). For DPPH and FRAP assay, the ethanol extract had higher than methanol extract and hot water extract (DPPH: 1.198 ± 0.040, 0.841 ± 0.061 and 0.811 ± 0.060 mg TEAC/mg extract, respectively and FRAP: 2.117 ± 0.026, 1.645 ± 0.033 and 1.279 ± 0.044 mg TEAC/mg extract, respectively, P<0.05). While, ABTS assay showed that hot water extract had significantly higher activity than methanol extract (6.893 ± 0.079 and 6.640 ± 0.141 mg TEAC/mg extract, respectively, P<0.05) as shown in table 2.

Table 2 Antioxidant activities from PCB extract in difference solvents.

Solvent	DPPH assay (mg TEAC/mg extract)	FRAP assay (mg TEAC/mg extract)	ABTS assay (mg TEAC/mg extract)
Hot water (80°C)	0.811 ± 0.060 ^b	1.279 ± 0.044 ^c	6.893 ± 0.079 ^b
Methanol	0.841 ± 0.061 ^b	1.645 ± 0.033 ^b	6.640 ± 0.141 ^c
Ethanol	1.198 ± 0.040 ^a	2.117 ± 0.026 ^a	9.886 ± 0.208 ^a
Ethyl Acetate	0.299 ± 0.014 ^c	0.628 ± 0.028 ^d	1.247 ± 0.057 ^d

Mean ± S.D (n=5) Different superscript letter in a column indicated the statistically different between samples (ANOVA, Turkey test, P<0.05)

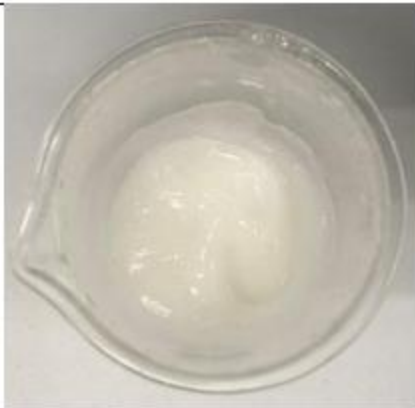
From the study of bioactive compound and antioxidant activities, the ethanol extract of PCB showed the most suitable for further study use as active ingredient in cosmetic products. Furthermore, the study of irritation test by close patch test in volunteer (n=7; female = 6; male =1) showed that the

**Figure 1** Irritation test in volunteer using negative control: distilled water (1) and the ethanol extract of PCB at 1 mg/ml (2) at day 0 (A) and day 1 at 0 min after moved patch test (B) and 30 min after moved patch test (C)

For development of facial deep serum with PCB extract, the base formula no.3 (with 3% w/w of polyacrylamide and C13-14 isoparaffin and Laureth-7) that showed the best results (good physical

texture and high customer stratification (3.86 ± 0.38 of 5 scores) with stability, was selected for further study. The PCB extract deep facial serum was developed into five formulas (F1A-F5A) depending on percentage of ethanolic PCB extract (1, 2.5, 5% w/w in formula). The results showed that the formula no. F4A with 5% w/w PCB extract had good physical property and greatest of customer stratification (4.00 ± 0.50 of 5 scores) with stability (Table 3).

Table 3 Physical properties of deep facial serum containing PCB extract Formula 4A (F4A)

Physical properties of facial deep serum containing PCB extract Formula 4A		
Color	White Opaque	
Viscosity	+++ 87,800 cP	
Odor	Good odor	
Stability	Good stability, pass accelerated test	
Satisfaction (Overall)	4.00 ± 0.50 (80%)	

Note: + = No viscous, ++ = low viscous, +++ = slightly viscous, ++++ = normal viscous, +++++ = high viscosity

In development of mild facial cleansing foam containing PCB extract, the base formula no. 2 (with 20% of disodium laureth sulfosuccinate, 10% of cocamidopropyl betaine and 10% cocamide DEA w/w in formula) that showed good physical property (foam texture) and high customer stratification (3.57 ± 0.79 of 5 scores) with stability was selected for further study. In addition, the mild facial cleansing foam containing PCB extract was continuously developed into three formulas (F1A-F3A) depending on percentage of ethanolic PCB extract (1, 3, 5% w/w in formula). The results showed that the formula no. F3A with 5% w/w PCB extract had good physical property (good foam texture) and greatest of customer stratification (4.00 ± 0.35 of 5 scores) with stability (Table 4).

Table 4 Physical properties of mild facial cleansing foam containing PCB extract Formula 3A (F3A)

Physical properties of mild facial cleansing foam containing PCB extract Formula 4A		
Color	Clear with yellow color	
Viscosity	50 cP (++)	
Odor	Good odor	
Stability	Good stability, pass accelerated test	
Cleansing	Good cleansing oil	
Efficacy of foam	Foam stability 92.9% Smooth foam small and firmly	
Satisfaction (Overall)	4.00 ± 0.35	

Note: + = No viscous, ++ = low viscous, +++ = slightly viscous, ++++ = normal viscous, +++++ = high viscosity

In clinical trial, the study of efficacy of deep facial serum and mild facial cleansing foam with PCB extract on skin moisture by skin analyzer (Cortex, DermaLab Combo, Denmark) (showed that skin had significantly greater skin hydration ($583.0 \pm 18.5\%$) and increase skin elasticity ($173.7 \pm 2.6\%$) in 3 day after use with comparing at beginning time (day 0) as shown in table 5 ($P < 0.05$) without irritation sign.

Table 5 Efficacy of deep facial serum and mild facial cleansing foam on skin moisture and elasticity

Day	Skin Moisture (%)	Skin Elasticity (%)
Day 0	100.0 ± 0.0 ^c	100.00 ± 0.0 ^b
Day 3	583.0 ± 18.5 ^a	173.7 ± 2.6 ^a
Day 7	617.9 ± 16.6 ^a	180.7 ± 2.8 ^a
Day 14	442.98 ± 23.1 ^b	179.9 ± 3.82 ^a

Mean ± S.D (n=7) Different superscript letter in a column indicated the statistically different between samples (ANOVA, Turkey test, p<0.05)

4. Conclusion

Parchment coffee bean extract (especially ethanol extract) had high antioxidant activity and bioactive compound. This PCB from agricultural waste can be used as cosmetic ingredient with antioxidant and moisture retention properties. It is utilization of waste from coffee production and industry.

5. Acknowledgements

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Formulation and characterization of microemulsion containing *Kaempferia galanga* oil as a sunscreen product

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Abstract

Sunscreen product contains agent that absorbs or reflects ultraviolet radiation and thus helps protect skin from sunburn. Some organic chemical sunscreens and inorganic particulates are toxic and can induce skin irritation. Sunscreen product from natural compound that can absorb UV light is preferable due to the skin compatibility. The absorption of UV radiation by *Kaempferia galanga* oil has been previously reported. In this study, microemulsion containing *K. galanga* oil was formulated using Tween 80 as a surfactant, propylene glycol (PG) as a co-surfactant, and water at a ratio of oil to surfactants at 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1. The formulation that contained the maximum amount of galangal oil and appeared transparent was further evaluated for globule size, size distribution, surface charge, type of microemulsion, and rheological behavior. The maximum content of galangal oil included in the microemulsion formulated by using Tween 80 was 72.73% and the oil content in the microemulsion formulated by using Tween 80 and PG was 63.64%. The size of microemulsion formulated by using Tween 80 as a surfactant and Tween 80 plus PG as a co-surfactant were 233.5 ± 16.3 nm, and 194.6 ± 3.4 nm, respectively. The polydispersity index of microemulsion prepared by using Tween 80 and Tween 80 plus PG were 0.272 ± 0.005 , and 0.314 ± 0.053 , respectively. The zeta potential values of microemulsion formulated from Tween 80, and Tween 80 plus PG were -13.40 ± 0.46 , and -9.61 ± 1.03 mV, respectively. Both formulations were found to be o/w microemulsion and showed a pseudoplastic (shear-thinning) rheological behavior. The viscosity of the microemulsion was decreased when PG was applied into the formulation. It can be concluded that galangal oil is possible to be prepared for microemulsion based sunscreen product and could be further developed for other skin formulations.

Keywords: *Kaempferia galanga*; Microemulsion; Sunscreen product; Ultraviolet; Formulation.

1. Introduction

Ultraviolet radiation is the main cause of dark spots, sunburn, premature wrinkles and skin cancer (Korac & Khambholja, 2011; Mishra, Mishra, & Chattopadhyay, 2012). Therefore it is important to protect the skin from ultraviolet radiation. Sunscreen products are popular but there are many allergic

reactions occurring against sunscreens chemicals (Korac & Khambholja, 2011; Latha, 2013). Some organic chemical sunscreens and inorganic particulates are toxic and can induce skin irritation (Latha, 2013). Natural compounds are therefore good choices because they are more compatible to the skin. *Kaempferia galanga* L. is a herb that has been recorded in Indian Ayurveda for treating coughs, asthma and helps the digestive system (Raina & Abraham, 2015). From the previous research, the oil extracted from the rhizome contains cinnamate derivatives, which has the same chemical structure as sunscreens agents in sunscreen products for UV protection (Athikomkulchai, 2007). Hence, *Kaempferia galanga* L. is a good candidate for a new sunscreens agent. However, the oil is not stable and should be prepared in the form of microemulsion. The process of producing microemulsion is simple, uncomplicated and requires no preparation energy. The creation of microemulsion depends on the type of surfactant and the ratio of oil, water and surfactant (Liu, 2013; Sisak, Daik, & Ramli, 2017). The purpose of this research was to study the effect of the type and ratio of surfactants and co-surfactants on the preparation of suitable microemulsion from *Kaempferia galanga* L. oil for further development in sunscreen products.

2. Materials and Methods

2.1 Materials

Kaempferia galanga L. oil was purchased from PT Haldin Pacific Semesta, Indonesia. Tween 80 and propylene glycol were purchased from Namsiang company, Thailand.

2.2 Effect of surfactant and co-surfactant

Surfactant and co-surfactant used in this study were tween 80 and propylene glycol, respectively. Microemulsions prepared by using Tween 80 alone was also compared to microemulsion prepared by the use of Tween 80 mixed with propylene glycol at 1:1.

2.3 Preparation of microemulsion

Preparation of the *Kaempferia galanga* L. oil microemulsion was firstly performed by determining the appropriate ratio of the components through pseudo-ternary phase diagram to analyze the optimal ratio between the oil, the surfactant and water. Water titration method was used to study the phase behavior of the prepared systems by controlling the volume of oil and a surfactant in a fixed quantity at 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:1 v/v. After that, water in a constant proportion (100 µl) was added to the mixture of oil and surfactant once at time. The mixture of oil, surfactant, and water was mix vigorously for 15 seconds by using a vortex mixer. The clarity, turbidity and precipitation of the microemulsion were recorded.

2.4 Particle size, Polydispersity index, Zeta-potential measurement

A dynamic light scattering technique was carried out to determine hydrodynamic diameter, polydispersity index (PDI), and zeta potential of microemulsion using Zetasizer Nano ZS (Malvern Instruments, UK) (Basheer, Noordin, & Ghareeb, 2013). The microemulsion was analyzed in triplicate at 25 °C with capillary zeta-potential cell.

2.5 Determining the type of microemulsion

The type of microemulsion was identified by staining method. Brilliant blue (water soluble dye) and Sudan IV (oil soluble dye) were used to judge the type of the microemulsions by staining the droplets of microemulsion with the dye (Xu, 2010). Microemulsion stained with the dye was observed under the light microscope. The pictures of the microemulsion were captured by using Cellsens standard software (Olympus BX53, Japan)

2.6 Rheology study

The rheological properties of the microemulsion were investigated using a rheometer (Thermo scientific, US). The temperature was maintained at 25 ± 0.5 °C throughout the experiment. Sample volume was 2 ml. Flow properties were investigated by measuring the dynamic viscosity (η) as a function of time for 50 reads in addition to measurement of viscosity as a function of shear rate (ranging from 0.1 s^{-1} to 100 s^{-1}) (Basheer et al., 2013).

3. Results and Discussion

3.1 Pseudo-ternary phase diagram

The formation of *K. galanga* oil microemulsion systems is represented by points on the graph. The turbid liquid and sedimentation are presented in other spaces outside the point as shown in Figure 1. The microemulsion formulations containing the most proportion of oil and transparent were selected for further studies. The formulations that gave clear appearance of microemulsion are shown in Table 1.

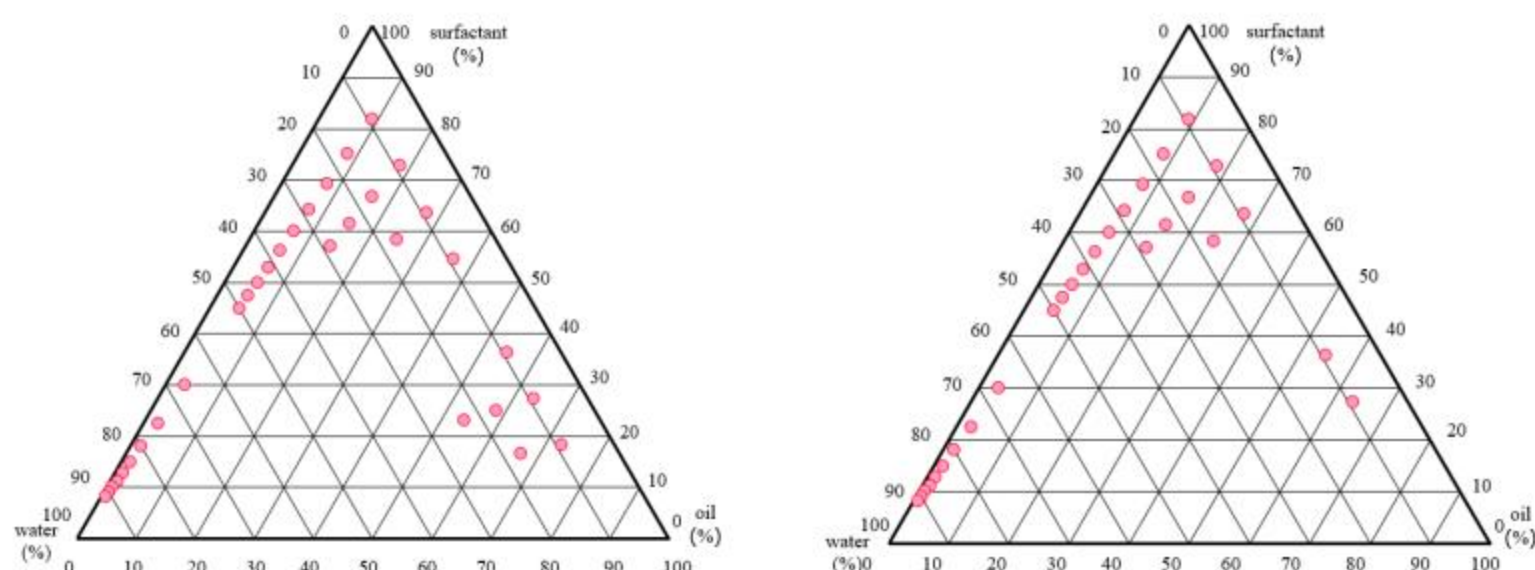


Figure 1 Pseudo-ternary phase diagram of *K. galanga* oil and Tween 80 (left) and Pseudo-ternary phase diagram of *K. galanga* oil with Tween 80 and propylene glycol at a ratio of 1:1 (right)

Table 1 The formulation of microemulsion containing the most proportion of oil yielding clear microemulsion.

Type of Surfactant / Co-surfactant	Oil (μl)	Surfactant / Co-surfactant (μl)	Water (μl)	Total volume (μl)	Oil (%)	Surfactant / Co-surfactant (%)	Water (%)
Tween 80	800	200	100	1,100	72.73	18.18	9.09
Tween 80: PG (1:1)	700	300	100	1,100	63.64	27.27	9.09

3.1 Particle size, Polydispersity index, Zeta-potential

The average particle size, PDI and zeta potential value of the microemulsion using 100% Tween 80 as a surfactant were $233.5 \pm 16.3 \text{ nm}$, 0.272 ± 0.049 and $-13.40 \pm 0.46 \text{ mV}$ respectively. The average particle size, PDI and zeta potential value of the microemulsion using Tween 80 and propylene glycol as a surfactant were $194.6 \pm 3.4 \text{ nm}$, 0.314 ± 0.053 and $-9.61 \pm 1.03 \text{ mV}$ respectively (Table 2). From the results, the particle size of the formulas with propylene glycol was smaller than those of microemulsion prepared by using Tween 80 alone. However, microemulsion containing Tween 80 only had narrow particle size distribution. The zeta potential of microemulsion prepared from Tween 80 alone was more

negative suggesting the better colloidal stability than the microemulsion containing Tween 80 and propylene glycol.

Table 2 Particle size, PDI and Zeta-potential values of *K. galanga* oil microemulsion.

Type of Surfactant / Co-surfactant	Oil (%)	Surfactant / Co-surfactant (%)	Water (%)	Particle size (nm)	Polydispersity index	Zeta-potential (mV)
Tween 80	72.73	18.18	9.09	233.5±16.3	0.272±0.049	-13.40±0.46
Tween 80: PG (1:1)	63.64	27.27	9.09	194.6±3.4	0.314±0.053	-9.61±1.03

Values are given as mean ± S.D. from triplicate experiments.

3.3 Type of microemulsion

The type of microemulsions was determined by observation under the microscopy. The pictures of microemulsion preparing from Tween 80 alone were shown in Figure 2 and the pictures of microemulsion preparing from Tween 80 and Propylene glycol were shown in Figure 3. The results show that droplets of microemulsion prepared from both types of surfactant and co-surfactant were stained with Sudan IV which is an oil soluble dye, whereas outside of the microemulsion droplets were stained with Brilliant blue. These results indicated that the two kinds of microemulsion were o/w.

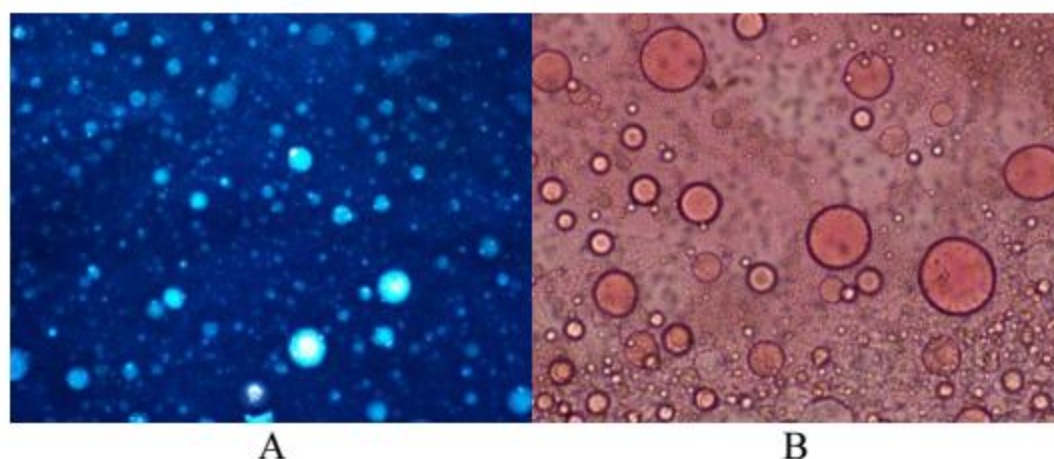


Figure 2 Microemulsion using Tween 80 alone as a surfactant (A) stained with Brilliant blue and (B) stained with Sudan IV.

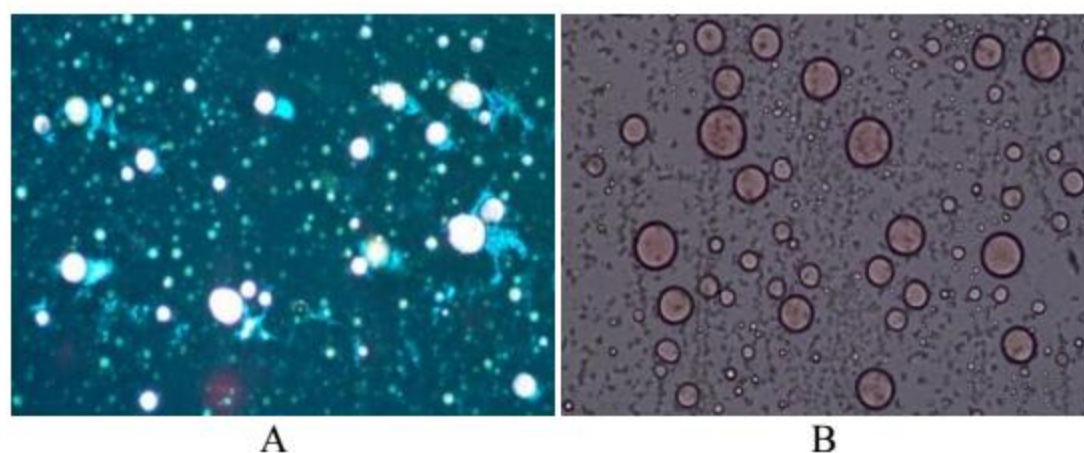


Figure 3 Microemulsion using Tween 80 and Propylene glycol as a surfactant (A) stained with Brilliant blue and (B) stained with Sudan IV.

3.4 Rheology

The rheological profiles of microemulsions are shown by the plot of viscosity (η) and the shear rate

(γ) ranging from 0.1 s^{-1} to 100 s^{-1} (Figure 4 and Figure 5). The viscosity of microemulsion prepared from Tween 80 alone and that of preparing from Tween 80 and propylene glycol was 3180.18 ± 691.25 , 391.93 ± 65.79 , 195.80 ± 26.69 , 41.31 ± 3.83 , $27.33 \pm 2.01 \text{ cP}$ and 1801.90 ± 83.56 , 160.93 ± 9.75 , 133.55 ± 8.82 , 46.30 ± 4.09 , $33.91 \pm 7.25 \text{ cP}$ at shear rates of 0.1 , 0.5 , 1 , 40 , 100 s^{-1} at 25°C , respectively. Both kinds of microemulsions were found to exhibit pseudoplastic (shear-thinning) flow. When the shear rate increases, the viscosity of the microemulsions decreases. The formulation containing propylene glycol had decrease viscosity in the formulation of microemulsion. The low viscosity of the microemulsion enhances the absorption of the microemulsion into the skin.

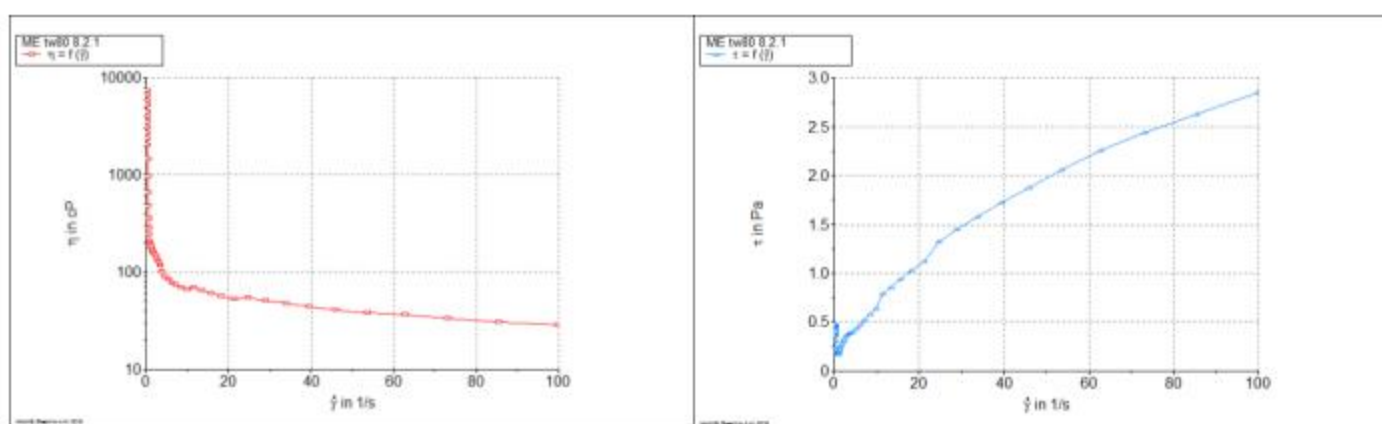


Figure 4 The rheological behavior of microemulsion prepared from Tween 80 alone.

