

Research Article

Improvement of Stability and Transdermal Delivery of Bioactive Compounds in Green Robusta Coffee Beans Extract Loaded Nanostructured Lipid Carriers

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The aim of this study was to develop green robusta coffee beans extract loaded nanostructured lipid carriers (NLCs) for enhancing dermal application and its efficiency. The green robusta coffee beans extract cultivated in Chumphon (CP) exhibited the highest antioxidant activity in the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay with IC_{50} of $34.1 \pm 0.9 \mu\text{g/ml}$, lipid peroxidation inhibition with percentage inhibition of 38.8 ± 1.7 , and ferric reducing antioxidant power (FRAP) assay with a FRAP value of $234.5 \pm 12.3 \text{ mM FeSO}_4/\text{g}$. The extract contained caffeine, chlorogenic acid, and caffeic acid as major compounds. The anti-inflammatory test indicated that CP could decrease the secretion of IL-6 in macrophage cells and caused no irritation to blood vessels on the irritation test by hen's egg test chorioallantoic membrane (HET-CAM) assay. The particle size of CP-loaded NLCs was $158.1 \pm 0.2 \text{ nm}$ with a narrow polydispersity index and showed no noticeable difference after the stability test. Entrapment efficacy of CP-loaded NLCs was found to be over 60%. Caffeine and chlorogenic acid in CP-loaded NLCs were released sustainably and penetrated deeper into the skin than the extract in a conventional emulsion. In conclusion, the CP-loaded NLCs can be further used in cosmetics for dermal applications due to good efficacy and safety.

1. Introduction

Coffee has been a major economic plant in Thailand for a long time. Thailand is the third largest producer of coffee in Asia. The most widely cultivated species is *Coffea canephora* or robusta, which accounts for 99% of coffee production in Thailand [1]. Conditions in the south of Thailand are perfect for growth, such as Chumphon, Nakhon Si Thammarat, Yala, and Krabi provinces. Geography has a strong effect on the characteristics and physiological properties of coffee beans, such as flavors, quality, and chemical content [2]. Despite the lower quality of robusta coffee beans, it is popular as instant coffee in the beverage industry [3]. Green coffee beans contain polyphenol compounds, such as chlorogenic acid

and its related compounds (caffeic acid, ferulic acid, and coumaric acid) which are used to inhibit the oxidative stress [4]. Phenolic compounds have attracted interest because of their antioxidant potential and anti-inflammatory activity [5, 6]. According to a previous scientific study, green coffee beans contain a higher amount of chlorogenic acid than roasted coffee beans and other plants [7]. Our preliminary study found that the green robusta coffee beans extract contained phenolic compounds that were unstable under light and temperature. The antioxidant activities of the extract declined at high temperature after a period of storage. These problems affected the stability of phenolic compounds, and thus nanostructured lipid carriers (NLCs) were selected to reduce this problem.

Nanotechnology involves the synthesis of nanoparticles with a particle size ranging from 20 to 200 nm having been applied in food, pharmaceutical, and cosmetic industries. Several lipid nanoparticles including liposomes, nanoemulsions, and solid lipid nanoparticles (SLNs) have been studied for decades. Liposomes can contain lipophilic drugs and hydrophilic drugs. However, they have many disadvantages including low drug loading, drug leakage, and fast release [8]. Nanoemulsions are optically transparent or slightly opaque emulsion with a small droplet size. Nanoemulsions are unstable due to Ostwald ripening, and controlled drug release is improbable due to their small particle size. SLNs are the first generation of lipid nanoparticles, and NLCs are modified from SLNs as the second generation of lipid nanoparticles. SLNs are composed of solid lipids whereas NLCs consist of liquid lipids and solid lipids. Therefore, NLCs show higher compound loading capacity than SLNs. In addition, the releasing profile can be controlled by delivering the active compound to the target organs and improving the stability of the active compound. NLCs are more feasible than the other lipid nanoparticles. Characteristics of NLCs formulation depend on formulation conditions, such as different lipids, surfactants, and methods of synthesis. Different types of lipids in NLCs formulation can be generated as imperfectly structured solid matrix. This type provides imperfect lipid matrix structure that presents the gap between triglyceride fatty acid chains and increases the ability of loading extracts [9]. The imperfect structure leads to many benefits such as the enhancement of the stability of compounds that are sensitive to light oxidation and hydrolysis reactions [10]. NLCs were also used to improve their stability of active compounds and used as a delivery system to control the release of active compounds. Therefore, NLCs can increase entrapment efficiency, drug loading, physical stability, and skin permeation, and they seem to be a valuable delivery system for loading the active compound in this study.

The purposes of this study were to develop green robusta coffee beans extract-loaded NLCs for the improvement of the stability and permeation of the active compounds through the skin barrier.

2. Materials and Methods

2.1. Plant Materials. Green robusta coffee beans (*Coffea canephora*) were obtained from different regions in Thailand, including Chumphon, Yala, and Chiang Rai. They were collected during May to June, 2016. All samples were deposited in the herbarium of the Faculty of Pharmacy, Chiang Mai University, Thailand.

2.2. Chemical Materials. Sorbitan oleate (Span® 80), 2,2-diphenyl-1-picrylhydrazyl (DPPH), caffeine, caffeic acid, and chlorogenic acid were purchased from Sigma-Aldrich Inc., USA. Linoleic acid and 2,4,6-tripryridyl-s-triazine (TPTZ) were purchased from Fluka Buchs, Switzerland. Dulbecco's Modified Eagle's medium (DMEM) and fetal bovine serum were obtained from Biochrom, Berlin, Germany. Thiazolyl blue

tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and lipopolysaccharide (LPS) were obtained from Sigma-Aldrich, Darmstadt, Germany. Penicillin/streptomycin, L-glutamine, and trypan blue were purchased from Invitrogen™, Grand Island, NY, USA. Sunflower oil and canola oil were purchased from Lam Soon CO., Ltd., Thailand. Acetic acid and acetonitrile were purchased from Labscan Ltd., Ireland. Cetyl alcohol, stearyl alcohol, Cremophor® A25, and Tween® 20 were purchased from United Chemical & Trading CO., Ltd., Thailand. Methanol was purchased from Labscan Asia Co., Ltd., Thailand. Acetate buffer and ferric chloride (FeCl₃) were purchased LOBA Chemie, India.

2.3. Extraction. The green robusta coffee beans were ground and dried in a hot air oven at $50 \pm 2^\circ\text{C}$ for 24 h. Those beans were ground with blender (600 W, Viva Collection Blender, Phillip) and reduced to the same size with a 120-mesh sieve shaker (125 μm) (Thomas scientific, USA). The ground beans were macerated with 50% ethanol (v/v) in water for 48 h, 3 cycles. After filtration through Whatman® filter paper number. 1, the solvent was evaporated by the rotary evaporator and dried to powder extracts by the spray dryer with outlet temperature of 70°C and inlet temperature of 120°C . The obtained three different extracts including the green coffee beans extract cultivated in Chumphon (CP), green coffee beans extract cultivated in Yala (YL), and green coffee beans extract cultivated in Chiang Rai (CR). The extracts were stored in amber glass bottles at $2 \pm 2^\circ\text{C}$ for further studies.

