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Contributed Paper

Anti-aging Cosmeceutical Product Containing of *Nymphaea rubra* Roxb. ex Andrews Extract

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ABSTRACT

The objectives of this study were to examine the biological activities of petal extract (PE) and stamen extract (SE) from *Nymphaea rubra* Roxb. ex Andrews, develop the extracts into a cosmeceutical product and evaluate the skin irritation as well as test the performance of the emulsion containing *N. rubra* extract. The petal and stamen extracts of *N. rubra* were conducted with 95% ethanol. The antioxidant activity, tyrosinase activities and total phenolic and flavonoid content of the extracts were determined. The anti-aging capability of the extracts were also tested by the inhibition of MMP-2, MMP-9 and hyaluronidase activities. The PE showed the highest antioxidant activity on lipid peroxidation inhibition and inhibition of tyrosinase activity together with the highest amount of total phenolic and flavonoid contents. Both the PE and SE showed high ability as antioxidants tested by DPPH and ABTS assays, which are comparable to ascorbic acid and trolox. Furthermore, they strongly inhibited MMP-2 and MMP-9, while the PE showed the highest *in vitro* hyaluronidase inhibition. The PE was then selected for incorporation into O/W emulsion, and the PE emulsion was stable under various tested conditions. According to the clinical evaluation, the PE emulsion was effective on skin wrinkle reduction and skin whitening with no irritation in 30 healthy volunteers after 60 days of twice daily application. Thus, the petal extract and PE emulsion possess potential anti-free radical, skin lightening and anti-aging properties.

Keywords: *Nymphaea rubra* Roxb. ex Andrews, anti-aging, biological activities, cosmeceutical.

1. INTRODUCTION

The outermost layer of the skin is the epidermis, where the pigment-producing cells, or melanocytes, are situated for production of melanin. Tyrosinase is the key enzyme in melanin synthesis. The catalysts of the tyrosinase enzyme include reactive oxygen species (ROS), UV radiation, inflammatory factors, DNA damage triggering of cytokines and other growth factors that can promote enzyme activity leading to enhanced melanin synthesis (1). The dermis layer of human skin is comprised of fibroblast cells that are organized to synthesize collagen and

elastin, which causes skin elasticity and softness. The main contributor to the aging process is the accumulation over time of reactive oxygen species (ROS) which induce the expression and activation of matrix metalloproteases (MMPs) (2). MMP-2 and MMP-9 are able to degrade collagen and elastic fibers. MMP-2 has the ability to hydrolyze gelatins, collagens (types I, IV, V, VII and XI), fibronectin and elastin, while MMP-9 has the ability to degrade gelatins, collagens (types III. IV, V and XIV) and elastin. These degradations result in wrinkle formation and skin aging (3). Moreover, the hyaluronidase enzyme may degrade hyaluronan (HA, hyaluronic acid), which also plays a role in the skin aging process (4).

In cosmetics products, arbutin, retinol and vitamin A are used as whitening and anti-wrinkle ingredients. However, it was reported that these agents may cause side effects such as skin irritation and hyperpigmentation (5, 6). The natural compounds, especially the polyphenols group from secondary plant metabolites, can also be used as antioxidant, whitening and anti-wrinkle agents in cosmetic products. They are easily found, cheap, proven to be safe and biocompatible. As a result, various studies have been conducted to identify new active materials for cosmeceutical applications.

Nymphaea rubra Roxb. ex Andrew, an aquatic plant, is known as the Red Indian Water Lily and belongs to the family Nymphaeaceae. This plant, which grows to heights of up to 6 or 7 feet and has leaves floating on the surface of various lakes, is generally seen planted in home ponds. *N. rubra* is naturally found in the temperate and tropical countries in Asia, such as Bangladesh, India, Taiwan and Thailand. It is commonly used for ornamental purposes and traditional medicines for the treatment of piles, bleeding noses and dysentery. In previous studies, *N. rubra* was noted for its insulin resistance and anti-hyperglycemic, anti-dyslipidemic, anti-inflammatory, anti-pyretic, hepatoprotective, antidiabetic and antioxidant capabilities. Moreover, polysaccharides from the stamen can be used as immunomodulators (7). The flower extract of *N. rubra* consists of phenolic compounds, which include rutin, quercetin, scopoletin and kaempferol (8). However, there is a lack of clarity regarding the cosmeceutical benefits of this plant, which has led researchers to study its antioxidant, skin whitening and anti-aging properties and the possibility to develop it into cosmeceutical products.

2. MATERIALS AND METHODS

2.1 Materials

2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), 2,2–diphenyl–1–picrylhydrazy (DPPH), 3,4-dihydroxy-L-phenylalanine, alcian blue 8GX**,** bis-acrylamide, ammonium thiocyanate (NH_4SCN), ammonium persulfate (APS), calcium chloride ($CaCl_2$), ellagic acid, Folin-Ciocalteu reagent, gallic acid (99.0%), hyaluronic acid, hyaluronidase enzymes from bovine testis (E.C.3.2.1.3.5), iron (ll) chloride tetrahydrate ($FeCl_2 \bullet 4H_2O$), linoleic acid, L-tyrosine, L-dopa, octylphenol ethoxylate (Triton X-100), quercetin, rutin, sodium acetate, sodium chloride*,* sodium chloride (NaCl), sodium nitrite ($NaNO_2$), and tyrosinase mushroom enzyme (25KU) were obtained from Sigma-Aldrich (Steinhiem, Germany). Aluminum chloride from Fluka (Buchs, Switzerland) was used. Bis-acrylamide, Coomassie Brilliant Blue R-250, protein maskers, sodium dodecyl sulphate, tetramethylethylenediamine (TEMED) and tris-HCl were purchased from Bio-Red Laboratories (Richmond, CA, USA). Dulbecco's Modified Eagle's Medium, fetal bovine serum (FBS), trypsin, penicillin and streptomycin were from GeneDireX, Inc. (Taiwan). Acetic acid, 95% ethanol, methanol, sodium carbonate (Na_2CO_3), potassium persulfate ($K_2S_2O_{8)}$, sodium hydroxide (NaOH) and disodium EDTA were from RCI Labscan (Bangkok, Thailand). Acetonitrile and ortho-phosphoric acid 85% were purchased from Merck (Darmstadt, Germany). Cyclomethicone, DL-alpha tocopherol, span 80, 2-phenoxyethanol, hydroxyethyl cellulose (HEC), Carbopol® Ultrez 21, triethanolamine, 1, 3-buthylene glycol, glycerin,

EDTA and tween 80 were purchased from Namsiang Co. Ltd (Bangkok, Thailand). A Finn Chamber® from SmartPractice, Phoenix, AZ (USA) was also used.

2.2 Plant Collection and Extraction

The petals and stamens of *N. rubra* were collected from Udon Thani province, Thailand, during December – February. Mature plants with a height of 1.5 to 1.7 meters together with the flowers having a diameter of 19 to 20 cm were selected. The specimens were authenticated by a botanist (Ms. Wannaree Charoensup) at the herbarium of the Faculty of Pharmacy, Chiang Mai University, Thailand (voucher specimen no. 023246). The petals and stamens were dried at 45 °C and pulverized into powders. Each sample was extracted by Soxhlet's apparatus using 95% (v/v) ethanol as a solvent with a ratio of 1:30. The rate of Soxhlet's extraction was three cycles of soaking/hour, which was performed for 17 h or until the extraction was complete. The filtrates were evaporated under vacuum until dried to obtain the petal extract (PE) and stamen extract (SE). Finally, these extracts were stored at 4 °C for further investigation.

