Research Article

การตั้งตำรับและการทดสอบคุณลักษณะครีมน้ำมันยางนาที่บรรจุในอนุภาคนาโนพอลิเมอร์

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บทคัดย่อ

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การศึกษานี้มีวัตถุประสงค์เพื่อตั้งตำรับและทดสอบลักษณะของครีมที่ประกอบด้วยน้ำมันยางนา สารสำคัญในน้ำมันยางนา คือ น้ำมันหอมและสารประกอบฟีนอลิก ซึ่งมีฤทธิ์ในการต้านอนุมูลอิสระ และสามารถตั้งตำรับเป็นผลิตภัณฑ์เครื่องสำอางได้ อย่างไรก็ตาม น้ำมันยางนามีความไวต่อแสง อากาศและออกซิเจนเพื่อเป็นการแก้ปัญหาดังกล่าวดังนั้นจึงเตรียมให้มันอยู่ในรูปแบบอนุภาคนาโนพอลิเมอร์ก่อนจะเตรียมเป็นรูปแบบครีม **วิธีการวิจัย**: ประการแรกเป็นการศึกษาสมบัติทางเคมีฟิสิกส์ของน้ำมันยางนา ได้แก่ ความสามารถในการเข้ากันได้กับตัวทำละลาย การทดสอบหาปริมาณสารประกอบฟีนอลิกรวมและความสามารถในการต้านอนุมูลอิสระ ประการที่สองเป็นการเตรียมอนุภาคขนาดนาโนเมตรของน้ำมันยางนาด้วยการบรรจุในอนุภาคนาโนพอลิเมอร์ ซึ่งพอลิเมอร์ที่ใช้ได้แก่ไคโตซานและเจลแลนกัมและเตรียมด้วยวิธีโคแอเซอร์เวชันเชิงซ้อน จากนั้นนำอนุภาคนาโนของไคโตซานและเจลแลนกัมที่บรรจุน้ำมันยางนาไปทดสอบคุณลักษณะ ประกอบไปด้วยค่าศักย์ไฟฟ้าซีตา ขนาดอนุภาคและประสิทธิภาพในการกักเก็บสารสำคัญ โดยตำรับที่ให้อนุภาคที่มีคุณลักษณะที่เหมาะสมจะนำมาเตรียมเป็นอิมัลชันชนิดครีมแบบน้ำมันในน้ำ (o/w) โดยตำรับครีมจะได้ทำการทดสอบลักษณะทางเคมีฟิสิกส์ก่อนและหลังการทดสอบเสถียรภาพในสภาวะเร่ง เช่นลักษณะทางกายภาพ กลิ่น ค่าความเป็นกรด-ด่าง ความหนืด การแยกชั้นและทดสอบหาปริมาณของสารประกอบฟีนอลิกรวม โดยการทดสอบความเสถียรภาพในสภาวะเร่งใช้วิธีการเก็บไว้ในอุณหภูมิร้อนสลับเย็นจำนวน 6 รอบ (โดยการเก็บที่อุณหภูมิ 45 ºC 24 ชั่วโมงและเก็บที่อุณหภูมิ 4 ºC 24 ชั่วโมงนับเป็นหนึ่งรอบ) **ผลการศึกษา**: ผลการศึกษาพบว่าน้ำมันยางนาความเข้มข้นร้อยละ 2 (ปริมาตรโดยปริมาตร) สามารถเข้ากันได้ดีกับเอทานอลและ dimethyl sulfoxide ที่สัดส่วนน้ำมันยางนา : ตัวทำละลาย เป็น 1:50 เมื่อนำน้ำมันยางนาละลายในเอทานอลมาทดสอบหาปริมาณของสารประกอบฟีนอลิกรวมมีค่าเท่ากับ 1.683 ± 0.057 mgGAE/mL ความเข้มข้นที่สามารถยับยั้งอนุมูลอิสระร้อยละ 50 มีค่าเท่ากับ 0.931 ± 0.025 mg/mL เมื่อทดสอบด้วยวิธี DPPH และสามารถต้านการเกิดปฏิกิริยา Lipid peroxidation ได้ร้อยละ 83.51 อนุภาคของไคโตซานและเจลแลนกัมที่บรรจุน้ำมันยางนามีขนาดเท่ากับ 128 ± 2.65 nm ค่าศักย์ไฟฟ้าซีตาเท่ากับ 14 ± 2.5 mV และมีประสิทธิภาพในการกักเก็บสารสำคัญที่ร้อยละ 27.82 ตำรับครีมพื้น ครีมควบคุมและครีมยางนาที่เตรียมจากตำรับที่ดีที่สุดซึ่งประกอบด้วย glycerol monostearate ให้ครีมที่มีเนื้อเนียนสวยและมีเสถียรภาพดีที่สุด มีค่าความเป็นกรด-ด่างเท่ากับ 4.6-5.4 และความหนืดเท่ากับ 22,745 ± 463 ถึง 29,337 ± 522 cP, ตามลำดับ หลังจากทดสอบเสถียรภาพในสภาวะเร่งพบว่าตำรับครีมยางนามีปริมาณของสารประกอบฟีนิลิกรวมร้อยละ 93.6 ซึ่งมีความแตกต่างอย่างมีนัยสำคัญทางสถิติกับตำรับครีมควบคุมที่มีปริมาณสารประกอบฟีนอลิกรวมเท่ากับร้อยละ 80.1 (p=0.036) **สรุปผลการศึกษา**: จากผลของการทดลองนี้สามารถสรุปได้ว่าตำรับครีมที่มีส่วนประกอบของน้ำมันยางนาบรรจุในอนุภาคนาโนพอลิเมอร์มีเสถียรภาพสูงเมื่อเทียบกับตำรับครีมควบคุม นอกจากนี้ระบบอนุภาคนาโนพอลิเมอร์ยังสามารถป้องกันสารออกฤทธิ์จากกระบวนการออกซิเดชันได้