2.4. Determination of the Antioxidant Activities

2.4.1. DPPH Radical Scavenging Activity Assay. Scavenging activity of extracts on DPPH radicals was investigated by following the method of Iwai et al. [11] with some modifications. Briefly, an aliquot of 180 μl DPPH in ethanol was mixed with 20 μl of each sample in 96-well plates and kept in the dark at room temperature for 30 min. The absorbance of each sample was measured at 520 nm using a multimode detector, Beckman Coulter® GmbH, Austria. The results were calculated as 50% inhibitory concentration (IC₅₀). Chlorogenic acid, caffeine, and caffeic acid were used as reference standards.

2.4.2. Lipid Peroxidation Inhibitory Assay. The lipid peroxidation process based on the ferric-thiocyanate method was detected by following the method of Osawa and Namiki [12] with some modifications. An aliquot of 50 μl of each extract (20 mg/ml) was mixed with 50 μl of linoleic acid in 50% DMSO (v/v) in water, 50 μl of 5 mM ammonium thiocyanate (NH₄SCN), and 50 μl of 2 mM ferrous chloride (FeCl₂). The mixture was placed in a hot air oven at $37 \pm 0.5^\circ\text{C}$ for an hour, and then the reaction was investigated. The control was prepared as same as the sample without linoleic acid. Chlorogenic acid, caffeine, and caffeic acid were used as reference standards. The absorbance of each extract was detected at 490 nm using a multimode detector. The

percentage inhibition was calculated by the following equation:

$$\% \text{ inhibition} = \left[\frac{(Ac - As)}{Ac} \right] \times 100, \quad (1)$$

where Ac is the absorbance of control, and As is the absorbance of the sample.

2.4.3. Ferric Reducing Antioxidant Power (FRAP) Assay.

The ferric reducing antioxidant power of each extract was detected by following the method of Ou et al. [13] with some modifications. The FRAP reagent consisted of 0.3 M acetate buffer of pH 3, 10 mM TPTZ dissolved in 40 mM of 37% HCl (v/v) in deionized water, and 20 mM ferric chloride solution with a ratio of 10 : 1 : 1. The concentration of each extract was 1 mg/ml dissolved in 20% (v/v) Tween 20 in water, and 20 μ l of each sample was added into 96-well plates and reacted with 180 μ l of FRAP reagent. The mixture was incubated at room temperature for 5 min. The dark blue solution was measured at 595 nm by a multimode detector. Ferrous sulfate ($FeSO_4$) was used as a standard with the equation

$$y = 0.1405x + 0.0287, \quad (2)$$

where y is the absorbance at 595 nm and x is concentration of $FeSO_4$ ($r^2 = 0.9926$). The results of ferric reducing antioxidant power were reported as the FRAP value by the following equation:

$$\text{FRAP value} \left(\frac{\text{mM } FeSO_4}{g} \right) = \frac{[(A - B) - 0.0287]}{0.1405}, \quad (3)$$

where A is absorbance of control of sample, and B is absorbance of blank of sample.

2.5. High Performance Liquid Chromatography (HPLC) Analysis.

HPLC analysis was performed by a C-18 column (250 \times 4.6 mm i.d., 5 μ m, Mightysil[®]) as a stationary phase. The mobile phase was isocratic elution that composed of (A) acetonitrile and (B) 1% glacial acetic acid (15 : 85, pH 3) with the flow rate of 1.0 ml/min at room temperature [14, 15]. Each extract was diluted in methanol with a concentration of 1 mg/ml and filtered through 0.45 μ m nylon syringe filters (Whatman Puradisc 25, USA). Ten microliters of extracts was injected into the HPLC column and was detected at 280 nm in triplicates. The reference standards were caffeine, chlorogenic acid, and caffeic acid. The assay method was validated before analyzing extracts (Table 1).

2.6. Determination of the Anti-Inflammatory Activity

2.6.1. Macrophage Cell Culture. The effect of the selected extract on the inflammatory process was examined by following the method of Mueller et al. [16]. The RAW 264.7 macrophage cells (American Type Culture Collection, ATCC-TIB-71) were seeded in 24-well plates with DMEM at a density of 2×10^6 cells per well and then incubated for 24 h at $37 \pm 0.5^\circ\text{C}$, 5% CO_2 , and 90% humidity. The extract was added on the following day and incubated for 2 h with the

TABLE 1: Methods of validation requirement: linearity, limit of detection (LOD), limit of quantitation (LOQ), and % RSD.

	Linearity	Limit of detection ($\mu\text{g/mL}$)	Limit of quantitation ($\mu\text{g/mL}$)	% RSD
Caffeine	$r^2 = 0.994$	1.237	4.124	1.825
Chlorogenic acid	$r^2 = 0.998$	0.488	1.627	1.627
Caffeic acid	$r^2 = 0.979$	0.630	2.101	1.998

same condition. LPS was added at the final concentration of 1 $\mu\text{g/ml}$ and continually incubated further for 24 h. The supernatant was collected after centrifugation at 13,500 rpm for 10 min before investigation of the IL-6 secretion. The IL-6 concentration in supernatants (100 μ l) was determined by ELISA according to the manufacturer's protocol (R&D Systems, Minneapolis, MN, USA). All incubation steps were performed at room temperature. The optical density at 450 nm, corrected by the reference wavelength 570 nm, was measured using a Genios Pro microplate reader (Tecan, Crailsheim, Germany). The negative control was untreated cells, and the positive control was cells incubated with ethanol and LPS.

2.6.2. Determination of the Cell Viability by MTT Assay.

The MTT assay is the method to measure the reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to formazan by mitochondrial succinate dehydrogenase. The viability of LPS-stimulated cells was measured by the MTT assay. MTT solution (5 mg/ml) was added to the cells after removing the supernatant for ELISA. Cells were incubated for 2 h at $37 \pm 0.5^\circ\text{C}$, 5% CO_2 , and 90% humidity. The supernatant was removed after incubation, and cells were lysed with lysis buffer (10% SDS in 0.01 N-HCl). The optical density was measured using a Genios Pro microplate reader at 570 nm and corrected by the reference at 690 nm. **2.6.3. Calculation of IL-6 Secretion for Anti-inflammatory Activity**

The calculated concentrations of cytokines were normalized to MTT values to reduce any variation from differences in cell density by following this equation:

$$\text{Normalized IL - 6 secretion} = \left[\frac{\% \text{IL - 6 secretion}}{\% \text{cell viability}} \right] \times 100. \quad (4)$$

The cells were treated with only LPS which was used as the positive control, and the amount of cytokine secretion was defined as 100%. The results of the extracts were calculated as a percent of cytokine secretion.