2.3 Antioxidant Activities

2.3.1 DPPH radical scavenging activity

The DPPH radical scavenging activity of PE and SE were determined using a modified method of Fukumoto and Mazza (2000) (9). Each 20 μL of extract dissolved in ethanol was added to 180 μL of 0.1 mmol/L DPPH radicals. After standing in the dark for 30 min, an absorbance at 517 nm was measured using a SPECTROstar nano (BMG LABTECH GmbH, Germany). Ascorbic acid and trolox at seven serial concentrations served as the standards. The DPPH radical scavenging activity of the extracts was calculated using the following equation: % Inhibition = [(Abs positive control-Abs negative control) – (Abs sample-Abs blank)] x 100/(Abs positive control-Abs negative control). In addition, IC_{50} was determined by its concentration with 50% inhibition on the DPPH radicals.

2.3.2 ABTS radical scavenging activity

The ABTS radical scavenging activity of the PE and SE were performed as previously described by Nenadis et al. (2004) (10) with some modification. The ABTS•+ solution was prepared by reaction of 5 mL of 7mM aqueous ABTS and 88 μL of 140 mM potassium persulfate ($K_2S_2O_8$), and then stored in the dark for 16 h at room temperature. Following this, 247 μL of ABTS·+ solutions were added to 3 μL of different concentrations of samples dissolved in ethanol and left standing in the dark for 6 min. The absorbance of the mixture was measured at 734 nm by SPECTROstar nano (BMG LABTECH GmbH, Germany). Trolox and ascorbic acid were used as the reference standards. The ABTS radical scavenging activity of the extracts were calculated by percentage of inhibition, and IC_{50} was determined by its concentration at 50% inhibition.

2.3.3 Lipid peroxidation inhibition activity

Lipid peroxidation inhibition activity of the extracts were determined by the ferric thiocyanate (FTC) method following Alam et al. (2013) and Manosroi (2015) (11, 12) with some modification. Different concentrations of 0.3 mL of extract dissolved in ethanol were mixed with 0.7 mL of DI water, 1.40 mL of 20 mM phosphate buffer pH 7.0 and 1.40 mL of linoleic acid in MeOH. Afterwards, 0.2 mL of AAPH was added to the mixture and incubated at 40 °C for 4 h. Following that, 5.0 μL of the mix sample was mixed with 250 μL of %75(v/v) MeOH, 2.5 μL of 10% (w/v) NH_4SCN and 2.5 μL of 20 mM $FeCl_2$ in HCl. After three minutes, the absorbance of the mixture was measured at 500 nm. α-Tocopherol was used as a reference standard. Lipid peroxidation inhibition activity of the extracts were calculated as in the DPPH radical scavenging activity.

2.4 Enzymatic Assays Tyrosinase Enzyme Activity

Tyrosinase activity, on both the L-tyrosine and L-dopa substrates, was determined using the dopachrome (2-carboxy-2, 3-dihydro-indole-5, 6-quinone) assay (12). In the assay, 70 μL of different concentrations of the extracts solution, 70 μL of 100 units mushroom tyrosinase solution in phosphate and 70 μL of 20 mM phosphate buffer (pH 6.8) were mixed and incubated at 37 °C for 10 min. Then, 70 μL 0.85 mM L-tyrosine or L-dopa solution was added to the mixture in a 96-well plate after being incubated at 37 °C for 20 min. The absorbance was measured at 450 nm for L-tyrosine substrate and 475 nm for L-dopa substrate by using a SPECTROstar nano (BMG LABTECH GmbH, Germany). Kojic acid and ascorbic acid were used as the reference standards. The inhibition of tyrosinase activity of the extracts were calculated with the following equation: % Inhibition = [(Abs positive control-Abs negative control) – (Abs sample-Abs blank)] x 100/(Abs positive control-Abs negative control), and IC_{50} was determined.

2.5 Determination of the Total Phenolic and Flavonoid Contents

The total phenolic content of the extracts was determined by the Folin-Ciocalteau reagent according to the method of Farasat et al. (2014) (13). Each extract was diluted with 95% (v/v) ethanol to a suitable concentration for analysis. 20 μL of extract solution was mixed with 100 μL of 10% (v/v) Folin-Ciocalteau reagent and 80 μL of 7.5% (w/v) Na_2CO_3, followed by incubation for 30 min. The absorbance was measured at 760 nm by a well reader (SPECTROstar nano, BMG LABTECH GmbH, Germany). The total phenolic content was quantified by the calibration standard curve received from various known concentrations of gallic acid [absorbance = 4.4493 gallic acid (mg) + 0.1561; R^2 = 0.9983]. The content was presented as mg of gallic acid equivalents (GAE)/g of the dried extracts.

The total flavonoid content of the extracts was determined by the modified aluminum chloride colorimetric method of Lizcano et al. (2010) (14). Each 35 μL of extract dissolved in ethanol was mixed with 7 μL of 5% (w/v) $NaNO_2$, 14 μL of 10% (w/v) $AlCl_3$. After 6 min, 35 μL of 1M NaOH and 110 μL of DI water were added. The mix solutions were incubated for 15 min at room temperature. The absorbance at 500 nm was measured. The total flavonoid content of the samples was presented as mg of quercetin equivalents (QE)/g of the dried extracts and quantified by the calibration standard curve received from various known concentrations of quercetin [absorbance = 0.3909 quercetin (mg) + 0.0754; R^2 = 0.9991].

2.6 Inhibition of MMPs Enzymatic Activity by Gelatin Zymography

The 3T3 skin fibroblast cells from mice were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and supplemented with 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 10% fetal bovine serum (FBS) at 37 °C in a humidified 5% CO_2 atmosphere. After cell confluence > 90%, the medium was changed to DMEM without serum for 24 h and harvested by centrifugation at 1200 x g for 5 min. Culture supernatants containing MMP-2 (72 KDa) and MMP-9 (92 KDa) were collected and stored at -80 °C.

The inhibition of MMP enzymatic activity was performed as described in Chaiyana et al. (2019) (15) with some modification. Gelatin was used to detect MMP-2 and MMP-9 activities in the culture medium. Briefly, the extract samples of the various concentrations were mixed with the medium and incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 and 48 h. Then, the loading buffer, including 1.5M tris-HCl (pH6.8), 30% (v/v) bis-acrylamide, 10% (v/v) SDS, 1% (v/v) gelatin, 10% (v/v) APS and TEMED that contains in SDS-PAGE, and a running buffer were performed at 100 - 150 Volts for 1.5 - 3 h. After electrophoresis, the gels were incubated

in a reaction buffer (1M Tris-HCL; pH 8.0, 5% (v/v) triton X-100, 5M NaCl and 1M $CaCl_2$) at 37 °C for 20 - 24 h. Then, the gels were added in the fixing buffer, comprised of 50% (v/v) methanol and 12% (v/v) acetic acid in DI water, for 30 min at 37 °C. After that, the gels were stained with Coomassie Brilliant Blue R-250 for 1 h and de-stained in the mixture of 7% (v/v) acetic acid and 40% (v/v) methanol in DI water until visualization of clear bands. The expression of MMP-2 and MMP-9 were analyzed using the ImageJ 1.51J8 program (Wayne Rasband, NIH, USA).

