CHAPTER IV

RESULTS AND DISCUSSION

This chapter presents the findings obtained from the study, which includes preparation of EPP, analysis of ellagic acid, antioxidant activities of EPP, the effects of process parameters on physicochemical properties of EPP loaded NLCs, the percentage of the ellagic acid incorporation efficacy, *in vitro* occlusion test, *in vitro* release study, ex *vivo* permeation study and stability studies of EPP loaded NLCs.

Preparation and standardization of EPP

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 as shown in Figure 13. Based on HPLC analysis, only ellagic acid was found as the major compound 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).

Figure 13 Ellagic acid rich pomegranate peel extract.

Analysis of ellagic acid

1. High performance liquid chromatography (HPLC) analysis for ellagic acid

The ellagic acid was determined in the extract by HPLC technique. The HPLC technique has been reported for identification and quantification of ellagic acid [37]. This HPLC method is simple and rapid. The elution of ellagic acid on reverse phase pentafluorophenyl (PFP) stationary phase using a mixture of methanol and 0.2% phosphoric acid aqueous solution at a ratio of 60:40 (v/v) as mobile phase resulted in reduced tailing and a narrower peak with a retention time of 5 min. The chromatogram of the RP-HPLC of ellagic acid was shown in Figure 14. I. Bala et al. reported that isocratic elution of ellagic acid using reverse phase C18 column resulted in tailing and broadening of the peak [41].

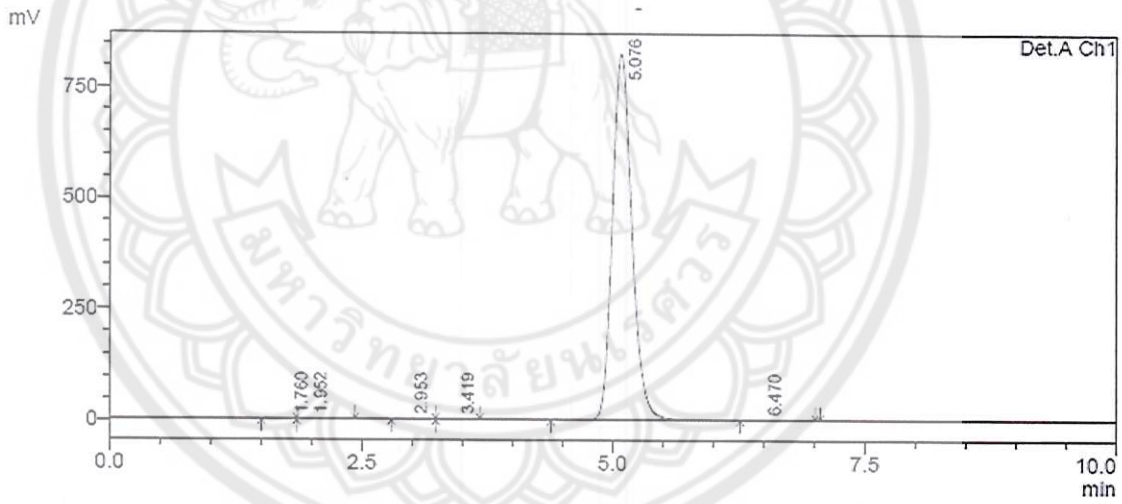

Figure 14 The RP-HPLC chromatogram of ellagic acid at 254 nm (50 µg/ml)

2. Calibration curve of standard ellagic acid by high performance liquid chromatography (HPLC)

The standard ellagic acid was determined by HPLC at wavelength 254 nm. Calibration curve of ellagic acid in the range of 0.1-50 µg/ml was constructed by its peak area versus the ellagic acid concentrations as shown in Figure 15. The calibration curve equation was $y = 134847x + 103949$ ($R^2 = 0.9991$)