2.7. Irritation Test by Hen's Egg Test Chorioallantoic Membrane (HET-CAM) Method.

The extract showed the highest antioxidant activity, which was determined for the potential to generate acute irritation by modified HET-CAM method [17–19]. All fertilized eggs were received from Faculty of Agriculture, Chiang Mai University. Eggs were kept 7–9 days in an incubator at $37 \pm 1^\circ\text{C}$ with $55 \pm 0.5\%$ relative humidity. On day 10, each eggshell was carefully opened with a dentist

rotary saw-blade, and white egg membrane was removed. The viable embryos with the yolk sac were returned to the incubator to protect them from cooling. The extract was prepared at a concentration of 0.5% w/v by dissolving in water. After that, 20 μ l of sample was dropped on the chorioallantoic membrane (CAM). The irritation reactions including vascular hemorrhages, vascular lysis, and vascular coagulation were observed using a stereo microscope within 5 min (300 sec) after adding the sample. Irritation score (IS) was calculated by the following equation:

$$IS = \frac{(301 - t(h))}{300 \times 5} + \frac{(301 - t(l))}{300 \times 7} + \frac{(301 - t(c))}{300 \times 9}, \quad (5)$$

where $t(h)$ is the time (sec) when the sign of vascular hemorrhages first occurred, $t(l)$ is time (sec) when the sign of vascular lysis first occurred, and $t(c)$ is time (sec) when the sign of vascular coagulation first occurred. The results were classified as no irritation when IS was 0–0.9, slight irritation when IS was 1.0–4.9, moderate irritation when IS was 5.0–8.9, and severe irritation when IS was 9.0–21.0. The blood vessel on CAM was continually investigated until 60 min for a long term irritation, and photographs were taken under the stereo microscope.

2.8. Preparation of NLCs Formulations

2.8.1. Development of NLCs. The major ingredients of NLCs are lipids, surfactants, and active compounds. Selected types of lipids affect the characteristics of NLCs formulation. The solubility of CP in lipids was determined by the test tube method [20]. The CP (1 mg) was weighed in the test tube. Each solid lipid or liquid lipid that is maintained at 5°C above their melting point was added in increments until CP was completely dissolved. The amount of lipids required to solubilize CP was determined. The high shear homogenization and ultrasonication technique is a modified method for preparing NLCs [21, 22]. The required hydrophilic lipophilic balance (rHLB) value of lipids was calculated for estimating the amount of surfactants in formulation. If the formulation uses amount of surfactants equal to the rHLB, the formulation with good stability and small particle size is obtained [23]. Briefly, the lipid mixture consisted of solid lipids and liquid lipids with the ratio of 1:6 (% w/w) that melted until 75°C. The Span 80 and Cremophor A25 which contained 5% w/w in placebo-NLCs (A1 and A2) and 7.5% w/w in placebo-NLCs (B1 and B2) were used as surfactants. Pre-emulsion occurred after mixing the oil phase and water phase with the high-shear homogenizer (Yellow line DI 25 basic, IKA Werke GmbH & Co. KG, Germany) at 8,000 rpm for 5 min. Pre-emulsion was then generated to NLCs using the high-intensity ultrasonic processor (Vibra cell™, Sonics & Materials, USA) with 400 W, 3 sec for the pulse on and off, 40% amplitude, and 5 min for placebo-NLCs (A1 and B1) and 15 min for placebo-NLCs (A2 and B2). All placebo-NLCs were gently stirred until being cooled down to room temperature. Compositions of NLCs are shown in Table 2.

In the next step, CP-loaded NLCs were prepared by adding the CP into the lipid phase before mixing other

TABLE 2: Compositions of NLCs formulations (values are given in % w/w).

Compositions (% w/w)	Formulation				
	Placebo- NLCs A1	Placebo- NLCs A2	Placebo- NLCs B1	Placebo- NLCs B2	CP- loaded NLCs
Cetyl alcohol	0.5	0.5	0.5	0.5	0.5
Stearyl alcohol	0.5	0.5	0.5	0.5	0.5
Sunflower oil	3.0	3.0	3.0	3.0	3.0
Canola oil	3.0	3.0	3.0	3.0	3.0
Span 80	2.81	2.81	3.27	3.27	3.27
Cremophor A25	2.19	2.19	4.25	4.25	4.25
CP	—	—	—	—	0.5
Sonication time (min)	5	15	5	15	15

components. The final concentration of the CP-loaded NLCs was 0.5% w/w.

2.8.2. Characterization of Prepared NLCs. Particle size, polydispersity index (PDI), and zeta potential of placebo-NLCs and CP-loaded NLCs were detected by photon correlation spectroscopy (Zetasizer® ZS, Malvern Instruments Ltd., UK). Each formulation was diluted with deionized water at a ratio of 1:100. All measurements were done in triplicate at $25 \pm 0.5^\circ\text{C}$.

2.8.3. Stability Study of NLCs Formulations. Placebo-NLCs and CP-loaded NLCs were kept in amber glass bottles and stored under various conditions using 6 cycles of heating-cooling (HC) method (changing between $4 \pm 0.5^\circ\text{C}$ for 48 h and $45 \pm 0.5^\circ\text{C}$ for 48 h), room temperature (RT or $30 \pm 0.5^\circ\text{C}$), $4 \pm 0.5^\circ\text{C}$ and $45 \pm 0.5^\circ\text{C}$ for 3 months. The characterization of NLCs formulations, the physical properties, and antioxidant activity were evaluated after storage.

2.9. Entrapment Efficiency and Extract Loading of CP-Loaded NLCs. Entrapment efficiency and extract loading were evaluated by the ultrafiltration method with some modifications [20]. In brief, CP-loaded NLCs were divided into 2 parts for analyzing entrapment efficacy: formulation for determining total CP and formulation for determining unloaded CP. 0.3 g of CP-loaded NLCs was dissolved in hexane and ethanol with a ratio of 6:4 and analyzed as total CP [W_{total}] in formulation by HPLC. Hexane and ethanol helped to dissolve NLCs formulation for analyzing total CP in formulation including unloaded CP and loaded CP in NLCs. Other part of CP-loaded NLCs (0.3 g) was weighted and added into the top of the ultra centrifuge filter (Amicon® Ultra-1.5 ml, NMWCO 10 kDa, Merck, Germany). The ultra centrifuge filter was then centrifuged at 7,000 rpm at 25°C for an hour. CP-loaded NLCs remained in the ultra centrifuge filter whereas the unloaded CP was dropped at the bottom of tube. The unloaded CP [W_{free}] was diluted with methanol and analyzed by HPLC. An amount of the active compound in the solution was determined by HPLC. Finally, the

percentage of entrapment efficiency (EE) and extract loading (EL) were calculated by the following equation.

$$\% \text{ EE} = \frac{[W_{\text{total}}] - [W_{\text{free}}]}{[W_{\text{total}}]} \times 100, \quad (6)$$

$$\% \text{ EL} = \frac{[W_{\text{total}}] - [W_{\text{free}}]}{[W_{\text{total}}] + [W_{\text{lipid}}] - [W_{\text{free}}]} \times 100,$$

where $[W_{\text{total}}]$, $[W_{\text{free}}]$, and $[W_{\text{lipid}}]$ are the amount of total CP, the amount of unloaded CP, and the amount of lipids used to prepare the formulation, respectively.