คำสำคัญ: น้ำมันยางนา, อนุภาคนาโนพอลิเมอร์, โคแอเซอร์เวชันเชิงซ้อน

Formulation and characterization of cream containing Yang-na oleoresin-loaded in polymeric nanoparticles

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Abstract

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The aims of this study were to formulate and characterize formulations consisting Yang-na oleoresin. Active compounds in Yang-na oleoresin are mostly essential oils and phenolic substances which posess antioxidant activities and are able to formulate a cosmetic cream. However, oleoresin is sensitive to light, air and oxygen. To overcome this obstacle, Yang-na oleoresin was loaded in polymeric nanoparticles before being incorporated into the cream. **Materials and methods:** Firstly, physicochemical properties of Yang-na oleoresin such as miscibility, total phenolic compounds (TPC) and antioxidant activities were investigated. Secondly, Yang-na oleoresin was loaded in polymeric nanoparticles using chitosan and gellan gum by complex-coacervation method. The chitosan-gellan gum (CS-GG) nanoparticles-loaded Yang-na oleoresin were characterized in terms of zeta potential, particle size and % entrapment efficiency (%EE). The suitable formulation was selected to incorporate into oil in water (o/w) cream base. Lastly, physicochemical characteristics of creams including appearance, odor, pH-value, viscosity, phase separation and amount of TPC were examined both before and after accelerated stability. The accelerated stability was conducted by heating/cooling for 6 cycles (by kept at 45 ºC for 24 hr and kept at 4 ºC for 24 hr/1 cycle). **Results:** Results of miscibility study revealed that Yang-na oleoresin was well dissolved in absolute ethanol and dimethyl sulfoxide in the ratio of Yang-na oleoresin: solvents of 1:50. The amount of TPC of 2% (v/v) of Yang-na oleoresin in absolute ethanol was 1.683 ± 0.057 mgGAE/mL. The 50% inhibitory concentration of Yang-na oleoresin was 0.931 mg/mL determined by $DPPH^{\bullet}$ assay whereas the % lipid peroxidation of Yang-na oleoresin was 83.51%. The particle size, zeta potential and %EE of the optimized CS-GG nanoparticles-loaded with Yang-na oleoresin were 128 ± 2.65 nm, 14 ± 2.5 mV and 27.82%, respectively. After accelerated stability, cream base, control cream and Yang-na cream prepared from a formulation which consisted of glycerol monostearate had a smooth texture and showed the suitable stability. The pH-value and the viscosity of these formulations were 4.6-5.4 and 22.745 ± 463 to 29,337 ± 522 cP, respectively. The amount of TPC containing in Yang-na cream was 93.6% which was significantly higher than that of the control cream (80.1%) (p=0.036). **Conclusion:** It was concluded that the formulation of cream containing Yang-na oleoresin-loaded polymeric nanoparticles had the highest stability compared to other formulations. Furthermore, the polymeric nanoparticle system was proven to be able to protect the active compounds against oxidation process.

Keywords: Yang-na oleoresin, polymeric nanoparticles, complex-coacervation

Introduction

Dipterocarpus alatus Roxb. ex G. Don is a tree belonging to the dipterocarpus species, which contains phenolic compounds, particularly phytochemicals in the group of sesquiterpenes, triterpenes, oligostilbenoid, coumarin derivatives, resveratrol and phytosterol (Aslam *et al.*, 2015). Other studies reported that the bark of the trees in the dipterocarpus species contains ε-viniferine, α-viniferine, vaticanol B and hopeaphenol (Hakim *et al.*, 2006; Zain *et al.*, 2011). These compounds showed varieties of therapeutic effects such as anti-HIV, anti-cancer, anti-inflammatory, anti-oxidant, anti-bacterial and anti-fungal activities (Aslam *et al.*, 2015). It has been reported that flowers of *D. alatus* contained the highest content of phenolic compounds (Nipaporn *et al.*, 2014). Moreover, resin in the bark of *D. alatus* consists of 60-80% gurjun essential oil such as α-gurjunene, β-gurjunene and sesquiterpenes. It has been used in cosmetics for color saturation or gloss enhancement (Burger, 2009). Yang-na oleoresin is a liquid of resin and contains essential oil as major phytochemicals (Burger, 2009). Phenolic compounds are extensively used in skin applications to reduce free radicals which cause skin aging, skin disease, skin damage such as wound and burns (Dzialo *et al.*, 2016). Yang-na oleoresin can be oxidized easily, resulting in instability of oil color and active compounds, and affecting the physicochemical characteristics of product. Thus, to overcome this obstacle, Yang-na oleoresin needs to be protected by encapsulating in a vesicle, particularly, nanoparticles. This strategy can be done by several methods and polymeric nanoparticle is one of available method.

