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Original Article

DEVELOPMENT OF ELLAGIC ACID RICH POMEGRANATE PEEL EXTRACT LOADED NANOSTRUCTURED LIPID CARRIERS (NLCS)

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ABSTRACT

Objective: This study aimed to access the anti-tyrosinase activity of ellagic acid rich pomegranate peel extract (EPP) and to prepare the EPP loaded nanostructure lipid carriers (NLCs) for topical application to promote active compound penetration efficiency.

Methods: EPP contained ellagic acid 12% (w/w) has been prepared and subjected to mushroom tyrosinase inhibitory assay. The EPP loaded NLCs have been developed using a warm microemulsion technique. The physicochemical properties, entrapment efficacy, *in vitro* release profile and *ex vivo* permeation study were investigated.

Results: The EPP possessed a strong anti-tyrosinase activity with IC_{50} values of 28.54 ± 1.34 µg/ml. At optimal condition, lyophilized EPP loaded NLCs showed spherical particles with a mean particle size of ~200 nm, polydispersity index of ~0.2 and zeta potential of ~ -34 mV. The high incorporation efficiency of ellagic acid, ~90%, was achieved. The *in vitro* release study showed a prolonged release of ellagic acid from the NLCs up to 12 h following the Higuchi's model. The *ex vivo* permeation study showed that the cream containing EPP loaded NLCs clearly promote the active compound penetration compare to EPP cream (control).

Conclusion: The prepared EPP showed strong anti-tyrosinase activity that suitable for use in cosmetics. NLCs were shown to be a promising delivery system for EPP to improved bioavailability of active ingredients. The penetration enhancing effect of NLCs was dependent on the types of oil and surfactant used in formulation.

Keywords: Pomegranate; *Punica granatum*; Ellagic acid; Nanostructured lipid carriers; Anti-tyrosinase

INTRODUCTION

Pomegranate (*Punica granatum* L.) is one of the ancient fruit that has been used in folk medicine, cosmetic and food supplement in many countries [1, 2]. The main compounds found in pomegranate peel are ellagitannins, gallotannins, gallic acid and ellagic acid [3-5] Ellagic acid (EA), Fig. 1, is one of the best investigated for variety of pharmacological activities [6-8]. It possessed antioxidant, anti-inflammatory, anti-tyrosinase, and anti-mutagenic activities. Fascinatingly, Yoshimura et al.. reported that EA effectively suppress UV-induced skin pigmentation in brownish guinea pig [5] suggesting that EA can prevent the build-up of skin pigmentation after sunburn. It can also be expected to improve the appearance of pigmented skin such as melasma, freckles or post-inflammatory pigmentation [9]. Therefore, EA is a promising skin-whitening active agent for cosmetics. Nevertheless, the use of botanical products is expanding rapidly worldwide. Recently, it has been reported that the pomegranate peel extracts containing 13% w/w ellagic acid possessed comparable antibacterial, anti-allergic and anti-inflammatory activities to the pure ellagic acid [10]. Comparing the manufacturing procedure of pure compound and crude extract, the latter is cheaper and easier to prepare and less time consuming. Moreover, a crude extract contains the original combination of active ingredients which is difficult to imitate in purified compounds. Thus, in this study, EPP was used instead of ellagic acid. In addition, the anti-tyrosinase activity of both ellagic acid and EPP was compared.

The main barrier for topical delivery is stratum corneum (SC), the topmost skin layer. It comprises of a multi-layered "brick and mortar" like structure, where the bricks are composed of keratin-rich corneocytes and the mortar is an intercellular matrix of a unique composition of lipids [11].

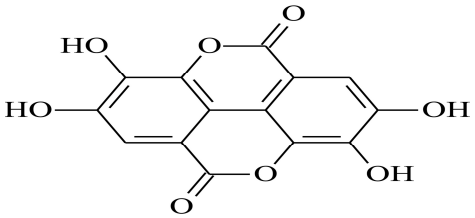

Fig. 1: Chemical structure of ellagic acid.

Thus, further investigation with a suitable cosmetic delivery system has to be carried out in order to enhance active compound penetration efficiency. Among modern drug delivery carriers, nanostructure lipid carriers (NLCs) are a promising colloidal carrier system. They made from biodegradable and biocompatible lipids that exist in the submicron size range and can be prepared by several methods. The advantages of NLCs are following; possibility of controlled drug release, protection of incorporated compound against chemical degradation, no toxicity of the carrier, avoidance of organic solvent and no problem with respect to large scale production [12-14]. In addition, NLCs possess occlusive property leading to skin hydration and subsequently enhance skin penetration of active ingredient [15, 16]. Pardeike et al.. reported that the NLCs containing cream significantly increased the skin hydration more than conventional cream with the same composition of cream [16]. Thus, in this study, we aimed to access the anti-tyrosinase activity of EPP and to prepare the EPP loaded NLCs for topical application to promote active compound penetration efficiency. The EPP loaded NLCs were prepared by a warm

microemulsion technique. The processing factors affecting the characteristics of EPP loaded NLCs were investigated, including their physicochemical properties, the efficiency of drug incorporation and the pattern of drug release. Moreover, the effect of types of oil and surfactant on permeation enhancing effect of EPP loaded NLCs was investigated by *ex vivo* using porcine ear epidermis.