2.10. Skin Permeation Study. CP-loaded NLCs and CP in conventional emulsion were evaluated for skin permeation by Franz diffusion cells. The porcine skin was shaved and subcutaneous fat was removed. After that, it was placed on top of the receiver chamber and the epidermis turned to the donor compartment whereas dermis was closed to the receiver chamber. Phosphate buffer (pH 5.5) was contained in the receiver chamber as receiver medium. The volume of receiver medium was 13 ml and the temperature was set at $32 \pm 2^\circ\text{C}$ and continuously stirred with a magnetic bar. 1 g of each formulation was added to the donor compartment. Receiver medium was withdrawn at 1, 2, 4, 6, 8, and 10 h. The porcine skin was turned into small pieces, immersed in methanol, and sonicated for 15 min. The concentration of chlorogenic acid and caffeine in viable epidermis and dermis (VED) was detected by HPLC. For the determination of chlorogenic acid and caffeine in the stratum corneum (SC), the skin was stripped with 20 pieces of an adhesive tape using a 3M scotch magic™ tape. Each tape was charged with a weight of 300 g for 10 sec. The first tape strip was discarded. The 2nd to 20th tape strips were extracted with methanol to quantify the amount of chlorogenic acid and caffeine in the stratum corneum. The percentage accumulative of chlorogenic acid and caffeine in SC, VED, and solution after 10 h was calculated by the following equation.

$$\% \text{ accumulative} = \left(\frac{A_{\text{sc}}, A_{\text{ved}} \text{ and } A_{\text{so}}}{A_{\text{total}}} \right) \times 100\%, \quad (7)$$

where A_{sc} , A_{ved} , and A_{so} are an amount of chlorogenic acid and caffeine in stratum corneum, viable epidermis and dermis, and solution, respectively. A_{total} is total amount of chlorogenic acid and caffeine in 1 g of formulation.

2.11. Statistical Analysis. All experiments in this study were done in triplicates. The results were presented as the mean \pm standard deviation (SD). Statistical analysis used the SPSS statistic version 17.0 program.

Statistical significance was evaluated by one-way ANOVA at the probability level of 0.05.

3. Results and Discussion

3.1. Green Robusta Coffee Beans Extracts. The percentage yields of green robusta coffee beans extracts and their

TABLE 3: Percentage of yields and physical characteristic of green robusta coffee beans.

Extracts	Yield (%)	Physical characteristic
CP	9.6 ± 0.7	Dry light green powder
YL	7.7 ± 0.2	Dry light green powder
CR	8.1 ± 0.7	Dry light green powder

TABLE 4: Antioxidant activities of green robusta coffee bean extracts detected by DPPH assay, lipid peroxidation inhibitory assay, and FRAP assay.

Samples	DPPH assay	Lipid peroxidation	FRAP assay
	IC ₅₀ ($\mu\text{g/ml}$)	inhibitory assay % inhibition	FRAP value (mM FeSO ₄ /g)
CP	34.1 ± 0.9^a	38.8 ± 1.7^a	234.5 ± 12.3^a
YL	44.2 ± 1.4^a	14.4 ± 0.4^b	231.8 ± 3.1^a
CR	48.6 ± 3.4^a	15.3 ± 0.7^b	211.7 ± 16.9^a
CA	7.1 ± 1.2^b	45.3 ± 0.4^a	234.5 ± 0.1^a
CGA	10.5 ± 1.3^b	34.1 ± 1.8^a	234.5 ± 0.1^a
CF	ND	ND	37.02 ± 10.7^b

Different letters within the same column mean significant difference ($p < 0.05$). ND: not detected, CP: green robusta coffee beans extract from Chumphon, YL: green robusta coffee beans extract from Yala, CR: green robusta coffee beans extract from Chaing Rai, CA: caffeic acid, CGA: chlorogenic acid, and CF: caffeine.

physical characteristics are presented in Table 3. All extracts were light green powders. The CP showed the highest percentage of yield as 9.6 ± 0.7 while the YL showed the lowest percentage of yield among all extracts. However, the results were not significantly different among all extracts grown in various locations.

3.2. Determination of Antioxidant Activity. Antioxidant activities of each extract by DPPH, lipid peroxidation inhibitory, and FRAP assays are shown in Table 4. Several assays for antioxidant activity were used to analyze various mechanisms of the antioxidants against free radicals. The CP demonstrated the best antioxidant activity among three extracts and caffeine in all assays. It showed the highest free radical scavenging activity of all extracts by the DPPH assay. The DPPH assay is based on the scavenging capacity of antioxidants by hydrogen donation and used to evaluate hydrophilic antioxidants, including chlorogenic acid and caffeic acid. These are phenolic compounds which are mostly found in green coffee beans [22]. Therefore, the scavenging capacity of extracts correlated with phenolic compounds in extracts. Those compounds consist of many hydroxyl groups that could scavenge reactive oxygen species (ROS) by donating hydrogen atoms [24]. The CP also showed better activity than chlorogenic acid which was detected by lipid peroxidation inhibitory assay. It can be assumed that CP could inhibit the oxidation reaction of unsaturated fatty acids by donating hydrogen atoms to stop the chain reaction. In addition, it possessed equal activity with chlorogenic acid and caffeic acid as shown by FRAP assay. The FRAP assay is based on the reduction of ferric iron (Fe^{3+}) complex to ferrous ion (Fe^{2+}) by electron donation of the antioxidant

