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Research Article

INVESTIGATION OF *IN VITRO* ANTIOXIDANT ACTIVITY AND CYTOTOXICITY ON HUMAN SKIN FIBROBLASTS OF *PLUCHEA INDICA* (L.) LESS. LEAVES EXTRACT AND DEVELOPMENT TO MICROEMULSION FOR SKIN COSMETIC PRODUCT

ITSARASOOK K*, PROMPAMORN P, TANGHIRANRAT J, PANICHAKUL T

Cosmetic Science Program, Faculty of Science and Technology, Suan Dusit University, Bangkok, 17000 Thailand.
Email: khwunjit_its@dusit.ac.th

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ABSTRACT

Objectives: The aim of this study was to determine the antioxidant activity and cytotoxicity on normal human skin fibroblasts of *Pluchea indica* (L.) Less. leaves extract and to prepare the extract into microemulsions for application in skin cosmetic ingredient.

Methods: The total phenolics and total flavonoids content in ethanolic (30%, 70%, and 95% ethanol) and aqueous extracts of *P. indica* leaves were determined by Folin-Ciocalteu assay and aluminum chloride method. The quantification of 4,5-O-dicaffeoylquinic acid in the extracts was determined by an ultra-high performance liquid chromatography method. The antioxidant activity of extracts was examined by 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), and ferric reducing antioxidant power (FRAP) assays. The cytotoxicity on human fibroblast cell was tested by sulforhodamine B assay. The oil in water microemulsion was prepared by pseudoternary phase diagram. The characterization of prepared-microemulsion including morphology, particle size, and polydispersity index (PDI) was studied and the physicochemical stability of microemulsions was evaluated.

Results: All *P. indica* leaves extracts contain a phenolic, flavonoid, and 4,5-O-dicaffeoylquinic acid contents. The 70% ethanolic extract possessed higher the antioxidant activities than 30% ethanolic, aqueous, and 95% ethanolic extracts, respectively, followed by DPPH, ABTS, and FRAP assay. Thus, the 70% ethanolic extract was selected to develop in skin cosmetic. The 70% ethanolic extract had not cytotoxicity on dermal human skin fibroblasts cell. The microemulsion loaded extract provided a mean particle size of 24.397±1.20 nm with minimum PDI of 0.397±0.05 which shown a spherical shape. The microemulsion loaded extract showed a good stability.

Conclusion: From all results, it has concluded that the 70% ethanolic extract of *P. indica* leaves contain phenolic, flavonoid, and 4,5-O-dicaffeoylquinic acid contents which possessed DPPH, $ABTS^{+}$, and FRAP activities. In addition, this extract had no cytotoxic to normal human skin fibroblasts. Finally, the microemulsion loaded extract showed a good characterization and physicochemical stability.

Keywords: *Pluchea indica* leaves extract, Antioxidant activities, Cytotoxicity, Microemulsion.

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INTRODUCTION

Free radicals are atoms or molecules which have an unpaired electron, at least 1 electron. Free radicals are unstable and reactive with neighboring molecules to make themselves more stable. As chain reaction, the neighboring molecules which lose or accept electron to become a new free radicals and then they will further react with another molecule to produce the new free radicals [1]. According to the ability to oxidize compounds, the free radicals are known as reactive species (RS). The RS are classified into two types; reactive oxygen species (ROS) including superoxide anion and hydroxyl radical and reactive nitrogen species (RNS). ROS are produced by both intrinsic and extrinsic factors effecting on skin disorder such as skin aging and skin hypigmentation. Under normal conditions, ROS are eliminated by the antioxidant defense systems such as antioxidant enzymes (glutathione [GSH] peroxidase, superoxide dismutase, and catalase) and non-enzymatic factors (Vitamin E, Vitamin C, GSH, uric acid, and ubiquinol) [2]. Whereas under abnormal conditions, the balance between the generation and elimination of ROS is broken, as a results of biomacromolecules; DNA, membrane lipids, and proteins are damaged by ROS-mediated oxidative stress [3]. These event can suppress by antioxidants [4].

In recent years, there are many researches investigation of new and safe antioxidants from natural sources to inhibit free radicals. Therefore, the extract from natural products with an anti-oxidant properties has been searched to treat a variety of skin conditions. Flavonoids are which mostly found in natural products. Many research found that flavonoids exhibit strong antioxidant activity in several mechanisms [5]. *Pluchea indica* (L.) Less., namely, Klu in Thai, belongs to Asteraceae family. This plant is an evergreen large shrub found abundantly in salt marshes and mangrove swamps in several countries in Asia including Thailand. *P. indica* has been used as a traditional medicine to treat diabetes, gallstone, hemorrhoids, fever, and to heal wounds. Moreover, the dried leaves are prepared in herbal tea as a health-promoting drink available in Thailand [6]. According to the previous studied found that leaves extract of *P. indica* are also showed a natural antioxidant [7-9] because they contain high amount of flavonoids and phenolic compounds; caffeoylquinic acid derivative (3-O-caffeoylquinic acid, 5-O-caffeoylquinic acid, 3,4-O-dicaffeoylquinic acid, 3,5-O-dicaffeoylquinic acid, and 4,5-O-dicaffeoylquinic acid) [10-11], and flavonol aglycones (quercetin, kaempferol, and myricetin) [12]. Others pharmacologically, they have been shown anti-inflammatory [13], hypoglycemic and anti-hyperglycemic activities [14], antibacterial [8], anti-alpha glucosidase [10] and anti-collagenase, MMP-2, and MMP-9 [15].

Microemulsions are one of the cosmetic delivery systems which can be defined as being optically isotropic and transparent in oil or water dispersions with diameters slightly greater than swollen micelles. The mean particle sizes of microemulsions are in the range of 10–200 nm. A microemulsion system consisted of water, oil, surfactant, and

co-surfactant that all are thermodynamically stable with low viscosity and Newtonian behavior. These components are usually optimized using pseudoternary phase diagrams which present the suitable amount of oil, water, and surfactant mixtures. Then, the microemulsion region can be identified in the phase diagram. Microemulsion has been reported to enhance the solubility and oxidation stability of ascorbyl palmitate. Therefore, microemulsion systems present attractive skin and hair formulations that can be used not only to investigate improvements in the stability of extract but also the rate of delivery of its active compounds through the skin barrier, thus obtaining better biological results [16,17].

Therefore, this present study was reported the investigation of the antioxidant activities including the cytotoxicity data from *P. indica* leaves extract used as a natural active ingredient for skin cosmetic industry. The antioxidant activities; 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid ($ABTS^+$), and ferric reducing antioxidant power (FRAP) assays of the *P. indica* leaves extract were investigated. In addition, the cytotoxicity of the extract was tested on normal human skin fibroblasts. Afterward, microemulsion loaded extract of *P. indica* leaves was prepared and all characterizations were also studied. Finally, the physicochemical stability of prepared microemulsion was determined.