MATERIALS AND METHODS

Materials

Pomegranates were purchased from Makro Public Co., Ltd. (Phitsanulok, Thailand). Porcine ears were obtained from Bangrakam slaughter house (Phitsanulok, Thailand). Standard ellagic acid was purchased from Sigma (Lot No. 020M1106V, MO, USA). Gelucire 44/14 and Compritol® 888 ATO were obtained from Gattefossé (Cedex, France). Poloxamer 188 was purchased from HiMedia Laboratories Pvt. Ltd. (Mumbai, India). Dioctyl sodium sulfosuccinate (AOT) was purchased from Fluka Chemie (Buchs, Switzerland). Lipoid® S 75 was obtained as a gift from Lipoid GmbH (Ludwigshafen, Germany). Glyceryl monostearate (GMS), Lexol (Medium chain triglyceride), isopropyl palmitate (IPP), isopropyl myristate (IPM), polyethylene glycol 400 (PEG 400) and Span 80 were purchased from Namsiang (Bangkok, Thailand). All other chemicals were of analytical grade. Regenerated cellulose membrane, diameter 76 mm, with a molecular weight cut off (MWCO) 100 kDa (Milipore® YM100) was purchased from Millipore Corporation (MA, USA).

Methods

Preparation and standardize of EPP

EPP was prepared and standardized to contain 12% w/w ellagic acid by previously described methods [17]. Briefly, the dried powder of pomegranate rind (0.5 kg) was extracted twice with 2 L methanol containing 10% (v/v) water under reflux conditions for 1 h. The pooled extracts were dried in vacuum. The crude extract was then suspended in 2% aqueous acetic acid and partitioned with ethyl acetate. The pooled ethyl acetate fractions were then evaporated to dryness in vacuo. Quantitative of ellagic acid using HPLC (Model LD10A Shimadzu, Kyoto, Japan) was performed by the previous report with some modifications [17]. Separation was achieved at 25°C on a 150 mm × 4.6 mm i.d. Luna 5 µm pentafluorophenyl column. The mobile phase consisted of methanol and 2% aqueous acetic acid (40-60% v/v) with isocratic elution at a flow rate of 1 ml/min. The injection volume was 20 µl. The quantitation wavelength was set at 254 nm.

Anti-tyrosinase activity by mushroom tyrosinase inhibitory assay

Tyrosinase inhibitory activity was tested according to method of Zheng et al.. with some modifications [18]. The sample was dissolved in methanol in a concentration ranging from 0.001-0.5 mg/ml. The sample (20 µl) was mixed with 165 µl 50 mM phosphate buffer (pH 6.5) in a 96-well microplate and 10 µl of 1 unit/µl mushroom tyrosinase was added into each sample. The mixture was incubated for 10 min at 37°C and finally 25 µl of 4 mM L-tyrosine was added. The enzyme reaction was monitored by measuring the change in absorbance at 510 nm of formation of the DOPA chrome using microplate reader (Model DTX880, Beckman Coulter Inc., Fullterton, Austria). The absorbance of the control was obtained by replacing the sample with phosphate buffer (pH 7.4). The absorbance of the blank was obtained by replacing the L- tyrosine with phosphate buffer (pH 7.4). Percent tyrosinase inhibition was calculated according to equation (1). Dose–response curves were obtained by plotting the % tyrosinase inhibition versus log concentration of samples using Prism program. The IC_{50} value, a concentration giving 50% inhibition of tyrosinase activity, was determined by interpolation of the dose–response curves.

$$\% \text{ tyrosinas e inhibition } = \frac{(A-B)}{A} x100 \quad (1)$$

Where: A = absorbance of control at 510 nm

B = absorbance of sample at 510 nm

Preparation and lyophilization of EPP loaded NLCs

The EPP loaded NLCs were prepared by a warm microemulsion technique that reported by Tiyaboonchai [19]. Briefly, the water phase consisted of 5% (w/w) AOT, 5% (w/w) poloxamer188, 15% (w/w) ethanol and deionized water added to 100% (w/w). The water phase was heated to ~75°C before added to the oil phase. The oil phase, consisting of 4-12% (w/w) GMS, 4-12% (w/w) Compritol® 888 ATO, 2-6% (w/w) gelucire 44/14, 5% (w/w) Lexol, and 3-7% (w/w) surfactant was heated to ~70°C. The obtained warm microemulsion was dispersed in cold water (2-3°C) under high-speed homogenization (Model T50 basic, IKA®, Staufen, Germany) at 9500 rpm for 30 min. Finally, the NLCs dispersion was washed two times with deionized water using an ultrafiltration cell system fitted with a membrane MWCO 100 KDa. After that, 3% (w/v) mannitol was added to the NLCs dispersion before frozen and lyophilized for 72 h at 1 x 10^{-4} Torr and −55°C.