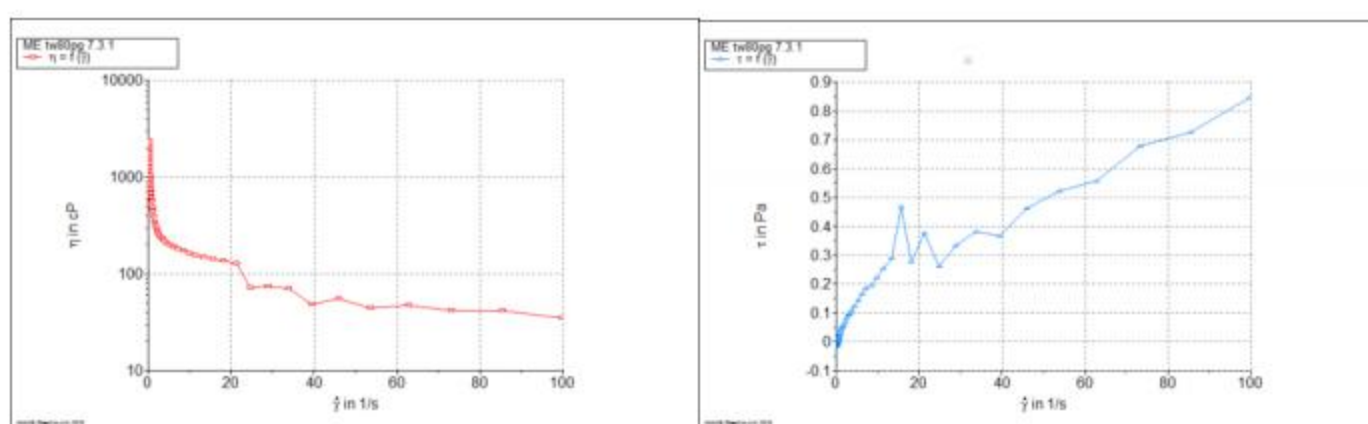


Figure 5 The rheological behavior of microemulsion preparing from Tween 80 and propylene glycol.

4. Conclusion

The microemulsions containing *K. galanga* oil yielded the high content of oil. The microemulsions prepared by using Tween 80 as a surfactant and Tween 80 and propylene glycol as a co-surfactant have acceptable particle size, PDI, and zeta potential. The microemulsion were identified as o/w microemulsions. The viscosity of the microemulsion was reduced when propylene glycol was applied in the formulation. It can be concluded that *K. galanga* oil is possible to be prepared for microemulsion based sunscreen product and could be further developed for other skin formulations in the future.

5. Acknowledgements

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Extraction of bioactive compounds from *Syzygium cumini* (L.) skeels

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Abstract

Java plum or *Syzygium cumini* (L.) Skeels is the wild plant with the edible deep purple sweet and sour ripe fruit. The plants are widely found in Thailand around June to July. From the reddish-purple color of the ripe fruit which represents the anthocyanin composition, this study was aimed to investigate the anthocyanin content from java plum extracts from multi-variation extraction factors. The extraction was performed by shaking method with 3 levels of 3 factors which are (A) the ratio of sample per solvent (1:5, 1:10, and 1:15 w/v), (B) ethanol percentage (45%, 75%, and 95%), and (C) solvent pH value (2, 5, and 8). The anthocyanin content was determined by pH different assay. It was found that in the extraction condition of 1:10 (w/v) sample per solvent with 45% ethanol at pH 5 provided the highest anthocyanin content (7.81 mg cyanidin-3-glucoside/g dried weight sample). The following anthocyanin content was found in the similar condition at the higher ethanol percentage, 75% (5.26 mg cyanidin-3-glucoside/g dried weight sample). The lowest anthocyanin content was obtained when the ratio of sample per solvent was minimized to 1:5 (w/v), (1.05 mg cyanidin-3-glucoside/g dried weight sample). The recent study could be the guideline for anthocyanin extraction of java plum to be used as the active compound. However, the bioactivity of the extract should be studied in the further study.

Keywords: Anthocyanin content; Java plum; Phenolic content; Radical scavenging; *Syzygium cumini* (L.) Skeels

1. Introduction

Syzygium cumini (*S. cumini*) (L.) Skeels; Syn.: *Eugenia jambolana* Lamarck, and *Eugenia cumini* (L.) Druce; Family: Myrtaceae), (java plum or jambolan) is the well-known edible plant in particular diabetes medicinal used. It is native from India widespread tropical tree nowadays in different regions of Brazil, as well as in Thailand. The java plum fruit looks like a black olive with a big purple seed and it has a sour taste (Tavares *et al.*, 2016). The major antioxidant capacity of the java plum is contributed by the anthocyanin compositions in fruit. The phenolic antioxidant compounds including glucoside, ellagic acid, isoquercetin, kaemferol and myrecetin were reported (Ayyanar and Subash-Babu, 2012). There were various studies found the potential of java plum as a source of phenolics and anthocyanins. Traves *et al.* (2016) reported the study of different part of java plum and found the variation of bioactive compounds existence in fruit skin and pulp which is phenolics and tannins, respectively. However, the separation is not practical in the industrial scale as well as the seed discarding is considered as a waste-production. Therefore the recent study chose to use of whole fruit to extract the multi-composition extracts. The extraction study of java plum with

various factor effecting to the extractability of bioactive compound were reported. The java plum extracts were studied for their antioxidant activities, and natural colorant (Aruna *et al.*, 2011; Sheikh *et al.*, 2011; Tobal *et al.*, 2012; Sari *et al.*, 2012). The java plum extract could be claims as the multi-functional agent according to their positive results on anti-inflammatory properties and antibacterial properties (Pavan Kumar *et al.*, 2010; Kaneria *et al.*, 2009, Migliato *et al.*, 2010). All the aforementioned bioactivities of jambolan have been at least partly ascribed to its phenolic constituents, mainly to the high content of anthocyanins in this fruit (Aqil *et al.*, 2012; Faria *et al.*, 2011). Therefore, the recent study purposed, from the previous literature of phenolic and anthocyanin main compounds in java plum, to study the effective extraction system of Thai java plum fruit. The study is goaled to be a guideline for the manufacturing java plum seed extract as a natural active ingredient in cosmetic that uniqueness for Thai cosmetics. The further determination of the consequent antioxidant activity and other cosmetic activities could be analyzed to evidence the effectiveness of the finding extraction system.

2. Materials and Methods

2.1 Chemicals and reagents

Commercial grade ethanol was purchased and distilled before use for the extraction. Hydrochloric acid was purchased from Fisher Scientific (Leicestershire, UK). Deionized water (DI H₂O) and ultrapure water (dd H₂O) was obtained from a MilliQ system (Millipore, Bedford, MA, USA). Folin-Ciocalteu reagent and gallic acid were purchased from Sigma-Aldrich Co., Ltd. (MO, USA).

2.2 Sample preparation

Java plum was purchased from Chiang Rai, Thailand in June to July 2018. The whole fruit of ripe java plums were washed and air dried for one day. Then the sample was completely dried in the hot air oven at 50 °C for 48 hours until obtain the constant dried weight. The java plum sample was powdered by hammer mill blender and kept at -20 °C until used.

2.3 Extraction

The java plum powder was extracted with hydroalcoholic solvent by shaking method. The extraction conditions were varied through the multi-variation design. The 3 factors with 3 levels of each were used in the study. Namely, the ratio of sample:solvent, ethanol concentration, and pH of the system. The factors were assigned as A, B, and C, respectively. The levels were -1, 0, and 1 of (A) the ratio of sample per solvent (1:5, 1:10, and 1:15), (B) ethanol percentage (45%, 75%, and 95%), and (C) solvent pH value (2, 5, and 8). After 24 hr of shaking at 150 rpm, RT, the samples were filtered by using vacuum funnel with No.1 filter paper. The filtrates were evaporated by using rotary evaporator (Eyela, CCA-1110, USA) and finally the sample was freeze-dried by using freeze dryer (#7960032 Labconco, MO, USA).

2.4 Total phenolic content

Total phenolic content (TPC) was determined by using Folin-Ciocalteu assay as per Brito *et al.* (2014) with some modifications. Each sample was weighed as 5 mg and dissolve with 1 mL of distilled water then pour into microcentrifuge tubes. Each sample was transferred in 96-well plate as 5 µl and diluted to 125 µl with distilled water, 25 µL of Folin-Ciocalteu's reagent was added and 150 µL of sodium carbonate solution was added. Then the reaction was incubated for 30 minutes at room temperature before detecting absorbance at 765 nm by UV-Visible spectrophotometer (Sánchez-Rangel, 2013). The results of TPC were expressed by mg gallic acid equivalent/g dry weight sample (µg GAE/ g dw sample).

2.5 Total Monomeric Anthocyanin pigment Content

The total monomeric anthocyanin concentration was performed by the pH differential method (Brito *et al.*, 2014). Briefly, each sample (0.5 ml) was separately taken in a test tube with buffer (ratio 1:4) either with pH 1.0 buffer (potassium chloride, 0.025M) or pH 4.5 buffer (sodium acetate, 0.4M). The absorbance was measured at 520 nm and 700 nm by UV-Visible spectrophotometer (Lee *et al.*, 2005). The results were calculated as the equation below;

$$\text{Anthocyanin pigment (cyanidin-3-glucoside g/L)} = (A \times MW \times DF \times 10^3) / \epsilon \times l$$

Where A = (A_{520nm} – A_{700nm}) pH 1.0 – (A_{520 nm} – A_{700 nm}) pH 4.5; MW (molecular weight) = 449.2 g/mol for cyanidin-3-glucoside (cyd-3-glu); DF = dilution factor established in D; l = pathlength in cm; ϵ = 26,900 L/ mol ⁻¹/cm⁻¹, molar extinction coefficient for cyd-3-glu; 10³ = factor for conversion from g to mg. The total monomeric anthocyanin was expressed as mg anthocyanin (cyanidin-3-glucoside)/ g dry weight sample (mg ATC/ g dw sample).

2.6 Statistical analysis

The results were analyzed by ANOVA using SPSS Statistics data editor One-way ANOVA: Post Hoc multiple comparisons by Duncan, 95% Confidence Interval and n = 3.

3. Results and Discussion

The results of extractable phenolic and anthocyanin content were shown in Table 1. The combination of extraction factors exhibited the variation in the bioactive compounds extraction. The effect of each factor is also discussed by setting other variations at the middle level. Firstly, the sample per solvent ratio (factor A) was at its best performance of phenolics extraction when using at level 0 and +1 as 1:10 and 1:15 (w/v) of 149.96±1.99 and 143.39±10.48 mg GAE/g dw sample when compared to the least sample: solvent ratio, 1:5 (w/v). The results were agreed with the ethanolic extraction of dried fruits of dog-rose, sea buckthorn and hawthorn by Predescu *et al.*, 2016 that the highest phenolic content was obtained by the higher ratio of solvent (1:10, w/v). Second parameter is the percentage of ethanol as the extractant (factor B). The trend of using different concentration 45%, 75%, and 95% possessed the bell-like curve of 135.32±3.15, 149.96±1.99, and 136.16±10.38 mg GAE/g dw sample, respectively showing the usefulness of the combination of water and ethanol in phenolic extraction. Lastly, the effect of pH of the system (pH 2, 5, and 8) was found owning the highest extractable phenolic content at the middle level of pH 5 (149.96±1.99, 71.76±1.28, and 135.50±1.36 mg GAE/g dw sample, respectively).

In conclusion for the extract ability on phenolic content of the system, the middle level of all factors was the most effective extraction condition. On the other hand, anthocyanin content extraction of the system showed the different performance. The highest anthocyanin content was found in the using of lower percentage of ethanol (45%, 7.81±0.00 mg ATC/ g dw sample). The recent result was similar with the blueberry anthocyanin extraction of Oancea *et al.* (2012). The extractable anthocyanin content was decreased along the increment of ethanol concentration (Oancea *et al.* (2012). The higher anthocyanin contents were found when pH is at the acidic condition of pH 2 and weak basic condition at pH 8 (4.51±0.00 and 4.01±0.00 ATC/ g dw sample, respectively). The mentioned pH effect also reported in Oancea *et al.* (2012) which 15% of 0.1 N HCl was used to acidify the ethnolic solvent. The anthocyanin content was lowered in the acid ethanolic solvent than 50% ethanol (Oancea *et al.*, 2012). The effect of sample per solvent ratio on anthocyanin content was similar to the extractable phenolic content which the highest is found at the middle level (1:10, w/v) than the lower ratio. However, the trend of sample:solvent ratio was not presented as a linear trend with slightly decrease at ratio 1:15. In term of the correlation of extractable phenolic and anthocyanin contents, the high level of linearity coefficient of 0.89 was found. The mentioned correlation is confirm the use of multivariation experimental design to determine the optimal extraction condition.

Table 1 Total phenolic and anthocyanin contents of java plum extracts from multivariation design

Extraction condition				Phenolic content (mg GAE/g dw sample)	Anthocyanin content (mg ATC/ g dw sample)
1	A-1	B0	C0	65.85 ±2.51 ^c	1.05 ±0.21 ^e
2	A0	B0	C0	149.96 ±1.99 ^a	3.90 ±0.00 ^c
3	A+1	B0	C0	143.39 ±10.48 ^a	2.70 ±0.42 ^d
4	A0	B-1	C0	135.32 ±3.15 ^b	7.81 ±0.00 ^a
5	A0	B+1	C0	136.16 ±10.38 ^b	1.80 ±0.28 ^e
6	A0	B0	C-1	71.76 ±1.28 ^c	4.51 ±0.00 ^b
7	A0	B0	C+1	135.50 ±1.36 ^b	4.01 ±0.00 ^b

Values are given as mean ± S.D. from triplicate.

Different letters in the same column indicate significant differences (P<0.05).

4. Conclusion

The findings of the recent results can concluded that the phenolic and anthocyanin extraction can be effectively extracted by the different system. Among the studied extraction system, the highest phenolic content can be obtained from the middle level of 1:10 (w/v) sample per solvent ratio of 75% ethanol at pH 5. While using the mentioned condition with lowering the ethanol concentration into 45% provided the highest anthocyanin content. The results will be used as the preliminary extraction condition for java plum antioxidant ingredient production in the further study.

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6. References

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Development of natural hair dye product

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Abstract

The objective of this research was to develop natural hair dye containing sappan extract. Sappan powder was extracted with 50% ethanol to obtain dark orangish brown viscous liquid. The developed emulsion consisted of 10% extract without (S1) and with 1% various mordants including aluminium sulfate (S2), zinc sulfate (S3) and aluminium chloride (S4) and dyed on bleached hair tress. The color staining efficacy of each formula was evaluated. All formulas (S1-S4) provided red shade on hair tress and formula with zinc sulfate (S3) gave strongest red color. Then, S3 was further developed by increasing zinc sulfate to 2% (S3-1). This formula showed higher dyeing efficacy than that of S3. The color retention efficacy of dyed hair with S3-1 was evaluated by shampooing 7 times and the results showed that the color fading of hair tresses slightly changed. Moreover, preference test was carried out on 11 volunteers. All volunteers were satisfied for the appearance, color of product, dying duration, dyeing efficacy, hair color and overall properties. 90.90% of volunteers were satisfied with spreading on hair and color retention. 81.82% of volunteers were satisfied hair smooth and not damage. However, only 54.54% of volunteers were satisfied with odor of the product.

Keywords: Hair dye; Natural hair dye; Natural extract; Sappan.

1. Introduction

For centuries the use of hair coloring was restricted to the fashion needs of a privileged few. Today, people in ever-greater numbers women and men alike change their hair color to make themselves more attractive. There are various reasons for this: to the fashion, to hide white hair, to lighten hair color or add an additional highlight, to remove the yellow look from gray hair, or to enhance the color of the natural gray, and so on. The principal dyes formulation can be classified in to three main categories: temporary coloring, semi-permanent coloring and permanent coloring (Ballarin *et al.*, 2007). The temporary and semi-permanent non-oxidative dyes are based on colorful molecules, named dye deposition, because the dye molecule only interacts with the hair cuticles. When there is a small penetration of the molecules into the hair cortex, they are named semi-permanent products and can be resistant up to six washes. The permanent oxidative are based on precursors, named oxidation dyes, whose color characteristics are developed by means of the interaction with an oxidizing agent, and present longer lasting color (Franca *et al.*, 2015). Contact allergy to hair dye ingredients, especially precursors and couplers such as *p*-phenylenediamine (PPD) and more which can lead to allergic reaction such as scalp puffiness, itching, swelling and difficulty in breathing (Rastogi *et al.*, 2006). They can also lead to development of some kind of cancer, hay fever, arthritis and leukemia etc. (Dominguez *et al.*, 2001). Therefore, natural hair dye products have been increasing interest. Sappan (*Caesalpinia sappan* L.) is a plant of Leguminosae family and has been used as a source of red color for hair dye. The red colorants in *C. sappan* were identified as brazilin, protosappanin and haematoxylin (Nirmal *et al.*, 2015).

However, problems with natural hair dyeing include weak color and difficulties with long-term deposition; therefore, natural dyes are not able to penetrate into the hair deep enough to protect dyed

hair from washing or fading. The addition of some substances, such as a mordant can improve hair dyeability. A mordant is a chemical which can itself be fixed on the fiber and also forms a chemical bond with the natural colorants. It helps in absorption and fixation of natural dyes and also prevents bleeding and fading of colors (Prabhu and Bhute, 2012). The developer can break chemical bonds and open the hair cuticle, which allows the dye molecules to penetrate deeper into the hair shaft and to bond with hair proteins (Boonsong *et al.*, 2012). In this study, aluminium sulfate, zinc sulfate and aluminium chloride were used as mordant to enhance staining efficacy of sappan hair dye.

2. Materials and Methods

2.1 Plant material

Sappan (*Caesalpinia sappan*) powder was purchased from Vejpongsoot, Bangkok.

2.2 Preparation of extract

The sappan was extracted with 50% ethanol in water in the ratio of 1 g: 5 ml (plant: solvent) for 1 hour by sonication method (Adirestuti *et al.*, 2018). The extract was filtrated by Whatman no.1 filter paper and the solvent was removed by using rotary evaporator to obtain crude extract. The pH value of the extract was measured by pH meter and color was measured by colorimeter.

2.3 Preparation of hair dye cream

Sappan cream as ingredients described in Table 1 was prepared by separately heated part A and B to 75°C and then mixed with homogenizer. The mixture was cooled down to 40-45°C and part C and D were added to cream base and mixed until homogeneous. Part E and F added into cream and mixed until homogeneous.

Table 1 The ingredients of hair dye cream

	Ingredients	S1	S2	S3	S4
A	DI water	q.s to 100	q.s to 100	q.s to 100	q.s to 100
	Butylene glycol	8.00	8.00	8.00	8.00
	Ceteareth-20, glyceryl				
B	Stearate, cetyl alcohol, cetearyl alcohol	22.00	22.00	22.00	22.00
C	Ammonium hydroxide 28%	10.00	10.00	10.00	10.00
D	Sappan extract	10.00	10.00	10.00	10.00
E	Vitamin C	1.00	1.00	1.00	1.00
F	Aluminium sulfate	-	1.00	-	-
	Zinc sulfate	-	-	1.00	-
	Aluminium chloride	-	-	-	1.00

2.4 Evaluation of dyeing efficacy

The human hair tresses were collected from barber shops and bleached with 50% hydrogen peroxide for 48 hours and then shampoo and dry with hair dryer. The bleached hair tresses were dyed with natural hair dye cream (S1-S4) for 30 minutes then shampooing and dried with hair dryer (Franca *et al.*, 2015). The color of dyed hair tresses was visually observed. Then, zinc sulfate was chosen as the best mordant and it was added in higher concentration (2%) in formula S3-1.

2.5 Stability testing

This study provides stability test of product were conducted heating-cooling cycles. The products were kept under 4°C for 24 hours and switched to 45°C for 24 hours. The processes were replicated for 7 cycles. After 7 cycles, product color, pH and viscosity values were measured and compared between before and after (IFSCC MONOGRAPH No.2, 1992).

2.6 Irritation test

Eleven healthy volunteers were participated in this study. The participants were briefed on the study procedures, and each was given written informed consent (Ethical Approve Number: REH-62306). The irritation test was done by closed patch test method. According to most hair dye products were applied for 30-45 minutes, in this study, the samples were applied on forearm for 30 minutes (Ho *et al.*, 2005). The test area was thereafter washed off by tap water and subsequently scored by MII technique (Baldissertoo *et al.*, 2018).

2.7 Sensory evaluation

Volunteers were answer satisfaction questionnaire after used product. There were ten parameters including appearance, odor of product, color of product, spreading on hair, dyeing duration, coloring efficacy, hair color, hair smoothness and not damage, long lasting color and overall satisfaction. Five scale of satisfaction were 5 = very satisfied, 4 = satisfied, 3 = adequate, 2 = unsatisfied, 1 = very unsatisfied. The results were calculated as percentage of acceptable satisfied.

3. Results and Discussion

3.1 Extraction

Sappan was extracted with 50% ethanol in water and obtained as dark orangish brown crude extract with 7.92 ± 0.92 yield percent based on plant weight. The pH of extract was 3.91 ± 0.02 .

3.2 Preparation of hair dye cream

Sappan creams (S1-S4) appeared as purple-red as shown in **Figure 1**. The pH of creams was range form 10.60-11.50. After being centrifuged at 5000 rpm for 10 minutes, formulas S1-S4 showed no separation therefore they were classified as stable formulas (IFSCC MONOGRAPH No.2, 1992). Which color of product are difference depend on mordant.

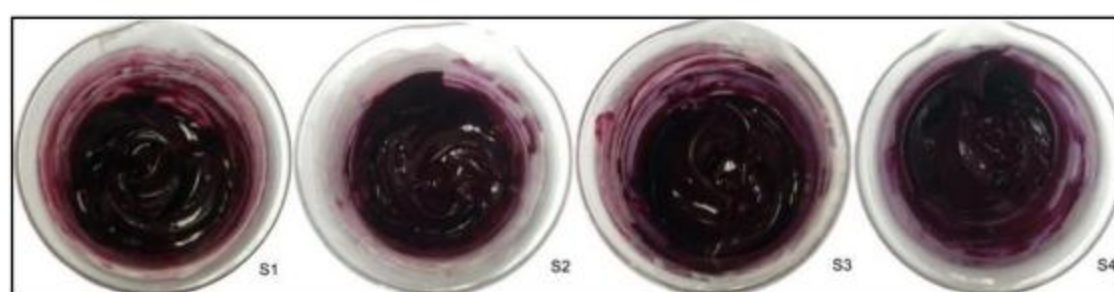


Figure 1 Appearance of sappan creams S1-S4

3.3 Evaluation of dyeing efficacy

The bleached hair tresses were dyed with natural hair dye creams (S1-S4) for 30 minutes then shampooing and dried with hair dryer. Color of dyed hair tresses was shown in **Figure 2**.



Figure 2 Appearance of 1) bleaching hair 2) dyed hair with S1 3) dyed hair with S2 4) dyed hair with S3 5) dyed hair with S4

S3 showed strongest red color on hair, this formula was chosen for further development by increasing zinc sulfate to 2% (S3-1). The result showed that S3-1 gave darker than S3 as shown in **Figure 3**, increasing zinc sulfate can improve color staining. Under microscope magnified 40x, the cuticle of the dyed hair were not damaged as shown in **Figure 4** and fixed on the fiber and also forms a chemical bond with the natural colorants. The color of dyed hair with S3-1 had no change after shampooing 7 times as shown in **Figure 5**. Zinc sulfate prevents bleeding and fading of colors (Prabhu and Bhute, 2012)



Figure 3 Hair dyed with 1) S3 2) S3-1

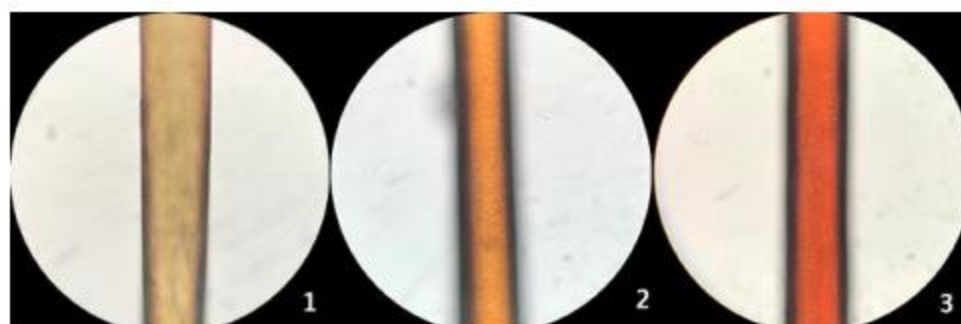


Figure 4 Structure of dyed hair under microscope 40x 1) Bleaching hair 2) S3 3) S3-1



Figure 5 Appearance of dyed hair with S3-1 1) before shampooing 2) after shampooing 7 times

3.4 Stability Testing

Selected S3-1 were proceeded to stability testing. The result of products stability in heating-cooling cycles was showed viscosity values and pH values between before and after was same, but the color of product changed from purple-red to purple-black, physical properties of product are unaccepted (**Figure 6 and Table 2**).



Figure 6 Appearance of S3-1 before and after heating-cooling cycles

Table 2 Stability of S3-1 before and after heating-cooling cycles between before and after

Condition	Parameter	Before	After
Heating-Cooling	Color	Purple-red	Purple-black
	pH	10.41 $\pm$ 0.02	10.61 $\pm$ 0.01
	Viscosity (Spindle no. 06, 150rpm)	5989 $\pm$ 62.21	6146 $\pm$ 98.66

Because of physical properties of S3 are unaccepted then cream base (S3-1B) and extract (S3-1E) were proceeded to stability test. The result of cream base (S3-1B) in heating-cooling cycles was showed viscosity values, pH values and color was same, physical properties of product are accepted **Figure 7 and Table 3**.