Polymeric nanoparticles are prepared by one or more types of polymers and have sizes ranging from 10-1000 nm (Rangari *et al.*, 2015). The matrix of polymeric nanoparticles can protect and control release of active compounds (Rangari *et al.*, 2015). Furthermore, polymeric nanoparticle gum is used to deliver lipophilic drugs into the stratum corneum (Guterres *et al.*, 2007). Natural polymers such as chitosan and gellan gum have gained more attention in creating nanoparticles because of their biocompatibility, biodegradability and safety (Osmalek *et al.*, 2014). Chitosan (CS) is a cationic polysaccharide which can form polyelectrolyte complexes (PECs) with oppositely charged polyelectrolytes (Picone and Cunha, 2013). Gellan gum (GG) is anionic polymer which became strong gel at low pH values and low concentration as 0.2% (w/v). The combination of CS-GG may create good quality nanoparticles because GG prevents the active compounds in a low pH environment whereas CS promotes the mucoadhesion and controls release of the bioactive compounds (Picone and Cunha, 2013). Picone and Co-workers (2013) prepared CS-GG nanoparticles by complex-coacervation method and reported that the mixing ratios of polymers affected to size and zeta potential of the particles. When the amount of CS increased, zeta potential was enhanced while the particle size was decreased (Picone and Cunha, 2013). In 2015, Scalia *et al.* reported the coating of resveratrol by chitosan and lipid microparticles could enhance *in vivo* human skin penetration. Moreover, CS-coated lipid microparticles provided a higher zeta potential than CS-uncoated lipid microparticles (64.2 ± 4.4 mV and -17.8 ± 4.8 mV, respectively) (Scalia *et al.*, 2015). According to the results mentioned above, the preparation of active compounds as nanoparticles showed several advantages due to their capability to diffuse through the superficial layers of stratum corneum and hair follicles. However, preparation of active compounds as nanoparticles (nanosuspensions and nanoemulsions) had various drawbacks, including a stability issue (larger particles, sedimentation and agglomeration) (Wu *et al.*, 2011). The emulsion is extensively used as a vehicle for delivering drugs to the body because of its advantageous characteristics such as enhancing the bioavailability and increasing the spreadability of active compounds (Waqas *et al.*, 2010). Furthermore, emulsion has an occlusive effect by hydrating the stratum corneum and inhibiting evaporation of eccrine secretions. Especially, as oil-in-water (o/w)

emulsions are widely used for facial cream, this can be easily washed off using water, leading to higher customer compliance (Singh *et al.*, 2011). Additionally, physicochemical characteristics of the emulsion are required to be concerned. These features directly affect stability of the formulations (Daudt *et al.*, 2015).

Thus, the aim of the study was to prepare and characterize a cosmetic cream containing Yang-na oleoresin-loaded in polymeric nanoparticles. The obtained creams were characterized by physicochemical characteristics in terms of appearance, pH-value, viscosity and phase separation both before and after accelerated stability. Furthermore, the amounts of total phenolic compounds (TPC) in the formulations were determined.

Materials and methods

1. Materials

The resin of *D. alatus* was supplied by Dr. Somporn Katekaew, Faculty of Sciences, Khon Kaen University. Gallic acid, Trolox, ascorbic acid, Folin-Ciocalteu reagent and 2,2 diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich, USA. Chitosan-low molecular weight and 2,2'-Azobis-(2-methylpropionamidine) dihydrochloride (ABAP) were obtained from Sigma-Aldrich, Germany. Gellan gum was purchased from Sigma-Aldrich, Japan and sodium carbonate was obtained from Loba Chemie Pvt. Ltd, India. All other chemicals were analytical grade.

2. Evaluation and characterization of Yang-na oleoresin

2.1 Miscibility study

The resin of Yang-na was filtered by cotton filter two times to separate the gum, resulting in liquid part as oleoresin (Yang-na oleoresin). Yang-na oleoresin was mixed with absolute ethanol, 80% ethanol in deionized water, dimethyl sulfoxide (DMSO), glycerin, polyethylene glycol 400 (PEG 400), propylene glycol (PG), Tween 80 and Span 80 in different ratios of Yang-na oleoresin: solvents as 1:1; 1:2; 1:4 and 1:8 (v/v). The resulting mixtures were vortexed for 10 min and equilibrated at room temperature for 24 hr. After centrifugation at 3,500 rpm at 25 ºC for 10 min, the mixtures were observed during the phase separation visually (Zhao *et al.*, 2010). The substances which can be miscible with Yang-na oleoresin were used to determine other properties of Yang-na oleoresin in further experiments.

2.2 Determination of total phenolic compounds

The concentration of total phenolic compounds in Yang-na oleoresin was determined by using the Folin-Ciocalteu method as described by Mansouri (2005). The results were expressed as milligram equivalent of gallic acid (mgGAE/mL). Briefly, the reaction medium was prepared in a plastic tube (1 mL) containing 10 µL of 2% (v/v) Yang-na oleoresin in absolute ethanol or DMSO, 790 µL of distilled water and 50 µL of Folin-Ciocalteu reagent with mild agitation for 3 min. Then, an aliquot of 150 µL of 20% (w/v) sodium carbonate solution was added and mixed thoroughly. All tubes were incubated in the dark for 2 hr. Later, an aliquot of 200 µL of each analysis was added to 96-well plate and the absorbance measured at 750 nm. The solution of gallic acid in concentrations of 100-1000 µM was used as standard curve (Mansouri *et al.*, 2005).

2.3 Antioxidant activities

DPPH$^{\bullet}$ free radical scavenging activity:

DPPH$^{\bullet}$ assay was used to evaluate the antioxidant of Yang-na oleoresin as described by Katalinic (2010) with slight modification (Katalinic *et al.*, 2010). Solution of 0.4 mM DPPH$^{\bullet}$ and 2% (v/v) of Yang-na oleoresin were dissolved in absolute ethanol separately. Then, each solution 50 µL was added in a 96-well plate. The mixtures were shaken and incubated in the dark at room temperature for 30 min before measuring the absorbance at 517 nm. Ascorbic acid and Trolox in the concentration of 1 mg/mL were used as standards and subjected to the same procedure for comparison. The results were expressed as the 50% inhibitory concentration (IC_{50}) and calculated using the following equation:

$$IC(\%) = \frac{(A_O - A_S)}{A_O} \times 100 \qquad \text{Equation 1}$$

Where A_S is the absorbance value of sample and A_O is the absorbance value of the blank (absolute ethanol).