The EPP loaded NLCs were prepared under different formulation as follows: types of oil were chosen from Lexol, oleic acid, olive oil, IPP and IPM; concentration of solid lipid (GMS, Compritol® 888 ATO, gelucire 44/14) was varied from 10% to 30% (w/w); types of surfactant were chosen from Lipoid s75, Tween 80 and Span 80; and concentration of surfactant was varied from 3% to 7% (w/w). The selection of these variables was based on preliminary experiments. All samples were prepared in triplicate.

Physicochemical characterization of the EPP loaded NLCs

The morphology of the EPP loaded NLCs was investigated using scanning electron microscopy (SEM) (Model 1455VP, LEO Electron Microscopy Ltd., Cambridge, UK). Each sample was coated with gold, using ion sputter, prior to the microscopic examination.

The mean particle size and particle size distribution were analyzed by dynamic light scattering using ZetaPALS® (Brookhaven Instruments Corporation, Holtsville, NY). The particle size analysis data was evaluated using the hydrodynamic diameter obtained by the auto-measuring mode at a fixed angle of 90°, the wavelength of 632.8 nm in 10 mm diameter cell. All samples were diluted with deionized water and run for 10 times of the measurement.

The zeta potential value was assessed by determining the particle electrophoresis mobility using ZetaPALS®. All samples were diluted with deionized water and run for 10 times of the measurement. The zeta potential value was calculated based on the Smoluchowski equation [20].

Determination of EPP incorporation efficiency

The content of EPP in NLCs was determined by centrifugation method. Twenty milligrams of lyophilized EPP loaded NLCs were accurately weighed and dissolved in 1 ml of ethanol. The dispersion was centrifuged at 80,000 rpm for 30 min. Then the amount of ellagic acid in the supernatant was determined from its absorption at 254 nm using HPLC as described above. Each of samples was determined in triplicate. The percentage of EPP incorporation was calculated by the following equation 2.

$$\% \text{ EPP incorporation} = \frac{(\text{Ellagic acid}_{\text{tested}})}{\text{Ellagic acid}_{\text{initial}}} x100 \quad (2)$$

Where: $\text{Ellagic acid}_{\text{tested}}$ = the amount of ellagic acid in EPP loaded NLCs

$\text{Ellagic acid}_{\text{initial}}$ = the initial amount of ellagic acid in EPP

Preparation of cream containing EPP loaded NLCs

The cream base was prepared by a beaker method. The oil phase and water phase were heated to 70-75°C. The water phase was added into oil phase, stirring until temperature cooling down to room temperature. Then lyophilized EPP loaded NLCs were added and mixed. The final product contained 0.05% (w/w) EPP was obtained. The cream containing non-encapsulated EPP, prepared by replacing the lyophilized EPP loaded NLCs with non-encapsulated EPP, was used as a control.

In vitro release study

In vitro release profile of EPP loaded NLCs was studied using vertical Franz diffusion cells (Model No. 57-951-061, Meditron, Vöelklingen, Germany) maintained at 32 ± 0.5°C. The diffusion area was 1.77 cm^2 with receptor volume of 11 ml. Cellulose acetate membrane was fitted between donor and receptor. The receptor medium consisted of a mixture of ethanol and 0.1M acetate buffer (pH 5.5) at a ratio of 1:1. EPP loaded NLCs cream containing 10.5 µg ellagic acid was evenly spread on the membrane surface. Half a milliliter of the receptor medium was taken at predetermined time intervals of 15, 30 min, 1, 3, 5, 7, 9 and 12 h. The amount of ellagic acid released was determined using HPLC at 254 nm as described above.

Ex vivo skin permeation study

Ex vivo permeation study of EPP loaded NLCs was studied using a vertical Franz diffusion cells as described before in the *in vitro* release study with some modifications. The porcine ear epidermis was fitted between donor and receptor. Full-thickness ear skin removing subcutaneous fat tissue was excised from porcine ear, obtained from the slaughter house. Then, epidermis was carefully separated from dermis, using a heat separation technique, after skin was immersed into a hot water, 60°C, for 2 min. A transepidermal water loss (TEWL) value of the separated epidermis was determined using a Tewameter (Model TM210, Courage and Khazaka electronic GmbH, Germany). A low TEWL value of less than 20 g/hm^2 was used as an indicator for undamaged epidermis. The study was carried out with the approval of Naresuan University Animal Ethics Committee, Phitsanulok, Thailand (no. 55 02 0001).