METHODS

Chemicals and reagents

The chemicals used were analytical reagent grade: ABTZ (ABTZ, Sigma-Aldrich USA), DPPH (DPPH, Sigma-Aldrich USA), Trolox (Sigma-Aldrich USA), Folin-Ciocalteu phenol reagent (Sigma-Aldrich USA), Sodium carbonate (Na_2CO_3, Univar Ajax Finechem New Zealand) Aluminium chloride ($AlCl_3$, Univar Ajax Finechem New Zealand), Potassium acetate (CH_3COOK; Univar Ajax Finechem New Zealand), Hydrochloric acid (Lab Scan Thailand), Ethanol (Lab Scan Thailand). HPLC grade: 4,5-O-dicaffeoylquinic acid (Sigma-Aldrich USA), Quercetin (Sigma-Aldrich USA), Gallic acid (Sigma-Aldrich USA), Methanol (Lab Scan Thailand), Phosphoric acid (Lab Scan Thailand), and Cosmetic grade: Tween 80 and propylene glycol (Namsiang Thailand).

Preparation of *P. indica* less. leave extract

The mature leaves of *P. indica* were collected in June 2018 from Chantaburi Province, Thailand and authenticated by the Department of Agriculture, Kasetsart University, Thailand. All fresh leaves were cleaned with tap water and dried in a hot air-oven at 60°C for 24 h. The dried leaves were ground using a grinder. The powdered leaves were kept in a sealed containers protected from light until use. Extracts of dried *P. indica* leaves were prepared by a maceration method in various concentrations of ethanol including 0% (DI water), 30%, 70%, and 95%. Briefly, 10 g of dried leaves powder were macerated in 100 ml of each solvent and then kept at room temperature for 24 h. The resulting solvent was then filtered through a Whatman No.1 filter paper. The marc was re-extracted again in the same method and done in triplicates. The pooled filtrate was concentrated using a vacuum rotary evaporator at 45°C. Each crude extract was weighed and kept in a tight container protected from light in refrigerator. The yield of each extract was calculated using the following equation:

$$\text{Yield (\%)} = (W1 \times 100)/(W2)$$

where W1 is the weight of *P. indica* leaves extract, and W2 is the weight of the dried *P. indica* leaves powder.

Quantitative assays

Total phenolic content

Total phenolic content of *P. indica* leaves extracts was determined by a Folin- Ciocalteu method [18], with slightly modification. Briefly, 500 µl of extract solution was mixed with 500 µl of Folin-Ciocalteu reagent and 10 ml of 7% sodium carbonate (Na_2CO_3) were added and vortex. After incubation for 60 min at room temperature, the absorbance of the incubated solution was measured at 760 nm. The amount of total phenolic was expressed as mg of gallic acid equivalents per gram of extract (mg GAE/g extract) from the calibration curve. This assay was performed in triplicate.

Total flavonoid content

Total flavonoid content of *P. indica* leaves extracts was assessed based on the formation of a complex flavonoid aluminum as previously studied [19], with slightly modification. Briefly, 100 µl of 2% aluminum chloride ($AlCl_3$) was mixed with the same volume of extract solution. Absorption measuring at 415 nm was read after incubation for 10 min at room temperature. The amount of total flavonoids was expressed as mg of quercetin equivalent per gram of extract (mgQE/g extract) from the calibration curve. This assay was done in triplicate.

4,5-O-dicaffeoylquinic acid content

The quantification of 4,5-O-dicaffeoylquinic acid in the *P. indica* extracts was determined using a ultra-high performance liquid chromatography (UHPLC) method. The separation was done in a HyPURITY C18 column (150 × 3 mm, 3 µm), mobile phases consisted of (A) 0.1% phosphoric acid in water, and (B) 0.1% phosphoric acid in methanol using a gradient elution and the flow rate was set at 0.8 ml/min with the controlled temperature at 30°C. DAD 190–800 nm detector was set at the wavelength of 330 nm and injection volume was 2.0 µl for sample and reference standard (4,5-O-dicaffeoylquinic acid).

In vitro antioxidant activities of *P. indica* leaves extracts

DPPH assay

The free-radical scavenging activity of *P. indica* leaves extracts was determined using DPPH assay as previously described [20,21]. Briefly, 150 µl of 0.2 mM DPPH solution was then mixed with 75 µl of various concentrations of the extract solution. The mixture was kept at room temperature for 30 min in the dark, and then the absorbance at 515 nm was measured using a microplate reader. This study was compared with the positive controls, L-ascorbic acid, and trolox. The radical scavenging activity was calculated as a percentage of DPPH decoloration using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = [1-(A_S/A_B)]\times 100$$

where A_S was an absorbance of DPPH with the tested sample and A_B was an absorbance of DPPH without the tested sample. The concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage plotted against the samples concentration. The study was run in triplicate.

$ABTS^+$ assay

The radical scavenging activity of *P. indica* leaves extracts was assessed spectrophotometrically by ABTS radical cation decolorization assay as previously studied [22], with slightly modification. Briefly, 7 mM of ABTS solution was mixed with 2.45 mM of potassium persulfate to produce an ABTS radical cation. After that, the mixture was kept in the dark at room temperature for 12–16 h. Then, the ethanol was used to dilute the $ABTS^+$ solution to an absorbance of 0.70±0.02 at 734 nm. The aliquot (25 µl) of various concentrations of the extract solution was added to 2 ml of $ABTS^{\bullet+}$ solution. The absorbance was taken at 734 nm using a microplate reader. Trolox and L-ascorbic acid was used as a reference compounds. The radical scavenging activity was calculated as a percentage of $ABTS^+$ decolorization using the following equation:

$$ABTS^+ \text{ radical scavenging activity (\%)} = [1 - (A_S/A_B)] \times 100$$

where A_S is an absorbance of $ABTS^+$ with the tested sample and A_B is an absorbance of $ABTS^+$ without the tested sample. The concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage plotted against the samples concentration. The study was done in triplicate.

FRAP assay

The ferric reducing power of *P. indica* leaves extracts was determined using the FRAP assay [23]. This method is based on the reduction, at low pH, of a colorless ferric complex (Fe^{3+}-tripyridyltriazine) to a blue-colored ferrous complex (Fe^{2+}-tripyridyltriazine [TPTZ]) by the action of electron-donating antioxidants. The reduction is monitored by measuring the change of absorbance at 593 nm. In brief, the FRAP reagent was prepared by mixing acetate buffer (300 mM, pH 3.6), a solution of 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3$ at 10:1:1 (v/v/v). Then, 190 µl of FRAP reagent was mixed with 10 µl of various concentrations of extracts. After incubation at 37°C for 30 min, the absorbance was taken at 593 nm using a microplate reader. The results were expressed as mg trolox equivalent per gram of extract from the calibration curve. This assay was performed in triplicate.