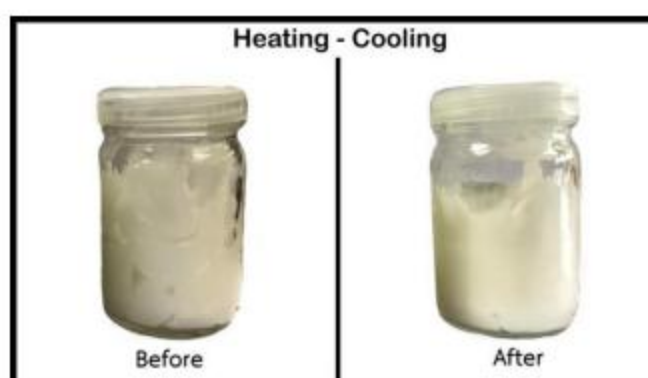


Figure 7 Appearance of S3-1B before and after heating-cooling cycles

Table 3 Stability of S3-1B before and after heating-cooling cycles

Condition	Parameter	Before	After
Heating-Cooling	Color	White	White
	pH	11.04 $\pm$ 0.02	10.79 $\pm$ 0.15
	Viscosity (Spindle no. 07, 200rpm)	11200 $\pm$ 72.11	14440 $\pm$ 1196

The result of products stability of extract (S3-1E) in heating-cooling cycles was showed pH values and color was same, physical properties of product are accepted **Figure 8 and Table 4**. The result suggested that product should mix before use.

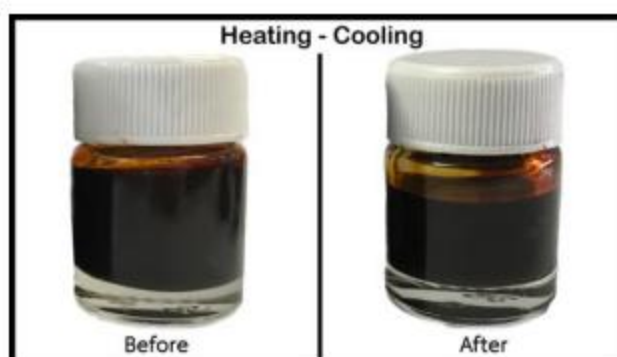


Figure 8 Appearance of S3-1E before and after heating-cooling cycles

Table 4 Stability of S3 -1E before and after heating-cooling cycles

Condition	Parameter	Before	After
Heating-Cooling	Color	Dark brown-orange	Dark brown-orange
	pH	4.48 ± 0.01	4.90 ± 0.01

3.5 Irritation test

Eleven healthy volunteers were participated in the irritation test which was done by closed patch test method. The samples were applied on forearm for 30 minutes. After the test area was washed off by tap water, MII scores were evaluated. The result found that MII value was 0 which the product was classified as non-irritant.

3.6 Sensory evaluation

Volunteers were answer satisfaction questionnaire after used product. There were ten parameters including appearance, odor of product, color of product, spreading on hair, time during use product, color staining efficacy on hair, satisfaction hair color, hair smooth and not damage, long lasting color and overall satisfaction. Five score of satisfaction were 5 = very satisfied, 4 = satisfied, 3 = adequate, 2 = unsatisfied, 1 = very unsatisfied. The results were expressed and percentage of volunteers who satisfied and very satisfied the product. All volunteers showed high satisfaction for color of product, time during use product, color staining efficacy, satisfaction hair color and overall satisfaction. More than 90% of volunteers were shown high satisfaction for spreading on hair and long lasting color. Moreover, 80% of volunteers were high in satisfaction for hair smooth and not damage. However, only 54.54% of volunteers were high in satisfaction for odor of product as shown in **Figure 9**.

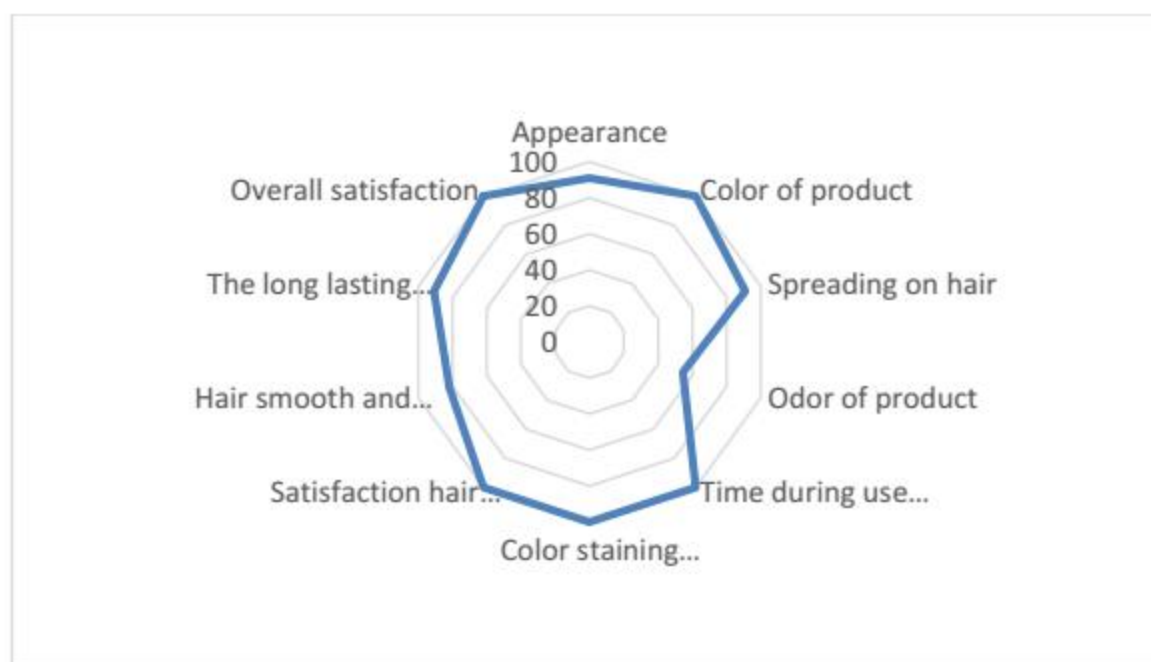


Figure 9 Percentage of satisfaction

4. Conclusion

The sappan powder was extracted with 50% ethanol and exhibited the orangish dark brown color. The emulsion was developed with 10% color extract and 1% different mordants including aluminium sulfate, zinc sulfate and aluminium chloride and evaluated for their color staining efficacy. Sappan cream (S1-S4) provided red color on hair shaft and formula containing zinc sulfate (S3) gave strongest hair color among all formulas. S3 was further developed by increasing zinc sulfate to 2% (S3-1) and this formula showed higher dyeing efficacy than S3. The color retention efficacy of S3-1 was evaluated by shampooing 7 times and the results showed that the red hair tresses had slightly changed. After stability testing, the color of product changed from purple-red to purple-black while the extract and slightly changed. Percentage satisfaction in satisfied (4) and very satisfied (5) showed that most of volunteers were high in satisfaction for color of product, time during use product, color staining efficacy, satisfaction hair color, overall, spreading on hair and long lasting color and hair smoothness. However, only 54.54% of volunteers were satisfaction for odor of product.

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Preliminary evaluation of microemulsions and nanoemulsions containing blended vegetable oils

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Abstract

Vegetable oils contains several crucial compounds that can provide remarkable properties for application in cosmetic products. There was several studies indicated that blending of various vegetable oils could improve stability, properties and sensory profile of these vegetable oil in products. In this study, seven common vegetable oils were selected. Sensory profiles of the blended oils were evaluated by 15 healthy volunteers aged between 18-25 years old. The most appropriate combination was jojoba oil, sweet almond oil, and macadamia nut oil at ratio of 3:2.5:0.5. This blended oil was mixed with 5% rosehip oil and it was used as an oil phase of microemulsions and nanoemulsions. Water titration method was used to construct a pseudo-ternary phase diagram. Tween 80, Span 80, and 1-butanol (2:1:1) were used as the mixture of surfactant and co-surfactant. The formation of microemulsions and nanoemulsions were preliminarily evaluated by observing bluish transparent and translucent liquid, respectively. The results showed that the formation of microemulsion began to form through 3.65–19.63% of oils, 32.85–76.92% of surfactants, and 3.85–63.50% of water. Nanoemulsion provided a boarder range with 1.67–72.58% of oil, 8.06–52.63% surfactants, and 15.25–83.33% of water. This study was prescreened and suggested for microemulsion and nanoemulsion formulation containing blended vegetable oils. The further identification of particle size and stability tests were suggested to perform.

Keywords: Microemulsion; Nanoemulsion; Rosehip oil; Vegetable oil

1. Introduction

In current cosmetic science, the role of natural ingredients is progressively in the spotlight due to the influence of wellness in eco-friendly and safety awareness. Likewise, skincare products consisting of fruits and vegetable oil is heavily sought after. Among the countless numbers of natural oils, rosehip oil (RHO) is now one of the standout preferable ingredients in cosmetology. RHO is an oil extracted from pseudo-fruits of various *Rosa* species e.g. *Rosa canina*, *Rosa damascene*, *Rosa gallica*, *Rosa moschata*, *Rosa Rubiginosa*, *Rosa rugose*, *Rosa woodsia*. *Rosa* genus widely grow in Europe, the Middle East, Asia and North America (Nilsson, 1997). RHO are known to be a source of antioxidant, anti-inflammatory and antimicrobial due to their high content of bioactive compounds including polyphenols, vitamins C, vitamin E, vitamin B and carotenoids (Özkan *et al.*, 2004; De la Iglesia *et al.*, 2010; Lopes *et al.*, 2014; Jimenez *et al.*, 2016). *Rosa canina* rose hips showed high concentrations of quercetin and ellagic acid, which was a potent tyrosinase and protein expression inhibitor involved in

melanogenesis. (Fujii and Saito, 2009; Tumbas *et al.*, 2012). Major phenolic compounds of *Rosa dumalis* was catechin, procyanidin B2 and epicatechin gallate, which are potent compounds to reduce oxidative stress (Demir *et al.*, 2014).

RHO possesses an irreplaceable profile as it contains diverse and high amounts of unsaturated fatty acids up to 80%. Essentially, those predominant unsaturated compounds in RHO are oleic acids (Omega 9, C18:1), linoleic acids (Omega 6, C18:3), and linolenic acid (Omega 3, C18:2) (Çelik *et al.*, 2010). These compounds are considered as essential fatty acids which our body is incapable of self-producing and plays a very important function in the renewal of skin cells and repair of damaged skin tissue. The omega series is believed to have interconnected with the skin's immune cells, as evidenced by the enhancement of wound healing (Cardoso *et al.*, 2004; Li, *et al.*, 2014). Fatty acids are used as a composition of cosmetic bases, allocated to improve protective layers and prevent water loss through the skin. (Zielinska, 2014).

RHO has been used in various cosmetic products as moisturizing, anti-aging, whitening, and anti-inflammation. It provides very outstanding sensory profiles among others. It is very light and has an immediate dry effect, leaving a silky touch with moisturized feel. Nevertheless, RHO is known as a high cost ingredient. Rapid rancidity is a major issue in RHO's shelf-life and quality. The degrading quality of oil, presents itself in the dispossession of physical appearance, odor, sensory profile, bio-activity, and also safety. Blending oil at particular ratios could improve stability, properties, sensory profile of vegetable oil in products, and increase their bioactivity, synergistically (Li *et al.*, 2014) at affordable prices. In this research, RHO was blended with common vegetable oils such as jojoba oil, apricot kernel oil, sweet almond oil, macadamia nut oil to obtain desirable sensory.

Cosmetic formulations in the form of emulsions are most often used for skin care products. As it is conducive to appropriate lipid supplementation and moisture, the main task of such formulations is to preserve the water-lipid equilibrium in epidermis. Emulsions can be classified based on the droplet size into microemulsion (5–100 nm), nanoemulsion (100–1000 nm), and macroemulsion (>1 µm). This study focused on developing nanoemulsion and microemulsion systems to improve stability of oils and skin penetration. Microemulsions are a thermodynamically stable system, the formations of microemulsion do not require high energy emulsification processes. With a proper selection of a certain surfactant and co-surfactant within a particular ratio, spontaneous formations are possibly occurred with the interactions in the interphase and by reducing interfacial tension. Providing improvement of stability against gravitational separation and Brownian motion, repel aggregation of droplet, aiming to extend long shelf-life (Tadros *et al.*, 2004). Microemulsion system is resulted in a lighter texture and lower in viscosity. This small droplet size of systems offers aesthetic appearance with increased chance of delivering bioactive molecules. Likewise, nanomemulsion could improve some similar aspects. Nanomemulsion is a kinetically stable but thermodynamically unstable system, a larger size of droplet means lower stability, cloudier in physical appearance. Microemulsion provides transparent system but nanoemulsion provides translucent or bluish-white due to the Tyndall effect. Nevertheless, both systems have the same composition at different ratio and similar structure of the droplet. Certainly, it is possible to prepare both emulsion types through water titration-method from the same components; oil, water, surfactant and co-surfactant (McClements, 2012). Construction of pseudo-ternary phase diagram could be used to investigate possibility of microemulsion and nanoemulsion formation of different compositions of oil, surfactant, co-surfactant, and water. To formulate the fine droplet particle through vegetable blending oil is a newer formulation of cosmetic attributes giving high moisturizing with rapid cutaneous absorption, able to present a see-through appearance. The recent innovations in the field of higher emulsion technology can bring the unique products with great commercial perception in a very competitive and lucrative global cosmetic market. Thus, this study aimed to prescreen and suggest for microemulsion and nanoemulsion formulation containing blended vegetable oils.

2. Materials and Methods

2.1. Materials

Rosehip seed oil was purchased from Chanjao Longevity Co. Ltd., Thailand. Apricot kernel oil, sunflower oil, sweet almond oil, and wheat germ oil were purchased from Desert Whale jojoba company, USA. Olive oil, jojoba oil, Span80 and Tween 80 were purchased from Namsiang Co. Ltd., Thailand. Macadamia nut oil was purchased from Southern Cross Botanicals Pty. Ltd., Australia. 1-butanol was purchased from QRAC.

2.2 Screening and investigation of vegetable oils profile

RHO and seven common vegetable oils, including apricot kernel oil, sunflower oil, sweet almond oil, wheat germ oil, olive oil, macadamia nut oil and jojoba oil, were screened for their sensory profile. The selection of oils was based on reviewing through the consideration of characteristic, cost-conventional and accessibility. The oils were continually investigated to identify as thick oil, medium oil, or light oil. Correspondingly, sensory profile was evaluated by researchers by scoring into 5 levels from + to +++++, referring to least desirable to most desirable within the following aspects: viscosity, spreadability, absorbability, and greasiness. The selection of oil used in blended formulation were focusing on score of absorbability, spread-ability, greasiness and oiliness at least three out of fifths.

2.3 Preparation and sensory evaluation of blended oils

RHO at 5% w/w was added into the blending oils containing various combination and ratios. Fifteen participants aged between 18-25 years old, both males and females with healthy skin condition; dry skin, oily skin, and normal skin were all included. Single-blinded sensory evaluation study was managed to determine preference on nine samples through study participants. The desirable in sensory evaluation were measured by scoring from 1 to 5, referring least desirable to most desirable. Evaluation was measured on these following parameters; odor, spreadability, absorbability, greasiness, and oiliness. The averaged results of sensory evaluation were interpreted and represented as a radar chart. The most preferable sample (the largest radar diagram area) was selected for further microemulsion and nanoemulsion formulation.

2.4 Construction of pseudoternary phase diagrams

The phase diagram was constructed in order to define appropriate ratios of each component which results in a great existence area of microemulsion and nanoemulsion. Phase diagram composed of water, Tween80:Span80: 1-butanol (2:1:1), and blended oil at different ratio. The ratio of surfactant and cosurfactant (S_{mix}) were kept constant. Then, various combinations of blended vegetable oil and S_{mix} were produced in nine different ratios as 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80 and 10:90. Subsequently, water was gradually added drop wise until reaching the identical checkpoint while the magnetic stirrer was set to vigorous stirring speed. Transparent, blueish translucent and opaque mixture were defined as microemulsions, nanoemulsions and macroemulsions, respectively. The quantity of each component were recorded and calculated. The region representing microemulsion and nanoemulsion formation were shaded on the diagrams.

3. Results and Discussion

3.1 Screening and investigation of vegetable oils profile

RHO was fixed at 5% w/w that was represented as an active oil ingredient and was blended with carrier oil to reduce cost, improve stability and sensory. Blending with other carrier oils is important to modify and fulfill its properties. The selection of vegetable carrier oils made is based on scientific review

and conventional sources. Nine of the selected common carrier oils were safflower seed oil, apricot kernel oil, sunflower oil, olive oil, sweet almond oil, macadamia nut oil, jojoba oil, and wheat germ oil. These ingredients were individually investigated for density type, physical appearance, and their sensory profile and the results are shown in Table 1.

As vegetable oils showed various properties within their own fatty acid contents, each oil was characterized by density type, color, viscosity, spreadability, absorbability, and greasiness. These parameters mainly impacted perception and preference of the blended product. The investigation of individual ingredients was made by scoring, + to +++++; with + being the least desirable and +++++ being the most desirable. According to the results, vegetable oils could be classified by the density of texture with light oil including rosehip oil (RHO), and jojoba oil. Sweet almond oil and apricot kernel oil were classified as light to medium oil. Olive oil, Macadamia nut oil, and Wheat germ oil were classified as thick oil. The difference in thickness was influenced by the chemical composition of the

Table 1 Properties and sensory profile of vegetable oil

Vegetable oils	Type	Color	Viscosity	Spreadability	Absorbability	Greasiness
Rose hip seed oil	Light	Clear	++++	++++	++++	++++
Safflower seed oil	Medium	Light yellow	+++	+++	+++	+++
Apricot kernel oil	Light-Medium	Light yellow	+++	+++	++++	++++
Sunflower oil	Medium	Light yellow	+++	++++	++	++
Olive oil	Thick	Greenish yellow	+	+	+	+
Sweet almond oil	Medium	Brownish yellow	+++	+++	++++	++++
Macadamia nut oil	Thick	yellow	+	+++	+++	+++
Jojoba oil	Light	Clear	++++	++++	++++	++++
Wheat germ oil	Thick	yellow	+	++	++	+++

*Scoring within 5 levels as + to +++++ which represent undesirable to most desirable.

oil, especially fatty acid composition, degree of substitution, structure and arrangement of compound. Higher amounts of long chain fatty acids tended to provide a heavier occlusive feel (Fasina *et al.*, 2006). The different carrier oils were selected to perform the variation of blending with RHO in various ratios. The selections were based on the vegetable oil's sensory profile. This study aims to achieve the superior sensory properties of great spreadability and absorbability with the least greasiness, providing softness and smoothness upon application. From the result, the light and medium carriers oil which scored below 3 (+++) out of 5 (+++++) levels on spreadability, absorbability, and greasiness parameters were excluded from further investigation. Remarkably, thick oils were restricted to scores of 2 (++) out of 5 (+++++) in absorbability and spreadability parameters. Because thick oils have high viscosity and difficulty penetrating the skin, it is necessary to use thick oils in a blending combination. Consequently, jojoba oil, apricot kernel oil, sweet almond oil, macadamia nut oil, safflower seed oil, and wheat germ oil were included for further blending.

3.2 The blended oils and sensory profiles

Various blended formulas were generated with different combinations. For the reason that mixing diverse types of vegetable oils could modify various properties. Not only able to alter fatty acid profiles and its metabolite compound, but also to give rise to levels of bioactive lipids and natural antioxidants, reinforcing the product's shelf-life and contributing to better quality oils (Li *et al.*, 2014). These could increase the product's efficacy at more affordable prices. Moreover, it assists in modifying the sensory profile and results in superior texture and feeling during and after applying the product. Formulation of

blended oils were shown in Table 2. As this study aims to achieve lightweight and sensual sensory, 5% RHO was mixed with various combination of carrier which were created by mixing light oil, medium oil, and thick oil in a ratio of 3:2:1. However, some formulas being an exception to this rule, due to personal consideration in order to investigate and compare. Jojoba oil has been mainly selected as the light oil as it is a liquid wax which has highly penetrating and closely resembles human sebum, offering good feeling and spreadability.

Apricot kernel oil and sweet almond oil are very similar in feeling as they do not have far different in fatty acid profile, Apricot kernel oil consists of about 6 % of palmitic acid, about 60 % of oleic acid, and about 30 % of linoleic acid while sweet almond oil is similar in approximate amount: 6.5% of palmitic acid, about 70 % of oleic acid, and about 20 % of linoleic acid (Cordain, 2010). However, the individual sweet almond oil tends to offer a softer feeling, so it was dominantly chosen as a medium oil component. For thick oil, individually, macadamia nut oil tends to offer greater benefits in all aspect, due to the significant difference in its properties. Sunflower oil and safflower seed oil were exceptional and given personal consideration as a combination replacing thick oil for its remarkable spreadability. Along these considerations, formulas 1 to 7 were first formulated and subsequently pre-evaluated. From the personal evaluation, the formula using jojoba oil as light carrier oil offered greater absorbability and spreadability over apricot kernel oil. Those containing sweet almond oil were found to offer a lighter silky sense than other medium oils. Macadamia nut oil left surprisingly soft and mellow texture with excessive oiliness, wheat germ oil as well. Safflower oil and Sunflower oil were significantly lighter and thinner but were greasy with excessive tackiness. From the screening, sample 2; the combination of jojoba oil: sweet almond oil: macadamia nut oil with the ratio of 3:2:1 had a tendency to provide the softest and the silkiest sensory but it was excessively oily and weighty in texture. Thus two more samples were improved based on the modification of sample 2. The modified Sample 8, jojoba oil and sweet almond oil were used at the same ratio but macadamia nut oil being replaced by apricot kernel oil, with the aim to reduce thickness and oiliness. In sample 9, the combination of ingredients was unchanged but the ratio altered by decreasing the proportion of macadamia oil. Sample 9 was modified with jojoba oil, sweet almond oil, and macadamia nut oil in the ratio of 3:2.5:0.5. However, as people have multiple types of skin, preference could be dissimilar in each individual. All samples were continually handed to 15 subjects with all types of skin to perform sensory evaluations. Evaluation was based on odor, spreadability, absorbability, oiliness and greasiness under 5 levels of scoring, from 1 to 5 which represent least desirable to most desirable. The sensory result of each sample was different. Records are portrayed in a statistical interpretation to represent the average sensory profile as seen in Figure 1.

Table 2 Formulation of blended oils

Samples	Oil Component	Ratio
1	Apricot Kernel oil: Sweet almond oil: Macadamia nut oil	3: 2: 1
2	Jojoba oil: Sweet almond oil: Macadamia nut Oil	3: 2: 1
3	Jojoba oil: Sweet almond oil: Wheat germ Oil	3: 2: 1
4	Jojoba oil: Sweet almond oil: Safflower seed Oil	3: 2: 1
5	Jojoba oil: Apricot kernel oil: Safflower seed Oil	3: 2: 1
6	Jojoba oil: Apricot kernel oil: Sweet almond oil	3: 2: 1
7	Apricot kernel oil: Sweet almond oil: Sunflower oil	3: 2: 1
8	Jojoba oil: Sweet almond oil: Apricot kernel Oil	3: 2: 1
9	Jojoba oil: Sweet almond oil: Macadamia nut oil	3: 2.5: 0.5

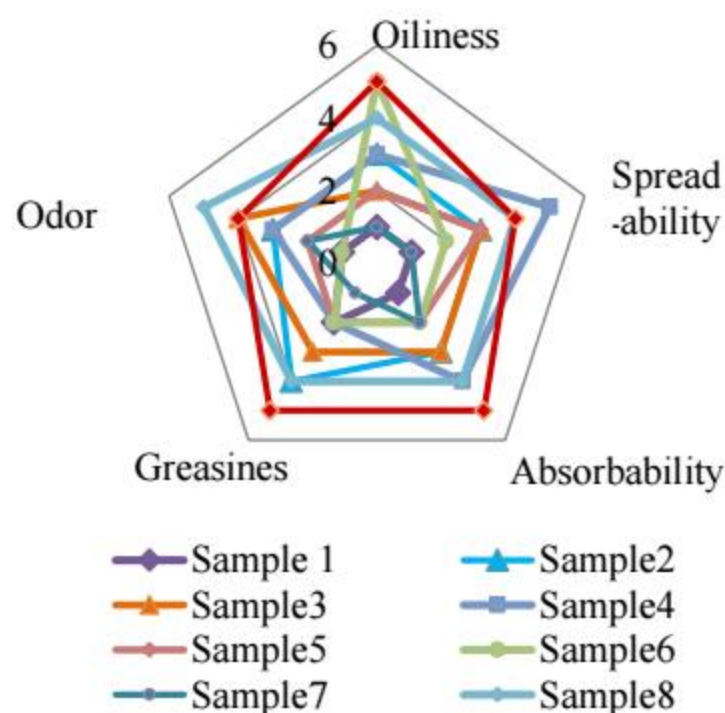


Figure 1 Sensory profiles of nine blended oil samples (n=15)

According to all subject's preference, the blended oil in all formula was reached in agreement as the most preferable sample which consist of Jojoba oil, Sweet almond oil, and Macadamia nut oil in the ratio of 3:2.5:0.5 (Sample 9), providing highest absorbability with a good control of greasiness and oiliness. As this blending formula dominantly contain Jojoba oil, a liquid wax ester, composing mainly monoester unsaturated fatty acid of gadoleic acid. Such unsaturated fatty acids were more effective enhancers. Result in good absorbability, smooth and non-greasy. Thus, sample 9 was selected for further formulation of nanoemulsion and microemulsion.