Statistical analysis

All data were expressed as mean ± SD. Calculation at least three different experiments. The data was analyzed by one-way analysis of variance (ANOVA). Differences were considered to be significant when *p* value equal or lower than 0.05.

RESULTS

Preparation and determination of total phenolic content of the extracts

Preparation of EPP using the method described above produced the extract with the yield of 7.78 ± 0.89% w/w compared to the weight of dried powder. The appearance of the extract was a dark brown powder. On the basis of HPLC analysis, ellagic acid was found as the major constituent in the ellagic acid rich extract. This method was capable of increasing the ellagic acid content in the extracts to 11.51 ± 0.23% w/w.

Anti-tyrosinase activity by mushroom tyrosinase inhibitory assay

The tyrosinase inhibitory activity of EPP was examined and compared with that of kojic acid (positive control) and ellagic acid (major constituent). Interestingly, EPP possessed strong anti-tyrosinase activity as shown in Table 1. Even though, EPP contained only ~12% w/w of ellagic acid, but the inhibitory activity was ~two times weaker than that of ellagic acid and seven times weaker than that of kojic acid. These data further implicate EPP as a potential anti-tyrosinase agent to be used as an excellent whitening agent for cosmetics.

Table 1: Anti-tyrosinase activity

Test sample	IC_{50} (µg/ml)
Kojic acid	4.49 ± 1.56
Standard ellagic acid	12.56 ± 0.86
EPP	28.54 ± 1.34

Preparation and characterization of EPP loaded NLCs

Morphology

Scanning electron micrographs revealed that lyophilized EPP loaded NLCs were spherical in shape with smooth surfaces and uniformly distributed, Fig. 2. In addition, the variation in process parameters had no effects on the appearance of the particles. The morphology of particles was found to be independent of the processing conditions (data not shown).

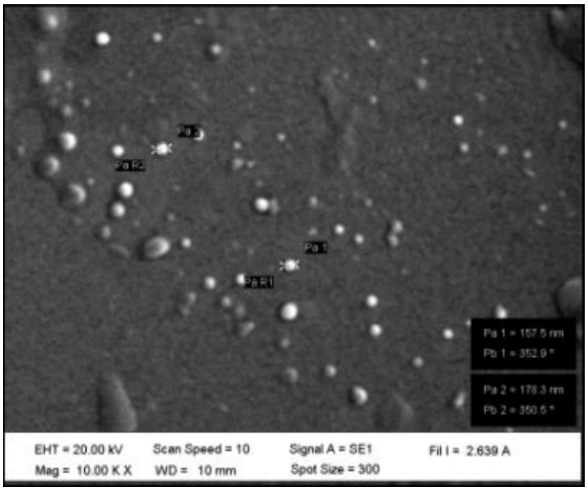

Fig. 2: Scanning electron micrograph of EPP loaded NLCs consisting of Lexol, Lipoid s75 and solid lipid 25% (w/w).

Mean particle size, size distribution and zeta potential

Mean particle size and polydispersity index (PI) value of EPP loaded NLCs were evaluated by dynamic light scattering. The PI value was used as a measurement of the size distribution. In general, PI value of < 0.1 indicates an unimodale size distribution, 0.1-0.3 indicates narrow size distribution, while PI value of > 0.3 indicates a higher heterogeneity [21]. The results revealed that the types of oil and surfactant showed no significantly influence on the mean particle size and size distribution of ~200 nm, and 0.2-0.3, respectively, Table 2-3. On the contrary, the concentration of solid lipid and surfactant were critical parameters for the mean particle size and size distribution of lipid nanoparticles.

The effect of solid lipid concentration on the mean particle size and size distribution was evaluated by varying from 10%, 15%, 20%, 25% to 30% (w/w) while using Lexol as an oil and 7% (w/w) of Lipoid s75. The results showed that when increasing solid lipid concentration from 10- 25% (w/w), the mean particle size and PI did not change significantly, ~170 nm and 0.27 respectively. Nonetheless, as the solid lipid increased to 30% (w/w), a larger mean particle size of 328 nm was observed, Table 4. This finding was in agreement with Tiyaboonchai et al. who reported that increasing the solid lipid concentration resulted in larger mean particle size and broader size distribution [19].

The effect of surfactant concentration on the mean particle size and size distribution was evaluated by varying the concentration of Lipoid s75 from 3-7% (w/w) while maintaining the solid lipid concentration at 25% (w/w) and using Lexol as oil. The results revealed that the mean particle size and size distribution of NLCs tended to decrease when the amount of surfactant increased (Table 5). As surfactant could reduce the interfacial energy on the surface of nanoparticles, thus, leading to decrease the particle size.