Cytotoxicity to normal human dermal skin fibroblasts (HDSF) cell

The sulforhodamine B (SRB) assay is used for cell density determination, based on the measurement of cellular protein content. The *P. indica* leaves extract was tested for cytotoxicity to normal HDSF cell by the SRB assay as previously described [24]. The cells (passage number 41) were seeded into each well of 96 well plate (1.0×10^4 cells/well) and maintained in the DMEM medium for cell attachment on the plate in 5% CO_2 at 37°C. Cells were then treated with five serial concentrations of the crude extract (0.0001–1.0 mg/ml) for 24 h. Sodium lauryl sulfate at the concentrations of 0.001–1.0 mg/ml was used as a positive control. After incubation, the adherent cells were fixed with 10% trichloroacetic acid and dyed with SRB for 30 min. After which the excess dye was removed by washing repeatedly with 1% acetic acid. The protein-bound dye was dissolved in 10 mM Tris base solution and the absorbance was measured on a microplate reader at 510 nm. The experiments were done in triplicate. The percentage of cell proliferation was calculated according to the following equation:

$$\text{Cell ability (\%)} = (A/B) \times 100$$

where A was the absorbance of the treated with sample and B was the absorbance of the control (untreated with sample).

Preparation of microemulsion

The oil in water microemulsion was prepared by pseudoternary phase diagram. The pseudoternary phase diagram consisted of oleic acid (1.0%w/w) as an oil phase, tween 80 (10–25%w/w) as a surfactant, and propylene glycol (5.0%w/w) as a co-surfactant and deionized water. The composition of the microemulsion was selected from the pseudoternary phase diagram that gave the largest microemulsion area. The extract was clearly dissolved in oleic acid and added into an internal phase of microemulsion.

Characterization of microemulsion

Physical appearances of free microemulsion and microemulsion loaded the extract were observed in terms of transparency. The color was measured using a colorimeter. The internal droplet size and polydispersity index (PDI) were evaluated using a Zetasizer Nano at 25°C. Viscosity was determined using a Brookfield while pH of microemulsions was determined using pH meter. Morphology of microemulsion was investigated by transmission electron microscope (TEM) using a negative staining technique.

Physicochemical stability investigation

The free microemulsion and microemulsion loaded extract were kept in air-tight containers under accelerated condition using six cycles of alternative heating-cooling (HC) (4°C for 48 h followed by 45°C for 48 h as 1 cycle), heating at 50°C for 30 days, cooling at 4°C for 30 days, and at room temperature (≈30±5°C) for 30 days. Physical appearances including phase separation and transparency of prepared microemulsion were observed. The color, droplet size, PDI, viscosity, and pH value were also determined at the end of each stability conditions.

Data analysis

All experimental measurements were triplicate performed. Result values were expressed as mean value ± standard deviation (SD). Statistical significance in this study was examined using student's unpaired *t*-test using QuickCalcs, GraphPad programs. The value of $p < 0.05$ was considered statistically significant.

RESULTS

Yield of crude extract, total phenolic, and flavonoid contents of extracts

All *P. indica* leaves extracts were dark-brown semisolids. The yields, total phenolic, and total flavonoid contents of each extract are shown in Table 1.

The yield results showed that the maximum value was obtained extract by maceration with 30% (v/v) ethanol which was 25.41% (w/w). The yield then tended to decrease when the ethanol concentration was 70% and 95%, respectively.

The content of total phenolic compound in the extracts was calculated with the calibration curve equation of gallic acid (0.025–0.5 mg/ml, $Y = 5.2234x+0.1618$, $R^2 = 0.9976$). The highest total phenolic content was obtained from a 30% ethanolic extract followed by 70% ethanolic extract, aqueous extract and 95% ethanolic extract as 175.91±4.06, 174.92±8.46, 126.58±3.59, and 54.61±0.53, respectively.

The amount of total flavonoid compounds of *P. indica* extracts was calculated with the calibration curve equation of quercetin (0.039–0.625 mg/ml, $Y = 1.8012x+0.1369$, $R^2 = 0.9995$) which found that 95% ethanol had a higher amount of total flavonoid (355.19±3.78 mgQE/g extract), followed by 70% ethanolic extract (344.83±10.19 mgQE/g extract), aqueous extract (271.73±9.87 mgQE/g extract), and 30% ethanolic extract (270.99±12.50 mgQE/g extract), respectively.

4,5-O-dicaffeoylquinic acid content

Caffeoylquinic acid (CQ) derivatives; 3-O-caffeoylquinic acid (3-CQ), 4-O-caffeoylquinic acid (4-CQ), 5-O-caffeoylquinic acid (5-CQ), 3,4-O-dicaffeoylquinic acid (3,4-CQ), 3,5-O-dicaffeoylquinic acid (3,5-CQ), and 4,5-O-dicaffeoylquinic acid (4,5-CQ) were previously reported as the major constituents in *P. indica* leaves which quantified in *P. indica* leaves extracted using HPLC. They studied found that *P. indica* leaves extract had higher 4,5-O-dicaffeoylquinic acid content than others caffeoylquinic acids [11]. Thus, this present study focused on the quantification of 4,5-O-dicaffeoylquinic acid in *P. indica* leaves extracts according to UHPLC. The chromatograms of 4,5-O-dicaffeoylquinic acid standard compound and *P. indica* leaves extracts are shown in Fig. 1.

From the chromatograms, the content of 4,5-O-dicaffeoylquinic acid in *P. indica* leaves extracts was in the range of 2.202–9.359 µg/ml which was calculated with the calibration curve equation of 4,5-O-dicaffeoylquinic acid (500–1500 µg/ml, $Y = 6371.69X$, $R^2 = 0.9995$) at 330 nm. Results found that 70% ethanolic extract (9.359 µg/ml) showed the higher amount of 4,5-O-dicaffeoylquinic acid than 30% ethanolic extract (5.205 µg/ml), aqueous extract (2.213 µg/ml), and 95% ethanolic extract (2.202 µg/ml), respectively. This result was similar to total phenolic content resulting (Table 1).