Lipid peroxidation assay:

A modified thiobarbituric acid reactive species (TBARS) assay was used to measure the lipid peroxide formed. Briefly, an aliquot of 50 µL of linoleic acid was added in a plastic micro-centrifuge tube (1 mL) followed by the addition of 50 µL of 2% (v/v) Yang-na oleoresin in absolute ethanol. Aliquot of 10 µL of 0.07M ABAP was added to the mixtures and vortex for 10 min. Thereafter, 150 µL of 20% (v/v) acetic acid (pH 3.5) was added to the mixtures and incubated in a hot air oven at 70 ºC for 1 hr. Aliquot of 20 µL of the resulting mixtures was added into 96-well plate with 160 µL of 75% (v/v) ethanol, adding 10 µL of 15% (w/v) ammonium thiocyanate and 10 µL of ferrous chloride (10 µM), respectively. Later, the mixtures were measured the absorbance at 500 nm (Roberto and Baratta, 2000). Ascorbic acid and Trolox in the concentration of 1.68 mg/mL were done the same method as Yang-na oleoresin in order to provide a comparison. The radical scavenging activity is expressed as %inhibitory lipid peroxidation (%LPO) at the concentration used.

3. Preparation and characterization of Yang-na oleoresin-loaded CS-GG nanoparticles

Yang-na oleoresin was loaded into CS-GG nanoparticles by a complex-coacervation method as described by Lathsamee *et al.* (2017). The stock solution of 1% (w/v) CS was prepared in 1% (v/v) acetic acid with agitation whilst 1% (w/v) GG was prepared by dissolved in deionized water at 80 ºC and stirred until the solution became transparent solution. CS and GG solution were equilibrated overnight and diluted with deionized water to obtain the final concentration of 0.01% (w/v). The particles were formed by addition of the mixture of 2% (v/v) Yang-na oleoresin and 2% (v/v) Tween 80 to CS solution by dropwise under vigorous mixing at 50 ºC for 5 min. Then, GG solution was added and continued mixing by magnetic stirrer at the speed of 1,000 rpm for 30 min. The mixing ratio of CS:GG (7:3 (v/v)) was used. The obtained nanoparticles were characterized by the zeta potential, particle size and entrapment efficiency (Lathsamee *et al.*, 2017).

3.1 Measurement of particle size and zeta potential

The dispersion of Yang-na oleoresin-loaded polymeric nanoparticles was diluted with deionized water at ratio of 1:3 (Yang-na oleoresin-loaded polymeric nanoparticles: deionized water), in order to measure zeta potential and particle size by zeta sizer (Model ZEN2600, Malvern Instruments Ltd, UK) and nano sizer (Model S90, Malvern Instruments Ltd, UK), respectively (Lathsamee *et al.*, 2017).

3.2 Entrapment efficiency

The dispersion of Yang- na oleoresin- loaded polymeric nanoparticles was centrifuged at 9,000 rpm, 4 ºC, 2 hr and collected in the supernatant. An aliquot of 1 mL of supernatant was then mixed with 2 mL of absolute ethanol and incubated at room temperature for 30 min. Subsequently, the mixture was sonicated and centrifuged at 9,000 rpm, 25 ºC, 30 min (Lathsamee *et al.*, 2017). Total phenolic compounds were determined from the supernatant using the Folin-ciocalteu method as described in the section 2.2.2, and gallic acid served as standard curve (Mansouri *et al.*, 2005). %Entrapment efficiency (%EE) was calculated using the equation as given below:

$$\%EE = \frac{\text{Quantity of total phenolic compounds entrapped}}{\text{Quantity of phenolic compounds added in CS-GG nanoparticles}} \times 100 \qquad \text{Equation 2}$$

4. Preparation and characterization of Yang-na creams

The cream formulations were prepared from various phase volume ratios of oil phases: water phases as 16:84; 20:80; 30:70 and 41:59. The compositions are shown in **Table 1**, the cream bases (without active ingredient) consisted of 4 formulations namely CB_1 to CB_4.

As shown in **Table 1,** both oil and water phase were heated to 70 ± 0.5 ºC and 75 ± 0.5 ºC, respectively. After the temperature reached the required level, the water phase was added into the oil phase with continuous stirring. Yang-na creams and control creams were prepared by addition of 10% (v/v) of Yang-na oleoresin-nanoparticles and 10% (v/v) of Yang-na oleoresin in deionized water into cream base and defined as YC_1-YC_4 and CC_1-CC_4, respectively.

Table 1 Compositions and concentrations of substances in cream formulations.

Compositions	CB_1	CB_2	CB_3	CB_4
Water phase				
DI-water (mL)	67	62	52	40.5
Yang-na oleoresin-loaded polymeric nanoparticles (% v/w)	10	10	10	10
Propylene glycol (g)	1	1	1	1
Paraben concentrate (g)	2	2	2	-
Tween 80 (g)	3.42	4.16	4.66	7
Oil phase				
Glycerol monostearate (g)	3.5	2	-	-
Stearic acid (g)	-	-	-	12.5
Lanolin (g)	-	5	8.5	9
Cetyl alcohol (g)	3.5	3.5	5	-
Isopropyl palmitate (g)	1.5	2	5.5	-
Sun flower oil (g)	1.5	-	-	-
Mineral oil (g)	-	2.5	6	13.5
Span 80 (g)	3.58	2.84	2.34	3
Green tea odor (mL)	3	3	3	3
Total	100	100	100	100
Phase volume ratio (w/w)	16/84	20/80	30/70	41/59
HLB of system	9.53	10.66	11.43	11.79
Surfactant (% w/w)	7	7	7	10

4.1 Physical appearance

After preparation for 24 hr, the obtained creams were examined for characteristics such as color, odor and following determination.

4.2 pH-value, viscosity and phase separation

The measurement of pH-value and viscosity were done by pH-meter (Model PL-600, M.R.C. Ltd, Israel) and Brookfield viscometer (Model DV- III Programmable Rheometer, LabX, USA), respectively (Ribeiro *et al.*, 2015; Singh *et al.*, 2011) . The viscosities of creams were measured at room temperature using spindle No. 96 and at a speed of 10 rpm for 30 sec. Moreover, the creams were determined in phase separation by centrifugation at 3,800 rpm, 25 ºC for 30 min (Model 6200- Kubota, Kubota Laboratory Centrifuges, Japan) (Dault *et al.*, 2015).