3.3 Construction of phase diagram

The achieved blending oil has been continually used as one of main components in the fine emulsion system cooperating with surfactant, co-surfactant, and water. In this construction of phase diagram, oil part was mixed with Smix at different ratios. Smix, combination of surfactant and co-surfactant were boundless in a selection. Even though many type of surfactant could be used in the nano system. However, for cosmetic applications, ionic surfactants are not desired due to toxicological concerns. Non-ionic surfactants are acceptable as the emergent lipid-base which stabilize interactions of the hydrophilic end of the surfactant with the aqueous phase through dipole and hydrogen bond interactions, the hydration layer of water managing the hydrophilic surface (Sarkhejiya *et al.*, 2013). Among non-ionic surfactants, blending between Span and Tween series were well synergistic to improve stability of droplet formations, due to the strength of packing and arrangement of those molecules. In this research, the selection of Tween 80 and Span 80 were used in the proportion of 2:1. According to the guideline of emulsifier selection, the combination of both high HLB emulsifier (Tween80) and low HLB emulsifier (Span80) in the ratio of 2:1 could provide the broad range of required HLB value which could cover the HLB value of blending oil as presented as 6.7. Moreover, non-ionic surfactants were often used in the system to avoid high risk of irritation. Co-surfactant ingredients were selected as 1-butanol. According to the study of microemulsion component, the alcohols co-surfactant as 1-butanol was cooperated well with sorbitan series surfactant (Basheer & Noordin, 2013). Thus, 1-butanol was considered to cooperate with blending surfactants in the ratio of 3:1. Hence, Tween80: Span80: 1-butanol was constructed in the

ratio of 2:1:1. Represented as Smix part continually used in water titration method, varying of ratios between Smix and oil in construction of phase diagram which could generate nanoemulsion and microemulsion. Nine different samples contributed to the different composition as shown in Table 3.

Table 3 Variation ratio between Smix and oil in construction of phase diagram

No.	Weight (g)			Ratio		Percentage	
	Oil	Smix	Total	Oil	Smix	Oil	Smix
1	0.50	4.50	5	1	9	10	90
2	1.00	4.00	5	1	4	20	80
3	1.50	3.50	5	3	7	30	70
4	2.00	3.00	5	2	3	40	60
5	2.50	2.50	5	1	1	50	50
6	3.00	2.00	5	3	2	60	40
7	3.50	1.50	5	7	3	70	30
8	4.00	1.00	5	4	1	80	20
9	4.50	0.50	5	9	1	90	10

Water titration method has been processed with all samples, to prescreening for an opportunity in the formation of microemulsion and nanoemulsion. During the titration, the phase behavior of nanoemulsion and microemulsion formation of the ternary mixtures prepared were characterized by visual observation for their phase separation and optical. The areas of nanoemulsion and microemulsion formation were shown in Figure 2. According to the result, the first two samples were presented the formation of microemulsion region. It shows that microemulsion systems were generated in the condition of a very high surfactant, very low in oil and water component, beginning to form through 3.65-19.63% of oil, 32.85-76.92% of surfactants, and 3.85-63.50% of water. However, such a condition was improper. As high amount of surfactant could be leading to high risk or irritation, high cost investment, and low in efficacy and nourish properties. As this formulation contained various types of vegetable oils, they could strongly effect and disturb the system to forming and packing firm droplet, resulting in requirement of high surfactant.

For nanoemulsion system, the shaded region was larger compared to microemulsion, indicating more opportunities in nanoemulsion formation. As results, it is able to start to form nano system with lowest amount of oil (1.67%), requiring 15.00% of surfactants and 83.33% of water. This combination provided advantages on cost-conventional due two low amount in blended oil and surfactants. Dominant amount of water could offer lighter touch, improving in sensory profiles. However, it may not reach the efficiency nourishment to the skin due to a very low amount of oil. Furthermore, low amount of oil (13.16%) require 52.63% of surfactants mixture and 34.21% of water. This combination would provide unproductive condition as it require highest amount of surfactants, leading to higher risk in irritation and disenchantment in cost-conventional. Also, nano system could reached with high amount of blended oil (72.58%) while require low surfactant composition (8.06%), and low amount of water (19.35%). This certain composition able to offer mild formulation product as low surfactant or emulsifier could provide a lower risk of irritation compared to higher surfactants, it also brings optimal nourishment toward skin due to high combination of blended essential oil. In the other hands, nano-formation was occurred in a combination. Each different of composition would offer different benefits, the selection of composition

on nanoemulsion formation is broad and adjustable which based on the priority of concern in product formulation.

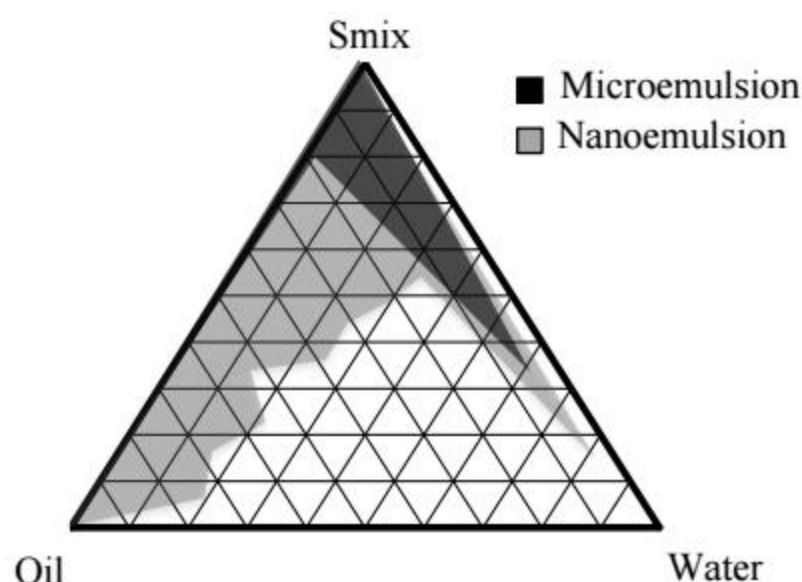


Figure 3 Pseudoternary phase diagram presenting microemulsion and nanoemulsion region by the construction using Smix as Tween80:Span80:1-butanol in the ratio of 2:1:1.

4. Conclusion

The blended vegetable oil which achieved the most desirable sensation were the combination of jojoba oil, sweet almond oil, and macadamia nut oil with ratio of 3:2.5:0.5. This blending oil was possibly applying to formulate as the innovated emulsion system. the construction of a pseudoternary phase diagram, determining the formation of nanoemulsion and microemulsion were managed by using Tween80, Span80, and 1-butanol (2:1:1) as the mixture of surfactant and co-surfactant. From the prescreening of microemulsion and nanoemulsion system using blended oil indicated that it was barely possible to formulate as micro-system. The recommended formulation was made on nanoemulsion system. Nanoemulsion able to start to formed with 1.67% of oil, 8.06% of surfactants, and 15.25% of water reached to high amount of oil (72.58%), 52.63% of Surfactants, and 83.33% of water. The selection of condition is broad and adjustable. However, the further suggestion of studies was to continually examined the stability of the nanoemulsion system through confirmation of the size of droplet, stability test toward the stability, influence of temperature, and oxidation or rancidity.

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Development of Acne Patch from *Curcuma longa* L. Extract

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Abstract

The aims of this study was to determine curcumin in *Curcuma longa* L. (Turmeric) extract, together with develop acne patch containing the extract formulation and evaluate the antimicrobial activity against *Propionibacterium acne* (*P. acne*). The acne patch product was then developed from the best formulation. An HPLC analysis showed that the ethanolic extract of *C. longa* contained curcumin 1.04 ± 0.03 %w/w. The acne patch formulation containing 0.5% and 1.0% of *C. longa* are opaque yellow solution with *C. longa* extract odor. The pH values of each formulation was 5.66 and 6.09, respectively. The *P. acnes* inhibitory activity tested by using agar disk diffusion method indicates that 1.0% of *C. longa* extract patch formulation had the highest clear zone against *P. acnes* with 21.33 ± 0.58 mm in diameter compared to other systems. The inhibition clear zone of standard curcumin (0.05%) and Clindamycin (0.05%) was 13.33 ± 0.58 mm and 18.00 ± 0.00 mm, respectively. In conclusion, the acne patch containing *C. longa* extract was successfully developed. The results are an essential information for further utilization of turmeric in cosmetic acne products.

Keywords: Acne patch/ *Propionibacterium acnes*/ Turmeric; Curcumin/*P. acnes*

1. Introduction

Acne (*Acne vulgaris*) is a skin disease that occurs naturally and is commonly found in all ethnicity and ages (Guzman *et al.*, 2018). Acne is caused by the excessive amount of oils on the skin produced by the sebaceous glands. Accumulation of the bacteria on the skin, *Propionibacterium acnes* (*P. acnes*), in time will cause inflammation on the skin (Webster, 2002).

Turmeric is a plant that is rich in vitamins and minerals. In addition, turmeric is an important ingredient found in essential oils (volatile oil) and curcumin (Curcuminoids). Volatile oils and curcuminoids contains curcumin, demethoxycurcumin, and bisdemethoxycurcumin. Extensive research found that curcumin provides antioxidants (Jayaprakasha *et al.*, 2002), anti-inflammatory (Jurenka, 2009), and antibacterial benefits (Antibacteria) (Gupta *et al.*, 2015). Turmeric has properties suitable for an active ingredient in acne treatment products. The aim of this study was to develop formulations containing turmeric extract in a form of an acne patch. Turmeric was extracted by using ethanol and was measured with High Performance Liquid Chromatography (HPLC) to determine its content. The turmeric extract was incorporated into a formulation and developed into an acne patch. An anti-bacterial performance test of the extract and formulation was performed by clear zone test method with *P. acnes* and Clindamycin as standard controller. It is believed that the prototype of the developed acne patches will effectively inhibit *P. acnes*. Further development of the formulation and product can be carried out to launch the product commercially.

2. Materials and Method

2.1 Preparation and extraction of turmeric

Turmeric ethanol extract was prepared by following the steps that is referenced from the Thai herbal pharmacopoeia (Department of Medical Sciences, 2016).

Turmeric was washed and cleaned, cut into small pieces, and then dried at 60°C for 48 hours. The turmeric is then coarsely blended with a blender. The coarse turmeric powder is used for extraction by ethanol. The turmeric powder is then soaked with 5% w/v ethanol solvent for 24 hours in a tight container. In the first 6 hours of extraction, the bottle was placed in a shaker, and was then set aside for another 18 hours. After 24 hours, the turmeric residue is filtered out with a filter paper. An evaporator was used to dry out the turmeric extract. The dry extract was stored at 20°C until use.

2.2 Preparation of the patch formulation

Two formulations were prepared with two concentrations of turmeric extract, 0.5% extract (Formula A) and 1.00% (Formula B). The ingredients are shown in Table 1. Part A is heated to 70°C - 75°C with continuous stirring. At the same time, Part B is also stirred for 10 minutes or until dissolved. Part B was gradually added to Part A when Part A was cooled to 40°C-45°C. The mixture was blended for 10 minutes before Part C was slowly added to PartAB. The mixture was stirred for an additional 5 minutes. After that, the formula was poured onto a fabric sheet to test the effectiveness of *P. acnes* inhibition. It was used to compare with standard curcumin at 0.05% concentration and clindamycin at 0.05% in the next step.

Table 1 Acne patch formulation.

Part	Ingredients	Formulation A (%w/w)	Formulation B (%w/w)
A	DI Water	88.25	88.75
B	Turmeric Extract	0.50	1.00
	PEG-40-hydrogenated castor oil	10	10.00
C	Benzalkonium chloride	0.20	0.20
	Polyaminopropyl biguanide	0.55	0.55

2.3 Test preparation

P. acnes DMST 14917 was incubated on the surface of the 5% blood agar media in anaerobic conditions at 37°C for 48 hours. After that, one colony is selected and cultured into the 5 mm Brain Heart Infusion broth and then cured in the same state. The turbidity is adjusted with 0.85% NaCl saline solution to have the same turbidity as the Mc. Farland No. 0.5 standard solution.

2.4 Medium preparation

Melted 5% blood agar culture medium that has a temperature of 45°C was poured into a petri dish (90 mm) at 25 mm/dish. The medium was set aside until solidified.

2.5 Turmeric anti-acne patch preparation and material composition

The most effective anti-bacterial formulation, Formulation B with 1.00% turmeric extract, has been chosen to develop into acne patches. One acne patch contains 50 μ l of the bulk. Each anti-acne patch is 1.0 cm in diameter and 40 gsm thick. Each patch has two sides – 1) spunlace layer and 2) skin-toned color dressing (Figure 5). The bulk is added onto the spunlace fabric side, which is then attached to a skin-toned adhesive dressing. One box contains 12 acne patches.

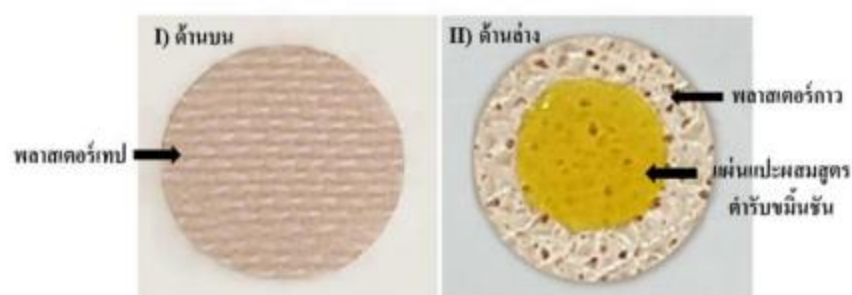


Figure 5 Acne patch material composition: I) Skin-toned adhesive dressing II) Spunlace fabric with bulk on skin-toned adhesive dressing

2.6 Turmeric anti-acne patch packaging design

Each box contains twelve patches, suitable for day and night time use. The packaging is designed to mirror the yellow color of turmeric. Yellow is known to be a fun, joyful, and unisex color. With this said, the product is suitable for all ages and sex.



Figure 6 Acne Patch Packaging Design

3. Results and Discussion

3.1 Analysis of curcumin content in turmeric extract with high performance liquid chromatography (HPLC)

Turmeric extract (25mg) was weighed out and dissolved in a volumetric flask. The volume was adjusted with 25 ml of ethanol. The solution with a concentration of 1 mg / ml was filtered with a 0.45 μ m filter before injected into the 10 μ l HPLC. The process was repeated three times.

The standard curcumin was prepared at a concentration of 1 mg/ml by weighing 10 mg of the standard curcumin and dissolved with 10 ml of ethanol solvent. Different concentrations of 5, 2, 5, 7, 10 and 12 μ g/ml, respectively, are filtered with a 0.45 μ m filter before injecting it into a 10 μ l HPLC. The process was repeated three times.

3.2 Analysis of curcumin content in turmeric extract

The standard graph obtained from the analysis of standard curcumin substances at concentrations of 2, 5, 7, 10 and 12 $\mu\text{g/ml}$ with a high performance liquid chromatography machine, is shown in Figure 2. The curcumin standard substance at this concentration has a linearity $y = 63704x + 10890$ and has a correlation coefficient (R^2) equal to 0.9944. This standard graph can be used to calculate the amount of curcumin in the substance. According to HPLC chromatogram analysis, it can be concluded that 1 mg/ml of turmeric extract contains a curcumin concentration of $10.44 \pm 0.27 \mu\text{g/ml}$ or equal to $1.04 \pm 0.03\%$ w/w as shown in Figure 1.

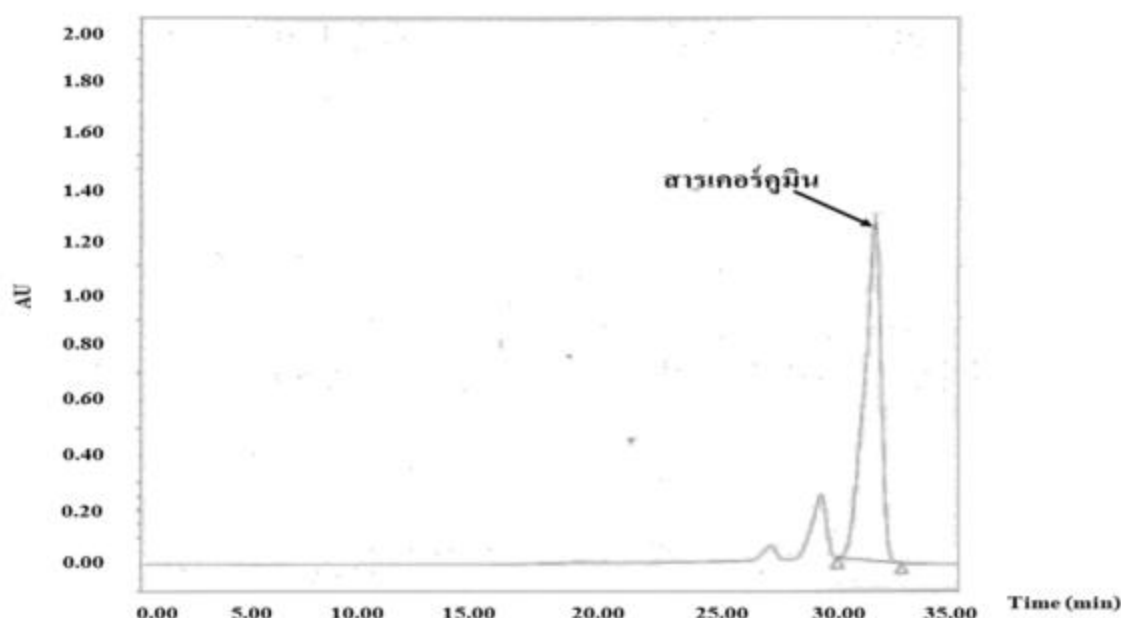
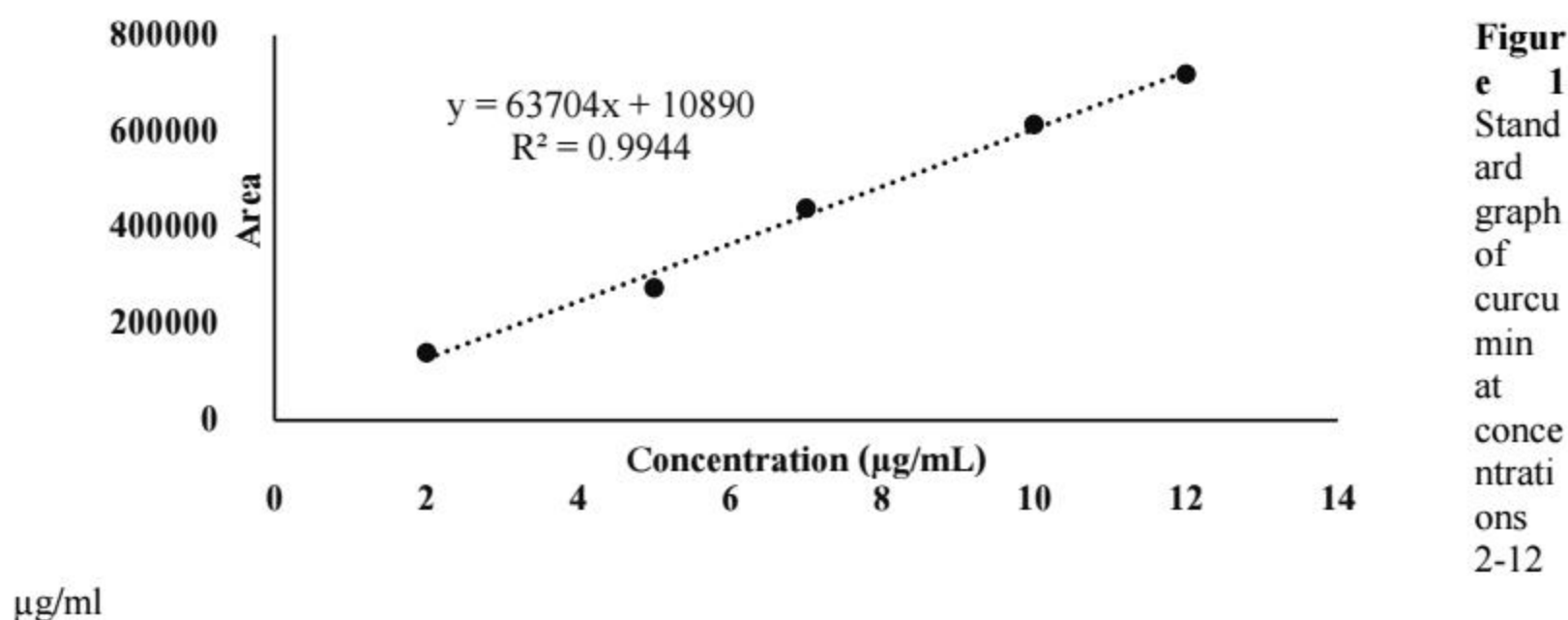


Figure 2 HPLC chromatogram of curcumin extract at a concentration of 1 mg/ml

3.3 *P. acnes* inhibition test using disk diffusion assay (CLSI M02-A11, 2012)

The antimicrobial activity of *P. acnes* in the two formulations was tested by using disk diffusion assay method. Onto a 6 mm (diameter) sterile paper disk, 20 μl (3 sheets per test substance) was added, and sterile distilled water was pipetted onto a 20 μl volume test sheet to be used as a solvent control sheet (blank). The test strips were dried under the Laminar flow cabinet for 10-15 minutes. With sterile forceps, the test strips were placed on the surface of the medium, then the test strips were pressed with the tip of a forceps to make sure that the test strip's surface is fully in contact with the medium. The test dishes were incubated in anaerobic conditions at 37°C for 24 hours. The size of the inhibition zone around the test plate was recorded in millimeters.

3.4 Formulation appearance

In preparation of the formulations containing Turmeric extract, Formula A with 0.5% w/w and Formula B with 1% w/w, it was found that both formulations are yellow and opaque in appearance (See Figure 3). The formulations also gives off a unique odor. The color, opaqueness, and odor resulted from the turmeric extract that was added. On the other hand, the base formulation (without turmeric extract) was colorless and odorless. The pH of the base formula has a pH of 4.23, while the formulation containing Turmeric extract 0.5% and 1% w/w has a pH value of 5.66 and pH 6.09, in order. It was found that the pH of the formula, 5-6, is suitable for the skin. The developed formula will be used to determine the inhibition level of bacterial virulence (*P. acnes*) in the next step.

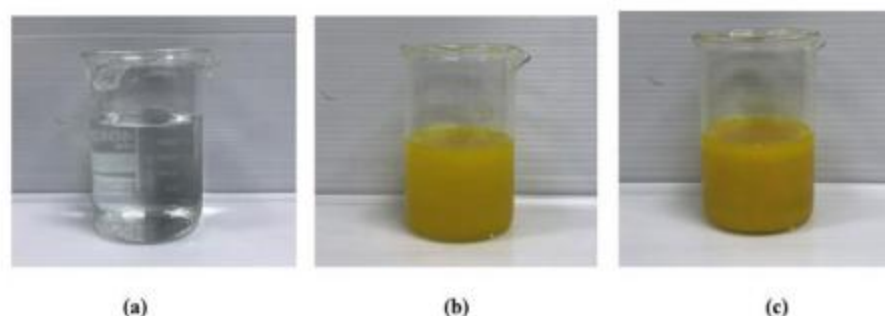


Figure 3 Solution Appearance

a) Base without turmeric extract b) Base with 0.5% turmeric extract c) Base with 1.00% turmeric extract

3.5 Determining *P. acnes* inhibition activity of acne patch

The antimicrobial activity of *P. acnes* of the two formulations was evaluated by comparing with the standard curcumin at 0.05% concentration and clindamycin (standard control) at 0.05% concentration by agar disk diffusion method. The growth of *P. acnes* was analyzed by observing the inhibition zone and measuring the size of the inhibition zone comparing the differences between the test sample and the standard controller, clindamycin. From this study, it was found that the inhibition activity of *P. acnes* may be the result of adding Turmeric extract to the formula. Formulas containing Turmeric extracts, contained *P. acnes* inhibition properties. However, in the base formula (formula without turmeric extract), *P. acnes* was not effectively inhibited. Formula B, containing 1.0% Turmeric extract, had the highest antibacterial activity compared to Formula A and the base formula. The results showed that the main active ingredient, Turmeric extract, is the anti-bacterial agent. The amount of Turmeric extract in the formulation also affected how well *P. acnes* was inhibited (Liu & Huang, 2013). Test results against *P. acnes* using agar disk diffusion test showed that the formula containing 1.0% turmeric extract had the best inhibitory effect on *P. acnes* bacterial growth, with an inhibitory zone of 21.33 ± 0.58 mm. The standard control formulation (Clindamycin 0.05%, the standard curcumin substance at a concentration of 0.05%, and the formula containing 0.5% turmeric extract had an inhibitory zone of 18.00 ± 0.00 mm, 13.33 ± 0.58 mm, and 10.33 ± 0.58 mm, respectively.