Table 1: Yield of crude extracts, total phenolic, and flavonoid contents of extracts

Pluchea indica leaves extracts	Yield (%w/w)	Total phenolic content (mgGAE/g extract)	Total flavonoid content (mgQE/g extract)
Aqueous extract	11.99	126.58±3.59*	271.73±9.87*
30% ethanolic extract	25.41	175.91±4.06	270.99±12.50*
70% ethanolic extract	14.70	174.92±8.46	344.83±10.19
95% ethanolic extract	11.87	54.61±0.53*	355.19±3.78

The total phenolic and total flavonoid contents values are given as mean±SD (n=3). Superscript (*) in the same column indicate significant differences ($p<0.05$; Student's t-test)

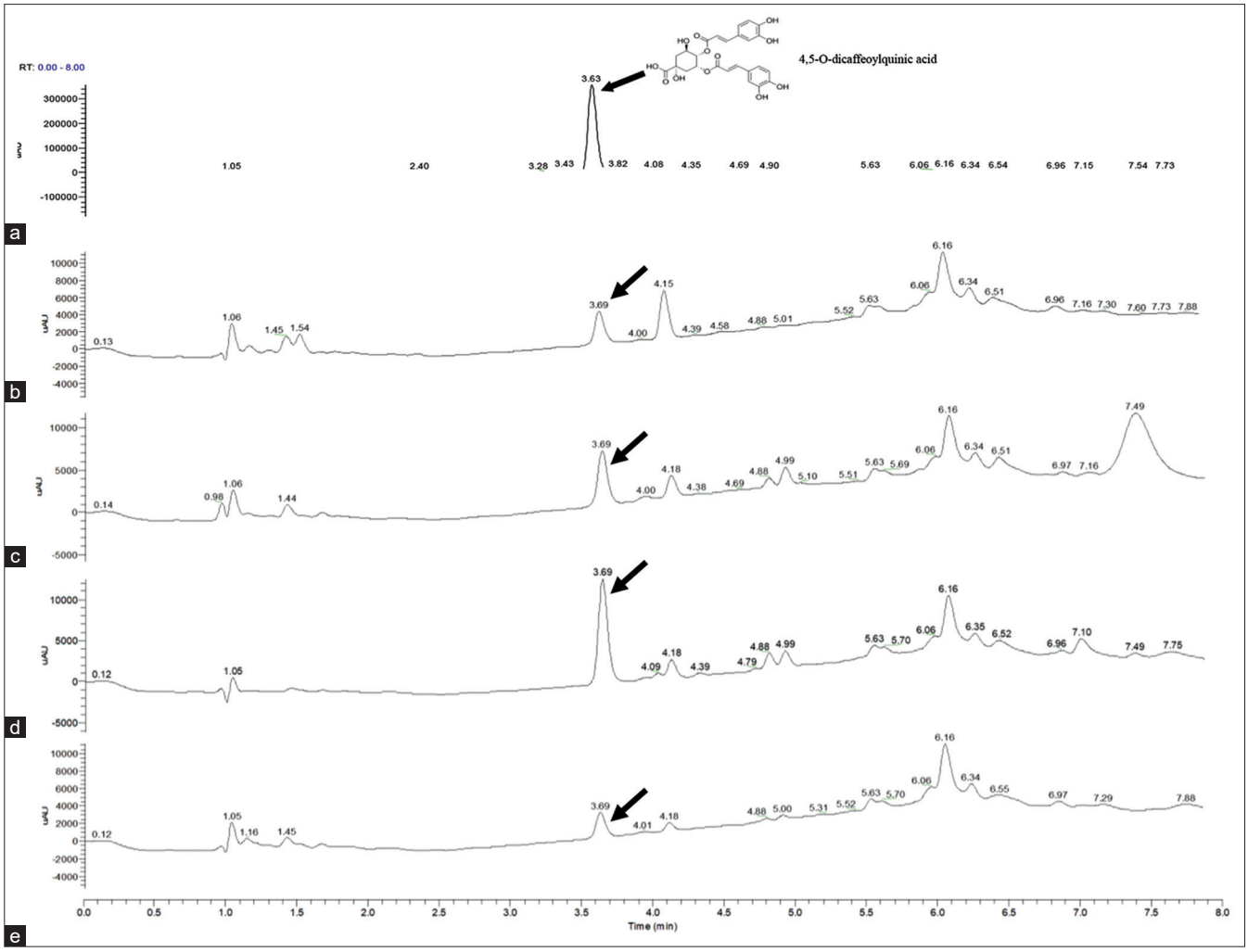

Fig. 1: High-performance liquid chromatography chromatogram of (a) 4,5-O-dicaffeoylquinic acid standard, (b) aqueous extract, (c) 30% ethanolic extract, (d) 70% ethanolic extract, and (e) 95% ethanolic extract

Antioxidant activity

The concentration providing 50% inhibition (IC_{50}) values from DPPH and $ABTS^{+}$ radicals scavenging and FRA*P* value of *P. indica* leaves extracts and positive controls are presented in Table 2.

The DPPH and $ABTS^{\bullet+}$ assays are widely used methods for the determine of the antioxidant activity of natural products, they both are spectrophotometric techniques based on quenching of stable colored radicals ($ABTS^{\bullet+}$ or DPPH) and show the radical scavenging ability of antioxidants even when present in complex biological mixtures such as plant extracts [25]. IC_{50} is the concentration of the samples required to inhibit 50% of free radical. Thus, the lower in IC_{50} value indicated that the higher of antioxidant activity. In this study, the 70% ethanolic extract possesses strong antioxidant activities based on DPPH and $ABTS^{\bullet+}$assays as IC_{50} value of 0.17±0.01 mg/ml and 2.04±0.03 mg/ml, respectively. This extract showed higher activities than other extracts.

The antioxidant activity of *P. indica* leaves extracts was accordingly estimated by FRAP assay, which is presenting of the sample reduction of Fe (III)/TPTZ complex to the ferrous form (blue color). This study, the 70% ethanolic extract showed the highest amount of ferric reducing power expressed as trolox (263.31±4.39 mgTE/g extract), followed by 30% ethanolic extract (250.17±6.06 mgTE/g extract), aqueous extract (157.62±2.47 mgTE/g extract), and 95% ethanolic extract (42.26±1.76 mgTE/g extract), respectively.