4.3 Accelerated stability testing

The obtained creams were submitted for 24 hr and taken to accelerated stability study by heating/cooling for 6 cycles (kept at 45 ºC for 24 hr and 4 ºC for 24 hr/1 cycle) according to previous reports (Estanqueiro *et al.*, 2014). The creams were then characterized by appearance, pH-value, viscosity and phase separation (Garbossa and Campos, 2016).

5. Determination of the amount of TPC in creams

The creams which showed best stability after accelerated stability were carried out to determine the amount of TPC. Briefly, 1 g of cream was mixed with absolute ethanol (2 mL) and 15 µL of 1M HCl. Thereafter, the mixture was sonicated at 45 ºC for 30 min, and centrifuged at 9,000 rpm at 25 ºC for 30 min (Dault *et al.*, 2015). The supernatant was collected and the amount of TPC was determined by Folin-ciocalteu method as mentioned in above section (2.2.2). The result was reported as percentage of TPC remaining in the formulation.

6. Statistical analysis

All experiments were repeated at least three times. Results were demonstrated by mean ± standard deviation. The statistical analysis was performed with $p<0.05$ as level of significance by using student's *t*-test, paired *t*- test and analysis of variance (one-way ANOVA test).

Results and discussion

1. Miscibility study

The miscibility study was used to screen compatibility of solvents/surfactants with Yang-na oleoresin. The solvents (absolute ethanol, 80% (v/v) ethanol in distilled water, DMSO, glycerin, polyethylene glycol 400 and propylene glycol) and the surfactants (Tween 80 and Span 80) were used in this study. The compatibility of Yang-na oleoresin with these substances was followed this order; tween 80=span 80>absolute ethanol>DMSO>80% (v/v) ethanol=propylene glycol=polyethylene glycol 400=glycerin as shown in **Table 2**. It was found that Yang-na oleoresin was able to miscible with tween 80 and span 80 because these substances contained hydrophobic and hydrophilic parts which were compatable with this oleoresin, creating a homogeneous high viscosity of the resultant with yellow color (Li *et al.*, 2012). In primarily, the mixtures of Yang-na oleoresin and tween 80/span 80 were used to determine the amount of TPC. However, the absorbance measurement was interfered with the color of tween 80 and span 80. Additionally, it had been reported that the low viscosity and low surface tension of solvents can improve the extraction rate, leading to high solubility of active compounds (Dai and Mumper, 2010). Tween 80 and span 80 were not then carried out in the study. The second order of miscibility results of absolute ethanol and DMSO was then considered. When the volume of these solvents was increased, the mixtures tended to be able to dissolve more. This explanation might be because absolute ethanol is a solvent containing 2-functional groups of non-polar and polar group. Thus, it could extract some bioactive compounds with a broad range of polarity (Sun *et al.*, 2015). In case of DMSO, it is an aprotic solvent with high polarity and can miscible with organic solvents, water and lipids. Moreover, DMSO has also been used as solvents for resins, fungicides, dyes and pigments (Kligman, 1965). Therefore, absolute ethanol and DMSO were selected to test compatibility with oleoresin at higher mixing ratios of 1:20 and 1:50. After mixing in these conditions, it was found that Yang-na oleoresin was able to miscible with both solvents in ratio of 1:50. Absolute ethanol and DMSO were therefore used as solvents to determine the amount of TPC and antioxidant activities of Yang-na oleoresin.

Table 2 Miscibility study of Yang-na oleoresin with solvents and surfactants

Ratios of Yang-na oleoresin to solvents/ surfactants (v/v)	Solvents/surfactants							
	Absolute Ethanol	80% Ethanol	DMSO	Glycerin	Polyethylene glycol 400	Propylene glycol	Tween 80	Span 80
1:1	+	+	-	+	+	+	+++	+++
1:2	+	+	-	+	+	+	+++	+++
1:4	+	+	+	+	+	+	+++	+++
1:8	++	+	++	+	+	+	+++	+++
1:20	++		++					
1:50	+++		+++					

+ + + clear; + + slightly turbid; + turbid and - immiscible

2. Total phenolic compounds in Yang-na oleoresin

According to the miscibility study, absolute ethanol and DMSO were used to dissolve Yang-na oleoresin. The amount of TPC was calculated from the calibration curve of gallic acid (Y=0.0001X+0.0056; R^2=0.9921). It was found that 2% (v/v) of Yang-na oleoresin dissolved in DMSO and absolute ethanol composed of TPC at 1.451 ± 0.096 and 1.683 ± 0.057 mgGAE/mL, respectively (**Table 3**). The amount of TPC in Yang-na oleoresin dissolved in absolute ethanol was significantly higher than the one dissolved in DMSO (p=0.022). This indicated that the extracting solvents affected to extract yield and the amount of TPC in plants (sun *et al.*, 2015), and is in accordance with Nipaporn *et al.* (2014) who studied effect of solvents (ethanol and water) on the amount of TPC in parts of *D. alatus*. It was found that ethanolic extraction provided a higher amount of TPC than water extraction (Nipaporn *et al.*, 2014).

Table 3 The amount of TPC in Yang-na oleoresin

Samples	mgGAE/mL (mean ± SD)
2% (v/v) Yang-na oleoresin/DMSO	1.451 ± 0.097
2% (v/v) Yang-na oleoresin/EtOH	1.683 ± 0.057*