2.7 Inhibition of Hyaluronidase Enzymes Activities

To assay the hyaluronidase enzymes activities according to the method of Chaiyana et al. (2019) (15) with some modification, extract samples of various concentrations were mixed with 0.1 g of hyaluronidase enzymes bovine testis (E.C. 3.2.1.3.5) and 20% (v/v) of 0.15M NaCl incubated at 37 °C for 24 or 48 h. Then, the loading buffer, including 1.5M tris-HCl (pH6.8), 30% Bisacrylamide, 10% (v/v) SDS, 1.7 mg/mL hyaluronic acid, 10% APS and TEMED that contains in SDS-PAGE, and a running buffer were performed at 100 - 150 Volts for 1.5 - 3 h. After electrophoresis, the gels were washed in 1M tris-HCl (pH8.0), 2.5% (v/v) triton X-100, 5M NaCl for 1 h at room temperature and distilled water and incubated in a reaction buffer pH 5 (0.2M sodium acetate-acetic acid (pH 5) and 0.15M NaCl) at 37 °C for 16 - 18 hr. The gels were stained with 0.5% (w/v) alcian blue 8GX and 3% (v/v) acetic acid in DI water, for 1 h and de-stained in the mixture of 50% (v/v) methanol and 1.0% (v/v) acetic acid in DI water until visualization of clear bands. The expression of hyaluronidase enzymes was analyzed by using the ImageJ 1.51J8 program (Wayne Rasband, NIH, USA).

2.8 HPLC Analysis of the Extracts

The phytochemical fingerprints of the PE and SE of *N. rubra* extracts were performed as previously reported (Gautam et al., 2014) (8) with some modification. Each sample was prepared by being dissolved in HPLC-grade methanol (2 mg/mL) and filtrated through a syringe filter nylon membrane (0.45 μm, 13 mm). The compounds were separated on a C18 column (4.6 I.D. x 250 mm, 5 μm particle, Inertsil™ ODS-3) at a flow rate of 0.5 mL/min. The mobile phases were comprised of 0.1% (v/v) phosphoric acid (pH 3) and acetonitrile. The gradient used 0 - 70 min, 90 - 59% of 0.1% (v/v) phosphoric acid (pH 3), and 70 - 75 min used 59% of 0.1% (v/v) phosphoric acid (pH 3). The detector was set at 369 nm. The components in the extracts were identified using the reference standards for quercetin and ellagic acid.

2.9 Stability Test of the Extract Solution

The extract with the highest value of bioactivities was selected for the stability evaluation. The extract solution was stored in accelerated condition using 6 cycles of heating-cooling cycling changing between 4 °C (48 h) and 45 °C (48 h) and room temperature with light exposure, room temperature in the dark, 45 °C and 4 °C for 3 months. After storage, they were evaluated for the differences with the initial stage regarding the physical properties, pH, viscosity, bioactivity and the content of ellagic acid.

2.10 Preparation of O/W Emulsion Containing PE

An O/W emulsion was prepared by the conventional hot process. The oil phase comprised of cyclomethicone, DL-alpha tocopherol, span 80 and 2-phenoxyethanol was heated up to 75 - 80 °C. The water phase consisting of hydroxyethyl cellulose (HEC), Carbopol® Ultrez 21, triethanolamine, 1, 3-buthylene glycol**,** glycerin, EDTA, tween 80 and water was heated to the same temperature. Then, the oil phase was added to the water phase with continuous stirring until the complete emulsion was formed. Next, 0.2% (w/w) of PE was added

to the emulsion at 45 °C before packaging. The emulsion base and PE emulsion were then tested for stability by storage at various conditions in the same way as the extract solution mentioned above.

2.11 Clinical Trials for Skin Irritation and Performance Testing

This study was approved by the Research Ethics Committee, Faculty of Pharmacy, Chiang Mai University. This ethics committee is organized and operates according to the GCPs and the relevant international ethical guidelines, as well as the applicable laws and regulations. The ethics committee approval number is 027/2561.

2.11.1 The skin irritation test on human volunteers

The skin irritation tests on human volunteers by patch tests (Finn chambers®) were carried out on the backs of 30 healthy volunteers. The patches were mounted with control (1% (w/v) sodium lauryl sulfate), 0.2% (w/w) of PE in solution, 1.0 g of the emulsion base and the PE emulsion. Each treated site was separately applied to marked regions on the back. The patches were removed after 48 h and washed with physiological saline. The erythema and edema on the skin were observed at 1 h, 24 h and 7 days after patch removal. The skin irritation was evaluated using the Drazie Scoring system and the PII value was calculated using the following equation (16): PII value = [(∑edema at 1 h/24h/7d) + (∑erythema at 1 h/24h/7d)]/ (number of the observations of treated sites) x (number of sample).

2.11.2 Performance test of products on volunteers

A total of 30 healthy volunteers with an average age of 30 - 50 years (11 males and 19 females) were selected for the study and informed consent was given. Performance tests of the formulation on volunteers by all samples and controls, including the emulsion base (A) and the PE emulsion (B), were applied on the lower forearm of the 30 healthy volunteers. The right forearm was covered with the emulsion base and separated from the untreated area, while the left forearm was covered with the PE emulsion twice a day (morning and evening) for 60 days. The overall efficiency was evaluated by the volunteers using the corneometer® for skin moisture content, the visiometer® for skin wrinkles, the cutometer® for skin elasticity and the mexameter® for color of skin. The results were expressed as percentage efficacy using the following equation: % efficacy = $(V_m-V_0) \times 100/V_0$, where V_0 was the value at the initial point (day 0), and V_m was the value at the measuring points (30 and 60 days).

2.12 Statistical Analysis

All assays were done in triplicate experiments. The data were expressed as the mean ± standard deviation (SD). One-way ANOVA (Tukey test) was applied for multiple comparisons and the paired t-test was conducted for comparisons of differences between the two samples by using SPSS (version 17.0, SPSS, Inc., Chicago, IL, USA). The significance level in the statistics was shown as $p < 0.05$.

3. Results and Discussion

3.1 Percentage Yield of Extracts

The PE and SE of *N. rubra* that were extracted by Soxhlet's apparatus with 95% (v/v) ethanol gave high percentage yields. The percentage yields of PE and SE were 44.92±1.3 and 29.57±2.0%, respectively. Thus, the PE showed a higher percentage yield than the SE.

3.2 Antioxidant Activities

3.2.1 DPPH radical scavenging activity

The PE and SE showed high ability regarding radical scavenging that is comparable to ascorbic acid and trolox, as shown in Table 1. The IC_{50} value of PE and SE were 0.0754±0.002 and 0.0833±0.001 mg/mL, respectively. From the study, the PE and SE were shown to consist of high contents of phenolic compounds. In general, phenolic and flavonoid constituents derived from

Table 1. The IC_{50} of biological activities of the PE and SE of *N. rubra*.