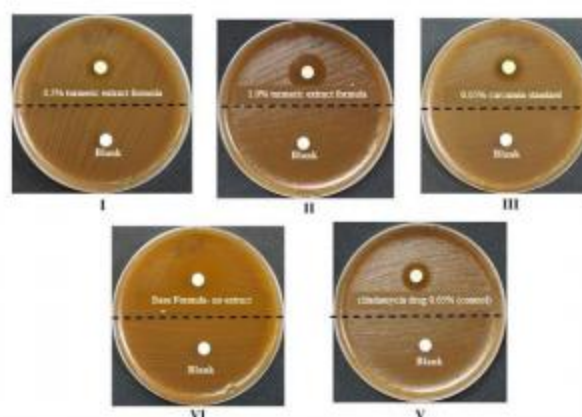


Figure 4 Anti-bacterial activity of *P. acnes* of I) 0.5% turmeric extract formula, II) 1.0% turmeric extract formula, III) 0.05% curcumin standard substance, IV) Base formula-no extract, and V) Clindamycin drug 0.05% (control)

4. Conclusion

Through ethanol extraction method, the Turmeric extract contains curcuminoid, a pharmacological active substance. The amount $1.04 \pm 0.03\%$ w/w Formula B with 1.00% Turmeric extract, is chosen to develop acne patches because the concentration of the extract provided the best antibacterial activity.

During development, both formulas (Formula A = 0.5% Turmeric extract, Formula B= 1.0% Turmeric extract), is found to have a dark opaque yellow color. It is also discovered that the two formulations contains a unique and distinct odor. The appearance and odor resulted from the yellow Turmeric extract is has been added into the formulation. The pH values of Formula A and Formula B is 5.66 and 6.09, respectively. Test results against *P. acnes* using agar disk diffusion test showed that the formula containing 1.0% Turmeric extract had the best inhibitory effect on *P. acnes* bacterial growth, with an inhibitory zone of 21.33 ± 0.58 mm. The standard control formulation (Clindamycin 0.05%, the standard curcumin substance at a concentration of 0.05%, and the formula containing 0.5% turmeric extract had an inhibitory zone of 18.00 ± 0.00 mm, 13.33 ± 0.58 mm, and 10.33 ± 0.58 mm, respectively. However, the bacterial inhibition activity may be from curcumin, Turmeric's main active ingredient, or may be from other important substances in Turmeric. This study does not rule out other substances in Turmeric that may contribute this anti-bacterial property.

To conclude, it is shown that turmeric provides anti-bacterial benefits, specifically *P. acnes* inhibition. This study is evidence that Thai agricultural product can be developed into a commercial product for both domestic and international market. With natural ingredients and products as an emerging trend, turmeric may be explored in other types of skincare products.

5. Acknowledgements

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Antimicrobial activity and GC-MS analysis in *Mucuna* seed extracts

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Abstract

The objective of this study was to evaluate the antimicrobial activity of *Mucuna* seed extracts and their bioactive compounds. Plant seeds of *Mucuna* species including *Mucuna pruriens*, *Mucuna monosperma*, *Mucuna interrupta*, *Mucuna gigantea* and *Mucuna revoluta* were used for extraction by soxhlet extraction method with different solvents (ethanol, ethyl acetate and hexane). Antimicrobial activity of seed extracts was investigated by broth micro-dilution assay against skin infecting microorganism, while bioactive compounds of the seed extracts were evaluated by Gas Chromatography-Mass Spectrometric (GC-MS) technique. The yields of seed extracts obtained by ethanol, ethyl acetate and hexane extractions were 1.95-4.83%, 0.45-0.67% and 4.85-8.04%, respectively. The ethyl acetate and hexane extracts of *M. pruriens* against *Staphylococcus aureus* were 4.10 mg/ml of MICs and MBCs, while ethyl acetate extract of *M. interrupta* against *Candida albicans* showed at 8.20 mg/ml of MICs and 16.40 mg/ml of MFCs. Ethanol extracts of *M. monosperma* possessed a broad spectrum of antimicrobial activity to *S. aureus*, *Staphylococcus epidermidis* and *C. albicans* at 4.10-8.20 mg/ml of MICs and 8.20-16.40 mg/ml of MBCs or MFCs. Antimicrobial activity were not found in any seed extracts of *Mucuna gigantea* and *Mucuna revolute*. After GC-MS analysis, main phytochemical compounds were identified as 9,12-Octadecadienoic acid/ Linoleic acid (29.41% and 34.06%) in both ethyl acetate and hexane extracts of *M. pruriens*, Bis(2-ethylhexyl) phthalate (24.30%) in ethyl acetate extract of *M. interrupta* and Dodecanamide/N-(2-hydroxyethyl)-lauramide (22.86%) in ethanol extracts of *M. monosperma*. Therefore, the results of the study indicate that the extracts of *M. pruriens*, *M. interrupta* and *M. monosperma* seeds exhibited antimicrobial activity which could be exploited as alternative antimicrobials for pharmaceutical and beauty treatments and cosmetic application.

Keywords: Antimicrobial activity; Gas chromatography; *Mucuna* seeds; Phytochemicals; Seed extracts.

1. Introduction

Infectious diseases caused by multidrug-resistant microorganisms have recently been a real global health concern. The microorganisms could reduce the efficacy of antibiotic drugs, consequently, leading to the frequency of therapeutic failure and even mortality in patients (Fankam *et al.*, 2017). Acne vulgaris is a prevalent infectious disease in the skin which causes both physical and psychological effects. *Propionibacterium acnes* is a gram-positive bacterium which is one of many crucial factors in pathogenesis of acne vulgaris. Moreover, some common aerobic commensal of normal skin, such as *Staphylococcus epidermidis*, *S. aureus* and *Candida albicans*, could be found and isolated from acne patients (Yousif & Dabbagh, 2016). Moreover, nowadays, more than 50% of *P. acnes* strains are resistant to topical antibiotics, causing them substantially less effective (Woodburn, 2018). Nowadays, medicinal plants are more focused than ever since the plants have the capability of providing many medicine and pharmacological benefits. Plants can produce numerous substances which may not be crucial for their primary metabolism, but necessary for an adaptive ability of a plant to prevent abiotic and biotic environmental conditions (Stefanovic & Comic, 2012). Therefore, crude extracts of medicinal plants could act as an alternative source of resistance modifying substances, such as alkaloids, tannins, polyphenols etc. These substances could function as potentials for antimicrobials and resistance modifiers (Gupta & Birdi, 2017).

The genus *Mucuna* belonging to the Fabaceae family includes about 150 species of annual and perennial legumes. *Mucuna* spp. have been reported to present L-dopa, hallucinogenic tryptamines and anti-nutritional factors. All parts of the *Mucuna* plant provide medicinal properties. In vitro and in vivo studies on *M. pruriens* extracts exhibited a wide variety of medicinal properties, including antidiabetic, antioxidant, neuroprotective and anti-inflammatory properties (Misra & Wagner, 2007). In addition, methanolic extracts of *M. pruriens* leaves have been shown to have mild activity against some bacteria in experimental settings (Ogundare & Olorunfemi, 2007). Additionally, methanol extract of *M. pruriens* seeds had the potential to kill *Vibrio harveyi*, *Vibrio cholera*, *Escherichia coli* and *Staphylococcus aureus* (Shanmugavel & Krishnamoorthy, 2015). Crude ethanolic extract of *M. pruriens* leaves could inhibit *Pseudomonas aeruginosa*, *Salmonella typhi* and *Shigella dysenteriae* (Abraham *et al.*, 2016). However, the antimicrobial activity of *Mucuna* seeds has been rarely reported, especially in acne-related microorganisms.

Therefore, the objective of this study was to evaluate the antimicrobial activity of *Mucuna* seed extracts and their bioactive compounds by GC-MS technique. *Mucuna* spp. used in this work were *Mucuna pruriens*, *Mucuna monosperma*, *Mucuna interrupta*, *Mucuna gigantea* and *Mucuna revoluta*. Microorganisms such as *S. aureus*, *S. epidermidis*, *P. acnes* and *C. albicans* were chosen for antimicrobial evaluation of *Mucuna* seed extracts since the microorganisms are skin infectious pathogen that reduce health and beauty of human skin.

2. Materials and Methods

2.1 Microorganisms and plant species

Skin infection microorganisms including three bacterial strains (*Staphylococcus aureus* TISTR746, *S. epidermidis* TISTR2141 and *Propionibacterium acnes* DMST14916) and one strain of yeast (*Candida albicans* TISTR10231) were used to evaluate antimicrobial activity. Seeds of *Mucuna* species used in the study included *Mucuna pruriens*, *Mucuna monosperma*, *Mucuna interrupta*, *Mucuna gigantea* and *Mucuna revoluta*.

2.2 Seed samples

Dry seeds of *Mucuna* species were collected from the herbal garden of Chiang Mai University and the traditional herbal medicine shops in Chiang Mai district, Thailand. The botanical specimens of the plants were identified by Miss Wannaree Charoensup, Plant botanist, Faculty of Pharmacy, Chiang Mai University. After collection, the seeds were washed repeatedly with tap water and air-dry.

2.3 Extraction of crude extracts

Air dried seeds of *Mucuna* species were individually made as a powder using an electrical blender. Each 50-g seed powder was extracted with different solvents including ethanol, ethyl acetate and hexane for 5 h in Soxhlet apparatus. Crude extracts were filtered through Whatman No.1 filter paper. The remaining extraction solvents in extracts were removed by a rotary evaporator and then kept in a refrigerator at 4 °C until use.

2.4 Antimicrobial activity assay

Antimicrobial activity of seed extracts was evaluated by broth micro-dilution technique. Negative and positive controls were 5% DMSO and Tetracycline, respectively. Seed extracts were diluted to 5% DMSO and used in the final concentration between 0.51-16.40 mg/ml. Briefly 10 µl of extracts was mixed in 100 µl of microbial cell solution (a density of 1×10^6 cfu/ml). The sample tests against *S. aureus*, *S. epidermidis* and *C. albicans* were incubated aerobically at 37 °C for 16-20 h, while the tests against *P. acnes* were incubated at 37 °C for 72 h under anaerobic condition. The inhibitory efficacy of seed extracts was investigated by method of resazurin reduction as an indicator of microbial growth. After complete incubation, 20 µl of 0.06% resazurin dye solution was added to each sample test and further incubated for 6 h.

The minimal inhibitory concentration (MIC) value was defined as the lowest sample concentration that could not affect the colorization of resazurin dye. The minimum bactericidal concentration (MBC) or minimum fungicidal concentration (MFC) was measured by using single streak plate method and was defined as the lowest sample concentration that completely kill bacteria or fungi (without any colony).

2.5 GC-MS analysis

Phytochemical compounds in *Mucuna* seed extracts were determined by Gas Chromatography-Mass Spectrometric (GC-MS) technique. GC system with Agilent 19091S-433 and HP-5MS column (30 m in length x 0.25 mm in diameter x 0.25 µm in thickness of film) were used for the determination. The oven temperature was programmed to be 290 °C at a rate of 10 min/ml, and helium gas were used as the carrier gas with a flow rate of 1 ml/min. Seed extracts in absolute methanol (~100 ppm) were injected using the spit sampling technique in the ratio of 1:10.

2.6 Identification of Chemical Constituents

Bioactive compounds in *Mucuna* seed extracts were identified according to GC retention time (RT) of eluted peaks on HP-5MS column and matching of mass spectra (MS) with database in the WILEY and NIST libraries.

3. Results and Discussion

3.1 Percentage yield of *Mucuna* seed extracts

Dried seed powders of five *Mucuna* species (50 g) were used to extract with the different extraction solvents. Among extraction yields of each extraction solvent, the highest yields of ethanol and hexane

extraction were found in *M. monosperma* at 4.83% and 8.04%, respectively while ethyl acetate extraction showed the highest efficacy to extract *M. pruriens* seeds at 0.67%. Moreover, to compare the extraction yields of three extraction solvents, mean percentage yields of ethanol, ethyl acetate and hexane extracts were 3.53-1.2%, 0.54-0.08% and 6.34-1.14%, respectively (Table 1). Hexane extracts showed the highest percentage yield. Most of the seed constituents were nonpolar in nature.

Table 1 Extraction yields of *Mucuna* seed extracts obtained by different extraction solvents.

<i>Mucuna</i> seeds	% Extraction yield		
	Ethanol	Ethyl acetate	Hexane
<i>Mucuna pruriens</i> (Mp)	3.96	0.67	4.85
<i>Mucuna monosperma</i> (Mm)	4.83	0.53	8.04
<i>Mucuna interrupta</i> (Mi)	1.95	0.52	6.53
<i>Mucuna gigantea</i> (Mg)	2.63	0.53	6.08
<i>Mucuna revoluta</i> (Mr)	4.27	0.45	6.19
Mean:	3.53	0.54	6.34
SD:	1.20	0.08	1.14

3.2 Antimicrobial activity of *Mucuna* seed extracts

The antimicrobial activities of *Mucuna* seed extracts were tested against four skin infection microorganisms by broth micro-dilution assay and were shown in Table 2. The inhibition of each microorganism by the seed extracts was investigated via monitoring the colorization change of resazurin dye after further 4-h incubation. The antimicrobial efficacies of *Mucuna* seed extracts were found in some extraction conditions, such as ethanol extraction of *M. monosperma* (ENE_Mm), ethyl acetate extraction of *M. pruriens* (EAE_Mp), hexane extraction of *M. pruriens* (HEE_Mp) and ethyl acetate extraction of *M. interrupta* (EAE_Mi) seeds. The MICs of ENE_Mm, EAE_Mp and HEE_Mp against *S. aureus* showed at 8.20, 4.10 and 4.10 mg/ml, while their MBCs were 16.40, 4.10 and 4.10 mg/ml, respectively. Moreover, ENE_Mn also revealed the antimicrobial activity to *S. epidermidis* and *C. albicans*, and the MICs and MBCs (MFCs) were 8.20 and 4.10 mg/ml of MICs and 16.40 and 8.20 mg/ml of MBCs/MFCs, respectively. EAE_Mi can inhibit *C. albicans* and the MIC and MFC were found at 8.20 and 16.40 mg/ml, respectively. However, no extraction condition showed the antimicrobial activity to kill *P. acnes*.

3.3 Bioactive compounds present in *Mucuna* seed extracts

CG-MS is the combination of two techniques including gas chromatography and mass spectrometry which is common technique to identify phytochemical constituents in plant extracts. GC chromatograms of ENE_Mm, EAE_Mi, EAE_Mp and HEE_Mp and were presented in Figure 1a-d, respectively.

After MS analysis, phytochemical compounds present in each *Mucuna* seed extract were further investigated. The identification and characterization of the compounds were based on their elution order in a GC column, and the retention time, name of the compounds and peak area (%) were then presented. Many phytochemical compounds were found in each seed extracts. Based on high amounts, the major compounds present in ENE_Mm were Dodecanamide/N-(2-hydroxyethyl)-lauramide (22.86%), Cytidine (14.60%), Bis(2-ethylhexyl) phthalate (13.45%) and Hexadecanoic acid (7.51%). EAE_Mi contained Bis(2-ethylhexyl) phthalate (24.30%) followed by Undecane (13.33%), Cyclotrisiloxane (12.69%) and 1,3,6,7-Tetramethyl-7,8-dihydrolumazine, 2-amino-6-cholo-3-cyano-5-(3-methyl-1-butenyl) pyrazine (12.35%).

The major compounds of EAE_Mp were 9,12-Octadecadienoic acid/ Linoleic acid (29.41%), Hexadecanoic acid/ Palmitic acid (27.90%) and 1,2,3-Propanetriol, monoacetate (13.82% + 11.63%). HEE_Mp had 9,12-Octadecadienoic acid (Z,Z)/ Linoleic acid (34.06%), Heptadecene-(8)-carbonic acid, 9-Octadecenoic acid/Oleic acid (18.38%), Hexadecanoic acid/ Palmitic acid (17.7%) and Octadecanoic acid/ Stearic acid (12.72%) as the top four major compounds.

Table 2 Antimicrobial activity of extracts of *Mucuna* seeds.

<i>Mucuna</i> seed extracts	<i>S. aureus</i>		<i>S. epidermidis</i>		<i>P. acnes</i>		<i>C. albicans</i>	
	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MBC (mg/ml)	MIC (mg/ml)	MFC (mg/ml)
Ethanol extraction								
<i>Mp</i>	-	-	-	-	-	-	-	-
<i>Mm</i>	8.20	16.40	8.20	16.40	-	-	4.10	8.20
<i>Mi</i>	-	-	-	-	-	-	-	-
<i>Mg</i>	-	-	-	-	-	-	-	-
<i>Mr</i>	-	-	-	-	-	-	-	-
Ethyl acetate extraction								
<i>Mp</i>	4.10	4.10	-	-	-	-	-	-
<i>Mm</i>	-	-	-	-	-	-	-	-
<i>Mi</i>	-	-	-	-	-	-	8.20	16.40
<i>Mg</i>	-	-	-	-	-	-	-	-
<i>Mr</i>	-	-	-	-	-	-	-	-
Hexane extraction								
<i>Mp</i>	4.10	4.10	-	-	-	-	-	-
<i>Mm</i>	-	-	-	-	-	-	-	-
<i>Mi</i>	-	-	-	-	-	-	-	-
<i>Mg</i>	-	-	-	-	-	-	-	-
<i>Mr</i>	-	-	-	-	-	-	-	-

Mp: *Mucuna pruriens*. *Mm*: *Mucuna monosperma*. *Mi*: *Mucuna interrupta*. *Mg*: *Mucuna gigantea*. *Mr*: *Mucuna revoluta*. Absent (-).

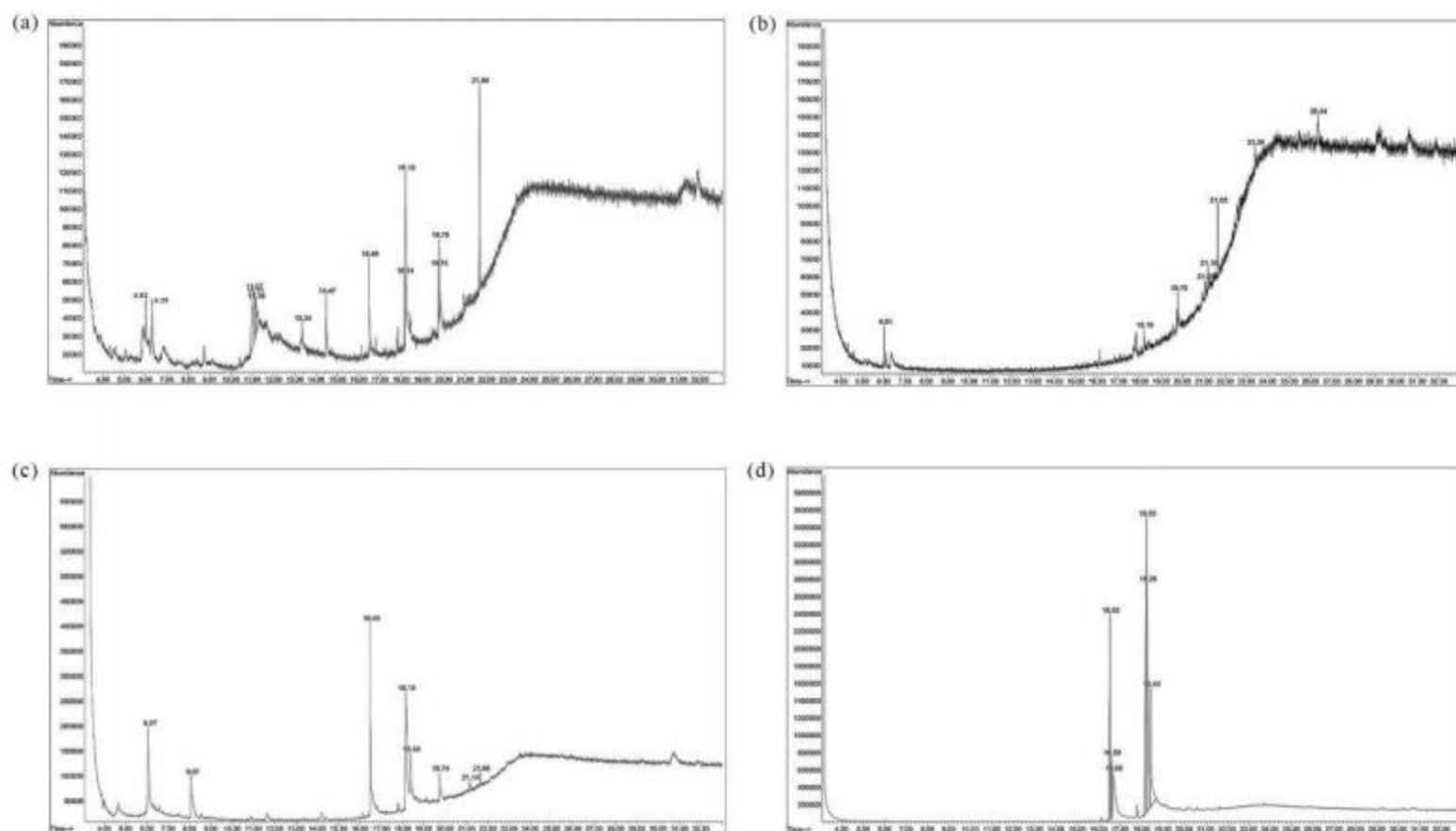


Figure 1 Seeds of *Mucuna* species. *Mucuna pruriens* (a), *Mucuna monosperma* (b), *Mucuna interrupta* (c), *Mucuna gigantea* (d) and *Mucuna revolute* (e).

4. Conclusion

This is the first report that antimicrobial activity and preliminary phytochemical screening in seed extracts of *Mucuna* species including *M. pruriens* (Mp), *M. monosperma* (Mm), *M. interrupta* (Mi), *M. gigantea* (Mg) and *M. revolute* (Mr) were evaluated. Some extraction conditions of these *Mucuna* seeds could provide the extracts that show antimicrobial activity, such as ENE_Mm, EAE_Mp, EAE_Mi and HEE_Mp. Among these extracts, ENE_Mm was the best candidate used for antimicrobials because this extract revealed a broad spectrum of antimicrobial activity against *S. aureus*, *S. epidermidis* and *C. albicans*. However, these extracts showed the lack of efficiency to kill *P. acnes*. Additionally, GC-MS analysis were used to evaluate phytochemical compounds in all antimicrobial seed extracts. Some phytochemicals, such as 9,12-Octadecadienoic acid/ Linoleic acid, Bis(2-ethylhexyl) phthalate and Dodecanamide/N-(2-hydroxyethyl)-lauramide, were expected to exhibit the antimicrobial activity.

5. Acknowledgements

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Formulation of facial toner containing northeastern vegetable extracts for anti-acne

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Abswtract

Toner is a clear solution that is applied on the face and wiped out to remove any remaining dirt from the facial wash. If cleaning the face is not sufficient clean, it may be the cause of acne. *Propionibacterium acnes* and *Staphylococcus aureus* are the most two common microbes that cause acnes. The objective of this research was to develop a facial toner containing some vegetable extracts with anti-acne property. Three vegetables from northeastern part of Thailand were screened for their anti-acne property including *Syzygium gratum* var. *gratum*., *Cratoxylum formosum* and *Centella asiatica*. The vegetables were dried, ground and extracted by maceration in 95% ethanol solution for 24 hr. The extracts were filtered and the solvent was removed by the rotary evaporator. The dried extracts were partitioned in hexane and ethyl acetate to remove chlorophyll in their compositions and freeze dried. Antimicrobial activity against *P. acnes* and *S. aureus* were investigated using Disc diffusion method. Minimum inhibitory concentrations of the extracts were evaluated by Broth dilution method. Results showed that *C. formosum* gave the highest inhibition zones against *P. acnes* and *S. aureus* 1.07 and 1.27 cm, respectively. Whereas *S. gratum* var. *gratum*. gave inhibition zone against *S. aureus* of 0.87 cm. The extract of *C. asiatica* had no inhibitory activity against both microbes. Therefore, the extract of *C. formosum* was selected for the formulation of facial skin toner. The developed toner had good physicochemical stability following 6 cycles of freeze thaw. Investigation of potential skin irritation of the toner by using HET-CAM assay model showed no sign of the irritation. In conclusion, the developed facial skin toner containing *C. formosum* extract for anti-acnes property has a potential to be furthered developed for commercialization.

Keywords: Anti-acne; Facial toner; *Propionibacterium acnes*; *Staphylococcus aureus*

1. Introduction

Acne vulgaris is one of the most common dermatological disease, affecting the majority of teenagers globally and the incidence is also frequently occurred in adults. Various factors including the presence of hormones, sebum, bacteria in the pilosebaceous units of dermis and keratinization of follicles have been associated with acne. The most two skin flora which trigger acne are *P. Acnes* and *S. aureus* (Fanelli, 2011). The pathogenesis of acne includes the induction of inflammatory responses, increased production of sebum, and hyperplasia of sebaceous glands, as well as changes in the lipid composition of sebum. Thus, insufficient skin cleaning may lead to inflammatory and noninflammatory of acne.