Table 2: Antioxidant activities of *Pluchea indica* leaves extracts

Samples	IC_{50} (mg/ml)		FRAP assay (mgTE/g extract)
	DPPH radical	$ABTS^{+}$ radical	
Aqueous extract	0.21±0.08	2.78±0.04	157.62±2.47*
30% ethanolic extract	0.18±0.01	2.28±0.05*	250.17±6.06*
70% ethanolic extract	0.17±0.01	2.04±0.03*	263.31±4.39*
95% ethanolic extract	0.81±0.05*	10.68±0.51*	42.26±1.76*
L-ascorbic acid	0.0178±0.0001*	0.57±0.02*	-
Trolox	0.023±0.01*	0.0065±0.000054*	-

Values are given as mean±S.D of triplicate. Superscript (*) in the same column indicate significant differences ($p<0.05$; Student's t-test)

Cytotoxicity to normal HDSF cell

From all the results as above, the 70% ethanolic extract was selected for investigation of cytotoxicity to normal HDSF cell. The obtained results are expressed as percentage of cell viability according to SRB assay and are shown in Fig. 2.

The result indicated that the *P. indica* extract at all concentrations used (0.001–1.0 mg/ml) had no effect on the viability of the human skin fibroblasts which was the percentage of in the range of 96.84–97.13. This demonstrated that the *P. indica* leaves extract was not toxic to human skin fibroblast *in vitro*, whereas sodium lauryl sulfate, positive control, showed statistically significant ($p < 0.05$) toxicity on normal human skin fibroblast at 0.1 and 1.0 mg/ml and it gave the percentage of cell viability were 32.28 and 8.10, respectively.

Microemulsion preparation

Pseudoternary phase diagram was used to prepare oil in water microemulsion. The composition of microemulsion was selected from the pseudoternary phase diagram that gave the largest microemulsion area as shown in Fig. 3. Optimal microemulsion formula consisted of oleic acid (1%w/w), tween 80 (20%w/w), propylene glycol (5%w/w), and deionized water (74%w/w).

Physicochemical characterization of prepared microemulsion

The physical appearance of free microemulsion was a yellowish transparent liquid and dark green transparent liquid for microemulsion loaded 0.085%w/w of *P. indica* leaves extract as shown in Fig. 4.

Table 3 shows the color (L*, a*, and b*) and pH values of free microemulsion and microemulsion loaded the *P. indica* leaves extract. The result found that after loaded the extract into microemulsion, the *L** and *b** values were significantly changed ($p < 0.05$), excepted *a** value which was not significantly changed ($p > 0.05$), similar to the evaluating by visual as Fig. 4 which showed that the color of microemulsion loaded extract appeared dark green. The pH values of both formulations are suitable for topical application when their pH values are in the range of 5–8 [26].

The obtained results of droplet size, PDI, and viscosity of free microemulsions and microemulsion loaded extract are shown in Table 4. After loaded the extract into microemulsion, the results showed that the mean droplet sizes, PDI, and viscosity were significantly increased ($p < 0.05$).

Fig. 5 presents the mean droplet sizes and distribution of free microemulsions and microemulsion loaded extract determined by nanoparticle analyzer. This results showed the increasing tendency of the mean droplet size and distribution after the extract was loaded into microemulsion.

The morphology of microemulsion loaded the extract assessed by TEM. The result indicated the spherical shape of internal droplet sizes of microemulsion loaded *P. indica* leaves extract as shown in Fig. 6.

Physicochemical stability study of microemulsion loaded *P. indica* leaves extract

The homogeneity of the microemulsions remained clear, the sedimentation and phase separation did not occur due to the high stable of microemulsions after stability testing. The physiochemical stability of microemulsion loaded the extract was performed under conditions; six cycles of alternative HC (4°C for 48 h followed by 45°C for 48 h as 1 cycle), heating at 50°C for 30 days, cooling at 4°C for 30 days, and at room temperature (RT, ≈30±5°C) for 30 days, results are shown in Table 5. The viscosity value did not significantly change ($p > 0.05$) compared with initial condition. The mean droplet size and PDI were a tendency of decreasing; however, it was still in the range of microemulsions formation. The color did significantly change ($p < 0.05$) in HC and room temperature conditions. The color of the microemulsion appeared darker than initial condition. The pH values significantly decreased ($p < 0.05$) compared with initial condition. For free microemulsion was stable at all storage conditions (data not show).

DISCUSSION

The effective extraction and proper assessment of antioxidants from medicinal plants are crucial to explore the potential antioxidant sources and promote the application in pharmaceuticals and/or cosmetic fields. In this study, *P. indica* leaves extracts were obtained using water and ethanol with different concentrations; aqueous (100% water), 30% ethanol, 70% ethanol, and 95% ethanol. The extraction resulting

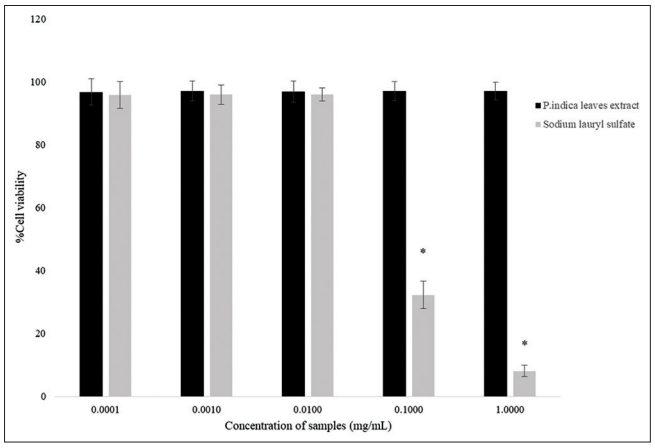

Fig. 2: Cytotoxicity of *Pluchea indica* leaves extract and sodium lauryl sulfate to normal human dermal skin fibroblasts cell. Each bar represents mean±S.D. (n=4). Superscript (*) above the average bars denote significant differences at p<0.05-Student's t-test