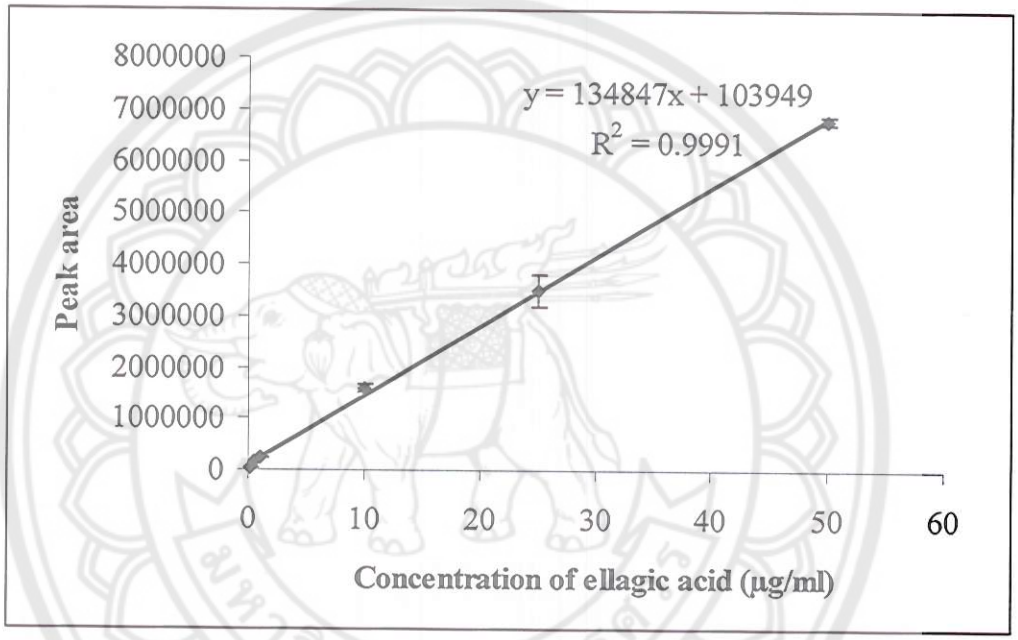

Figure 15 Calibration curve of ellagic acid by HPLC at 254 nm

Antioxidant activity of EPP

TBARs assay was used to measures the capacity of antioxidant compounds to inhibit the oxidation process. This method is a simple spectrophotometric assay that measures a chromogen that is produced by the reaction of thiobarbituric acid (TBA) with malondialdehyde (MDA), which is an end product of lipid peroxidation [28]. Oxidative modification of lipids can be induced by metal and pro-oxidant agents and gave the end products of lipid peroxidation that is MDA. MDA can react with TBA and produced MDA-TBA formed that exhibited pink color. The pink color of MDA-TBA formed can be measured by microplate reader at 510 nm. When antioxidant compounds donate hydrogen to free radical while itself become a stable radical that

incapable of continuing the propagation chain cycle and the end products are reduce with the loss of pink color.

In this study, the lipid peroxidation inhibition of EPP was examined and compared with that of trolox (positive control) and ellagic acid (marker). As showed in Table 4, trolox, ellagic acid and EPP showed lipid peroxidation inhibition with the IC_{50} values of 7.80 ± 0.85, 36.92 ± 0.64 and 24.35 ± 1.24 µg/ml, respectively. The inhibitory activity of EPP was about three times weaker than that of trolox and higher than that of ellagic acid. The EPP was contained about 12% w/w of ellagic acid; it showed high lipid peroxidation inhibition efficiency. This may be due to the synergistic effect of other compounds that present in this extract. M.Viuda-Martos et al. reported that the pomegranate compounds, including punicalagin isomer, ellagic acid and anthocyanins (delphinidin, cyaniding and pelargonidin 3-glucosides, and 3, 5-diglucosides) are known for their properties to scavenge free radicals and to inhibit lipid oxidation *in vitro* [42]. The antioxidant activity of pomegranate extracts seems to be due to the presence of polyphenols, which may act in a similar fashion as reductones by donating the electrons and reacting with free radicals to convert them to more stable product and terminate free radical chain reaction. Many researchers reported that antioxidant activity in natural products may be used to aid the human body reducing oxidative damage. In the last year, it has been reported to many fruits, vegetables and herbs were investigated for their antioxidant activity [26, 27].

Table 4 IC_{50} values of trolox, standard ellagic acid and EPP determined by TBARs assay

Test sample	IC_{50} (µg/ml)
Trolox	7.80 ± 0.85
Standard ellagic acid	36.92 ± 0.64
EPP	24.35 ± 1.24