*significant difference (p=0.022) compared to Yang-na oleoresin in DMSO

3. Antioxidant activity by DPPH• assay

According to the result of TPC, Yang-na oleoresin in absolute ethanol was chosen. An antioxidant activity of Yang-na oleoresin was evaluated by DPPH• assay and expressed as 50% of inhibitory concentration (IC_{50}) using Trolox and ascorbic acid as references. **Figure 1** shows that Yang-na oleoresin dissolved in absolute ethanol, Trolox and ascorbic acid showed the IC_{50} of 0.931± 0.025 mg/mL, 0.266 ± 0.034 mg/mL and 0.113 ± 0.011 mg/mL, respectively. It was found that Yang-na oleoresin showed the antioxidant capacity lower than Trolox and ascorbic acid with statistical significance (p<0.001). According to the research by Ud-Daula (2016), the antioxidant activity of active compounds associated with the presence of phenolic compounds. Moreover, the research of Balasundram (2006) reported that the antioxidant capacity of active compounds associated with their structure. Especially the number of hydroxyl groups which can donate hydrogen atom to free radicals (Ud-Daula *et al.*, 2016; Balasundram *et al.*, 2006). Yang-na oleoresin consisted of α-gurjunene and β-gurjunene as major compounds with inactive antioxidant activity

(Ruttanamongkol *et al.*, 2014). However, the structure of ascorbic acid composed of 4-OH groups, it can inhibit the free radicals by hydrogen donation and chelation of metal ions (Fe^{+2}) (Brewer, 2011). Meanwhile, Trolox is a derivative of vitamin E which contains a hydroxyl group to donate a hydrogen atom to free radicals (Lúcio *et al.*, 2009). These reasons may explain why Yang-na oleoresin showed very weak antioxidant capacity compared to that of the synthetic antioxidants.

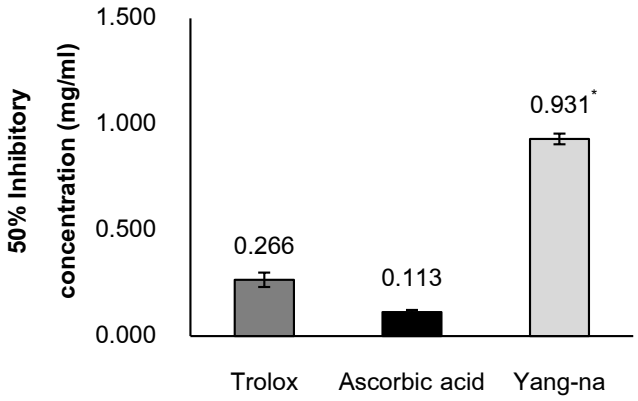

Figure 1 Antioxidant activity of Yang-na oleoresin compared to Trolox and ascorbic acid by DPPH• assay

*significant difference (p<0.001) compared to Trolox and ascorbic acid

4. Lipid peroxidation assay

Lipid peroxidation is an accumulated effect of reactive oxygen species (ROS) that could deteriorate biological systems. Antioxidants inhibit the lipid peroxidation by donating hydrogen atom from the hydroxyl group to lipid peroxyl radical (LOO•) and converting it into a lipid hydroperoxide (Lúcio *et al.*, 2009). The concentration of Yang-na oleoresin used in the determination was 1.683 mg/mL which was in accordance with the controls. The results were expressed as %lipid peroxidation inhibition (%LPO inhibition) as presented in **Figure 2**. 2% (v/v) of Yang-na oleoresin dissolved in absolute ethanol (containing the amount of TPC as 1.683 mgGAE/mL) inhibited lipid peroxidation of 83.51±1.67%. It was significantly higher than Trolox (p=0.042) but comparatively lower than ascorbic acid (p=0.54). It has been reported that the terpenoid components of essential oils can react rapidly with peroxyl radicals which related with Yang-na oleoresin which is an essential oil (Amorati *et al.*, 2013; Aslam *et al.*, 2015). However, ascorbic acid consisted of 4-OH groups while Trolox composed of a hydroxyl group to donate a hydrogen atom to LOO• (Lúcio *et al.*, 2009).

5. Preparation of Yang-na oleoresin-loaded CS-GG nanoparticles

According to Lathsamee *et al.* (2017), Yang-na oleoresin was prepared as nanoparticles by loading in polymeric nanoparticles using a complex-coacervation method. The obtained Yang-na oleoresin nanoparticles had the size of 128 ± 2.65 nm and PDI of 0.53 ± 0.02. The zeta potential of the control (dispersion of Yang-na oleoresin in deionized water and Tween 80) and Yang-na oleoresin-loaded CS-GG nanoparticles were -10.6 ± 3.2 mV and 14 ± 2.5 mV, respectively. The change in surface charge of Yang-na oleoresin-loaded CS-GG nanoparticles indicated the efficient adsorption of the cationic polysaccharide on the surface of the particles. Moreover, the determination of %EE of Yang-na oleoresin-loaded CS-GG nanoparticles was found to be 27.82 ± 1.52%.

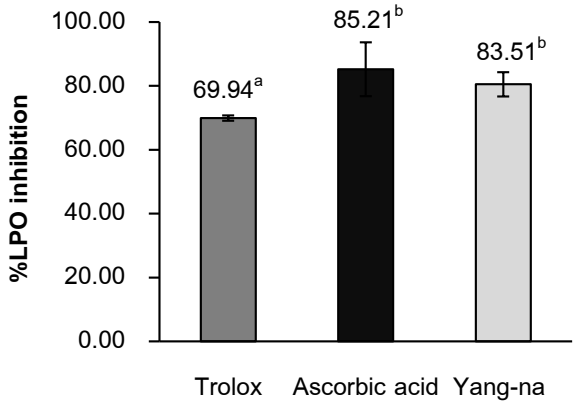

Figure 2 Antioxidant activity of Yang-na oleoresin compared to Trolox and ascorbic acid by lipid peroxidation assay

(a: significant difference p= 0.042 compared to Yang-na; b: not significant difference p= 0.54 compared between Yang-na and ascorbic acid)

6. Preparation of Yang-na cream formulations

Table 4 shows viscosity and physical characteristic of creams. In the cream base group (CB_1-CB_4), the viscosity increased when oil phase was increased, except CB_4. The CB_1-CB_3 had the viscosity ranging from 26,119 ± 1,483 to 40,366 ± 1,701 cP whereas CB_4 had the viscosity of 32,243 ± 409 cP. The pH-values were in the range of 5.3-5.7 which was suitable for human skin (Waqas *et al.*, 2010). Furthermore, CB_1-CB_3 had a light and smooth texture with a white color and green tea odor. After testing a phase separation by centrifugation, only CB_1 was stable. Other formulations were broken, indicating the amount of oil and some compositions in formulations affected on viscosity and appearance of the creams. Phase volume ratio of CB_4 was 41: 59 which was the highest ratio compared to others. Moreover, CB_4 composed of high amount of surfactant (10% (w/w)) and stearic acid as shown in **Table 1**. It has been reported that the ointment base having high amount of stearic acid may show evidence of drying out (Arthur, 2000). This reason might why CB_4 showed poor texture and hardness than other creams.