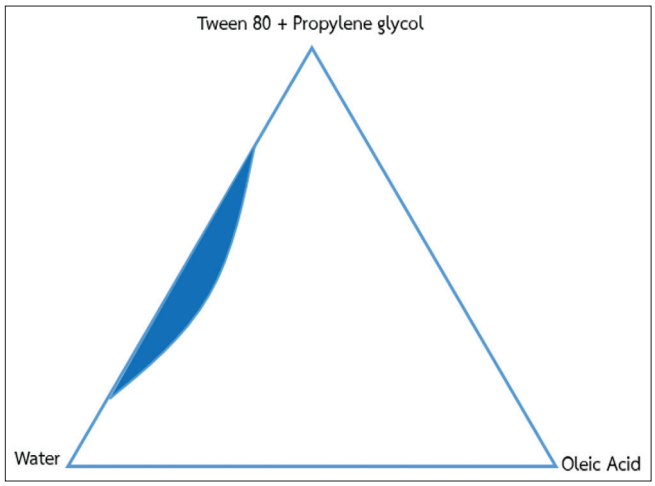

Fig. 3: Pseudoternary phase diagram of microemulsion

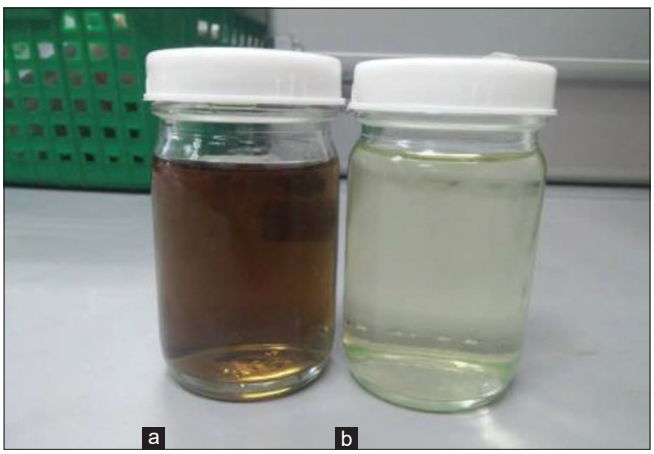

Fig. 4: The physical appearance of (a) microemulsion loaded extract and (b) free microemulsion

showed the crude extract obtained by a 30% ethanol found the highest percent yield. This might due to the combination between water and ethanol serve the extraction of compounds that are soluble in water and ethanol in *P. indica* leave such as phenolic acid and flavonoid compound. Other compounds may have been extracted and contribute to higher yield.

Table 3: L*, a*, b*, and pH value of free microemulsion and microemulsion loaded the *Pluchea indica* leaves extract

Formulations	Color			pH
	*L**	*a**	*b**	
Free microemulsion	+10.37±0.06	+2.21±0.09	−0.17±0.04	6.08±0.01
Microemulsion loaded extract	+8.29±0.76*	+2.27±0.07	+0.26±0.09*	6.08±0.01

Values are given as mean±S.D of triplicate. Superscript (*) in the same column indicate significant differences ($p<0.05$; Student's t-test)

Table 4: The mean droplet size, polydispersity index, and viscosity of free microemulsion and microemulsion loaded the *Pluchea indica* leaves extract

Formulations	Droplet size (nm)	Polydispersity index (PDI)	Viscosity (cP)
Free microemulsion	11.25±1.20	0.257±0.001	16.3±0.30
Microemulsion loaded extract	24.40±1.20*	0.397±0.050*	18.3±0.60*

Values are given as mean±S.D of triplicate. Superscript (*) in the same column indicate significant differences ($p<0.05$; Student's t-test)

The content of total phenolic compound in the extracts was calculated with the calibration curve equation of gallic acid based on Folin–Ciocalteu assay. The extract obtained by a 30% ethanol had a highest amount of total phenolic. This demonstrated that the total phenolic content in *P. indica* leaves extract might contained mainly of caffeoylquinic acid (CQ) derivatives; 3-O-caffeoylquinic acid (3-CQ), 4-O-caffeoylquinic acid (4-CQ), 5-O-caffeoylquinic acid (5-CQ), 3,4-O-dicaffeoylquinic acid (3,4-CQ), 3,5-O-dicaffeoylquinic acid (3,5-CQ), and 4,5-O-dicaffeoylquinic acid (4,5-CQ) [11]. This studied extract had higher content of total phenolic compound than previously reported [7,8,27,28].

The amount of total flavonoid compounds of *P. indica* extracts were calculated with the calibration curve equation of quercetin determined by aluminum chloride assay. Obtained resulted found that 95% ethanolic extract had the highest amount of total flavonoid. These implied that this ethanol-water (5%) solvent could extracted the flavonoid compounds from *P. indica* leaves such as quercetin, kaempferol, and myricetin more than other solvents which contain higher percentage of water. These resulted were similar to previously reported [12]. In addition, this present showed a higher a flavonoids contents than previous studied [27,28].

The present report concluded that *P. indica* leaves extract had higher amount of total phenolic and flavonoids than previous studied may

Table 5: Physicochemical stability of microemulsion loaded the extract after storage under test conditions

Conditions	Color			Droplet size (nm)	PDI	Viscosity (cP)	pH
	*L**	*a**	*b**				
Initial	+8.29±0.76	+2.27±0.07	+0.26±0.09	24.40±1.20	0.397±0.05	18.3±0.60	6.08±0.01
4°C	+7.12±0.05	+1.91±0.11*	+0.53±0.06*	10.64±0.60*	0.137±0.06*	17.5±0.92	6.00±0.02*
50°C	+8.41±0.13	+2.01±0.09*	+0.28±0.05	10.67±0.19*	0.190±0.04*	17.0±0.73	5.57±0.01*
HC	+16.93±0.34*	+0.05±0.04*	+0.29±0.22	16.84±0.19*	0.215±0.01*	17.2±0.95	5.78±0.01*
RT	+10.22±0.67*	+2.03±0.13*	−0.07±0.13*	14.04±0.09*	0.280±0.02*	18.0±0.45	5.92±0.02*

Values are given as mean±S.D of triplicate. Superscript (*) represents significant differences when compared with initial condition at $p<0.05$ (Student's t-test)

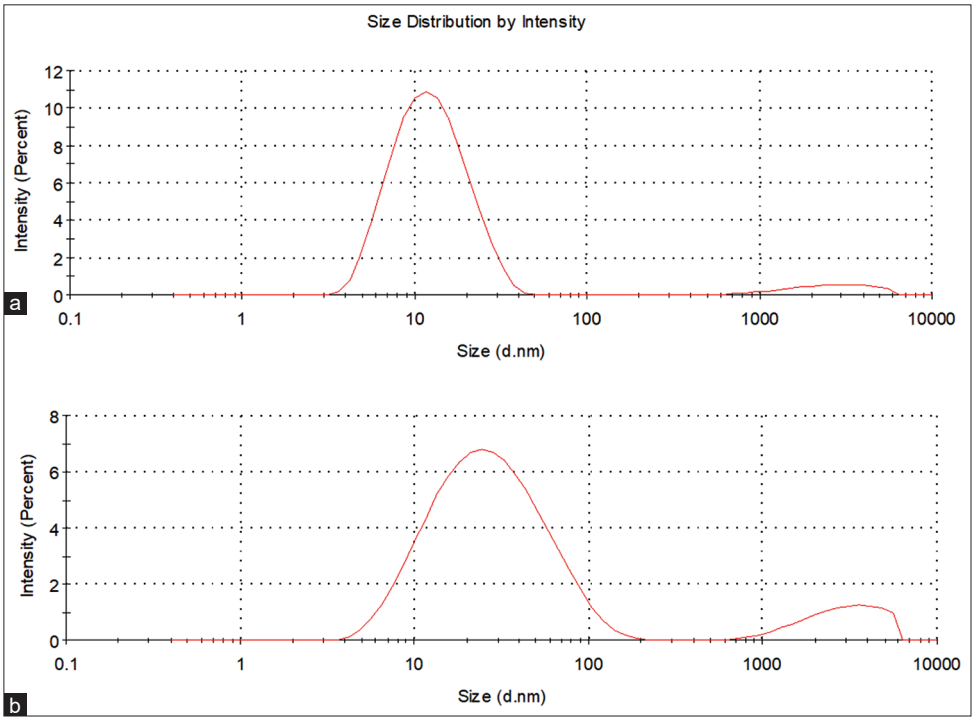

Fig. 5 Mean droplet sizes and distribution of (a) free microemulsions and (b) microemulsion loaded extract