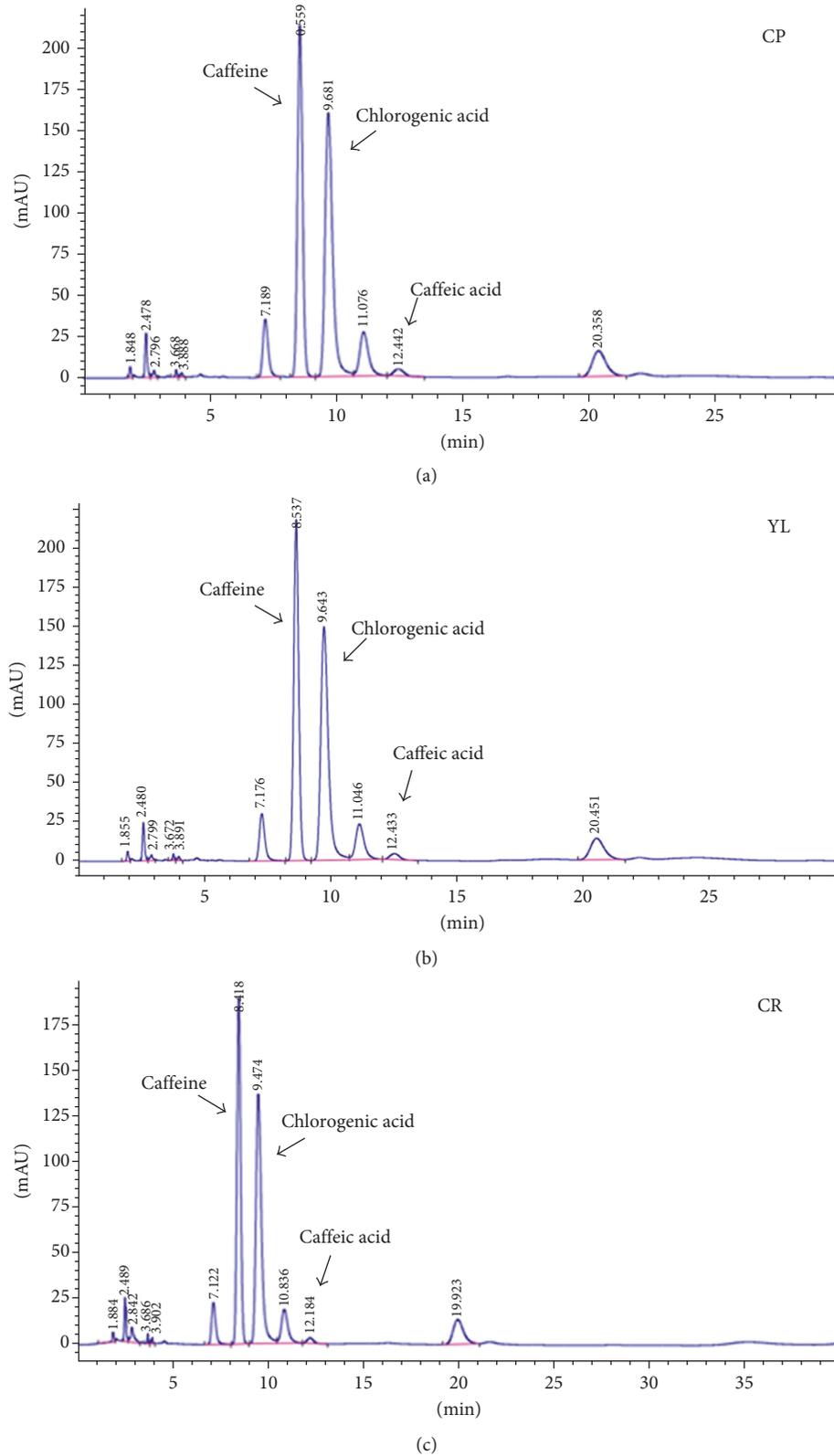


FIGURE 1: HPLC chromatograms of extracts: (a) CP, (b) YL, and (c) CR using HPLC at a wavelength 280 nm.

agent [25, 26]. Due to the high FRAP values of all extracts, it could be assumed that they had the ability to reduce Fe^{3+} to Fe^{2+} as well as act as a chelating agent.

3.3. High Performance Liquid Chromatography (HPLC) Analysis. Caffeine, chlorogenic acid, and caffeic acid could be clearly identified by HPLC with retention times of 8.049,

9.491, and 12.051 min, respectively. All extracts showed the major peak at the same standard retention time (Figure 1). It could be noticed that the major compounds in green robusta coffee beans were found in all extracts. However, different contents of caffeine and phenolic compounds in green coffee beans depended on temperature, weather, and location of plant growth [27]. Generally, robusta coffee is grown in the southern part of Thailand at elevations of 800 to 1,200 m above the sea level in an open area with full sunlight. It could be assumed that the CP was grown in a suitable location. Therefore, the level of chlorogenic acid content was higher than other extracts. Plants from different locations consisted of the same natural compounds, but the amounts of the compounds were different.

3.4. Anti-Inflammatory Activity. Interleukin 6 (IL-6) acts as a proinflammatory cytokine and is secreted by T cells and macrophages to stimulate the immune response. In this study, the effect of CP on the expression of inflammatory cytokines was evaluated. Anti-inflammatory activity was determined by studying the inhibition of the secretion of IL-6 on RAW 264.7 macrophages cells. The results are shown in Figure 2. All concentrations of CP were not toxic to the cells. Concentrations of CP between 0.05 and 0.1 mg/ml could decrease the IL-6 secretion. The IL-6 secretion in the presence of a high concentration of CP was not significantly different when compared to caffeine and the untreated control without LPS induction. It indicated that the extract had the ability to inhibit inflammatory activity due to a high amount of the caffeine content in the extract. Caffeine has been well known as a strong anti-inflammatory agent [28].

3.5. Hen's Egg Test Chorioallantoic Membrane (HET-CAM) Assay. The results of the irritation test using the HET-CAM assay are shown in Table 5. For the positive control, 1% w/v SLS in deionized water was used to induce an irritation effect on the chorioallantoic membrane (CAM). SLS is usually found in cosmetic products and causes acute skin irritation. SLS showed moderate irritation and vascular lysis. Solution of CP 0.5% w/v in deionized water presented the same result as NaCl 0.9% w/v in deionized water which was used as the negative control. The CP did not affect blood vessels and capillaries after 60 min of observation. The surfaces of the CAM and blood vessels are presented in Figure 3. It can be concluded that the CP was safe for usage in cosmetic products as an active ingredient since it showed no irritation. The HET-CAM assay is a model for predicting the irritation effect on the conjunctiva. However, it has been used to assume the irritation of many types of cosmetic formulations [29, 30].

3.6. Preparation of Placebo-NLCs and CP-Loaded NLCs

3.6.1. Development of Placebo-NLCs. The solubility of CP in lipids is shown in Figure 4. The CP could dissolve in liquid lipids following this order: canola oil > sunflower oil > rice bran oil > camellia oleifera seed oil. Solubility of CP in solid lipids followed this order: cetyl alcohol > stearyl alcohol > glyceryl

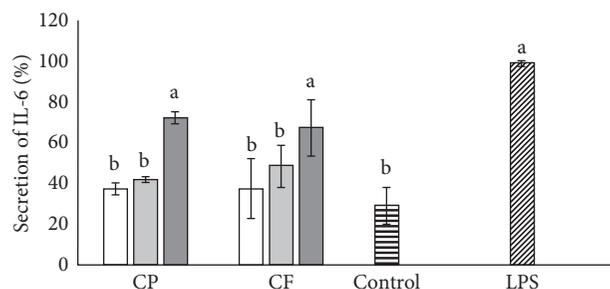


FIGURE 2: The influence of various concentrations of CP (□ 0.10 mg/ml, □ 0.05 mg/ml, and ■ 0.01 mg/ml), caffeine (CF) (□ 0.10 mg/ml, □ 0.05 mg/ml, and ■ 0.01 mg/ml), and RAW 264.7 cells without LPS (control) and cells induced with LPS (LPS) Different letters mean significant differences ($p < 0.05$).