Table 2: Effect of types of oil on the mean particle size, polydispersity index (PI) and zeta potential of EPP loaded NLCs.

Types of oil	Mean particle size (nm ± SD)	PI ± SD	Zeta potential (mV) ± SD
Lexol	169 ± 11	0.27 ± 0.01	-36.5 ± 2.1
Oleic acid	176 ± 3	0.26 ± 0.04	-33.4 ± 1.9
Olive oil	188 ± 2	0.34 ± 0.02	-33.9 ± 2.6
IPP	175 ± 5	0.26 ± 0.04	-36.8 ± 2.1
IPM	183 ± 7	0.27 ± 0.05	-35.9 ± 3.5

Table 3: Effect of types of surfactant on the mean particle size, polydispersity index (PI) and zeta potential of EPP loaded NLCs.

Types of surfactant	Mean particle size (nm ± SD)	PI ± SD	Zeta potential (mV) ± SD
Lipoid s75	169 ± 11	0.27 ± 0.01	-36.5 ± 2.1
Span 80	197 ±12	0.32 ± 0.05	-33.1 ± 3.1
Tween 80	225 ± 11	0.32 ± 0.04	-34.1 ± 2.4

Table 4: Effect of solid lipid concentration on the mean particle size, poly dispersity index (PI) and zeta potential of EPP loaded NLCs.

Solid lipid (% w/w)	Mean particle size (nm ± SD)	PI ± SD	Zeta potential (mV) ± SD
10	162 ± 6	0.27 ± 0.04	-35.4 ± 2.2
15	169 ± 2	0.28 ± 0.05	-35.4 ± 3.4
20	212 ± 8	0.27 ± 0.05	-32.6 ± 1.9
25	169 ± 11	0.27 ± 0.01	-36.5 ± 2.1
30	328 ± 15	0.32 ± 0.03	-34.3 ± 2.1

Table 5: Effect of surfactant concentration on the mean particle size, polydispersity index (PI) and zeta potential of EPP loaded NLCs.

Surfactant (% w/w)	Mean particle size (nm ± SD)	PI ± SD	Zeta potential (mV) ± SD
3	324 ± 8	0.34 ± 0.02	-34.7 ± 1.9
5	277 ± 5	0.27 ± 0.03	-37.4 ± 1.8
7	169 ± 11	0.27 ± 0.01	-36.5 ± 2.1

Zeta potential is a measurement of the electric charge at the surface of the particles. There were no significant differences in the resulting zeta potentials of nanoparticles when varying the preparation parameters. The zeta potential of all formulations was ~-34 mV, suggesting the adsorption of anionic surfactant, the Lipoid s75 and AOT, at the lipid nanoparticles surface.

Determination of EPP incorporation efficiency

Considering that ellagic acid was found as a major compound in EPP, thus, the EPP incorporation efficiency was investigated based on the amount of ellagic acid determined by HPLC.

The types of oil and surfactant, and concentration of solid lipid and surfactant were critical factors determining the EPP incorporation efficiency. Formulation with Lexol showed the highest EPP incorporation efficiency, ~90%; follow by formulation with IPP and IPM, ~70%. This result may be explained by the chain length of oil.

Lexol with the shortest chain length, $C_{6\text{-}12}$, may facilitate more space to accommodate active compounds as compared to IPP and IPM with longer chain length of C_{17} and C_{19}, respectively. Nevertheless, when formulated with oleic acid and olive oil, the incorporation efficiency was decreased to ~40-50%, Fig. 3A. The high viscosity of oleic acid and olive oil could lead to a significant loss of EPP during the preparation process.

When varying the types of surfactant, formulation with Lipoid s75 and Span 80 showed the highest incorporation efficiency, ~90%, while those formulated with Tween 80 resulted in the lowest incorporation efficiency, ~60%, Fig. 3B.

This could be attributed to the hydrophobic properties of surfactant. Hydrophilic lipophilic balance (HLB) value of Lipoid s75, Span 80 and Tween 80 are 4.0, 4.3 and 15, respectively. Therefore, ellagic acid, a hydrophobic substance, possessed high affinity to the hydrophobic surfactant leading to high incorporation efficiency. This obsrevation was in agreement with Müller et al.. who reported that the incorporation efficiency was proportional to the solubility of active substance in the lipid phase [14]. As expected, when the amount of solid lipid and surfactant increased, the incorporation efficiency tended to increase, Fig. 3C and D. These might be a result from a higher incorporation of ellagic acid into lipid component of microemulsion on the primary formed nanoparticles as the amount of lipid and surfactant increased.