Sample	IC_{50} of DPPH assay (mg/mL)	IC_{50} of ABTS assay (mg/mL)	IC_{50} of lipid peroxidation inhibitory activity (mg/mL)	Tyrosinase inhibition assay	
				IC_{50} on L-tyrosine substrate (mg/mL)	IC_{50} on L-dopa substrate (mg/mL)
PE	0.0754 ± 0.002^{a}	0.2730 ± 0.006^{c}	28.5758 ± 2.781^{e}	3.2853 ± 0.361^{h}	11.9861 ± 1.016^{k}
SE	0.0833 ± 0.001^{b}	0.2836 ± 0.020^{d}	$35.2165\pm1.704^{e,f}$	$4.3729\pm0.400^{h,i}$	$14.7013\pm0.606^{k,l}$
Standards					
Ascorbic acid	$0.0501\pm0.002^{a,b}$	$0.1553\pm0.003^{c,d}$	-	$0.1573\pm0.038^{h,i,j}$	$0.0815\pm0.025^{k,l,m}$
Trolox.	$0.0504\pm0.001^{a,b}$	$0.1359\pm0.010^{c,d}$	-	-	-
α-tocopherol	-	-	$4.4301\pm0.447^{e,f}$	-	-
Kojic acid	-	-	-	$0.01404\pm0.001^{h,i,j}$	$0.0573\pm0.003^{k,l,m}$

- = not applicable. Each value represented the mean ± s.d. (n = 3). Values with the same alphabetical superscripts along columns are significantly different ($p < 0.05$) as analyzed by one-way ANOVA.

plants are highly effective on radical scavenging (17). Phenolic compounds exhibit radical scavenging ability by donating hydrogen atoms or electrons with free-radicals of oxidant compounds. The efficacy of phenolic compounds as an antioxidant is determined by the stability of the phenoxyl radical formation (18, 19). The ability against DPPH radicals of the PE and SE may be related to the presence of phenolic compounds.

3.2.2 ABTS radical scavenging activity

The IC_{50} value of the PE and SE were 0.2730 ± 0.006 and 0.2836 ± 0.020 mg/mL, respectively. The PE and SE exhibited high ability against ABTS radicals when compared with ascorbic acid and trolox (Table 1), which is also based on their high phenolic content.

3.2.3 Lipid peroxidation inhibition activity

The PE showed significantly ($p < 0.05$) higher lipid peroxidation inhibition activity than the SE (IC_{50} value of 28.5758 ± 2.781 and 35.2165 ± 1.704 mg/mL, respectively), but lower than α-tocopherol (IC_{50} value of 4.4390 ± 0.447 mg/mL), as shown in Table 1. This method is suitable for the lipophilic compounds. Polyphenols are more hydrophilic due to the hydroxyl group in their structure, which limits the interaction with lipids (20). The results in this study agreed with previous studies, in which tamarixetin and isorhamnetin were more lipophilic as shown by their high ability of scavenging free radicals on lipid peroxidation compared with hydrophilic compounds such as quercetin and its polar derivatives (21).

3.3 Enzymatic Assays Tyrosinase Enzyme Activity

The tyrosinase inhibition is the key target for skin-lightening. The PE showed significantly ($p < 0.05$) higher inhibition of tyrosinase on both L-tyrosine and L-dopa substrates than SE. However, both extracts were less active than ascorbic acid and Kojic acid (Table 1). The results may depend on the flavonoid content that showed inhibition on oxidizing of L-tyrosine or L-dopa substrates with tyrosinase. Moreover, the structure of the flavonoid at position 3-hydroxy-4-keto moiety and their antioxidant properties can inhibit tyrosinase by chelating with copper in the active site of the enzyme (1, 22).

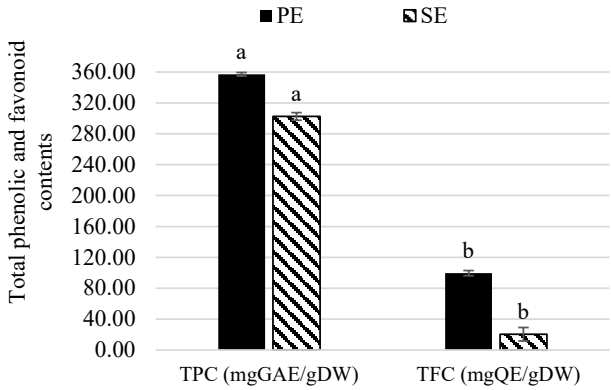

Figure 1 Total phenolic and total flavonoid content of the petal and stamen extracts of *N. rubra* by Soxhlet's apparatus. Results are the means of three replicates. The same alphabetical superscripts indicate significant difference between PE and SE ($p < 0.05$) as analyzed by t-test.

3.4 Total Phenolic and Flavonoid Contents

The levels of phenolic and flavonoid contents were calculated from the regression equation of the calibration curve and presented as mg GAE/g DW and mg QE/g DW. The PE gave significantly ($p < 0.05$) higher total phenolic and flavonoid contents than the SE, as shown in Figure 1. The high contents of phenolic and flavonoid may be dependent on the solvent polarity. The phenolic and flavonoid were extracted with more polar solvents (12). Ethanol showed effective ability to extract sterol, flavonoid, phenolic, and alkaloid contents (23). Previous studies also reported that the aqueous ethanol extract of *Merremia borneensis* showed the higher contents of phenolic and flavonoid than the hexane extract (24).

3.5 Inhibition of MMP Enzymatic Activity by Gelatin Zymography

The inhibition of matrix metalloproteinases (MMPs) is related to the anti-aging properties. MMP-2 and MMP-9, also known as 72 kDa gelatinase A/type IV collagenase and 92 kDa gelatinase B/type IV collagenase, may degrade collagen and elastin (3). For MMP-9 activity, the concentration at 1.25 mg/mL of the PE and the SE exhibited a strong inhibition of MMP-9 at 48 h of incubation with % inhibition of 91.3±2.0 and 87.6±5.5%, respectively, and at 48 h of incubation, they showed a significantly ($p < 0.05$) higher % inhibition of MMP-9 than at 24 h (Figure 2A). In this study, both the PE and SE presented a dose dependent on MMP-2 activity inhibition. Interestingly, both the PE and SE presented a high % inhibition (greater than 75%) on MMP-2 at 5.00 mg/mL with a low intensity band expression on the gelatin zymogram, as shown in Figure 2B, and were effective after 24 h of incubation. However, the PE and SE were not significantly different regarding the ability of inhibition of both the MMP-9 and MMP-2 activities. Polyphenols have been previously reported as providing strong inhibition of the gelatinolytic activity of MMPs such as MMP-9 and MMP-2 with different mechanisms, including the blocking of enzymatic activity and the reduction of gene expression. Moreover, they can directly inhibit MMP levels through the activation of enzymes and nuclear receptors, or by the inhibition of the transcription factors. The inhibition of MMPs of polyphenol and antioxidant compounds may be the indirect result of ROS scavenging. The ROS may induce enhancement of MMP-9 through the activation of the MAPK pathways and transcription factors (25, 26). The extracts might attenuate the collagen degradation by MMP-2 and MMP-9, which are