Treatment of acne with synthetic chemical medicines, such as antibiotics and steroids, may result in mild to severe side effect. Therefore, alternative and complementary recipe, such as herbal extracts

have been investigated but still lack of scientific data to support their efficacy. From our earlier research, some vegetables of northeastern regions exhibit some interesting activities for skin such as antioxidant and antimicrobial activity. Some of vegetables from northeastern part of Thailand including; *Syzygium gratum* var. *gratum* and *Cratoxylum formosum* contain abundant phenolic compounds and possess high antioxidation activity (Prasitpuriprecha *et al.*, 2009; Yingngam *et al.*, 2014). In addition, *Centella asiatica* has been used in post-acne formulation due to the wound healing activity (Somboonwong *et al.*, 2012).

Toner has a characteristic of clear solution that becomes an increasingly popular used to apply on skin and wiped out to remove residual dirt. Toner with the composition of some vegetable extracts that exhibits some antimicrobial activity will be the value added to the product. The objective of this study was to develop a facial toner containing some vegetable extracts with anti-acne property. The three vegetables; *Syzygium gratum* var. *gratum*., *Cratoxylum formosum* and *Centella asiatica* were investigated for their anti-acne property.

2. Materials and Methods

2.1 Materials

Fresh *Syzygium gratum* var. *gratum*., *Cratoxylum formosum* and *Centella asiatica*. harvested on July to August were purchased from Charoensri Market, Warinchamrab District, Ubon Ratchathani. *Propionibacterium acnes* DMST 14916 and *Staphylococcus aureus* ATCC 25927 were provided by Department of Medicinal Sciences, Ministry of Health, Thailand. Standard 5-*O*-caffeoylquinic acid was purchased from Acros Organics Company (New Jersey, USA). Acetonitrile HPLC grade and formic acid AR grade were purchased from Carlo Erba Company (Milan, Italy).

2.2 Plant extract

Plants were dried and ground to fine powder. Dried pulverized powders of the plants (150 g) were extracted with 95 % ethanol (2000 mL) at room temperature for 24 hours. The crude extract was filtered and organic solvent was removed using rotary evaporator (Buchi, Switzerland) at 50 °C under pressured of 150 mbar. After that the crude extracts were dissolved with deionized water. Chlorophyll was removed by partition method with hexane and ethyl acetate, respectively. The organic solvents were taken away under rotary evaporator and the extracts were dried out by freeze dry method.

2.3 Antimicrobial activity test

The antimicrobial activities of the -extracts were evaluated using inhibition zone to *P. acnes* and *S. aureus* by Disc diffusion method as described by Prasitpuriprecha *et al.*, (2009). *P. acne* and *S. aureus* were cultured on thioglycollate agar by using anaerobic jar and Muller Hilton agar, respectively. The turbidities of the microbial suspensions were adjusted to 1.5×10^8 CFU/mL compared with that of the McFarland no.0.5 at the wavelength 625 nm. Disc diffusion antimicrobial test was conducted by transferring the microbials on to brain heart infusion (BHI) agar and incubating at 37°C for 24 hours. Tetracycline and ampicillin were served as the positive controls.

Minimum inhibitory concentrations (MIC) of the extracts were conducted by broth dilution method. The microbials were cultured in BHI broth with the extracts of 12.5 to 200 mg/mL in 37°C for 24 hours. Tetracycline was served as the positive controls.

2.4 High performance liquid chromatography analysis

The sample was quantitative analyzed by HPLC (Dionex Ultimate 3000, Thermo Fisher Company, USA) with C-18 column (250 x 4.6 mm, 5 µm diameter). Mobile phase was concentration gradients between acetonitrile and 0.1 % formic acid at 0.7 mL/min. Chromatogram of was detected by UV-DAD at wavelength 325 nm.

2.5 Toner formulations

The toner formulation was prepared in composition of crude extract (5 mg/mL), co-surfactant, humectants and deionized water. The formulation was investigated in term of stability and skin irritation.

2.6 In vitro eye and skin irritation test

Eye and skin irritation of crude extract and toner formulation were evaluated by Hen's Egg Test on the Chorioallantoic Membrane (HET-CAM) assay followed ECVAM (1999) protocol. 0.1 N NaOH and 0.9% sodium chloride solution served as positive and negative control, respectively.

2.7 Stability test

The stability of formulation was investigated by heating-cooling stability test. The toner was stored for 48 hours per cycle at 5 and 40°C for 6 cycles. Color change and pH of formulation were evaluated as physical stability. Active compound in the extract was analyzed using HPLC.

3. Results and Discussion

3.1 Vegetable extraction

After remove chlorophyll and organic solvents, the yields of the extraction as shown in Table 1. *C. formosum* and *C. asiatica* gave higher yield than *S. gratum*.

Table 1 Extraction yield of vegetables

Vegetable	Extraction amount (g)	% yield based on dry weight
<i>S. gratum</i>	3.73	2.49
<i>C. formosum</i>	6.76	4.51
<i>C. asiatica</i>	6.59	4.39

3.2 Antimicrobial activities

The antimicrobial activities investigated by disc diffusion method against *P. acne* and *S. aureus* which are the common microbials trigger acne. *C. formosum* demonstrated significantly higher activity against both microbials with the clear zones were more than 1 cm (Table 2) whereas *S. gratum* shown clear zone against only *S. aureus* more than 0.7 cm and no clear zone in the plate of *P. acne*. *C. asiatica* did not show antimicrobial activity against both *P. acne* and *S. aureus*.

Table 2 Antimicrobial activities represented as clear zone (cm) of disc diffusion test and MIC by broth dilution method.

Extract / control	Clear zone (cm)		MIC (mg/mL)	
	<i>P. acne</i>	<i>S. aureus</i>	<i>P. acne</i>	<i>S. aureus</i>
<i>S. gratum</i>	0.00 ± 0.00	0.87 ± 0.06	N/A	N/A
<i>C. formosum</i>	1.07 ± 0.06 *	1.27 ± 0.06 *	25	25
<i>C. asiatica</i>	0.00 ± 0.00	0.00 ± 0.00	N/A	N/A
Tetracycline	2.37 ± 0.21	2.47 ± 0.06	+	+
Ampicillin	0.47 ± 0.06	3.87 ± 0.12	-	-

Mean ± S.D. (n=5) Positive control of MIC was 100 µg/mL tetracycline; N/A = not available

* Significant difference among crude extracts in the same column at $p < .05$

MIC of the extracts tested by broth dilution method demonstrated the lowest concentration of *C. formosum* at 25 mg/mL which both *P. acne* and *S. aureus* broth did not express turbidity. On the other hand, all concentration of *S. gratum* and *C. asiatica* shown turbidity in the broth represented that the test concentration of them did not available inhibition concentration.

From these results *C. formosum* extract was selected for further toner development.

3.3 HPLC analysis the active compound in *C. formosum*

C. formosum ethanol extract contained 5-*O*-caffeoylquinic acid (Yingngam et al, 2014). Caffeoylquinic acids are phenolic compound which has been proven antioxidant activity (Yingngam et al, 2015; Liang and Kitts, 2016). This compound composes in many fruits, herbs and vegetables and possesses anti-inflammatory activity and protective against oxidative stress. The extract in this study was analyzed for the amount of 5-*O*-caffeoylquinic acid by HPLC. Chromatograms of *C. formosum* extracts containing standard compound are shown in Figure 1. The compound was extract by ethanol of higher amount than water. The biomarker composed in crude ethanol extract was 94.33 ± 0.73 mg/g crude extract.

Although 5-*O*-caffeoylquinic acid was not investigated for its antimicrobial against *P. acne* in this study, the activity against *S. aureus*, *Escherichia coli*, *Enterococcus faecium*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *Candida albicans* has been reported earlier which showed the strongest activity against *Klebsiella pneumonia* (Bajko et al., (2016). This research aimed at the potential development of *C. formosum* extract to be a facial toner with anti-acne property.

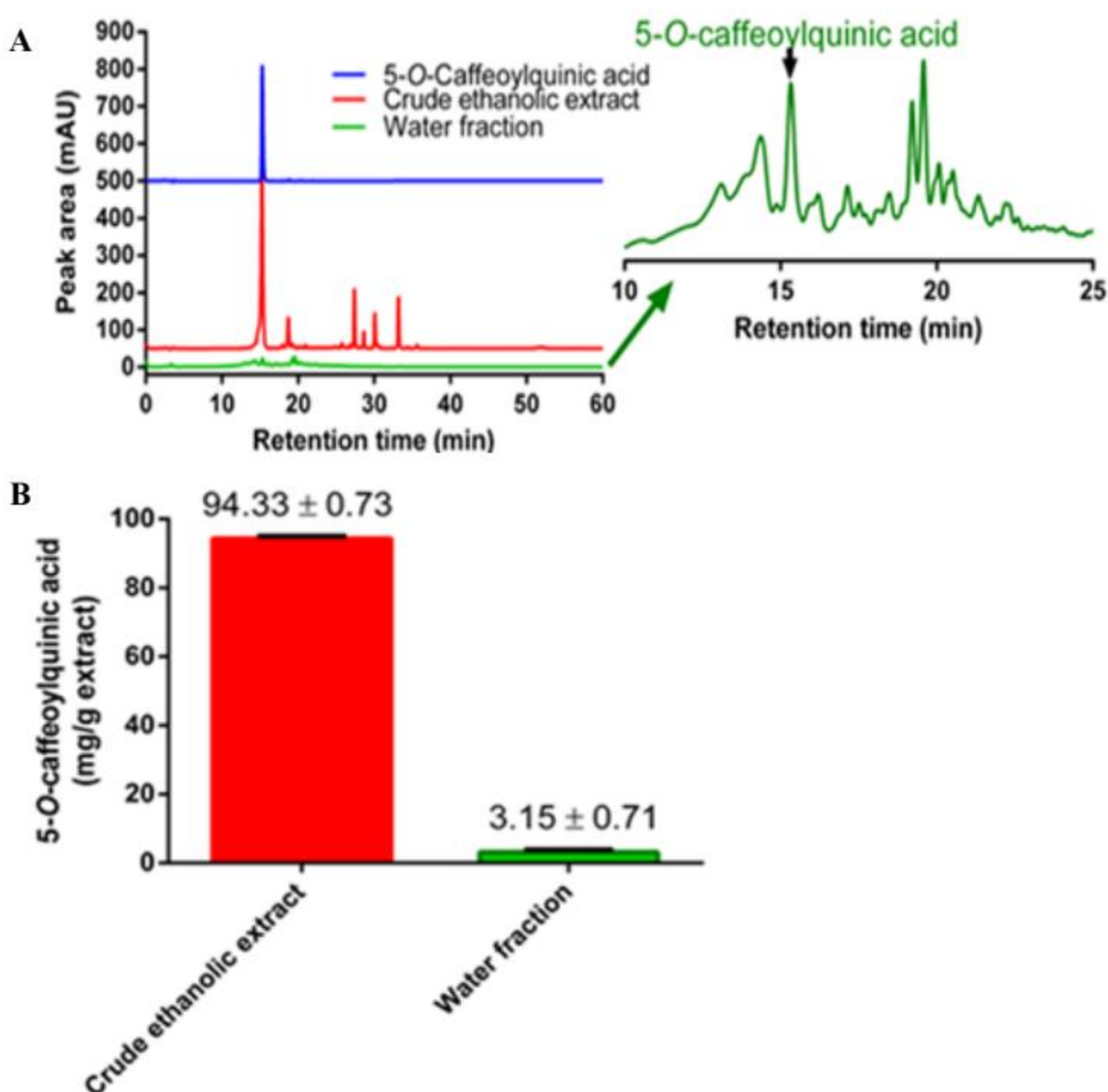
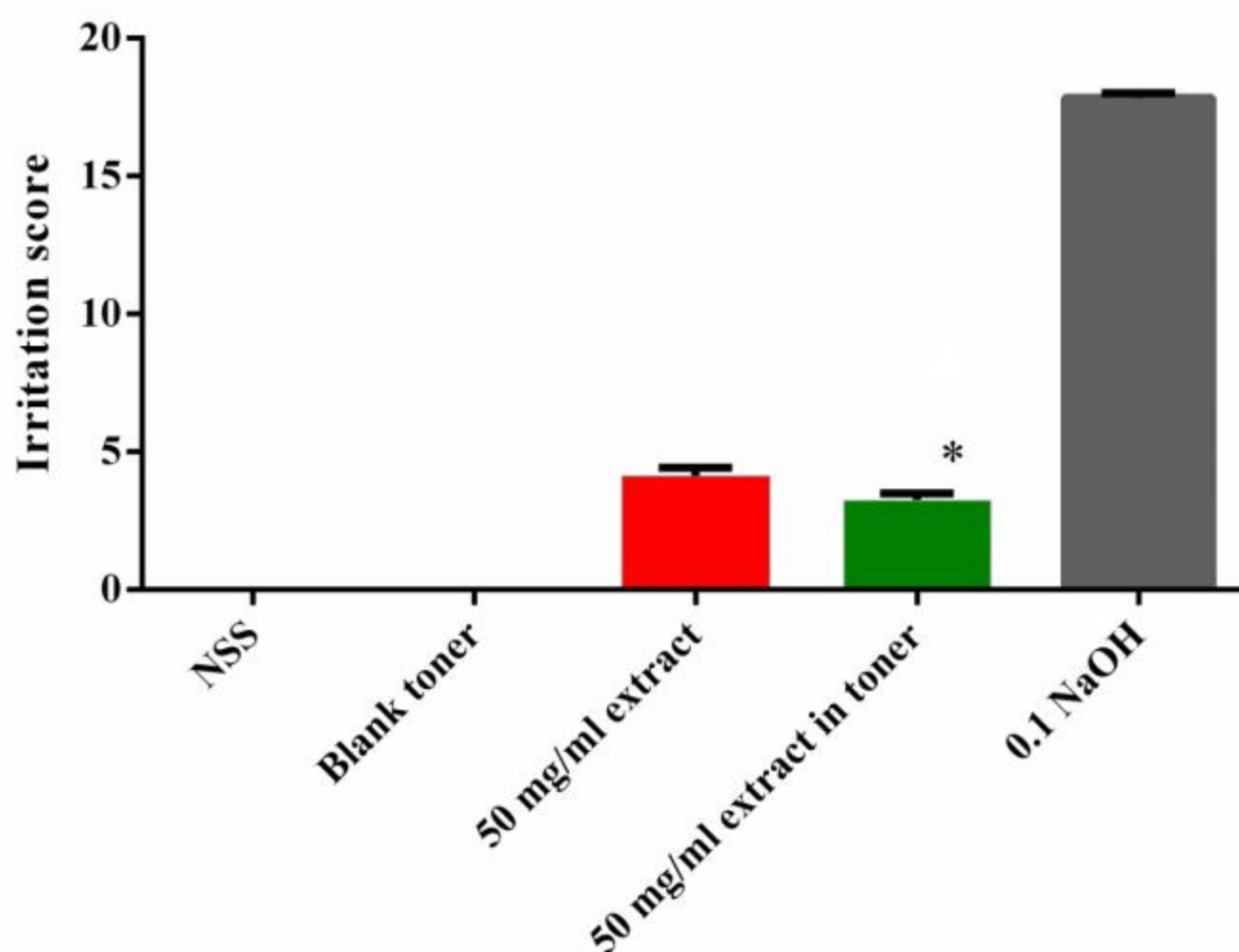


Figure 1 HPLC chromatograms(A) and amount (B) of standard 5-*O*-caffeoylquinic acid in *C. formosum* extract**3.4 Eye and skin irritation tests**

Face skin and eye are high sensitive organs to physical and chemical stimulants. HET-CAM is the *in vitro* test mimic vascular system irritation includes lysis, hemorrhage and coagulation; and calculated to irritation score. All test samples showed hemorrhagic effect in the hen's egg without blood vessel lysis and coagulation. Toner of *C. formosum* exhibited significantly lower irritation than the free extract compound (Figure 2).

**Figure 2** Irritation score of *C. formosum* extract and toner containing *C. formosum* extract**3.5 Toner formulation stability**

Toner contained *C. formosum* extract shown clear solution with low pH (pH 3). After 6-cycle freeze-thaw testing, the physical properties were not change (Table 3) indicate the stability of the formulation. Simultaneously, concentration of 5-*O*-caffeoylquinic acid in toner was not different after freeze-thaw for 6 cycles (data not shown).

Table 3 Physical properties of toner contained *C. formosum* extract before and after freeze-thaw method

Physical properties	Before	After
Appearance	Clear	Clear
Color	Brown	Brown
pH	3	3

4. Conclusion

The vegetables from northeastern part of Thailand, *C. formosum* extract demonstrated an antimicrobial activity against *P. acnes* and *S. aureus*. The developed facial skin toner containing *C.*

formosum extract for anti-acne property had a potential to be developed as commercial cosmetic product. Clinical trial should be further studied to see the risk and benefit of the product before commercialization.

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Efficacy of body wrap and slimming lotion for cellulite treatment

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Abstract

Cellulite refers to the dimple-like skin texture on thighs, hips or buttocks that leads to cosmetic problems. Currently, there are many methods that have been used for cellulite reduction. This study aimed to evaluate the efficacy of body wrap and slimming lotion containing L-carnitine, caffeine, *Fucus vesiculosus* and *Centella asiatica* extracts for cellulite treatment. The subjects in this study were 12 women who had cellulite problems at grade 1 to 3. The subjects were applied slimming lotion twice-daily plus receiving control body wrap 3 sessions/week for 4 weeks on their right thigh (treatment A). In addition, they were applied control lotion twice-daily plus receiving slimming body wrap 3 sessions/week for 4 weeks on their left thigh (treatment B). The control and slimming lotion were suitable for skin with no irritation. The satisfaction of lotions was evaluated by 5 points Likert scale. Most of the subjects were more satisfied with control lotion than slimming lotion. Skin efficacy was assessed by 5 parameters; standard visual scale, thigh circumference, skin surface (smoothness and roughness) by visioscan® VC 98, skin elasticity by cutometer® MPA580, and skin moisture by corneometer® CM 825. The results showed that treatment A improved all parameters more than treatment B at week 4 compared to those at baseline, which implied the improvement of cellulite severity. It can be concluded that twice-daily using slimming lotion was more effective for cellulite treatment than applying slimming body wrap 3 sessions/week.

Keywords: Body wrap; Cellulite treatment; Skin efficacy; Slimming lotion; Thigh

1. Introduction

Cellulite refers to a skin texture that is similar to orange peel surface waves, which occur in some areas of the body. It appears often in females than males because cellulite is directly related to the hormones estrogen (Christensen, 2014). The accumulation of cellulite is commonly found around the waist, hips, and thigh areas (Leelapornpisid, 2003). The studies report that cellulite associated with changes in the structure of the fat (fat lobules) in the subcutaneous fat layer, the wall of the connective tissue (connective tissue septa), changes of the blood vessels (vascular change), and lymph and inflammatory-related factor (Pugliese, 2007). The causes of cellulite are metabolic imbalance, genetic factors, hormonal factors, diet and lifestyle which play an important role in causing excess fat (Yupakorn, 2010). Cellulite treatment is currently available to control the factors that cause cellulite such as exercise, massage, or the use of treatment tools. In addition, the use of topical products such as creams or gels are the most popular method because it does not cause pain and scars. It can be seen that there are many beauty business nowadays. With the growth of this business, products and tools that help to reduce cellulite have been developed (Paksri, 2015).

Anti-cellulite agents are divided into 4 groups; 1) agents that increase microvascular flow, 2) agents that reduce lipogenesis and promote lipolysis, 3) agents that restore the normal structure of the

dermal and subcutaneous tissue, 4) agents that prevent or destroy free-radical formation (Hexsel and Soirefmann, 2011; Rona *et al.*, 2006). Most anti-cellulite products focus on improving the structure of the skin and connective tissue, and some ingredients have been described to adjust the storage of fat in adipocytes; for examples, caffeine is well-known that it stimulates the release of triglycerides which can stimulate the transfer of free fatty acids into the mitochondria to produce energy (Roure *et al.*, 2011). L-carnitine is generated in the body and can be found in the liver and kidneys. It increases the use of fat by transporting fatty acids into the mitochondrion, which is the source of cellular energy. Brown seaweed extract (*Fucus Vesiculosus*) can improve the circulation of blood vessels. It accelerates the excretion of waste. *Centella Asiatica* extract is used to tighten skin (Leelapornpisid, 2003). It helps to increase microvascular flow and has antioxidant and anti-cellulite properties (Hashim, 2011). Body lotions are alongside creams (Malgorzata *et al.*, 2015). They can be used as a medium and convey the substance that has the properties to the breakdown of fat. They combined with massage and a foil wrap or plastic wrap is another method which is widely used for shaping a spectacular skin due to the tight skin, tissue, the pressing skin and tissue to the liquid, and leaning out of the body to drive toxic substances. It also helps to reduce the appearance of orange peel and the skin flabby or sagging skin (Williams, 2006). The massage may increase the efficiency of anti-cellulite topical products because the massage helps increase the absorption of the cream or lotion (Leelapornpisid, 2003). Therefore, this study aimed to evaluate the efficacy of body wrap and slimming lotion containing L-carnitine, caffeine, *Fucus vesiculosus* and *Centella asiatica* extracts for cellulite treatment. The skin moisture, skin elasticity, skin surface image, visual scale, tight circumference and body mass index, were investigated as parameters for cellulite treatment.

2. Materials and Methods

2.1 Materials

Chemicals and material used in this study were the extract of Caffeine, L-carnitine (Chanjao Longevity Co., Ltd., Bangkok, Thailand) *Fucus Vesiculosus* extract and *Centella Asiatica* extract (S. Chemipan, Bangkok, Thailand) and plastic sheet (MMP Co., Ltd., Bangkok, Thailand).

2.2 Screening questionnaire for development of slimming product

There were 50 subjects aged between 18-23 years old from Mae Fah Luang University (MFU) responded the screening questionnaire. Questionnaire composed of questions about slimming products, slimming body wrap, and cellulite grade of the respondents. The results from questionnaire were useful for developing new product.

2.3 Formulation of slimming lotion

Control lotion and slimming lotion were prepared by cold process method. Overall oil phase (part A) was mixed with water phase (part B) with continuous homogenizing at room temperature. Then, part C was added into the formulation under homogenizing until homogeneous lotion. The control lotion was prepared by the same method without caffeine, L-carnitine, *Fucus vesiculosus* extract and *Centella asiatica* extract as shown in Table 1. The physical appearances of lotions were observed by naked eye. A pH meter (pH, Mettler Toledo) was used for the evaluation of pH value. A viscosity was evaluated by viscometer (Brookfield/ RVDV2T extra, USA) with a needle No. # 5, speed around 40 rpm for 30 seconds.

2.4 Skin irritation test

The skin irritation test was performed in 12 subjects who had no history of allergy and abnormal skin. Close patch tests (Finn chambers®) were used on inner arm of the volunteer, which contained test substances (20µL) for 24 hours. Then subjects were observed for any irritating reaction (erythema and edema) at 24 hours after removal of the patch. If there is redness to observe in the next 72 hours, the change of the skin will be recorded. The data collection of the skin irritation was analyzed using Draize scoring system to calculate the primary dermal irritation index (PDII) (Jirova *et al.*, 2008). The test substances were 1% sodium lauryl sulphate (SLS) as a positive control, distilled water as a negative control, control lotion and slimming lotion.

Table 1 Formulation of control lotion and slimming lotion

Part	Ingredient	Content (% w/w)		Function
		Control lotion	Slimming lotion	
A	Polyacrylamide & laureth-7	5.2	5.2	Emulsifier
	Hydrogenated polydecene			Emollient
B	Butylene glycol	90.7	84.7	Humectant
	Glycerin			Humectant
	DI Water			Diluent
	Phenoxyethanol			Preservative
	Allantoin	-	2	Soothing agent
	Carbopol 21			Stabilizer
	Caffein			Active
	L-carnitine			Active
C	<i>Fucus Vesiculosus</i> extract	-	1	Active
	<i>Centella asiatica</i> extract	-	2	Active
	Cyclopentasiloxane	4.1	4.1	Emollient
	Dimethicone			Occlusive
	Vitamin E acetate			Antioxidant
	Fragrance			Fragrance

2.5. Evaluation of anti-cellulite efficacy

2.5.1 Study design

Figure 1 showed the flowchart of the study enrollment. Experimental research with before - after design. A total of 12 healthy females who had a cellulite grade I- III on thighs were recruited. Grade I cellulite is visible when the skin is pinched or muscles are contracted. Grade II cellulite is visible while standing. Grade III cellulite is visible orange peel or mattress appearance while lying down with the presence of stage II plus raised areas and nodules (Nootheti *et al.*, 2006). Moreover, they were enrolled in the study on the following inclusion criteria; no history of cosmetic product allergy, no high blood pressure or heart condition, and not pregnant. Subjects who were not using slimming method or any cellulite treatment for a washout period of 6 months. No other products were used on each thigh during the trial period.

2.5.2 Treatment

Treatment A, right thigh was applied slimming lotion twice daily plus plastic wrapping with control lotion for 3 sessions/week within 4 weeks (total of 12 treatments). Treatment B, left thigh was applied control lotion twice daily plus plastic wrapping with slimming lotion for 3 sessions/week within 4 weeks (total of 12 treatments). Before using a body wrap, the subjects were instructed to apply the lotion (control lotion and slimming lotion) on their thighs by 3 techniques; 1) gently massage by effleurage technique (massage strokes along the length of the weight of the hand above the knee to the thigh), 2) thumb stroking technique (massage rolled legs), 3) pinching technique (use an alternating hands lift small amounts of tissue between the first finger and thumb in quick and gentle pinching movements), and effleurage technique again, respectively (Brown, 2010) (Figure 2). Each step was repeated 10 times and then applied a plastic wrap 3 rounds (Jaipakdee *et al.*, 2015) for 30 minutes by the professional massage therapist (Ward and Cartwright, 2004).