TABLE 5: Irritation score and irritation assessment from HET-CAM assay.

Samples	Irritation score	Irritation assessment
CP (0.5% in aqueous solution)	0.0	No irritation
Negative control (0.9% NaCl)	0.0	No irritation
Positive control (1% SLS)	6.4 ± 1.3	Moderate irritation

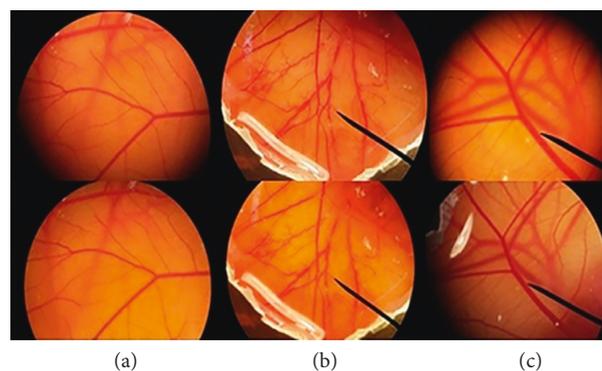


FIGURE 3: Photographs of CAM after 5 min (upper) and after 60 min (lower) exposure to (a) negative control (0.9% NaCl), (b) positive control (1% SLS), and (c) CP in aqueous solution (0.5%).

monostearate > beeswax > carnauba wax. From the solubility results mentioned above, canola oil, sunflower oil, cetyl alcohol, and stearyl alcohol were selected as the oil phase for NLCs preparation. Furthermore, the solubility of CP was evaluated in different lipid blends. The solubility of blended canola oil and sunflower oil and cetyl alcohol blended with stearyl alcohol were 1.37 and 0.78 mg/ml, respectively. Therefore, canola oil (HLB 7) and sunflower oil (HLB 15) were used as liquid lipids and cetyl alcohol (HLB 15.5) and stearyl alcohol (HLB 15.5) were used as solid lipids in formulation. Span 80 (HLB 4.3) and Cremophor A25 (HLB 17) were selected for using as surfactants in NLC formulation. The required hydrophilic-lipophilic balance value of lipids in formulation was 11-12, which is appropriate to produce stable NLCs.

The characteristics of placebo-NLCs are presented in Table 6 including the particle size, polydispersity index

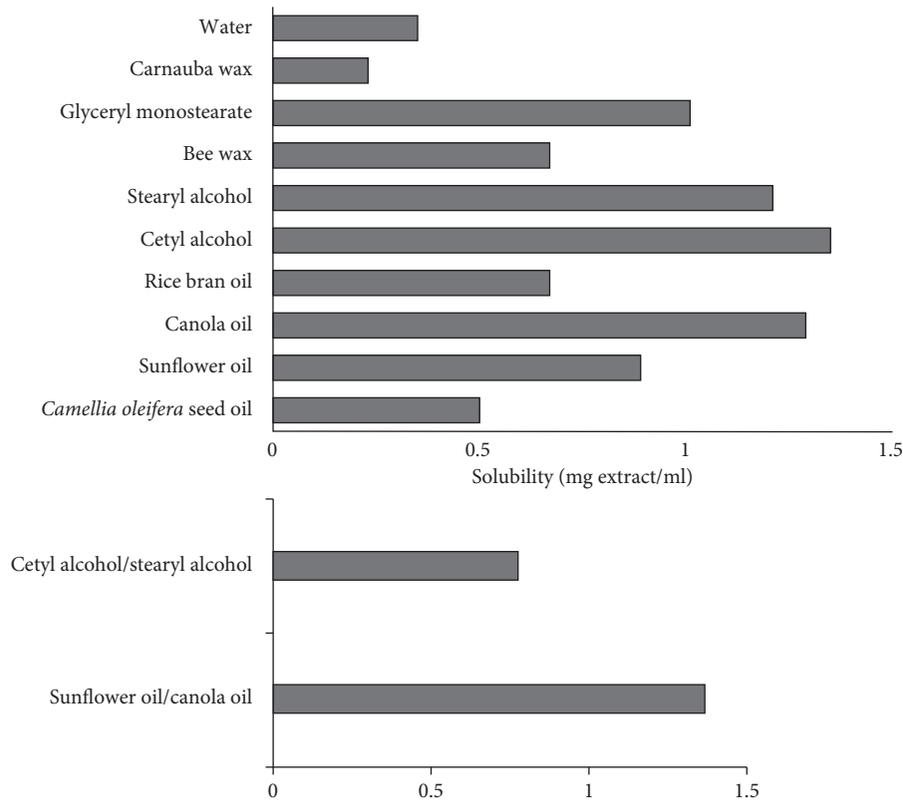


FIGURE 4: Solubility of CP in lipids.

TABLE 6: Mean particle size, polydispersity index (PDI), and zeta potential of NLCs with different ultrasonication time and surfactant concentrations.

Placebo-NLCs	Ultrasonication time (min)	Surfactant (% w/w)	Particle size (nm)	PDI	Zeta potential (mV)
A1	5	5	219.5 ± 0.5 ^a	0.3 ± 0.02 ^a	-21.7 ± 0.5 ^a
A2	15	5	150.7 ± 1.5 ^b	0.3 ± 0.04 ^a	-20.7 ± 0.2 ^a
B1	5	7.5	194.9 ± 2.6 ^c	0.2 ± 0.02 ^b	-30.5 ± 0.4 ^b
B2	15	7.5	152.4 ± 0.9 ^b	0.2 ± 0.01 ^b	-28.9 ± 0.8 ^b

Different letters within the same column mean significant difference ($p < 0.05$).