Yang-na creams and control creams were defined as YC_1 to YC_4 and CC_1 to CC_4, respectively. The results showed that all of these formulations had pH-values around 5.3-5.8, the viscosities were in the range of 12,716 ± 984 to 38,648 ± 4,295 cP as shown in **Table 4**. The pH-values were not significantly different between the control and Yang-na groups. The pH-values of Yang-na creams slightly higher than the control creams, due to different forms of Yang-na oleoresin incorporated in the creams. YC_1 and CC_1 had a higher viscosity than other formulations within Yang-na creams and control creams, respectively. CC_1 and YC_1 were obtained from CB_1 containing glycerol monostearate as a thickening agent in the formula. This might be a reason why the viscosities of CC_1 and YC_1 were higher than other formulations in the groups.

Figure 3 shows the organoleptic of Yang-na creams and control creams. It was found that all creams appeared white texture and had green tea odor. YC_1-YC_3 and CC_1-CC_3 showed smooth texture whereas YC_4 and CC_4 showed viscous texture. After the centrifugation to characterize the phase separation, YC_2-YC_4 and CC_2-CC_4 occurred the phase separation except for YC_1 and CC_1 which were in good accordance with the results of cream bases.

Table 4 Viscosity and physical characteristic of creams after preparation for 24 hr

Formulations	pH (mean ± SD)	Viscosity (cP) (mean ± SD)	Phase separation
CB_1	5.7 ± 0.17	26,119 ± 1,483	-
CB_2	5.6 ± 0.05	35,117 ± 716	+
CB_3	5.7 ± 0.05	40,366 ± 1,701	+
CB_4	5.3 ± 0.05	32,243 ± 408	+
CC_1	5.5 ± 0.05	38,648 ± 4,295$^{\#}$	-
CC_2	5.5 ± 0.05	28,400 ± 2,110	+
CC_3	5.6 ± 0.05	28,025 ± 1,081	+
CC_4	5.3 ± 0.15	26,775 ± 1,594	+
YC_1	5.8 ± 0.05	32,868 ± 1,011$^{\#\#}$	-
YC_2	5.8 ± 0.05	29,212 ± 1,194	+
YC_3	5.8 ± 0.05	27,619 ± 845	+
YC_4	5.3 ± 0.05	12,716 ± 984	+

Phase separation: + Yes/- NO ($^{\#}$significant difference (p<0.001) compared to other formulations in control groups; $^{\#\#}$ Significant difference (p<0.001) compared to YC_4)

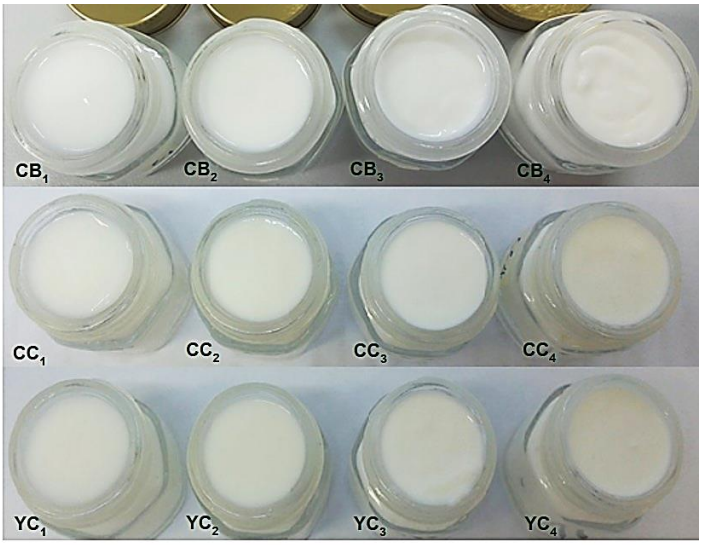

Figure 3 Appearance of cream bases (CB_1-CB_4), control creams (CC_1-CC_4) and Yang-na creams (YC_1-YC_4) after preparation

7. Accelerated stability test

After the test, the result accorded with the centrifugation test after preparation. The phase separation appeared in the formulations of CB_2-CB_4, CC_2-CC_4 and YC_2-YC_4 as shown in **Figure 4**. These creams were not therefore subject to further experiments. Meanwhile, the formulations of CB_1, CC_1 and YC_1 were not changed in the visual aspect. These creams had a white and smooth texture with a green tea odor. Thus, these formulations were used to evaluate characteristics including pH-value, viscosity and phase separation. The viscosities of CB_1, CC_1 and YC_1 were 22,745 ± 463, 28,775 ± 2250 and 29,337 ± 522, respectively (**Table 5**). YC_1 showed significant higher viscosity than CB_1 (p=0.002) but no significant difference was found when compared to CC_1. The viscosity of formulations (CB_1, CC_1 and YC_1) compared between before and after accelerated stability showed a significant decreased (p<0.041). Babcock (1931) reported that the aging cream at a low temperature (5 ºC) increased its viscosity. The greatest increase took place during the first 24 hr of aging. An increasing viscosity due to aging was greater for creams with high butterfat content than creams with low butterfat content. Moreover, the increase in viscosity due to aging was greater with raw creams than with heated creams. The heating lowered the cream viscosity and did not restore to its original viscosity (Babcock, 1931). The pH-values before and after accelerating test of CB_1, CC_1 and YC_1 were statistically significant decrease (p<0.01). According to Gallarate (2013), changes in pH-values of creams could be associated with degradation of some oil phase components, yielding free fatty acids (Gallarate *et al.*, 2013). However, these creams still had good texture and high spreadability and non-greasy after applied to the skin. These results were in good accordance with the phase separation study which showed that CB_1, CC_1 and YC_1 were stable and no phase separation was observed.