2.5.3. Product and service satisfaction test

The satisfaction test was conducted by questionnaire which answered by 12 subjects. For the part of product satisfaction, the questions were considered product characteristics. The levels of satisfaction were evaluated by 5 points Likert scale. The assessment contained 5 levels which were

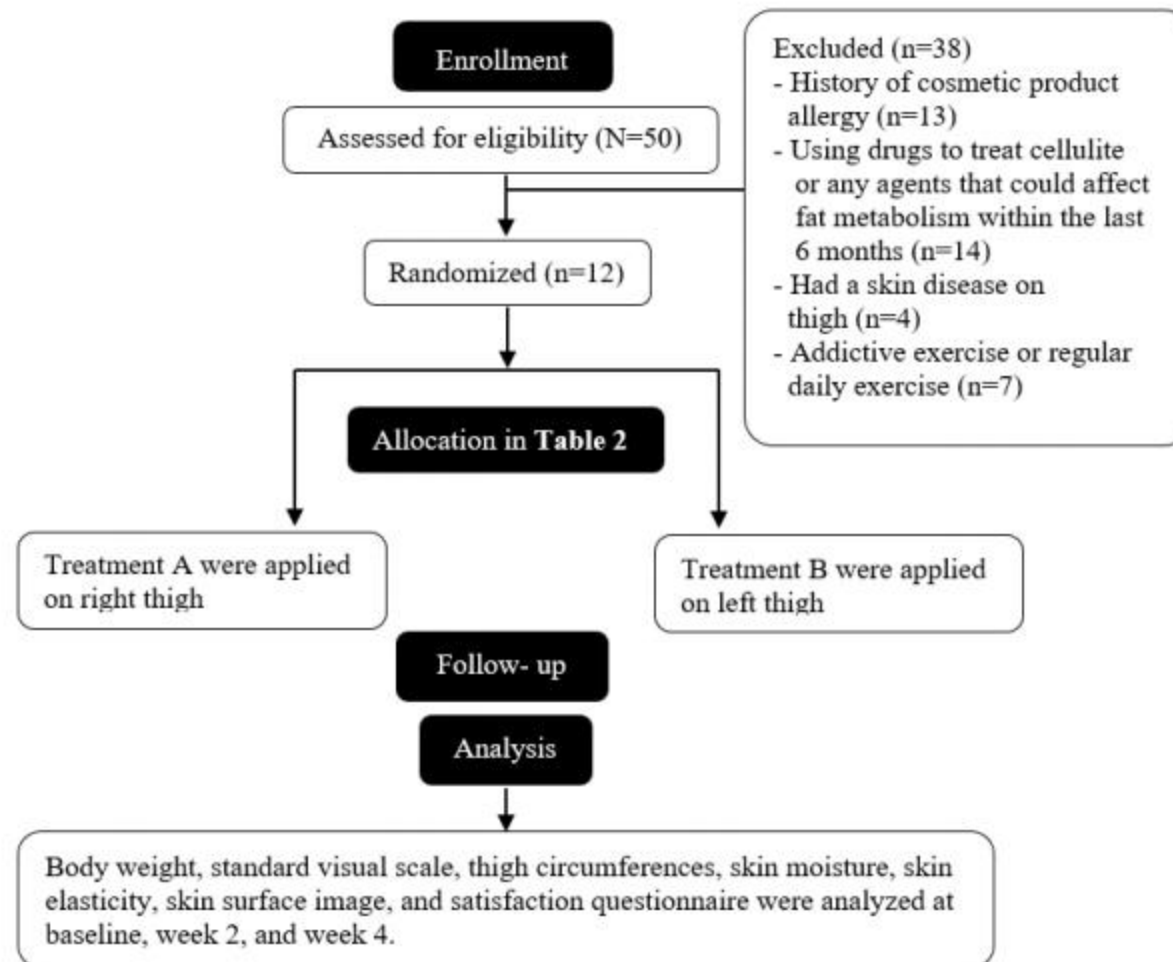


Figure 1 Flowchart of study enrollment.

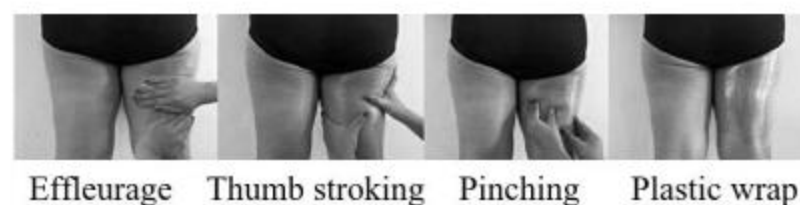


Figure 2 The massage steps for cellulite treatment

very high satisfying, high satisfying, medium satisfying, low satisfying and very low satisfying as following:

Average value	Satisfaction level
4.21-5.00	Very high
3.41-4.20	High
2.61-3.40	Medium
1.81-2.60	Low
1.00-1.80	Very low

2.5.4 Assessments

Skin efficacy assessments were analyzed at baseline, week 2, and week 4 including body weight, a standard visual scale, thigh circumferences, skin moisture, skin elasticity, skin surface image, and satisfaction questionnaire.

The thigh circumferences were measured by measuring tape. Subjects were standing in standardized upright position (Dupont *et al.*, 2014). The thigh circumference measurements were taken on both legs at 18 cm and 28 cm from the superior pole of the patella for the lower and upper thigh (Rao *et al.*, 2005).

The degree of skin elasticity and moisture was measured by cutometer® MPA580 (Courage & khazaka, colong, germany) and corneometer® CM 825, respectively at baseline, week 2, and week 4. Subjects stayed at control room $20 \pm 2^\circ\text{C}$ for maintaining balance of body temperature. The relative humidity of air at 40-60 percent determines the area to be tested. The tested skin is 4×4 square centimeters for measuring the quality of the skin (Suwansichon *et al.*, 2015). A cutometer® MPA580 and corneometer® CM 825 were used 3 times on thighs for averaging the result (Panyakaew, 2015). For cutometer® MPA580, R2 values is indicated skin elasticity. Corneometer® CM 825 showed the

degree of moisture (arbitrary unit) which were very dry (<35), dry (35 to 50), and sufficiently moisturized (>50).

The visioscan® VC 98 showed the image of the skin appearance. It analyses the grey level distribution and allows the calculation of skin parameters to quantitatively and qualitatively describe the skin surface as an index: skin smoothness (SEsm) and skin roughness (SEr) (Fanian *et al.*, 2013).

2.6. Statistical analysis

Data were expressed as mean value $\pm$ SD (standard deviation). The measured different values of efficacy were analyzed by a paired t-test and $P < 0.05$ was considered as statistically significant. Statistical analysis was performed using the SPSS system ver. 21.0.

3. Results and Discussion

3.1 Screening questionnaire for development of slimming product

From the Table 2, the results presented that people have ever used a slimming product (28 %) and never used a slimming product (72 %). The most preferred ingredients in slimming product was plant extracts (71.4 %), followed by synthetic chemical (21.4%) and animal extracts (7.1 %). The most favorite dosage form was lotion (66%), followed by cream (64%), spray (18.4%), mask and oil (6.1%). The most favorite odor was fresh (46%), followed by natural (42%), relax (34%), romantic and flower (26%), and fruit (14%). Most of subjects interested in using body wrap because they want to reduce body fat (52%), firming (50%), nourishing skin (28%), stimulating blood circulation (26%) and detox (18%). As the results of questionnaire were shown the most of subjects interested in body wrap treatment (58%). The results of questionnaire were useful for developing slimming product in this study.

3.2 Formulation of slimming lotion

The result of slimming lotion and control after the fresh preparation showed white shine texture with no phase separation, and precipitate. The pH and viscosity values of control were 6.24 and 10,504 cP, while the slimming lotion were 5.57 and 7,865 cP, respectively.

3.3 Skin irritation test

Skin irritation test was observed from 12 healthy female volunteers. The tested skin area with DI water (negative control), control lotion and slimming lotion did not appear any erythema and edema formation in any subjects. 1% SLS showed slightly irritation. The results suggest that the slimming lotion and control lotion were considered non-irritating products (PDII = 0).

3.4 Evaluation of anti-cellulite efficacy

The efficacy parameters such as skin moisture, skin elasticity, skin surface image, visual scale, thigh circumference, and body mass index were recorded at baseline (before treatment), after 2 weeks and 4 weeks of treatment. The mean values of the body weight and BMI did not change significantly during the trial period (Table 3). Although, the food consumption and lifestyle of subjects were not controlled.

The standard visual scale graded by 2 independent investigators improved precisely when compared between at baseline and week 4 at right thigh (treatment A) and left thigh (treatment B). It showed that right thighs and left thighs were changed by observing with naked eye at week 4.

The means of thigh circumference of right thighs at baseline, week 2 and week 4 were 57.83, 56.58, and 54.75 cm, respectively, while the left thighs were 57.83, 56.96, and 55.13 cm, respectively (Figure 3). The mean circumference of both thighs decreased significantly by 3.08 cm (right thigh) and 2.70 cm (left thigh) at week 4 compared to baseline ($P < 0.05$). Thus, treatment A had efficacy more than treatment B.

Visioscan acquired images which were subsequently analyzed with visioscan. The resulting photograph showed the differences in skin photography of the skin surface on left thigh by applying treatment B and right thigh by treatment A before and after 2 weeks, and 4 weeks (Figure 4). The skin surface on both thighs were significantly improved in terms of smoothness and roughness after treatment. Based on the comparison of the right thighs with treatment A, the average skin roughness

(SEr) of the baseline, week 2, and week 4 were 1.01 ± 0.21 , 0.96 ± 0.21 , and 0.92 ± 0.21 , while the SEr of the left thighs with body wrap were 1.01 ± 0.2 , 0.98 ± 0.19 , and 0.93 ± 0.18 , respectively. The comparison of the right thighs with treatment A, the skin smoothness (SEsm) of the baseline, week 2, and week 4 were 28.68 ± 1.77 , 28.73 ± 1.77 , and 28.77 ± 1.76 , while the left thighs with treatment B were 29.05 ± 1.14 , 29.09 ± 1.14 and 29.12 ± 1.14 (Table 3). Since, the slimming and control lotions consist of humectants; butylene glycol and glycerin that help to prevent moisture loss thereby retaining the skin's natural moisture. Hydrogenate polydecene, dimethicone and cyclopentasiloxane are emollients that provide skin softening and smoothing properties. In addition, emollients showed very good spreadability on the skin and provided a satiny and non-greasy feeling to the skin. Typically, they are non-allergic and non-irritant. Vitamin E acetate has excellent antioxidant, skin moisturizing and soothing properties (Barel *et al.*, 2014). Therefore, both formulas provided smoother skin and improved skin roughness.

Table 2 General information of slimming products and body wrap behavior of 50 MFU students from questionnaires

Parameter	Frequency (N=50)	Percent (%)
Ever use a slimming product or not?	36	72
Never	14	28
Ever		
*Ingredient	10	71.4
Plant extracts	6	21.4
Synthetic chemical	2	7.1
Animal extracts		
*Dosage form	33	66
Lotion	32	64
Cream	9	18.4
Spray	3	6.1
Mask	3	6.1
Oil		
*Odor of product	23	46
Fresh	20	42
Natural	17	34
Relax	13	26
Romantic	13	26
Flower	8	14
Fruit		
Ever use a body wrap or not?	46	92
Never	4	8
Ever		
Interested body wrap or not?	29	58
Interest	21	42
No interest		
Benefits of body wrap	27	52
Reducing body fat	25	50
Firming	14	28
Nourishing skin	12	26
Stimulating blood circulation.		
Detox	9	18

* Subjects can answer more than one choice

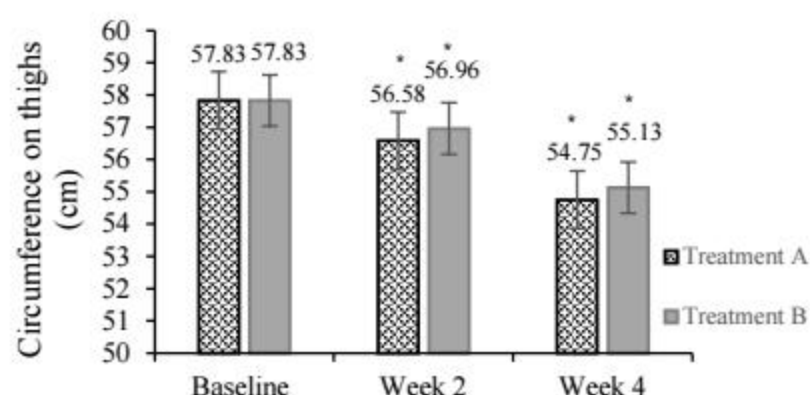


Figure 3 Thigh circumferences at baseline, week 2, and week 4

The skin elasticity (R2) can be measured by cutometer®. The means of skin elasticity raised by 0.03 on right thigh and 0.02 on left thigh at week 4 compared to baseline (Figure 5). So that, treatment A had higher efficacy than treatment B. The results suggested that skin elasticity was increased on both thighs with no significant difference ($P>0.05$) between baseline and week 4. The skin elasticity did not change obviously in either right thigh or left thigh because of the age of the subjects (18-23 years). The ages associate with the theories of skin aging and hormone. The skin begins to lose more collagen, according to ages and higher level in relation with the hormone. The skin is an organ for various hormones, especially estrogen (Thornton, 2013) which helps increase the collagen, increase the thickness of the skin and strengthen blood vessels (Agius, 2007), in subjects' ages > 35 years (Panyakaew, 2015) when using lotion that composed of emollients and humectants ingredients which help boost skin elasticity show a little change due to the fact that age of the hormone was complete.

Table 3 Comparative analysis of body weight, BMI, skin roughness (SEr) and skin smoothness (SEsm) between treatment A and treatment B

Parameter	Baseline	Week 2	Δ Change ¹ (mean $\pm$ SD)	Week 4	Δ Change ² (mean $\pm$ SD)
Body weight	61.7 $\pm$ 14.61	61.72 $\pm$ 14.1	0.02 $\pm$ 0.64	61.71 $\pm$ 13.93	0.01 $\pm$ 1.28
BMI	24.16 $\pm$ 5.91	24.16 $\pm$ 5.71	0 $\pm$ 0.23	24.18 $\pm$ 5.70	0.02 $\pm$ 0.47
Treatment A					
SEr	1.01 $\pm$ 0.21	0.96 $\pm$ 0.21*	-0.05 $\pm$ 0.03	0.92 $\pm$ 0.21*	-0.09 $\pm$ 0.04
SEsm	28.68 $\pm$ 1.77	28.73 $\pm$ 1.77*	0.05 $\pm$ 0.05	28.77 $\pm$ 1.76*	0.09 $\pm$ 0.06
Treatment B					
SEr	1.01 $\pm$ 0.2	0.98 $\pm$ 0.19	-0.03 $\pm$ 0.02	0.93 $\pm$ 0.18*	-0.08 $\pm$ 0.04
SEsm	29.05 $\pm$ 1.14	29.09 $\pm$ 1.14	0.02 $\pm$ 0.01	29.12 $\pm$ 1.14*	0.06 $\pm$ 0.07

¹ Δ change was calculated as result from 2 weeks of treatment - result from baseline.

² Δ change was calculated as result from 4 weeks of treatment - result from baseline.

*Different significantly when compare at baseline ($P<0.05$).

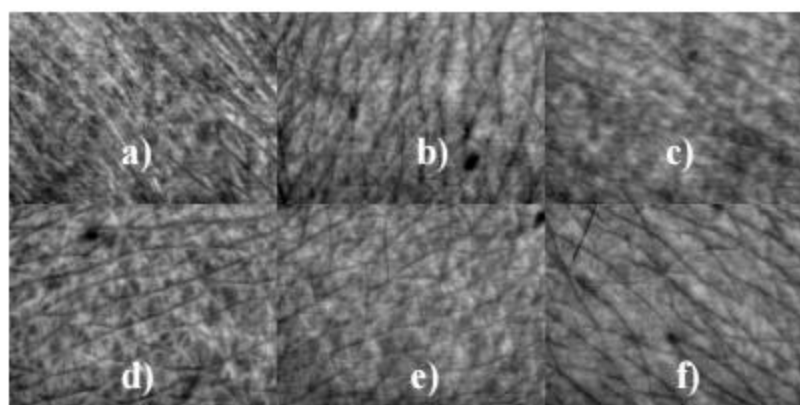


Figure 4 The visioscan images of the skin surface on left thigh (a,b,c) by applying treatment B and right thigh (d,e,f) by treatment A before and after 2 weeks, and 4 weeks.

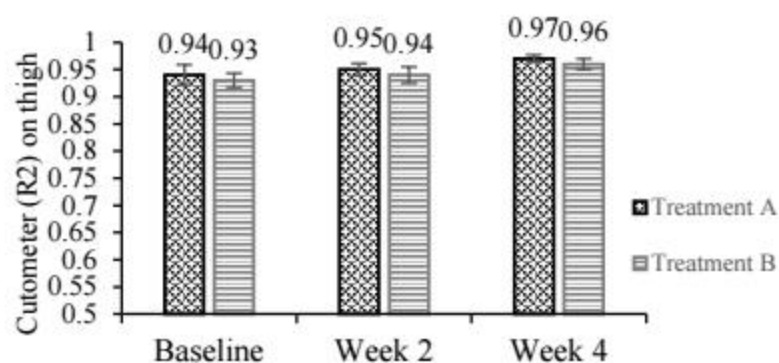


Figure 5 Skin elasticity measured by Cutometer on right thigh and left thigh increased at week 2 and week 4 compared to baseline.

Skin moisture was measured by corneometer®. This was accomplished by measuring the electrical capacitance of skin (which is related to water content in the stratum corneum). The skin moisture increased significantly on both thighs. The means of skin moisture on right thighs increased markedly by 19.23 and 38.1 at week 2 and 4 compared with baseline, while the left thighs increased by 15.4 and 30.37 at week 2 and 4 compared with baseline, respectively (Figure 6). Thus, treatment A had efficacy more than treatment B. Both treatments presented many moisturizing ingredients such as butylene glycol, glycerin, hydrogenate polydecene, cyclopentasiloxane, dimethicone and vitamin E acetate that help to increase skin moisture.

At the end of the study, standard visual scale, thigh circumference, skin surface (smoothness and roughness), skin elasticity, and skin moisture increased at week 4 compared to those at baseline which indicated to improve cellulite severity. Since, the clinical trials of topical product contain caffeine help inhibit phosphodiesterase (PDE) so that cAMP is not converted to AMP, allowing it to promote the breakdown of fat, L-carnitine is a conditionally-essential amino acid necessary for energy and fat metabolism. It is synthesised in the liver by the amino acids lysine and methionine when into body distribution to surround tissue can turn fat to energy cause decrease cholesterol and triglyceride. *Fucus vesiculosus* extract is a brown marine algae that contains sulfated polysaccharides, iodine compounds, and alginic acid. It is reported to produce contraction of the dermal connective tissue through the increased expression of integrin molecules. An increasing of dermal density is the likely mechanism by which this agent improves cellulite. It also has a stimulating effect on vascular flow. *Centella asiatica* extract contains triterpenes that accelerate lysine and proline (two fundamental amino acids in collagen structure) metabolism, increase tropocollagen synthesis, and stimulate connective tissue mucopolysaccharide exchange for cellulite treatment (Maslen and Bird, 2006; Foster, 2004). The use of plastic sheet for wrapping body also contributes to the treatment of cellulite, because it can remove the toxins from the body to drive toxins. The lymphatic flow helps the lymphatic flow better and helps cleanse the toxins at the same time. It also helps to reduce the orange peel and the skin flabby or sagging skin (Williams, 2006). Moreover, massage before applying body wrap is also used to remove interstitial fluid and accelerate lymphatic drainage, which leads to the diminution of cellulite (Byun *et al.*, 2015).

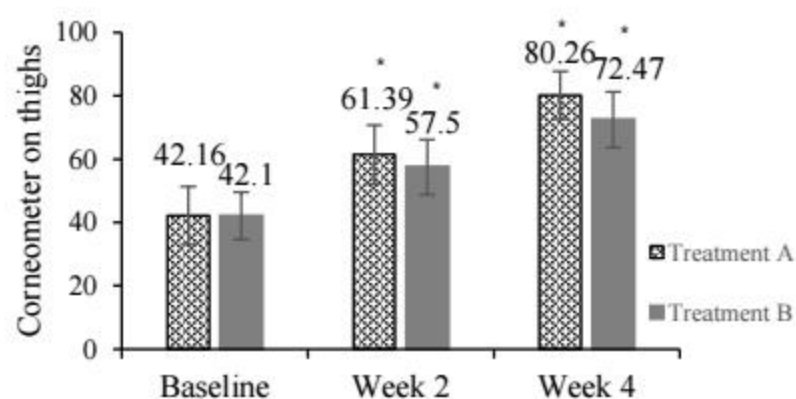


Figure 6 Skin moisture measured by Corneometer on right thigh and left thigh increased significantly at week 2 and week 4 compared to baseline ($P < 0.05$).

Table 4 The results of product satisfaction of slimming lotion and control lotion

Character	Control lotion		Slimming lotion	
	Score	Level	Score	Level
Texture	3.58±0.79	High	4.33±0.77*	Very high
Color	3.75±0.75	High	4.25±0.86	Very high
Smell	4.75±0.45	Very high	4.66±0.49	Very high
Spread	3.58±1.31	High	3.08±1.16	Medium
Absorption	3.16±1.33	Medium	3.25±0.96	Medium
Occlusive	4.33±0.77	Very high	3.58±0.79*	High
Moisture	4.33±0.65	Very high	3.83±0.83*	High
Smooth	4.16±0.71	High	3.25±0.86*	Medium
Stickiness	3.16±1.94	Medium	3.16±1.69	Medium
Overall	4.33±0.65	Very high	3.91±0.66	High

*Significantly difference at $P < 0.05$ when compared with control lotion

3.5 Satisfaction test

The results of questionnaires regarding the subjective satisfaction (n=12) after using the products for 4 weeks, which compared between the slimming lotions (applied on right thigh) and control lotion (applied on left thigh) were shown in Table 4. The texture and color of the products were in high level in control lotion and very high level in slimming lotion. The smell of both products was in very high level. The occlusive and moisture of products were in very high level in control lotion and high level in slimming lotion. The spread and smooth on skin were in high level in control lotion and medium level in slimming lotion. The absorption and stickiness of products were in medium level both in control lotion and slimming lotion. The overall satisfaction of control lotion was in very high level, whereas slimming lotion was in high level. From the satisfaction results, most of the subjects were more satisfied with control lotion more than slimming lotion because the viscosity of control lotion was more than slimming lotion this made the score of occlusive and moisture of control lotion were in very high level. The spread and smooth on skin were high level in control lotion lead to the subjects preferred control lotion.

4. Conclusion

This study intends to evaluate the effectiveness of slimming lotion and body wrap for cellulite treatment. Treatment A was applied slimming lotion containing L-carnitine, caffeine, *Fucus vesiculosus*, and *Centella asiatica* extracts for twice-daily and plus using body wrap with control lotion for 3 sessions/week within 4 weeks. Treatment B was applied control lotion twice-daily and plus using body wrap with a slimming lotion for 3 sessions/week within 4 weeks. The results of efficacy test showed that standard visual scale, thigh circumference, skin surface (smoothness and roughness), skin elasticity, and skin moisture of treatment A improved more than treatment B at week 4 compared to those at baseline. Therefore, treatment A (twice-daily using slimming lotion) is more effective treatment for cellulite than treatment B (slimming body wrap 3 sessions/week).

5. Suggestions

There are some limitations in this study such as the small number of subjects (N=12), and short-term efficacy tests. Therefore, additional clinical data are required to determine the long-term efficacy and large number of subjects. The comparison between control lotion and slimming lotion application as well as the comparison between body wrap with control lotion and slimming lotion should be separately performed.

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Anti-inflammatory activity of grape seed extract as a natural sun protection enhancer for broad-spectrum sunscreen

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Abstract

Photoaging effect of solar radiation can be prevented by application of broad-spectrum sunscreens onto skin. Recently, the combinations of the synthetic sunscreens with natural antioxidants have gained an interest due to their abilities to protect the skin from ultraviolet (UV)-induced reactive oxygen species generation. Grape seed extract (GSE) is a source of polyphenol antioxidants with anti-inflammatory properties accumulating for anti-aging capability. This study is aimed to access the effect of GSE on nitric oxide (NO) inhibition using lipopolysaccharide (LPS)-stimulated RAW 264.7 cells and its protective capacity against UV and visible light radiation. The anti-inflammatory effect of GSE was determined by nitric oxide inhibition assay using gallic acid as the positive control. The UV-visible absorption spectrum of GSE alone and in combination with UV-filters, including octyl methoxycinnamate (OMC) and Avobenzone were examined in a range of 200-800 nm by UV-spectrophotometer. The results showed that GSE at concentration 100 µg/ml significantly inhibited production of NO in LPS -stimulated RAW 264.7 cells demonstrating promising anti-inflammatory properties. In addition, GSE showed the ability to absorb broad range of the solar spectrum including UV and blue light. Moreover, combinations of GSE with UV-filters broaden the absorption spectra of both OMC and Avobenzone from UVB and UVA, respectively, to the visible spectrum of longer wavelengths. This study demonstrates promising results that could become the basis for development of sunscreen containing GSE with broad-spectrum action.