***In vitro* release study**

Ellagic acid possesses very poor aqueous solubility. Thus, to provide sink condition 50% (v/v) ethanol in acetate buffer was chosen as a receptor medium.

The *in vitro* release studies from both cream containing free EPP and cream containing EPP loaded NLCs demonstrated a prolong released characteristic following Higuchi's square root model, Fig. 4.

All formulations showed a biphasic pattern with a burst release during the first 1 h, followed by a prolong release up to 12 h. However, a faster release rate of ellagic acid from EPP cream was observed compared to EPP loaded NLCs creams. In addition, the effect of types of oil and surfactant on release kinetic of ellagic acid was investigated.

A faster release rate was observed with Lexol-based NLCs, while IPP and IPM-based NLCs showed a similar released profile, Fig. 4A. When varying types of surfactant, Lipoid s75-based NLCs showed a faster release rate than Span 80-based NLCs, Fig. 4B.

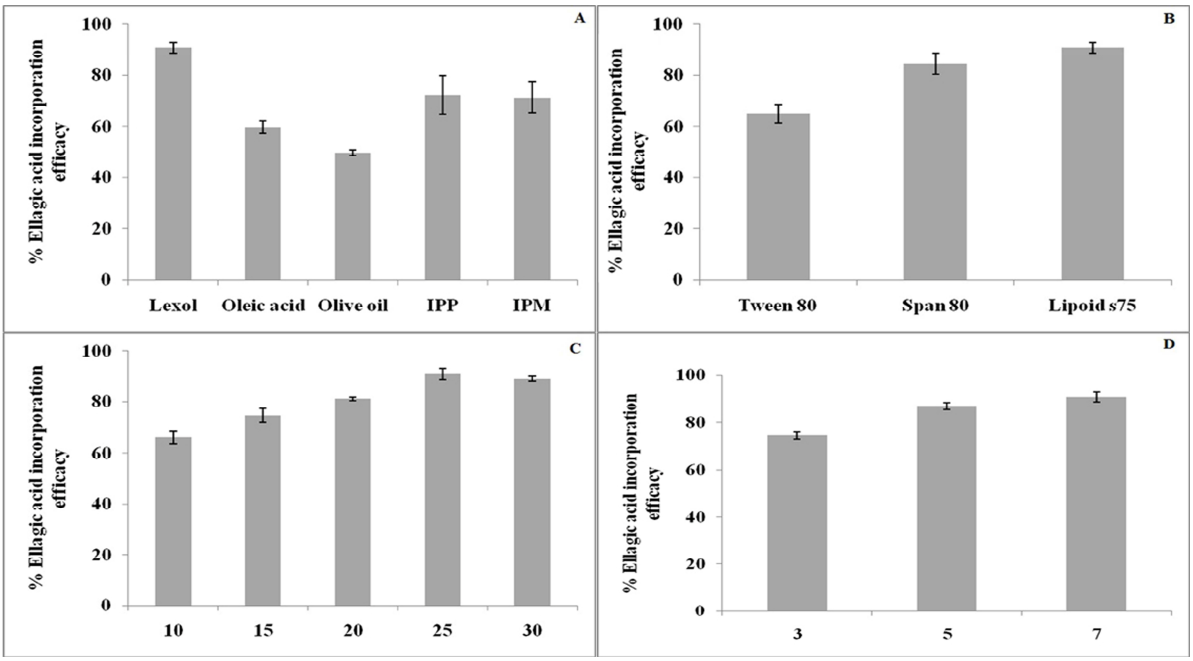

Fig. 3: Incorporation efficacy of ellagic acid in NLCs with different (A) types of oil; (B) types of surfactant; (C) solid lipid (% w/w); (D) surfactant (% w/w).