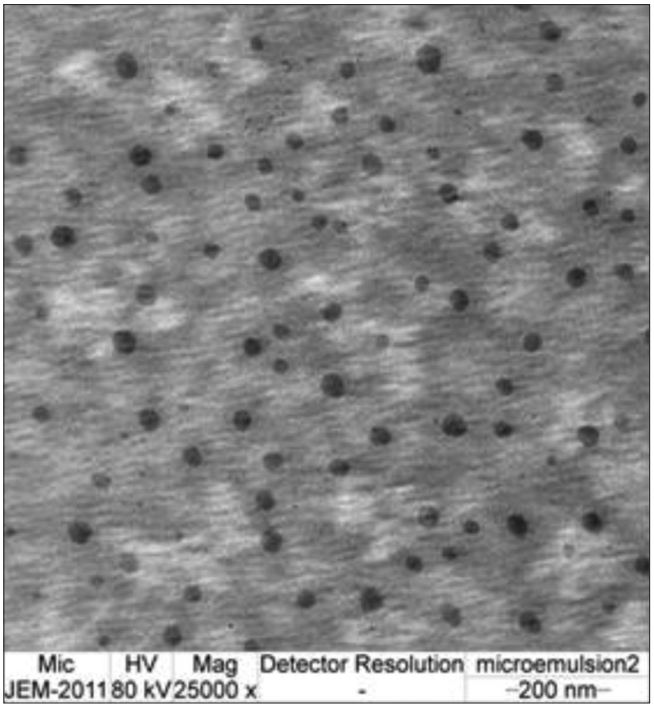

Fig. 6: Transmission electron microscope of microemulsion loaded the *Pluchea indica* leaves extract

be attributed to differences in extraction or the aforementioned pre-harvest factors such as climate, geography, or agronomic practices.

The quantification of 4,5-O-dicaffeoylquinic acid in *P. indica* leaves extracts according to UHPLC. Results found that the extract obtained by 70% ethanol had the highest content of the major compound, 4,5-O-dicaffeoylquinic acid. This result was similar to total phenolic content resulting.

Antioxidant properties of *P. indica* leaves extracts were determined through different methods including DPPH, ABTS, and FRAP methods. Our results found that the 70% extract showed a higher DPPH and $ABTS^{+}$ radical scavenging activity than other extracts as well as FRA*p* value, and all extracts showed lower than reported previously [27,28]. However, free-radical scavenging activity of the extract that obtained in this study may result from high phenolic and flavonoid contents. Thus, the antioxidant properties of natural extract may be attributed to the main components of herbal extract which may act as an antioxidant [29]. All finding together indicated that the 70% ethanolic extract contained high level of phenolic and flavonoids as much as a major active, 4,5-O-dicaffeoylquinic acid, and it demonstrated the highest antioxidant activities. Therefore, this extract was selected to further study for applied in skin cosmetic products.

The cytotoxicity of 70% ethanolic extract to normal HDSF cell was investigated by SRB assay. Our study showed that the *P. indica* extract had no effect on the viability of the human skin fibroblasts. This demonstrated that the extract was not toxic to human skin fibroblast *in vitro*.

The physicochemical characterization of free microemulsion and microemulsion loaded extract; the color, pH values, viscosity, droplet size, and PDI were studied. Resulted the L^*, a^*, and b^* values were similar to evaluating by visual which showed that the color of microemulsion loaded extract appeared dark green. The extract did not affect the pH values. The pH values of both formulations are suitable for topical application when their pH values are in the range of 5–8 [26]. The extract incorporation did significantly affected the viscosity of unloaded microemulsion ($p < 0.05$) but it was found that both prepared-microemulsion exhibited Newtonian behavior since their viscosity values were not changed by increasing the shear rate (rpm).

The extract incorporation affected the droplet size and PDI of microemulsion ($p < 0.05$). This results based on the solubility of the extract, it might mainly be localized in the external phase and surfactant layer between internal phase and external phase of the microemulsion. This might have resulted in larger droplet size. PDI value describes the homogeneity of the droplet size. This resulted were similar to the previous studied [30]. Both PDI values were smaller than 0.5. Therefore, these results indicate that droplet sizes have appropriate homogeneity.

The homogeneity of the microemulsions remained clear, the sedimentation and phase separation did not occur due to the high stable of microemulsions after stability testing. The physiochemical stability of microemulsion loaded the extract was performed under tested conditions. The viscosity value did not significantly change under test conditions ($p > 0.05$). This implies that the microemulsion loaded extract exhibited Newtonian flow behavior. The mean droplet size and PDI were a tendency of decreasing; however, it was still in the range of microemulsions formation. The color did significantly change in HC and room temperature conditions ($p < 0.05$), but it did not affect to microemulsion form. The pH values significantly decreased ($p < 0.05$). This might be affect from the acidic compounds in the extract released to external phase. For free microemulsion was stable at all storage conditions (data not show).

CONCLUSION

The *P. indica* leaves extract which extracted by 70% ethanol found that it contained high a phenolic, flavonoid and 4,5-O-dicaffeoylquinic acid contents which possessed DPPH, $ABTS^{+}$, and FRAP activities. In addition, this extract also showed a complete lack of any toxicity toward normal human skin fibroblasts. The optimal microemulsion formula consisted of the 70% ethanolic extract of *P. indica* leaves (0.085%), oleic acid (1.0%), tween 80 (20.0%), propylene glycol (5.0%), and deionized water. The microemulsion loaded the extract showed a good stability under all conditions tested. All finding indicated that *P. indica* leaves extract can be prepared in microemulsions as a carrier for the herbal extract delivery system. Further investigation will evaluate skin penetration and skin irritation of microemulsion loaded *P. indica* leaves extract along with the performance of clinical trials in skin cosmetic products.