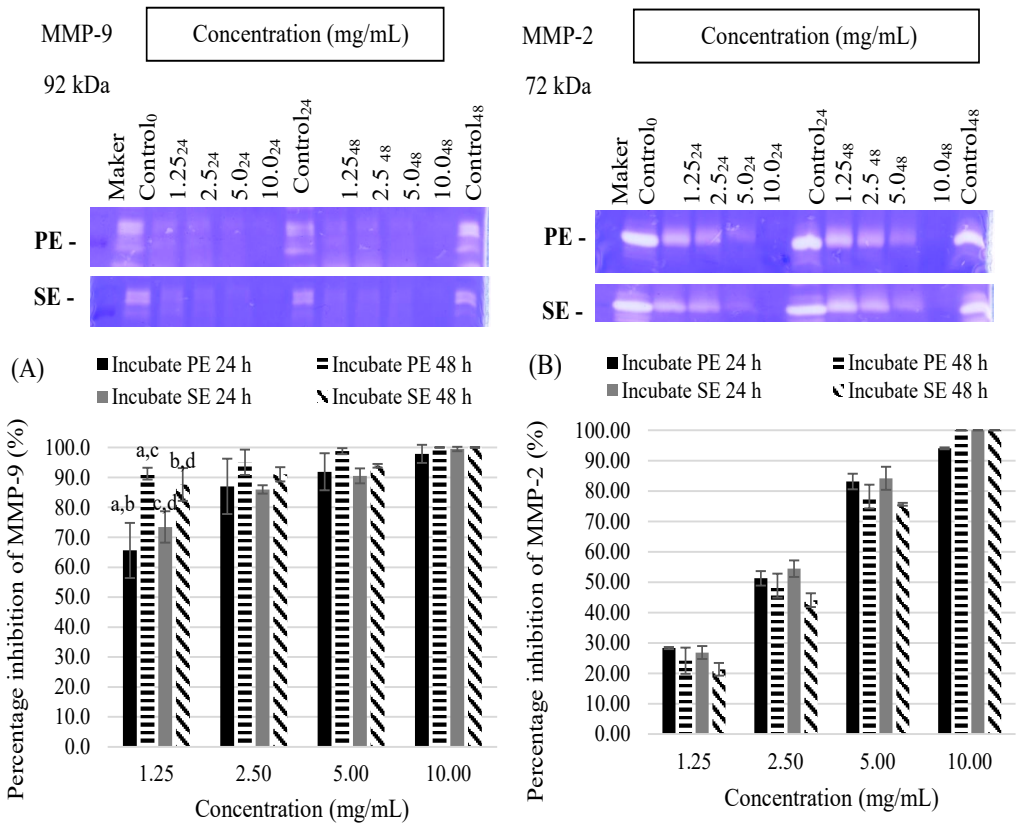

Figure 2. The zymograms and percentages of inhibition on MMP-9 (A) and MMP-2 (B) of the PE and SE. The results are the means of three replicates. The same alphabetical superscripts indicate significant differences ($p < 0.05$) between the PE and SE in the same concentration at 24 and 48 h of incubation that were analyzed by one-way ANOVA.

thought to be at least in part due to the polyphenol content and antioxidant effects.

3.6 Inhibition of Hyaluronidase Enzymes Activities

The enhanced activation of the hyaluronidase enzyme, which is an extracellular matrix degrading enzyme, is capable of breaking down hyaluronan (HA, hyaluronic acid). This plays a role in skin dryness, wrinkles and skin aging (4). With merely 0.125 mg/mL, the PE in 48 h of incubation presented the highest hyaluronidase enzymes inhibition with no expression of band intensity on zymograms, which was 94.2±4.6% inhibition (Figure 3). The inhibition effect of the extracts may be caused by the presence of flavonoids that have been reported to inhibit hyaluronidase activities. The flavonoid structure that performs enzyme inhibition consists of a double bond between carbons at positions 2 and 3, unsubstituted hydroxyl groups, and a ketone group (27).

3.7 HPLC Analysis of the Extracts

Figures 4A - 4C show the HPLC chromatogram of the PE, SE and reference standard. Both extracts presented the retention time of 36.575 min, 36.182 min, 59.944 min and 59.557 min, which were the same as the reference standards, ellagic acid (Rt of 36.633 min) and quercetin (Rt of 59.931 min). According to the previous research,

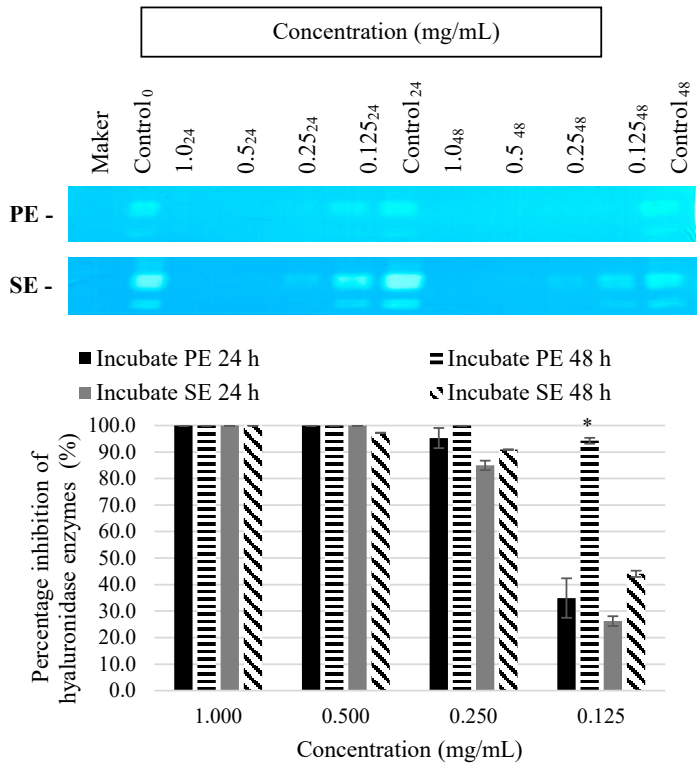

Figure 3. The zymograms and percentages of inhibition on hyaluronidase enzymes of the PE and SE of *N. rubra*. The results are the means of three replicates. The (*) symbol indicates a significant difference ($p < 0.05$).

it was reported that the chloroform fraction from the ethanolic crude extract of *N. rubra* flowers in India was comprised of quercetin, rutin, scopoletin, and kaempferol (8); whereas, this study only found the quercetin. The region of the plant, the climate, and the harvesting period may have an effect on the chemical composition of the extract. In addition, the main compound of the PE and SE of *N. rubra* was identified as ellagic acid. However, the PE has a higher ellagic acid content than the SE at the same concentration with % relative peak areas of 30.25±1.2% and 24.50±0.8%, respectively (Figures 4A and 4B). Thereby, the ellagic acid as a major compound may be one of bioacitivity components in the PE and SE of *N. rubra*, and the unknown peaks in the HPLC chromatograms of PE and SE were further studied.

3.8 Stability Test of the Extract Solution

The PE presented the highest level of bioactivities that was selected for stability study. The PE solution was a brown solution that did not change in color and pH value at various storage conditions for 3 months. However, when stored at 45 °C, the color became darker and precipitated at 3 months. The PE solution showed 97.4±0.5% of inhibition on DPPH radicals together with 82.3±1.9% and 52.5±0.5% inhibition of tyrosinase enzyme activities (monophenolase and diphenolase activities) at the initial stage. After storage with heating-cooling cycling and 45 °C, 4 °C, room temperature with light exposure and in the dark for 3 months, it presented a slightly reduced antioxidant capacity as tested by DPPH with 94.7±0.6%, 90.9±0.6%, 94.1±0.3%, 93.4±0.9% and 92.2±0.7%