(PDI), and zeta potential. An important factor of NLCs preparation is the technique, the final particle size could be affected by the influence of process parameters on sonication including duration and amplitude [31]. All placebo-NLCs had obtained a particle size less than 220 nm, confirming that the particles were produced in the nanosize range. Placebo-NLCs A2 and placebo-NLCs B2 were obtained in a smaller size than placebo-NLCs A1 and placebo-NLCs B1 with a significant difference at $p < 0.05$. From the results, it could be explained that the particle size dramatically decreased with higher sonication time. Ultrasonication was used to break down the colloidal to nanoscale by amplitude. Higher amplitude and longer ultrasonication time provided higher energy sound wave to break down the colloidal. In this study, the amplitude of ultrasonication was 40% that is suitable to produce nanoparticles. Ultrasonication time of 20 min or longer in other placebo-NLCs (data not shown) produced the same particle size as the formulation of 15 min of ultrasonication time with a wide PDI. Ultrasonication time of about 15 min regularly provided nanoscale, while 1

to 5 min produced a final particle size of more than 150 nm. Therefore, an appropriate ultrasonication time leads to the production of small particle size because it directly influenced the final particle size of the dispersion. Surfactants in formulation also affected the particle size of placebo-NLCs. Increasing the surfactant concentration from 5% to 7.5% in placebo-NLCs B1 resulted in a smaller particle size than placebo-NLCs A1 due to the decreased interfacial tension [32]. The high amount of surfactant decreased the surface tension of lipid drops, breaking down lipid drops to a smaller size. In addition, a suitable amount of surfactant concentration could prevent coalescence of the placebo-NLCs. Cremophor A25 and Span 80 might affect the surface charge that decreased zeta potential value, but it could generate stable formulation by steric effect [33]. The polydispersity index of placebo-NLCs remained in a range of 0.2 to 0.3.

Placebo-NLCs were evaluated for their stability after 3 months. Placebo-NLCs B2 exhibited good stability with a constant particle size after storage at 4°C and room temperature. The particle sizes of the other NLCs were slightly

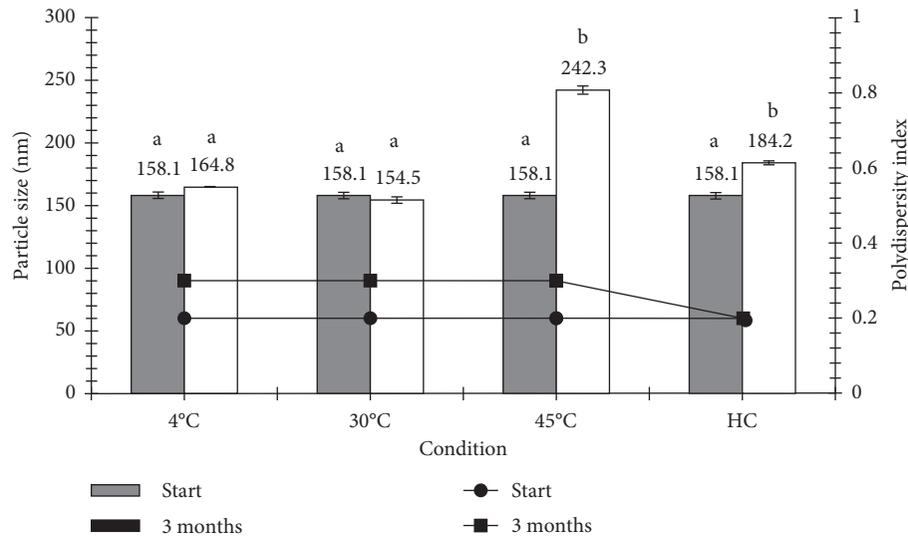


FIGURE 5: Mean particle size and PDI of CP-loaded NLCs before and after the stability test at different conditions. Different letters mean significant difference ($p < 0.05$).

increasing from the starting time. These results clearly indicated that placebo-NLCs B2 had good properties for loading CP.

3.6.2. Preparation and Stability Study of CP-Loaded NLCs. CP 0.5% w/w was loaded in the lipid phase to prepare NLCs and kept at various conditions, heating-cooling (HC): $4 \pm 0.5^\circ\text{C}$, $30 \pm 0.5^\circ\text{C}$, and $45 \pm 0.5^\circ\text{C}$ for 3 months. The physical appearances of CP-loaded NLCs including color and pH value did not change during storage. The results of the stability study are shown in Figure 5. The particle size, zeta potential, and PDI were 158.1 ± 2.7 nm, -29.2 ± 0.1 mV, and 0.2 ± 0.01 , respectively. Formulations after HC and at 45°C significantly increased the particle size ($p < 0.05$) whereas 4°C and room temperature did not affect the particle size of CP-loaded NLCs. Usually, an increase of the particle size and PDI are parameters of instability in the colloidal system [34]. It could be indicated that CP-loaded NLCs slightly changed at high temperature for the long storing period but no coalescence or phase separation occurred.

3.6.4. Antioxidant Activities of CP-Loaded NLCs after Stability Test. Antioxidant activity after storage was evaluated by DPPH and FRAP assays. The results are shown in Figure 6. CP-loaded NLCs exerted nearly 90% of inhibition by the DPPH assay in all conditions with no significant difference ($p < 0.05$) from the starting values. The results from the FRAP assay were also similar to the starting values especially at 4°C , but FRAP value slightly decreased under other conditions. The temperature had affected the antioxidant activity of CP-loaded NLCs due to degradation of phenolic compounds after storage. Chlorogenic acid and polyphenols are sensitive to temperature [35]. However, NLCs could help decrease the degradation of phenolic compounds in formulation leading to the preservation of the antioxidant potential.

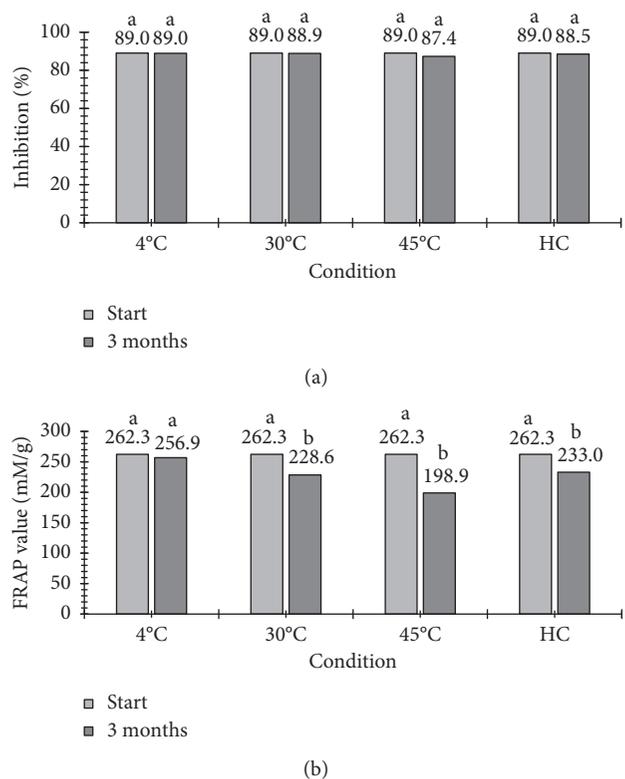


FIGURE 6: Antioxidant activities of CP-loaded NLCs by (a) DPPH and (b) FRAP assays before and after stability test at 4°C , 30°C , and 45°C and heating-cooling (HC) cycles. Different letters within the same condition mean significant difference ($p < 0.05$).