Table 5 The physical characteristic of YC_1, CC_1 and CB_1 after accelerated stability

formulations	pH (mean ± SD)			Viscosity (cP; mean ± SD)			Phase separation
	Before	After	%C	before	After	%C	
CB_1	5.7 ± 0.17	5.4 ± 0.26	5.2	26,119 ± 1,483	22,745 ± 463$^{\#}$	12.9	-
CC_1	5.5 ± 0.05	4.6 ± 0.10	16.4	38.648 ± 4,296	28,775 ± 2,250$^{\#}$	25.5	-
YC_1	5.8 ± 0.05	4.9 ± 0.15	16.4	32,868 ± 1,011	29,337 ± 522$^{\#**}$	10.7	-

Phase separation: + Yes/- No and %C-%Change ($^{\#}$significant difference (p<0.041) compared between the viscosity before and after accelerated stability of each sample; **significant difference (p=0.002) compared to CB_1)

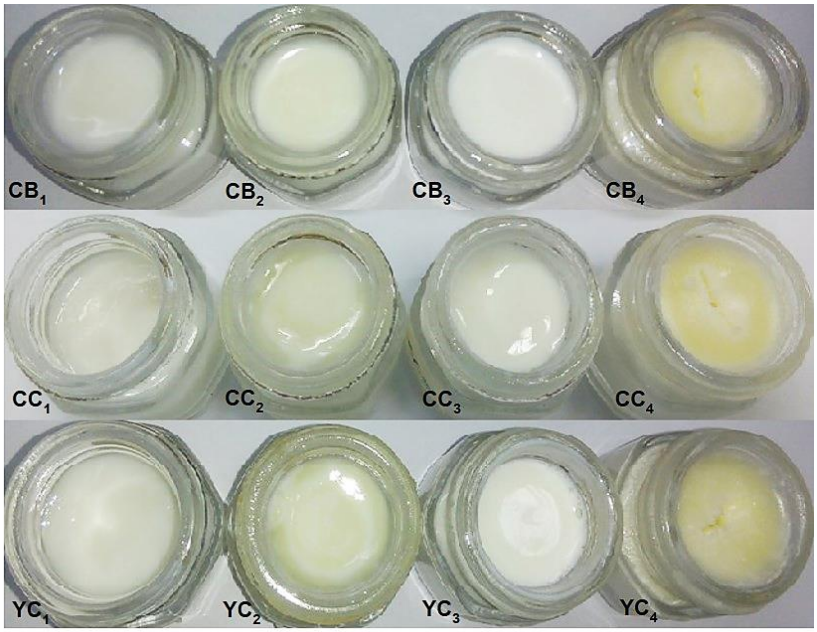

Figure 4 Appearance of cream bases, control creams and Yang-na creams after accelerated stability

8. Determination of the amount of TPC in creams

According to the results of phase separation and accelerated stability test, the creams (CB_1, YC_1 and CC_1) were carried out to determine the amount of TPC by ethanolic extraction at pH of 2.5. **Table 6** shows the amount of TPC in the formulations before and after accelerated stability. After the preparation, the amount of TPC in YC_1 and CC_1 were 95.9 ± 9.95 and 84.8 ± 8.02, respectively. After the test, the amount of TPC in YC_1 was slightly decreased to 93.6 ± 6.49% while that of CC_1 was obviously decreased as 80.1 ± 3.82% (p=0.036). In the CC_1, Yang-na oleoresin was not loaded in polymeric nanoparticles; thereby phenolic compounds are degraded easily by the light and high temperature. This might lead to a decrease in the amount of TPC. In consideration of this result, it can be concluded that the loading of Yang-na oleoresin in polymers can protect the active compounds from the environment which caused the oxidation process and decreased in a capacity of active compounds therapeutically.

Table 6 The amount of TPC in creams after preparation and accelerated stability

Formulations	TPC (before accelerated stability)		TPC (after accelerated stability)		%Change
	mg/mL (mean ± SD)	% (mean ± SD)	mg/mL (mean ± SD)	%(mean ± SD)	
YC_1	0.161 ± 0.01	95.9 ± 9.95^{A}	0.157 ± 0.01	93.6 ± 6.49^{A}	2.2
CC_1	0.142 ± 0.01	84.8 ± 8.02^{B}	0.136 ± 0.04	80.1 ± 3.82^{B*}	3.9

A: not significant difference (p=0.797) compared between YC_1 before and after accelerated stability; B: not significant difference (p=0.423) compared between CC_1 before and after accelerated stability; *significant difference (p=0.036) compared to YC_1

Conclusion

The Yang-na oleoresin was loaded into polymeric nanoparticle successfully. The obtained nanoparticles had a size in nanometer at 128 ± 2.65 nm with positive charges at 14 ± 2.5 mV and %EE of 27.82%. The formulations of cream base, control cream and Yang-na cream developed with glycerol monostearate were considered stable and suitable for skin. After the stability test, there was no change in the appearance, color and odor of these formulations. No phase separation which was confirmed by centrifugation test was observed. The pH-values of the formulations were within the range of skin pH (5.3-5.7), the viscosity was suitable for skin application. The cream composed of Yang-na oleoresin in the nanoparticles form had an amount of TPC higher than the control cream with a significant difference of both before and after accelerated stability. It can protect the TPC in the formulation compared with the control formulation. These results supported the hypothesis that polymeric nanoparticles could protect the active compounds from the environment compared to the control.

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