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AUTHORS' CONTRIBUTIONS

Khwunjit Itsarasook analyzed the laboratory work, analyzed the data, and wrote the manuscript. All authors read and approved the manuscript and especially edited and final revised by Dr. Piyanuch Prompamorn.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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REFERENCES

1. Cornelli U. Antioxidant use in nutraceuticals. Clin Dermatol 2009;27:175-94.
2. Shindo Y, Witt E, Packer L. Antioxidant defence mechanisms in murine

epidermis and dermis and their responses to ultraviolet light. J Invest Dermatol 1993;100:260-5.
3. Butterfield DA, Castenga A, Pocernich CB, Drake J, Scapagnini G, Calabrese V. Review: Nutritional approaches to combat oxidative stress in Alzheimer's diseases. J Nutr Biochem 2002;13:444-61.
4. Masaki H. Role of antioxidants in the skin: Anti-aging effects. J Dermatol Sci 2010;58:85-90.
5. Ferreyra RM, Viña SZ, Mugridge A, Chaves AR. Growth and ripening season effects on antioxidant capacity of strawberry cultivar. Selva Sci Hortic 2007;112:27-32.
6. Suriyaphan O. Nutrition, health benefits and applications of *Pluchea indica* (L.) Less leaves. Mahidol Univ J Pharm Sci 2014;41:1-10.
7. Noridayu AR, Hii YF, Faridah A, Khozirah S, Lajis N. Antioxidant and antiacetylcholinesterase activities of *Pluchea indica* Less. Int Food Res J 2011;18:925-9.
8. Srimoon R, Ngiewthaisong S. Antioxidant and antibacterial activities of Indian Marsh Fleabane (*Pluchea indica* (L.) Less). KKU Res J 2015;20:144-54.
9. Thoobbucha N, Petchlert C. Free radical scavenging capacity and modulative effect on antioxidant enzyme activity of *Pluchea indica* Less. Tea leaf extract. Naresuan Phayao J 2015;8:74-9.
10. Arsiningtyas IS, Gunawan-Puteri MD, Kato E, Kawabata J. Identification of α-glucosidase inhibitors from the leaves of *Pluchea indica* (L.) Less. a traditional Indonesian herb: Promotion of natural product use. Nat Prod Commun 2014;28:1350-3.
11. Kongkiatpaiboon S, Chewchinda S, Vongsak B. Optimization of extraction method and HPLC analysis of six caffeoylquinic acids in *Pluchea indica* leaves from different provenances in Thailand. Rev Bras Farmacogn 2018;28:145-50.
12. Andarwulan N, Batari R, Sandrasari DA, Bolling B, Wijaya H. Flavonoid content and antioxidant activity of vegetables from Indonesia. Food Chem 2010;121:1231-5.
13. Buapool D, Mongkol N, Chantimal J, Roytrakul S, Srisook E, Srisook K. Molecular mechanism of anti-inflammatory activity of *Pluchea indica* leaves in macrophages RAW 264.7 and its action in animal models of inflammation. J Ethnopharmacol 2013;146:495-504.
14. Pramanik KC, Bhattacharya P, Biswas R, Bandyopadhyay D, Mishra M, Chatterjee TK. Hypoglycemic and antihyperglycemic activity of leaf extract of *Pluchea indica* Less. Orient Pharm Exp Med 2006;6:232-6.
15. Ohtsuki T, Yokosawa E, Koyano T, Preeprame S, Kowithayakorn T, Sakai S, *et al*. Quinic acid esters from *Pluchea indica* with collagenase, MMP-2 and MMP-9 inhibitory activities. Phytother Res 2008;22:264-6.
16. Acharya DP, Hartley PG. Progress in microemulsion characterization. Curr Opin Colloid Interface Sci 2012;17:274-80.
17. Kreilgaard M. Influence of microemulsion on cutaneous drug delivery. Adv Drug Deliv Rev 2002;1:77-98.
18. Liu M, Li XQ, Weber C, Lee CY, Brown J, Liu RH. Antioxidant and antiproliferation activities of raspberries. J Agric Food Chem 2002;50:2926-30.
19. Meda A, Lamien CE, Romito M, Millogo J, Nacoulma OG. Determination of the total phenolic, flavonoid and proline contents in Burkina Fasan honey, as well as their radical scavenging activity. Food Chem 2005;91:571-7.
20. Itsarasook K, Ingkaninan K, Viyoch J. Artocarpin-enriched extract reverses collagen metabolism in UV-exposed fibroblasts. Biologia 2014;69:943-51.
21. Yakaew S, Itsarasook K, Ngoenkam J, Jessadayannamaetha A, Viyoch J, Ungsurungsie M. Ethanol extract of *Terminalia chebula* fruit protects against UVB-induced skin damage. Pharm Biol 2016;54:2701-7.
22. Tlili N, Mejri H, Anouer F, Saadaoui E, Khaldi A, Nasri N. Phenolic profile and antioxidant activity of *Capparis spinosa* seeds harvested from different wild habitats. Ind Crops Prod 2015;76:930-5.
23. Sulaiman SF, Yusoff NA, Eldeen IM, Seow EM, Sajak AB, Ooi KL. Correlation between total phenolic and mineral contents with antioxidant activity of eight Malaysian banana (*Musa* sp.). J Food Compos Anal 2011;24:1-10.
24. Vichai V, Kirtikara K. Sulforhodamine B colorimetric assay for cytotoxicity screening. Nat Protoc 2006;1:1112-6.
25. Sánchez-Moreno C, Larrauri JA, Saura-Calixto F. A procedure to measure the antiradical efficiency of polyphenols. J Sci Food Agric 1998;76:270-6.
26. Poomanee W, Chaiyana W, Wickett RR, Leelapornpisid P. Stability and solubility improvement of Sompoi (*Acacia concinna* Linn.) pod extract by topical microemulsion. Asian J Pharm Sci 2017;12:386-93.
27. Vongsak B, Inson N, Jaisamut S. Effect of *Pluchea indica* extracts from different habitats and seasons on free radical scavenging capacity. J Pharm Sci 2017;41:169-72.
28. Vongsak B, Kongkiatpaiboon S, Jaisamut S, Konsap K. Comparison of active constituents, antioxidant capacity, and α-glucosidase inhibition in *Pluchea indica* leaf extracts at different maturity stages. Food Biosci 2018;25:68-73.
29. Soradech S, Kusolkumbot P, Reungpatthanaphong P, Thubthimthed S. Investigation of DPPH radical scavenging, antioxidant and melanogenesis stimulating activities of various pigment extracts from Thai herbal plants. Res J Pharm Biol Chem Sci 2016;7:393-9.
30. Saraf S, Sahu S, Kaur CD, Saraf S. Comparative measurement of hydration effects of herbal moisturizers. Pharmacogn Res 2010;2:146-51.