Keywords: Grape seed extract; Nitric oxide; Anti-inflammatory; Anti-aging; Sunscreen.

1. Introduction

Solar ultraviolet (UV) radiation has broad spectrum, ranging from 200 to 400 nm, including UVC (200–290nm), UVB (290–320), and UVA (320–400nm) (Rai *et al.*, 2012). Due to the ability of longer waves to reach the earth in the higher extent and penetrate through the dermal layers of skin to subcutaneous tissue, UVA rays attributed more to the photoaging and less to carcinogenic effects compare to UVB. However, UVA radiation can also cause oxidative stress by generation intracellular reactive oxygen species (ROS) indirectly damaging DNA strand (Giampieri *et al.*, 2012).

The exposure of skin to UV radiation is also known to promote the skin inflammation, that manifests as sunburn erythema, edema, thickening of the epidermal skin layer and hyperpigmentation (Choi *et al.*, 2019). These symptoms play a crucial role in premature skin aging contributed to the UV

light-induced cellular inflammatory pathway. In almost all cell types, the irradiation of the cells with UV light induces activation of transcription factor NF- κ B that plays a central role in regulating inflammatory, immune and anti-apoptotic responses. NF- κ B activation induces the expression of proinflammatory cytokines (IL-1), IL-6 and tumor necrosis factor (TNF)- α , prostaglandin (PG) E₂ and nitric oxide (NO) (Preedy, 2014).

Nitric oxide is an intercellular mediator that is overproduced by inducible nitric oxide synthase (iNOS) expressed in macrophages in response to inflammatory factors or lipopolysaccharide (LPS) that cause cytotoxicity of the skin cells (Rai *et al.*, 2012). The overproduction of NO in keratinocytes upon UVB (290-320 nm) and UVA (320-400 nm) irradiation was reported in several studies (Roméro-Graillet *et al.*, 1997; Chang *et al.*, 2003; Chen *et al.*, 2009). Holliman *et al.*, (2017) has shown the dose-dependent increase of NO in keratinocyte, fibroblasts, melanocytes and endothelial cells after irradiation with UVA light.

However, recently, the attention from studying the harmful effects of UVA and UVB light to the human skin has been brought to the blue light. The blue light, being a part of the visible light (400-760 nm) that takes up about 40% of solar radiation, covers the range of wavelengths 400-495 nm. Although, the low doses of blue light were proposed for the treatment of hemodynamic disorders, it was found that prolong exposure in the higher doses can produce the cytotoxic effect to keratinocytes and induce the generation of nonenzymatic NO in the skin (Liebmann *et al.*, 2010; Opländer *et al.*, 2013). In addition, Vandersee *et al.*, (2015) has shown the blue light-induced generation of ROS in accordance with the degradation of carotenoid concentration in the human skin.

The majority of existing nowadays sunscreen products do not provide sufficient protection of the skin against the solar radiation beyond UV range. In this regard, it became necessary to develop the sunscreen product providing an effective photoprotection in the UV-visible solar spectrum.

Besides the traditional approach of topical sunscreen application, the plants rich in polyphenols have gained the attention of cosmetic sciences by demonstration of multiple functions in photoprotection routes. Phenolic compounds are known to be potent free radical scavengers and inhibitors of several inflammatory mediators, including nitric oxide, possessing by that anti-inflammatory activity (Kwon *et al.*, 2011; Choi *et al.*, 2011).

Grape seed extract (GSE) is a source of polyphenol antioxidants with anti-inflammatory properties accumulating for anti-aging capability. The dietary feeding of proanthocyanidins derived from grape seeds to SKH-1 hairless mice significantly inhibited UVB-induced depletion of endogenous antioxidant defense enzymes and inhibited lipid peroxidation, protein oxidation, and nitric oxide (Sharma *et al.*, 2007). The suppression of UV-induced erythema is a useful variable to indirectly evaluate the anti-inflammatory effect of topically applied formulations. In the study of Hughes-Formella *et al.*, (2007), the effectiveness of cream and lotion contained oligomeric proanthocyanidins in combination with dietary supplementation showed the reduction of erythema after exposure to UV radiation suggesting that topical application of exogenous antioxidants have a potential to reduce the harmful effects of free radical damage triggered by UV irradiation.

Considering the bioactive and photoprotective properties of GSE, the addition of this extract to the synthetic sunscreens may improve the overall effectiveness of sun protection products. Therefore, the aim of this study was to access the effect of GSE on nitric oxide (NO) inhibition using lipopolysaccharide (LPS)-stimulated RAW264.7 cells and its protective capacity against UV and visible light radiation.

2. Materials and Methods

2.1 Materials

Grape seeds were provided by Village Farm & Winery (Nakhon Ratchasima, Thailand). Octyl methoxycinnamate (OMC) was purchased from Namsiang, Bangkok, Thailand. Butyl methoxydibenzoylmethane (avobenzene, AVO) was obtained from DSM Nutritional Ltd., Basel, Switzerland. Roswell Park Memorial Institute medium (RPMI 1640), fetal bovine serum (FBS) and penicillin-streptomycin solution (10,000 U/mL) were from GIBCO™, Thermo Fisher Scientific, MA, USA. Lipopolysaccharides (LPS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

(MTT) and Griess reagents were obtained from Sigma-Aldrich, St. Louis, Missouri, USA.

2.2 Methods

2.2.1 Preparation of grape seed extract

Dried grape seeds were crashed into fine powder using Fitz Millcomminutor (Dietz-motoren GmbH&Co.KG, Stuttgart, Germany). The ground grape seed powder was macerated in 95% ethanol for 1 week. The filtrate was collected and concentrated by Buchi R-3 rotary vacuum evaporator (Flawil, Switzerland) at 40°C (Yarovaya & Khunkitti, 2019).

2.2.2 Cell culture

The RAW 264.7 cells were cultured in the sterile 96 well-plate in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin 100 units/ml and streptomycin 100 µg/ml). The cells were incubated in a CO₂ incubator at 37°C with 5% CO₂ and 95% relative humidity (RH) for 12-16 hr. The cells were sub-cultured every 2-3 days or when the confluence reached 70-80%.

2.2.3 Cytotoxicity assay

The RAW 264.7 cells were seeded in a sterile 96 well-plate (2.5×10^4 cells/well) and incubated in the CO₂ incubator for 24 hr. The culture medium was removed and the cells were washed twice with phosphate buffer solution (PBS). The cells were treated with GSE at various concentrations (0.1-1000 mg/ml) and placed for 24 hr incubation. The cell viability was determined by MTT assay. Briefly, the culture medium was removed and 50 µl of 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution diluted with FBS-free media were added into each well. The cells were incubated at the CO₂ incubator for 4 hr. The culture medium was removed and formed formazan crystals were dissolved with 100 µl/well dimethyl sulfoxide (DMSO). The absorbance was measured at 550 nm by Varioskan flash microplate reader (Thermo Fisher, Vantaa, Finland). The percentage of cell viability was calculated using the following formula:

$$\% \text{ cell viability} = \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100, \quad (1)$$

where $\text{Abs}_{\text{control}}$ is the absorbance of cells treated with medium contained LPS, and $\text{Abs}_{\text{sample}}$ is the absorbance of cells treated with sample and medium contained LPS (Somchit *et al.*, 2018).

According to the result of this test, the concentrations of GSE procuring cell viability more than 80% were selected for inhibition of nitric oxide production experiment (Yazdimamaghani *et al.*, 2019).

2.2.4 Inhibition of nitric oxide production in LPS-stimulated RAW 264.7 cells

The effect of GSE on the nitric oxide inhibition was assessed by measuring generated NO upon the stimulation of RAW 264.7 cells with lipopolysaccharides (LPS). The cells were seeded in a sterile 96 well-plate (2.5×10^4 cells/well) and incubated for 12-16 hr. The culture medium was removed following by the repeated twice wash of cells with PBS. In this experiment, the cells were divided into four treatment groups: 1) untreated negative control containing culture medium and 1% antibiotics; 2) treated control with LPS 1 µg/ml; 3) treated with LPS 1 µg/mL and GSE at various concentrations (0.1-1000 mg/mL); 4) gallic acid (GA) was used as a positive control. Cells were incubated for another 24 h. The reduction of nitrite in culture medium were determined by mixing 100 µl of culture medium with 100 µl of Griess reagent (2% (w/w) sulfanilamide and 2% (w/w) N-1-[naphthyl] ethylenediamine dihydrochloride in 4% (w/w) H₃PO₄). The reaction was performed at room temperature for 10 minutes. The absorbance of the was measured at 540 nm. The % nitric oxide production was calculated as following:

$$\% \text{ nitric oxide production} = \frac{\text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100, \quad (2)$$

where $\text{Abs}_{\text{control}}$ is the absorbance of cells treated with medium contained LPS, and $\text{Abs}_{\text{sample}}$ is the absorbance of cells treated with sample and medium contained LPS (Bak, Truong, Kang, Jun, & Jeong, 2013).

2.2.5 UV absorption spectrum

The samples of 3% (w/w) GSE, 3% (w/w) Avobenzone, 7.5% (w/w) OMC, and the mixture of 7.5% (w/w) OMC and 3% (w/w) Avobenzone each with 3% (w/w) GSE in 95% ethanol were prepared. The absorbance was measured in the range of 200-800 nm by Shimadzu UV-1650PC spectrophotometer (Shimadzu Corporation, Tokyo, Japan).

2.2.6 Statistical analysis

Data from three independent experiments were presented as the mean $\pm$ standard deviation (SD). The statistical analysis between the mean of each group was performed using the One-way analysis of variance (One-way ANOVA) and Tukey test by Statistical Package for Social Sciences (SPSS version 17.0, SPSS Inc., Chicago, IL, USA). The statistical significance was considered at $P < 0.05$.

3. Results and Discussion

The cytotoxic effect of GSE on RAW264.7 cells was evaluated by MTT assay. The GSE did not show any impact on the cell growth or viability at the concentration range of 0.1-100 $\mu\text{g/ml}$ (Figure 1). However, the significant cytotoxicity was achieved in the concentration of 1000 $\mu\text{g/ml}$ (cell viability $< 63.5\%$). According to these results, the non-toxic concentrations of GSE (0.1-100 $\mu\text{g/ml}$) were selected for subsequent inhibition of nitric oxide production experiment.

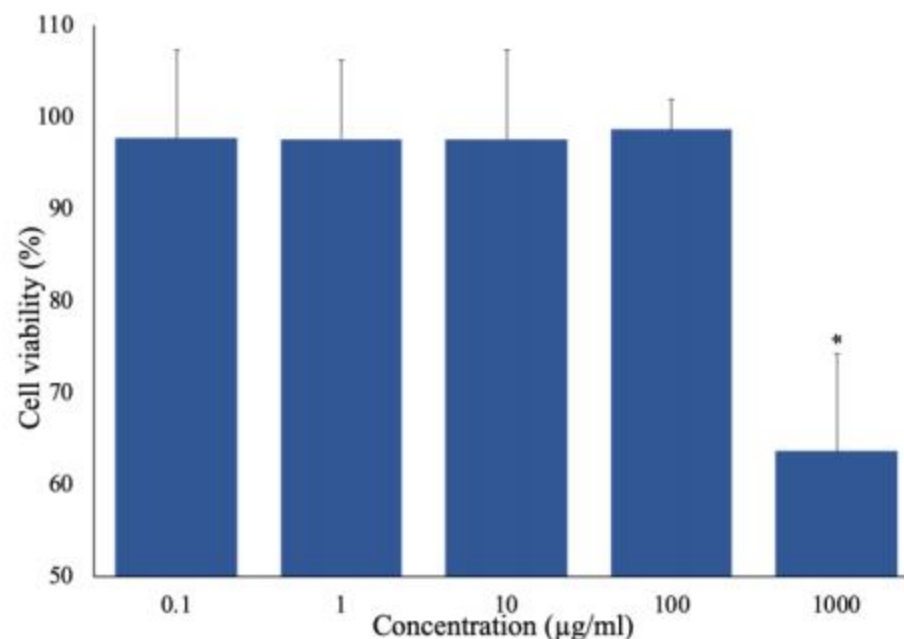


Figure 1 Cell viability of GSE in RAW264.7 macrophages.

* significantly different ($P < 0.05$)

The inhibitory effect of GSE on nitric oxide production in macrophages stimulated with LPS was comparable to that of GA, used as a positive control, in the concentration range of 0.1-10 $\mu\text{g/ml}$ (Figure 2). The increased concentration of both GSE and GA up to 100 $\mu\text{g/ml}$, significantly reduced the production of nitrite in RAW264.7 cells compare to LPS-induced cells. Moreover, the cells treated with 100 $\mu\text{g/ml}$ of GSE (20.26%) significantly reduced the NO production compared to GA (72.25%).

In addition, the RAW264.7 cells viability was intact after inhibition of NO production by GA in all tested concentrations compare to LPS-induced cells (Figure 3). Although, 100 $\mu\text{g/ml}$ of GSE significantly reduced cell viability from that of LPS-induced cells, it was above the accepted percentages (81.7%) and considered as non-toxicity (Yazdimamaghani *et al.*, 2019). This result indicates that GSE at concentration of 100 $\mu\text{g/ml}$ significantly inhibited NO production of RAW264.7 cells, demonstrating promising anti-inflammatory properties.

In agreement with our results, previous *in vitro* and *in vivo* studies have reported the ability of extracts from grape seeds to inhibit NO production in RAW264.7 cells. Terra *et al.*, (2007) showed a dose- and time-dependent inhibition of NO in LPS- and IFN- γ induced RAW264.7 macrophages by grape seed proanthocyanidin extracts. The significant reduction of NO production was found when the cells were treated during stimulation and for a longer period of time. *In vivo* determination of anti-inflammatory effect of proanthocyanidins from grape seeds demonstrated significant reduction in NO

production in croton oil-induced ear swelling in mice and carrageenan-induced hind paw edema in rats (Li *et al.*, 2001). The treatment of RAW264.7 macrophages with wild grape seeds extract showed the significant inhibition of NO in concentration of 30 $\mu\text{g/ml}$, thereby confirming its potent anti-inflammatory activity (Bak *et al.*, 2013).

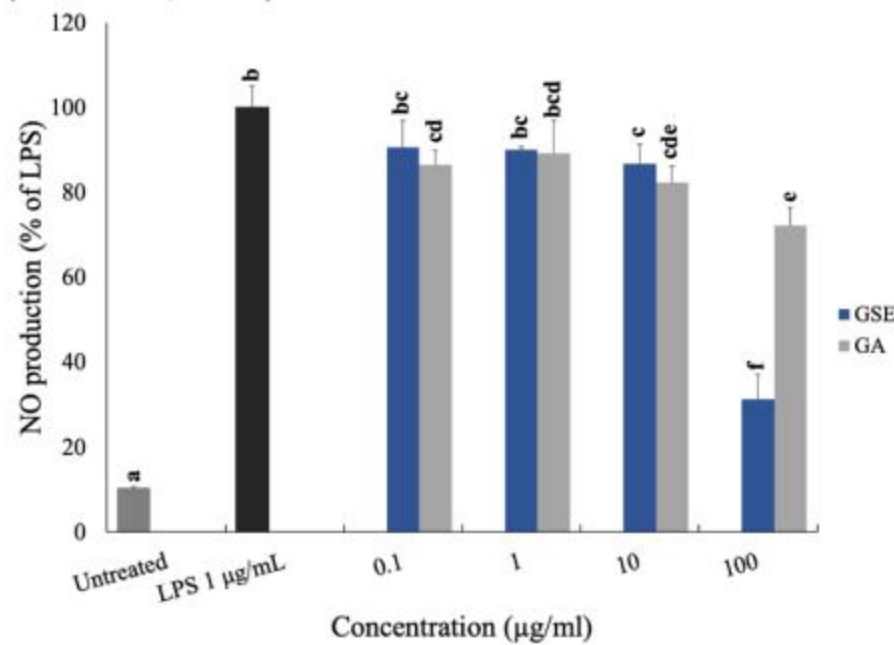


Figure 2 Effect of GSE and GA on LPS-induced nitric oxide in RAW264.7 macrophages. Value with different alphabets (a-f) indicated significant differences ($P < 0.05$).

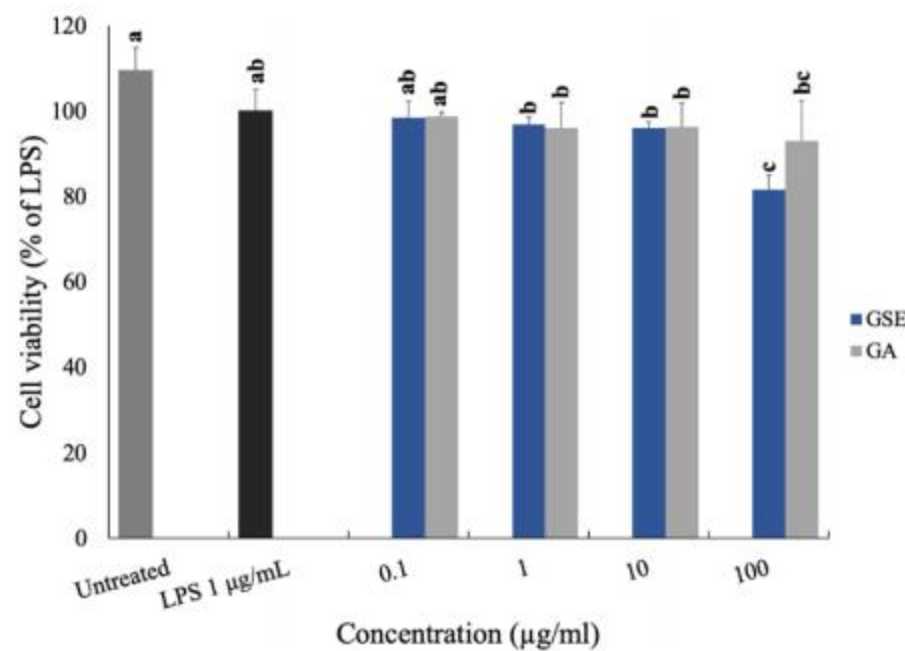


Figure 3 Cell viability assay after NO experiment. Value with different alphabets (a-c) indicated significant differences ($P < 0.05$).

Skin exposure to UV light causes inflammatory response including erythema contributing to the chronic damage of the skin that manifests as the premature skin aging (Pandel *et al.*, 2013). In the study conducted by Rhodes *et al.*, (2001), it was found that PGE2 and NO acted as the main inflammatory mediators of erythema induced by UVB light. The ability of antioxidants to inhibit the production of NO has been proved by several studies. The mechanisms of inhibition are most likely related to the ability of GSE to deactivate of NF κ B by the reduction of the intracellular redox status consequently inhibiting the production of iNOS (Xie *et al.*, 1994; Terra *et al.*, 2007). Another possible mechanism associated with the ability of GSE to regulate the transcription molecules of iNOS activated by LPS treatment directly (Bak *et al.*, 2013). Thus, rich in antioxidant extracts, including GSE, exerting the anti-inflammatory activities may be used as the agents preventing the photoaging of the skin.

The UV-visible absorption spectrum in 200-800 nm range of GSE, OMC, AVO and the mixtures of OMC and AVO with GSE dissolved in ethanol are shown in Figure 4. Although the low-energy tail of absorption spectrum of OMC extends up to 400 nm in UVA region, it is generally used as a UVB filter absorbing the solar spectrum in a range of 280-320 nm (Hanson *et al.*, 2015). Compare to OMC, Avobenzone absorbs a broader spectrum of UV light (290-400 nm) with the absorption maximum at

357 nm and is incorporated into the sunscreen products as the UVA filter. Grape seed extract showed the ability to absorb broad range of the solar spectrum including UV and blue light. The addition of GSE to UV-filters has broaden the absorption spectra of both OMC and Avobenzone from UVB and UVA, respectively, to the visible spectrum of longer wavelengths. This finding indicates that the ability of GSE to extend the absorption spectra of UV filters to the visible light may represent an effective tactic for development of the broad-spectrum sunscreen. This is in accordance with the result of the study of Martincigh & Ollengo, (2016) demonstrating the increase of absorption capacity of AVO after addition of GSE that extended its UV spectrum to the wider range of wavelengths. They have also reported an effective photostabilizing potential of GSE on both OMC and AVO.

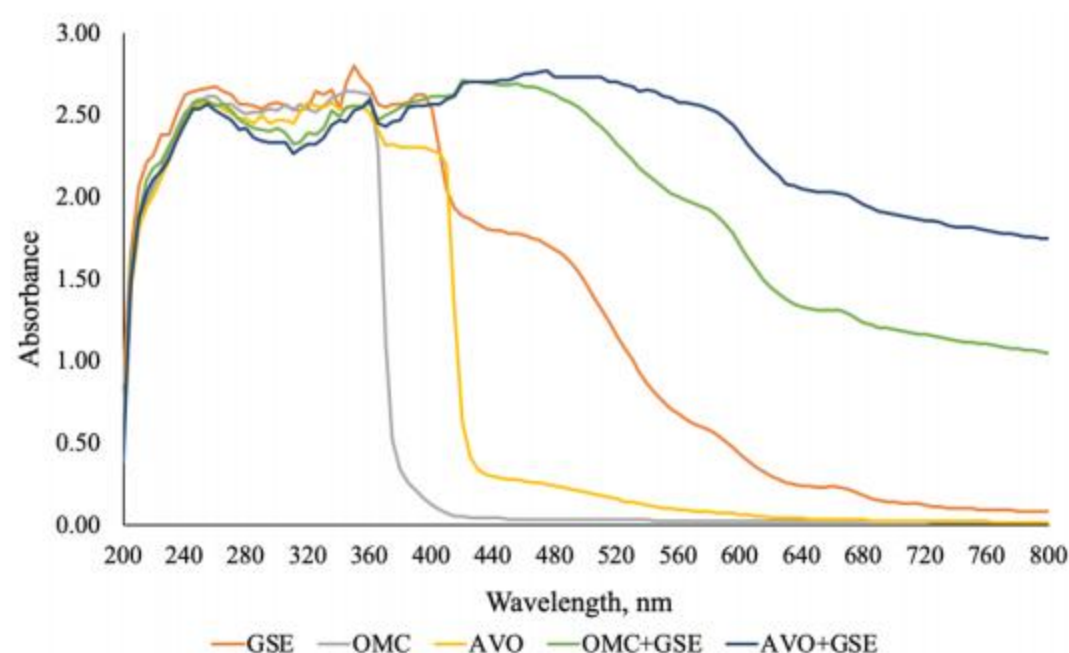


Figure 4 UV-visible spectra (200-800 nm) of grape seed extract (GSE), octyl methoxycinnamate (OMC), Avobenzone (AVO), and mixtures of OMC and AVO with GSE dissolved in ethanol.

The ability of GSE to act as photoprotector is related to the aromatic conjugated structure of polyphenols that absorb radiation in the UV–vis spectral region. Several studies have also proved the ability of polyphenolic compounds to act synergistically with the synthetic sunscreens boosting up the sun protection (SPF) value (Afonso *et al.*, 2014; Galanakis *et al.*, 2018). Ramos *et al.*, (1996) explained the increase of SPF values by synergism of natural polyphenols and sunscreen agents due to the structural analogy. In our previous study we have found, that the ability of GSE to absorb the light was concentration-dependent. The results showed that 3% and 5% w/w GSE absorbed broad UV spectrum covering the blue light in a higher extent than that of 1%. Sun protection factor (SPF) and persistent pigment darkening (PA) increased up to 5 units and 1 unit, respectively, when 3% of GSE was added to the counter-brand product. However, when 3% GSE was added to the cream base, the SPF/PA value (1.29/1.19) did not reach a sufficient level for UV-protection (Yarovaya & Khunkitti, 2019). Similar result was obtained in the *in vivo* SPF testing of rich in polyphenolic compounds green tea extract, which SPF was found to be 1.05 (Camouse *et al.*, 2009). Another recent study showed that addition of 1% grape seed extract to the sunscreen lotion contained chemical and physical UV filters increased SPF value and demonstrated high antioxidant activity (Limsuwan & Amnuakitt, 2017). Although, GSE is not a good candidate to be used as a single agent in sun protection products, it can improve overall photoprotection of sunscreen products increasing the absorbing capacities and boosting SPF values of UV filters, as well as improving their photostability.

4. Conclusion

The prevention of skin aging can be achieved by application of the topical UV-protection product and by inhibition of inflammation using components with anti-inflammatory action. According to the obtained results in our study, GSE was able to reduce the production of NO in LPS-induced RAW264.7 cells and extend the absorption spectrum of the most commonly used UV filters in the modern sunscreens products, octyl methoxycinnamate and avobenzone, to the visible spectrum of longer wavelengths. Thus, it can be assumed that GSE can represent a good candidate for improvement of the skin protection against UV and blue light-induced photodamages and

inflammation. This study demonstrates promising results that could become the basis for development of broad-spectrum sunscreen containing GSE with anti-aging action.

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