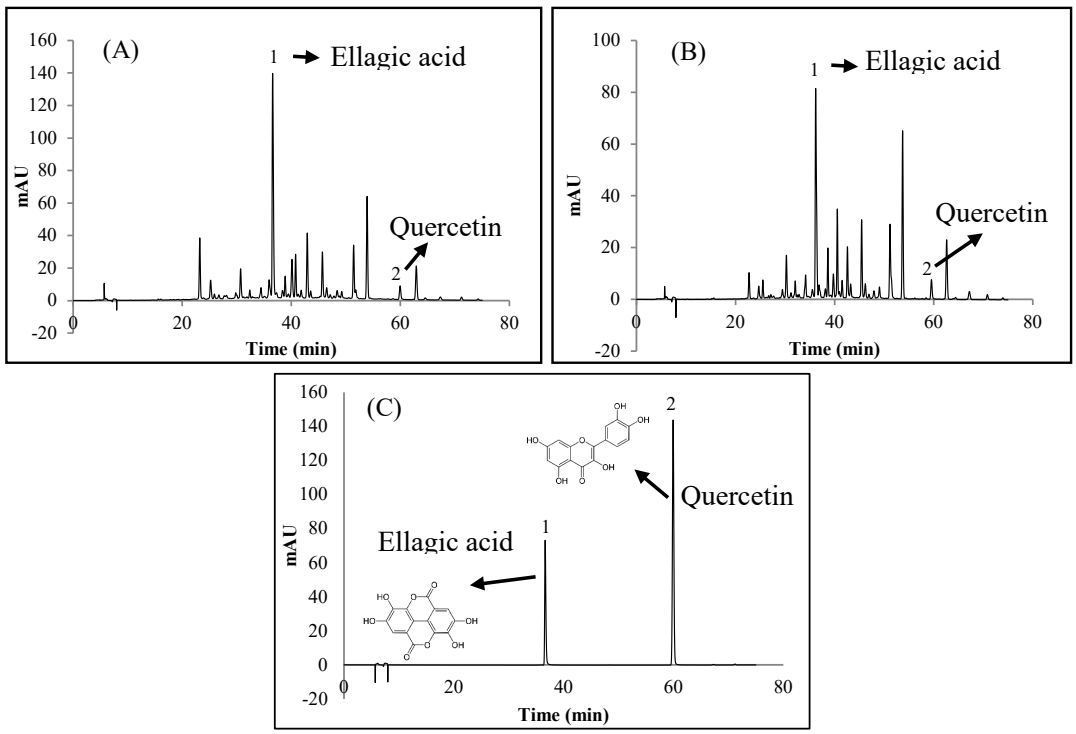

Figure 4. Representative finger printing of the HPLC chromatogram of the PE (A) and SE (B) of *N. rubra*, ellagic acid and quercetin (C).

of inhibition, respectively. The inhibition of the tyrosinase enzyme at monophenolase activity was 62.9±2.1%, 66.4±2.4%, 73.7±2.8%, 67.2±2.1% and 68.2±1.1% and diphenolase activity was 38.8±2.0%, 36.4±1.2%, 42.3±2.4%, 39.5±1.4% and 40.8±0.9% at 3 months, respectively. After 3 months, the ellagic acid content changed from 85.80±1.98 μg/mL at the initial stage to 64.41±2.84, 61.98±1.94, 74.17±1.38, 64.72±2.18 and 73.58±0.81 μg/mL, respectively. The stability of the PE solution decreased with high temperature. Therefore, the extract must be kept away from heat.

3.9 Development of Emulsion Containing PE

3.9.1 Product formulation

The PE was selected for development into emulsion at a concentration of 0.2% (w/w). The freshly prepared emulsion base and PE emulsion were white and light yellow homogeneous creams with pH 5 and a viscosity value of 1.879 and 1.857 Pa.s, respectively. In addition, they provided smooth skin, easy spreading and high permeation when applied onto the skin.

3.9.2 Stability test of the formulation

The physicochemical properties such as color, pH, and viscosity of the emulsion base and PE emulsion were not changed with the on phase separation after 3 months in all storage conditions. At the initial point, the PE emulsion showed 95.2±0.2% of inhibition on the DPPH assay together with 76.6±0.5% and 63.2±1.0% inhibition of tyrosinase enzyme activities. After storage with the heating-cooling cycling and 45 °C, 4 °C, and room temperature with light exposure and the dark for 3 months, it presented a slight decrease

in antioxidant capacity as tested by DPPH with 93.9±1.0%, 90.2±0.2%, 90.4±0.34%, 89.8±0.5% and 90.1±0.3% of inhibition, respectively, as well as the inhibition of the tyrosinase enzyme at monophenolase activity with values of 63.9±2.1%, 59.7±0.9%, 69.0±0.6%, 65.2±0.6% and 66.0±0.7%, and diphenolase activity that was 56.4±0.7%, 50.8±0.6%, 69.0±0.6%, 65.2±0.6% and 66.0±0.7% at 3 months, respectively. After 3 months, the ellagic acid content changed from 69.05±1.32 μg/mL at the initial point to 55.37±0.81, 49.04±1.32, 63.01±2.78, 57.12±2.81 and 57.45±2.55 μg/mL, respectively. These results demonstrated that the high temperatures affect the bioactivities and ellagic acid content of the PE emulsion.

3.10 The Skin Irritation Test on Human Volunteers

The skin irritation was evaluated using the Drazie scoring system and calculated in terms of primary irritation index (PII) values (16). The results presented no irritation from the PE of *N. rubra*, the emulsion base and the PE emulsion on 30 healthy volunteers, as shown in Table 2. This results indicated that 0.2% (w/w) PE of *N. rubra* and PE emulsion were safe for application on skin. The previous study also reported that *N. rubra* rhizome extract was not toxic in both the acute and sub-acute toxicity tests (7).

3.11 Performance Test of PE Emulsion in Human Volunteers

3.11.1. Skin moisture content

The PE emulsion showed the highest increase of skin moisture content after 60 days of application. Its percentage efficiency value was significantly higher than that of the untreated skin and emulsion base at both 30 and 60 days (Figures 5A and 5B). However, the male volunteers after application of the PE emulsion for 60 days presented a significantly higher percentage efficiency than the females (Figure 5B). This result was in accordance with the previous report that stratum corneum hydration is stable in women during their lifetime, while it decreases over time in men beginning at the age of 40 years. In general, the lifestyle of most males aged 30 - 50 years is to not use skin care products, which can cause a high increase of skin moisture content after application of the PE emulsion. Moreover, transepidermal water loss (TEWL) in men was significantly lower than the loss in women of the same age (28).

3.11.2 Skin pigments (melanin and erythema)

According to the results, the PE emulsion presented the highest decrease of both melanin and erythema contents after 60 days of application (Figures 6B and 6D), which showed significantly ($p < 0.05$) higher percentage efficiency values than the untreated skin and when using the emulsion

Table 2. Evaluation of response categories of skin irritation (Primary irritation index (PII)).

Treated sites	∑erythema at 1h/24h/7d	∑edema at 1h/24h/7d	PII value	Reaction
1% (w/v) SLS	83	22	3.5	Moderate irritation
Untreated	5	0	0.17	Negligible
0.2% (w/w) PE	1	0	0.03	Negligible
Emulsion base	0	0	0	Negligible
PE emulsion	3	0	0.1	Negligible

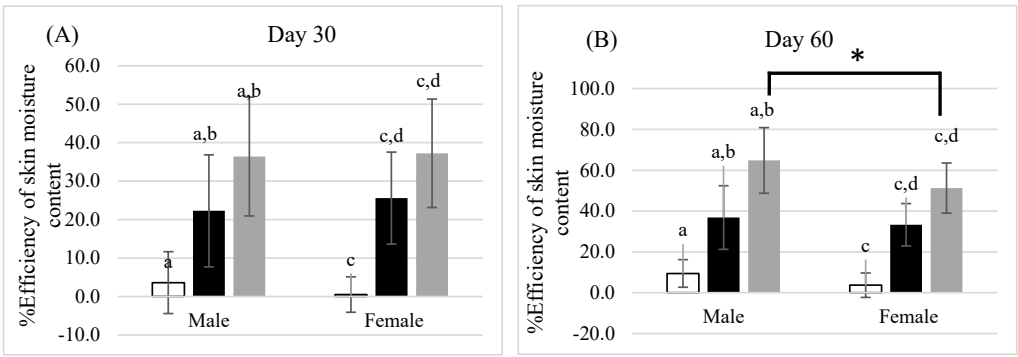

Figure 5. The percentage efficiency values on skin moisture content after application of the emulsion base and PE emulsion in male and female volunteers at day 30 (A) and day 60 (B). The same alphabetical superscripts indicate significant differences ($p < 0.05$), and the symbol (*) indicates a significant difference ($p < 0.05$) between males and females.