3.7. Entrapment Efficacy and Extract Loading of CP-Loaded NLCs. Entrapment efficacy of chlorogenic acid and caffeine in CP-loaded NLCs was found to be 73.2% and 62.9%, respectively. Drug loading capacity of chlorogenic acid and caffeine in CP-loaded NLCs was found to be 73.5% and

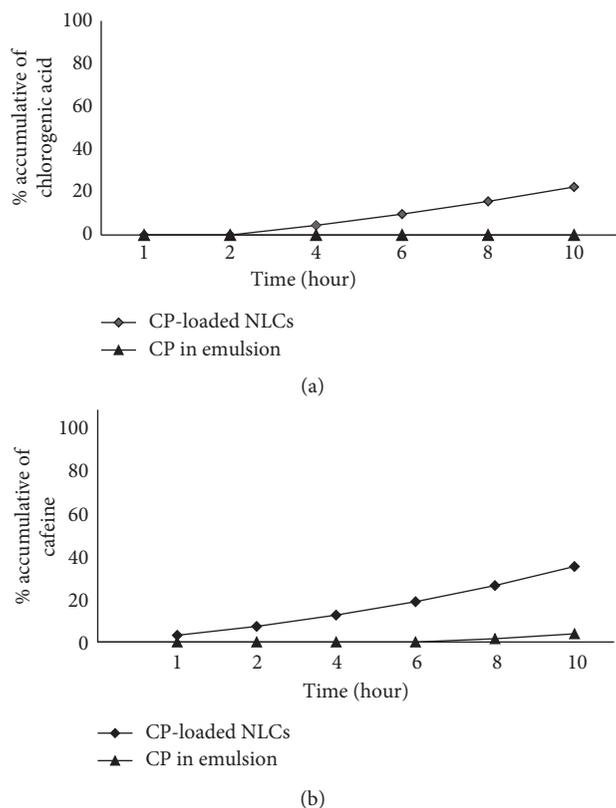


FIGURE 7: Percentage of accumulative amount of (a) chlorogenic acid and (b) caffeine from CP-loaded NLCs and conventional emulsion after 10 h.

68.8%, respectively. The mixture of hexane and ethanol could effectively extract the active compound from the lipid matrix for analyzing the amount of the total compound. The extract was entrapped into the lipid matrix due to the structure of NLCs consisting of medium chain triglycerides with free spaces capable of holding a certain amount of the extract. Entrapment efficacy depends on the amount of the lipid, the solubility of the compounds in lipid, and the concentration of the surfactant [23, 36]. Therefore, increasing the concentration of the lipid matrix and the solubility of the extract in the lipid phase can generate higher encapsulation efficiency.

3.8. Skin Permeation Study by Franz Diffusion Cells.

Chlorogenic acid and caffeine in the CP were detected as a marker in the skin permeation study. The percentage of accumulative amounts of chlorogenic acid and caffeine between CP-loaded NLCs and conventional emulsion in the receiver chamber after 10 h is shown in Figure 7. Amounts of chlorogenic acid and caffeine remaining in the viable epidermis and dermis (VED) and stratum corneum (SC) from CP-loaded NLCs and conventional emulsion are also shown in Figure 8. NLCs-loaded CP exhibited higher accumulative amount of chlorogenic acid and caffeine than conventional emulsion. Skin samples were extracted after the end of the permeation experiments. The amounts of chlorogenic acid and caffeine from NLCs-loaded CP penetrated into the

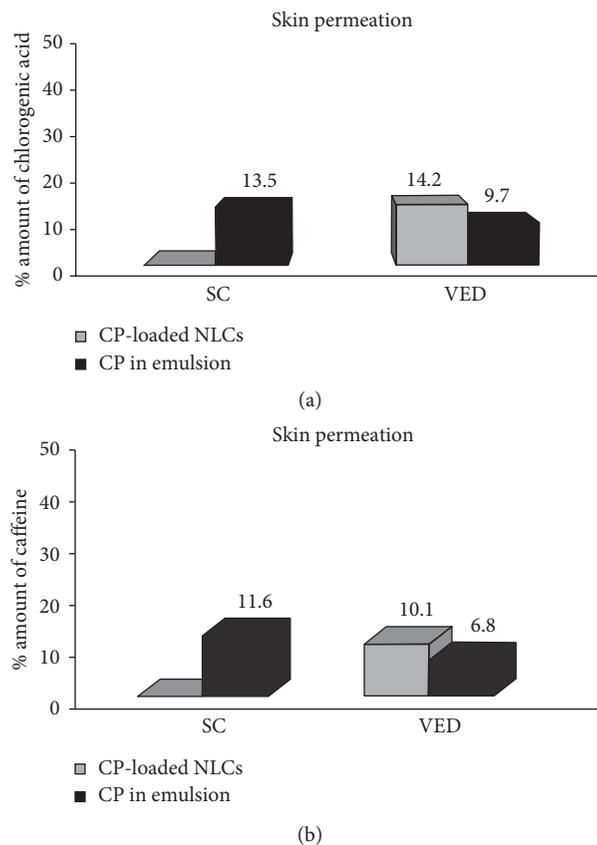


FIGURE 8: Amount of (a) chlorogenic acid and (b) caffeine in the stratum corneum (SC) and viable epidermis and dermis (VED) after 10 h.

viable epidermis and dermis and receiver solution deeper than conventional emulsion. Furthermore, adhesive tapes were performed as the amounts of chlorogenic acid and caffeine in stratum corneum (SC). Chlorogenic acid and caffeine from CP-loaded NLCs could not be detected on the stratum corneum whereas conventional emulsion showed a high content of chlorogenic acid and caffeine. The result showed that CP-loaded NLCs could penetrate and deliver the active compound into the skin better than conventional emulsion. In addition, NLCs-loaded CP possessed the property of enhanced skin penetration of the active compound due to small particle size, film former, and occlusive effect [9, 37]. Conventional emulsion was not able to form film with larger particle size. Those results proved that the particle size and occlusive properties of NLCs formulation achieve a higher penetration of the active compound through the skin.

4. Conclusion

Green robusta coffee beans extracts from three different locations in Thailand, including Chumphon, Yala, and Chiang Rai contained caffeine and phenolic compounds including chlorogenic acid and caffeic acid. CP exhibited the highest antioxidant activity shown by DPPH and FRAP assays which correlated to a high amount of chlorogenic acid

and caffeine in the extract. IL-6 secretion is decreased by CP indicating an anti-inflammatory potential. Growing coffee at suitable attitude and location was an important factor that related to biological activities of the extract. CP did not irritate the chorioallantoic membrane of hen's eggs, thus can be safely applied to the skin. The CP-loaded NLCs was successfully formulated to improve the stability of the extract and increase skin permeation efficacy. Therefore, the CP-loaded NLCs can be further used in cosmeceutical applications.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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