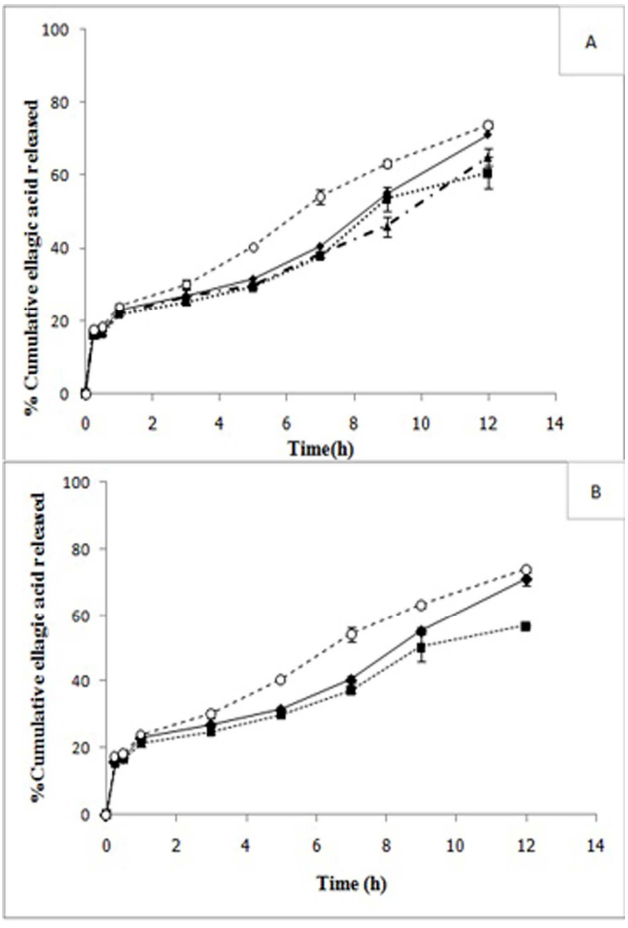

Fig. 4: Release profile of EPP from cream containing EPP and cream containing EPP loaded NLCs formulated with (A) different types of oil (Lexol, IPP, IPM and EPP cream), and (B) different types of surfactant (Lipoid s75, Span 80 and EPP cream).

***Ex vivo* permeation study**

The *ex vivo* permeation studies from both model EPP loaded NLCs contained cream and free EPP contained cream demonstrated a similar permeation profile with a lag time of 1 h, Fig. 5. All preparations demonstrated biphasic kinetics, with a high initial flux of ellagic acid during the first 3 h followed by a reduced flux over the 3–12 h period. The results indicated that the types of oil and surfactant play an important role in ellagic acid permeation through pig skin. Interestingly, for IPP and IPM-based NLCs, the observed ellagic acid permeation was similar to EPP contained cream, but lower than Lexol-based NLCs, Fig. 5A. A similar trend was observed when varying types of surfactant, Lipoid s75-based NLCs showed the highest amount of ellagic acid permeation, while Span 80-based NLCs and EPP contained cream illustrated similar amount of ellagic acid permeation, Fig. 5B. Thus, the result indicated that only NLCs formulated with Lexol and Lipoid s75 showed permeation enhancing effect compared to cream base formulation. As evidence from the initial flux of ellagic acid during the first 3 h, ~0.7 µg/cm^2 of ellagic acid was permeated from Lexol and Lipoid s75-based NLCs, which was higher than that from EPP cream, ~0.4 µg/cm^2 (p<0.05 t-test). From a therapeutic standpoint, a high initial flux can be considered as an advantage as a sufficient amount of active ingredients are rapidly released from the cream to the skin to exert an initial therapeutic effect followed by a controlled release of the remaining active ingredients form the NLCs.

DISCUSSION

Extraction process of pomegranate peel developed by Panichayupakaranant et al. showed high ellagic acid content up to 12% (w/w). In addition, it possessed a strong antioxidant activity with IC_{50} value of 5.8 µg/ml as determined by DPPH radical scavenging assay (Panichayupakarananta et al.., 2010b). In this study, we further examined biological activity of EPP and found that it possessed strong anti-tyrosinase activity with IC_{50} of 28.54 ± 1.34 µg/ml which close to IC_{50} of standard ellagic acid, 12.56 ± 0.86 µg/ml. The strong antitysinase activity may be due to the synergistic effect of other phenolic and flavonoid compounds presented in the crude extract, especially punicalagin, punicalin, quercetin and kaemperol [5, 22]. Their hydroxyl group could chelate the copper ions at the active site of the tyrosinase enzyme leading to inhibition of enzymatic activity [22]. Thus, the prepared EPP showed high potential application as whitening agent.