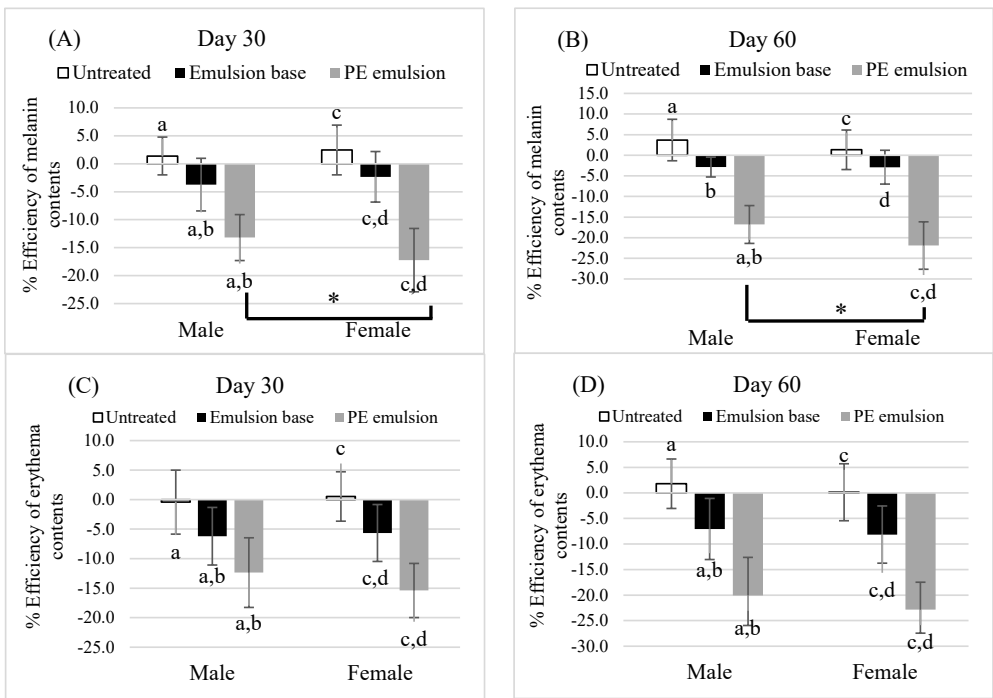

Figure 6. The percentage efficiency on skin pigment after application of the emulsion base and PE emulsion: (A) Melanin content after 30 days, (B) Melanin content after 60 days, (C) Erythema content after 30 days and (D) Erythema content after 60 days. The same alphabetical superscripts indicate significant differences ($p < 0.05$), and the symbol (*) indicates a significant difference ($p < 0.05$) between males and females.

base at 30 and 60 days (Figures 6A - 6D). In this study, the application of the PE emulsion for 30 and 60 days in female volunteers showed a significantly ($p < 0.05$) higher percentage efficiency value of melanin content than the males (Figures 6A and 6B). There has been previous research conducted on Asians in which the skin melanin index was significantly higher in male subjects (28). This may affect the ability of reducing the melanin content in the males after application of the PE emulsion.

3.11.3 Skin elasticity

After 60 days of treatment, the PE emulsion showed the highest increase of skin elasticity. In the percentage efficiency values on skin elasticity, the PE emulsion presented a significantly ($p < 0.05$) higher value than the untreated skin and that with the emulsion base after 30 and 60 days of application. Moreover, the percentage efficiency values after 30 and 60 days of males and females were not significantly different, as shown in Figures 7A and 7B.

3.11.4 Skin wrinkles

The PE emulsion presented the highest reduction of skin roughness (R3 and R5), skin surface and volume after 60 days of application. The percentage efficiency values of all skin wrinkles parameters of the PE emulsion were significantly higher than the untreated skin and the emulsion base at 30 and 60 days of application. The male and female sample groups showed no significant differences in percentage efficiency on all skin wrinkles parameters after 30 and 60 days, as shown in Figures 8A - 8H. Furthermore, the depth levels of skin roughness and wrinkles after being treated with PE emulsion in the human volunteers were obviously reduced when compared with those before treatment, the untreated skin and the emulsion base at 30 and 60 days (Figure 9).

In this study, the skin moisture and elasticity were improved after treatment with the PE emulsion, which showed the skin pigmentation and wrinkles reducing properties on 30 healthy volunteers for 60 days. The enhancement of the skin moisture content of the PE emulsion was a moisturizing effect of an occlusive mechanism or as humectants including glycerin and butylene glycol, which are applied in order to delay the dryness and provide skin moisturizing in emulsions (29). The increase in skin moisture may be due to the swelling effect of the phenolic compounds in the PE on the corneocytes of the skin surface. It also

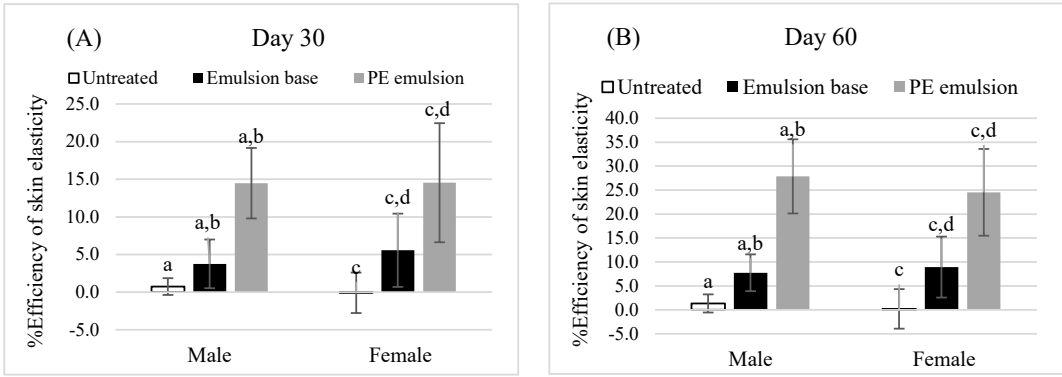

Figure 7. The percentage efficiency on skin elasticity after application of the emulsion base and PE emulsion in male and female volunteers at day 30 (A) and day 60 (B). The same alphabetical superscripts indicate significant differences ($p < 0.05$).