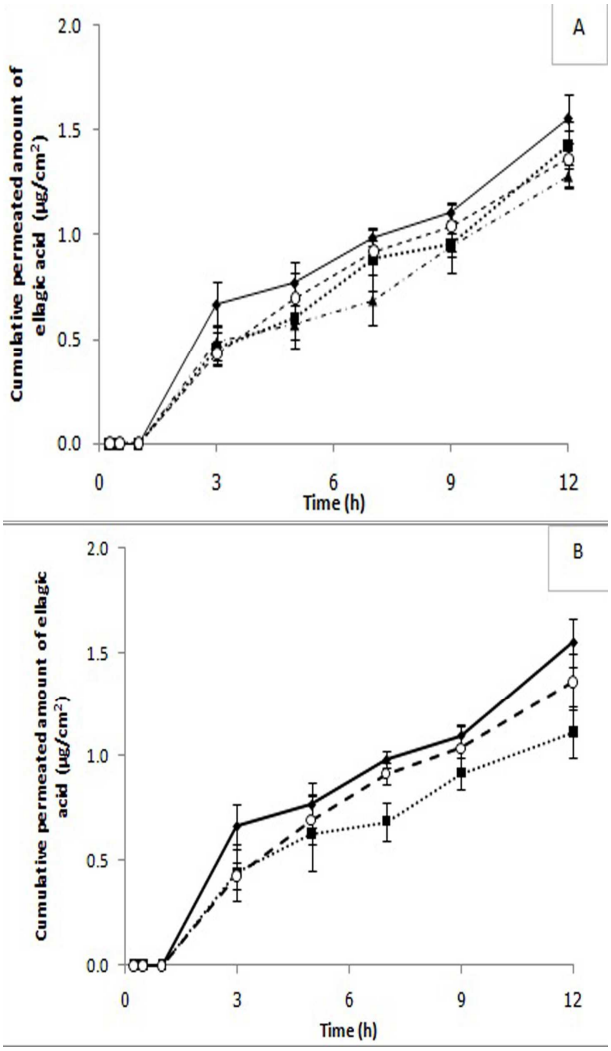

Fig. 5: Permeation profile of EPP from cream containing EPP and cream containing EPP loaded NLCs formulated with (A) different types of oil (◆ Lexol, ■ IPP, ▲ IPM and ○ EPP cream), and (B) different types of surfactant (◆ Lipoid s75, ■ Span 80 and ○ EPP cream).

To promote skin hydration and active compound penetration efficiency, NLCs were fabricated as a topical delivery system for EPP using a warm microemulsion technique. EPP loaded NLCs were successfully prepared by a warm microemulsion technique. An oil-in-water microemulsion was spontaneously obtained as recognized by a clear solution after adding the heated water phase into the oil phase of the same temperature. The NLCs were obtained immediately when dispersing the warm microemulsion into cold water with the aid of a homogenizer. The cold water facilitated rapid lipid crystallization and prevented lipid aggregation [23]. White powder of EPP loaded NLCs were obtained after freeze-drying. They could be easily re-dispersed in water and in the model cream base. The optimal preparation conditions are the following; 25% (w/w) of solid lipid, Lexol and Lipoid s75 were chosen as oil and surfactant, respectively. These experimental conditions result in the formation of nanoparticles of ~200 nm with a polydispersity index of 0.2 and a zeta potential of ~-34 mV. A drug entrapment efficiency of up to 90% could be achieved. The bioavailability of topical application could be enhanced by using NLCs. The small particle size ensures close contact with stratum corneum leading to a film formation on the skin surface which exerts an occlusive effect. Thus, stratum corneum hydration is increased and influence percutaneous absorption [15, 16]. In this study, the effect of types of oil and surfactant used in the NLCs on permeation enhancing effect were investigated. Even though, the *in vitro* release study suggesting that EPP cream showed a faster release rate of ellagic acid than cream containing EPP loaded NLCs. This can be explained by the ellagic acid can penetrate from cream to the medium directly, while for the EPP loaded NLCs, ellagic acid had to release from the particles into cream base before penetrate into medium. Nevertheless, *ex vivo* skin permeation revealed that cream containing EPP loaded NLCs showed higher ellagic acid permeated than EPP cream. The types of oil and surfactant were critical factors determining permeation rate of ellagic acid. The effect of types of oil on the percutaneous permeation profile was associated with the entrapment efficiency. The Lexol-based NLCs showed the highest ellagic acid incorporation efficiency compared to IPP and IPM-based NLCs. The higher drug loading would result in an increase driving force for drug partitioning from the nanoparticle matrix into the stratum corneum [24]. Additionally, the appearance of surfactant is related to the solid state of the lipid matrix. Lipoid s75 is a solid and Span 80 is a liquid. Thus, lipid matrix of Lipoid s75-based formulation possessed more crystallinity than that of Span 80-based formulation resulting in the higher occlusion effect [25]. This finding was in agreement with our previous work. *Ex vivo* penetration of nile red loaded NLCs was assessed using porcine ear epidermis and investigated with cryosection and fluorescence microscopy. The skin treated with nile red loaded NLCs showed higher amount of dye in the viable epidermis as compared to the nile red solution [26].

CONCLUSION

The prepared EPP containing ellagic acid of ~12% w/w showed strong antioxidant and anti-tyrosinase acitivities that suitable for use in cosmetics. Nanostructures lipid carriers were successfully developed to improved bioavailability of active ingredients. The present study clearly establishes that penetration enhancing effect of NLCs is dependent on the types of oil and surfactant used in formulation which affecting on the entrapment efficiency and lipid matrix crystallinity, respectively. At optimal condition, the developed NLCs system shows an attractive potential for the use as topical delivery system. Advantages of this system are (1) ease of manufacturing and mild preparation condition, (2) use of biodegradable lipid and avoiding organic solvent, (3) control drug release, (4) high entrapment efficiency, and (5) enhance active ingredient penetration efficiency.

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