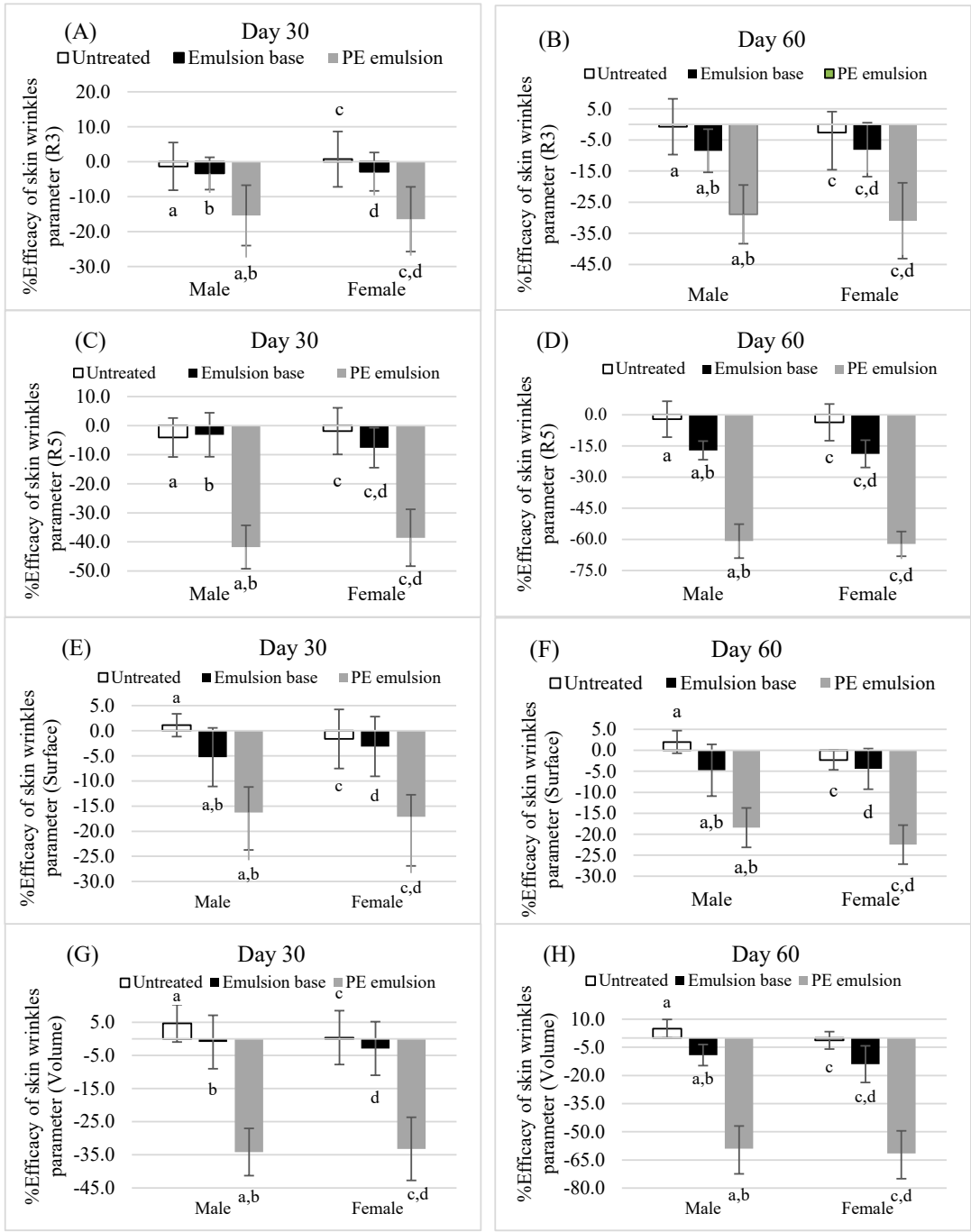

Figure 8. The percentage efficiency of the skin wrinkles parameters after application of the emulsion base and PE emulsion: (A) Roughness; R3 after 30 days, (B) R3 after 60 days, (C) Roughness; R5 after 30 days, (D) R5 after 60 days, (E) Surface after 30 days, (E) Surface after 60 days, (G) Volume after 30 days and (H) Volume after 60 days. The same alphabetical superscripts indicate significant differences ($p < 0.05$).

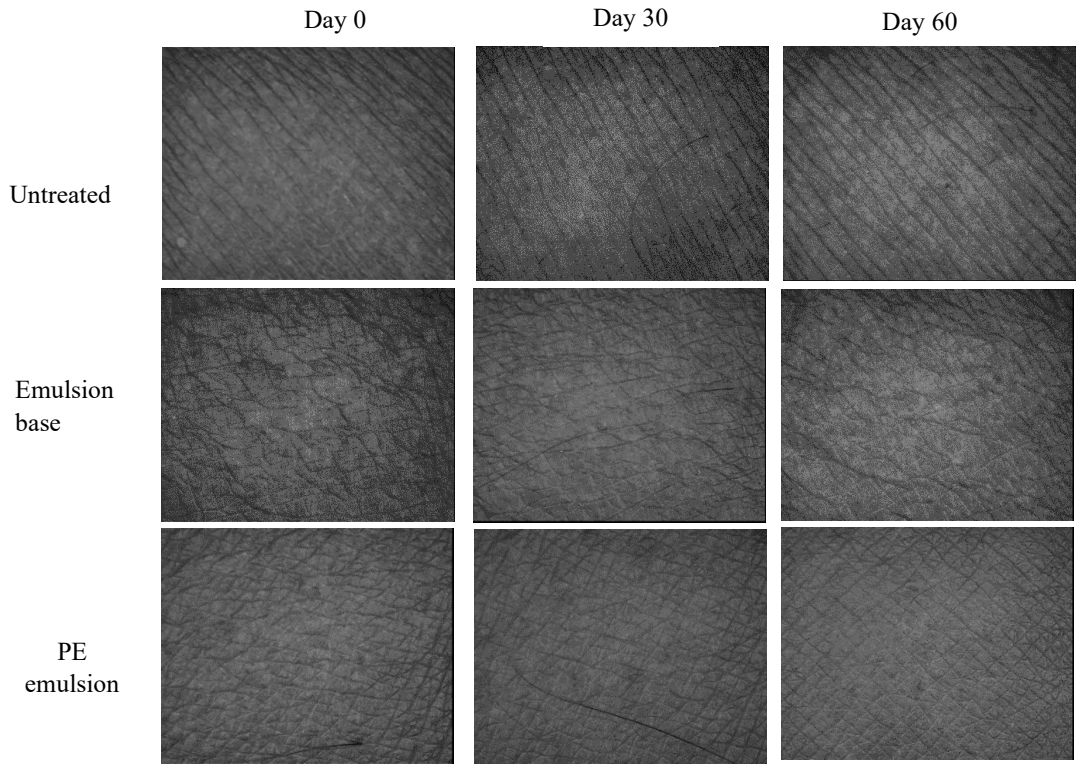

Figure 9. The depth levels of skin roughness and wrinkles of human volunteers on untreated skin and skin after treatment with the emulsion base and PE emulsion for 0, 30 and 60 days by Visiometer®.

increased the extracellular collagen that induced more elastic skin and lower amounts of skin wrinkles (30). The skin lightening and anti-aging effects from the clinical trials were in accordance with our previous *in-vitro* biological activities studies. The antioxidants property from the PE may prevent the oxidative effects on skin from UV-irradiation that contributes to the activation of melanogenesis. In addition, antioxidants can inhibit the reactive oxygen species (ROS) in the skin that may induce melanogenesis by activating tyrosinase and also cause the aging process (1).

4. Conclusions

The petal extract of *N. rubra* presented the highest biological activities, including antioxidant, skin lightening and anti-aging activities with *in vitro* studies. The product containing the PE (PE emulsion) was found to be stable under various storage conditions. This formulation was shown to be able to improve skin moisture and elasticity. Furthermore, that the PE emulsion could decrease skin pigmentation and provide the skin anti-aging effects with no skin irritation on 30 healthy volunteers for 60 days of twice daily application was confirmed.

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