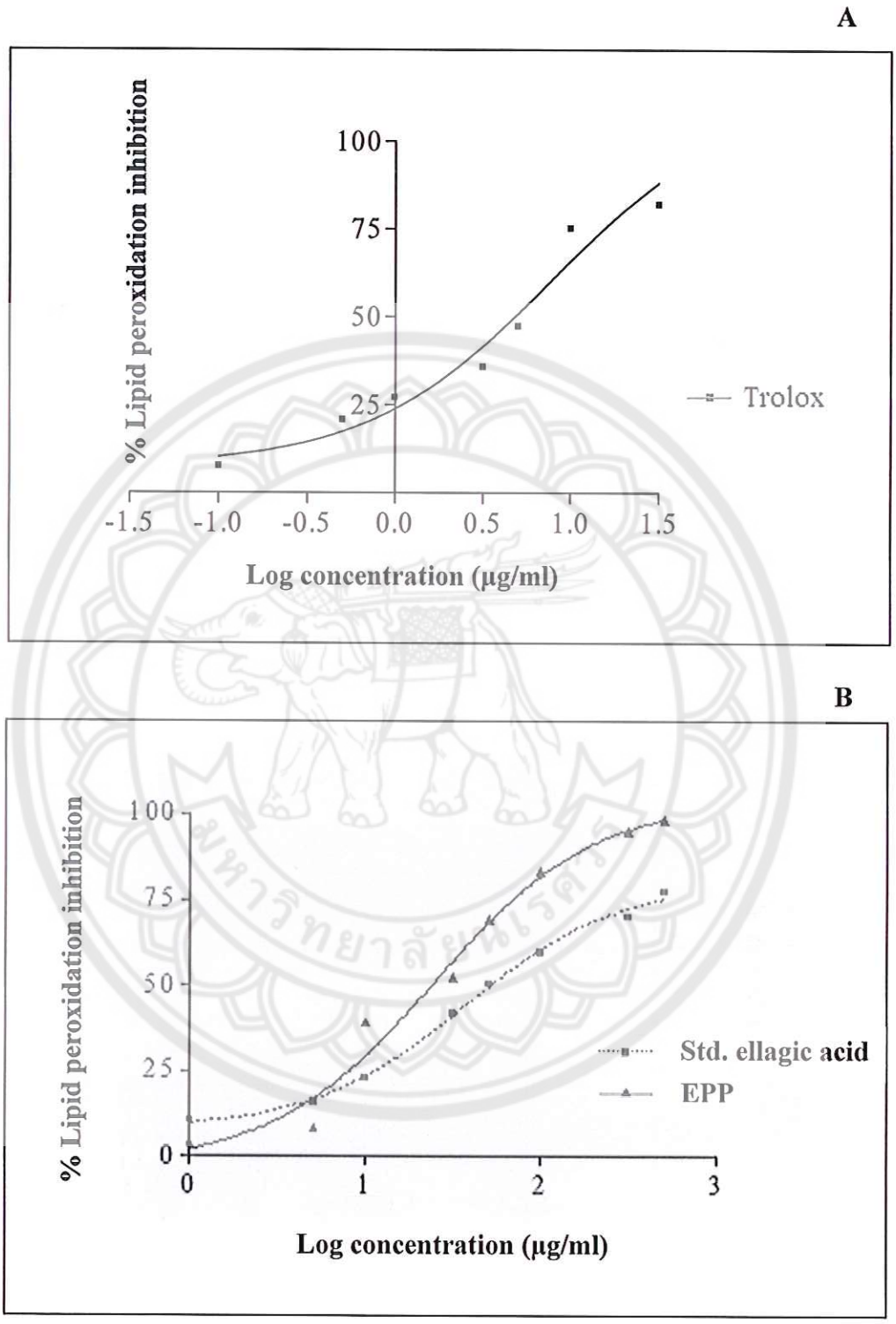

Figure 16 Lipid peroxidation inhibitory activities of (A) trolox, (B) standard ellagic acid and EPP determined by TBARs assay

Antityrosinase activity of EPP

Mushroom tyrosinase assay is a method used to determine antityrosinase activity of compounds. This assay is the determination of dopachrome content occurred due to the reaction of tyrosine substrate and tyrosinase enzyme, which is orange pigment intermediate that can be detected by microplate reader at 510 nm. When some compounds are added to this reaction mixture and the dopachrome disappears, it means that the added compound could block the activity of tyrosinase enzyme.

In this study, the tyrosinase inhibitory activity of EPP was examined and compared with that of kojic acid (positive control) and ellagic acid (marker) which are known tyrosinase inhibitors. Ellagic acid, a naturally occurring polyphenol, has been reported to prevent pigmentation caused by sunburn [43, 44]. Shimogaki et al. reported that when ellagic acid was topically applied, it suppressed UV-induced skin pigmentation of brownish guinea pigs [44]. As showed in Table 5, kojic acid, ellagic acid and EPP showed antityrosinase activity with the IC_{50} values of 4.49 ± 1.56, 12.56 ± 0.86 and 28.54 ± 1.34 μg/ml, respectively. The inhibitory activity of EPP was about seven times weaker than that of kojic acid and two times weaker than that of ellagic acid. It has been reported that ellagic acid has a high affinity for copper at the active site of tyrosinase and inhibit its activity [5]. Recently, ellagic acid was shown to inhibit skin pigmentation resulting from UV irradiation. It is believed that ellagic acid suppresses tyrosinase activity; tyrosinase is one of the key enzymes involved in melanogenesis in melanocytes. Tyrosinase catalyses the oxidation process of tyrosine to dihydroxy-phenylalanine (DOPA) (hydroxylation), and from DOPA to DOPAquinone (oxidation). Tyrosinase is know to be a metalo-enzyme, containing copper at an active site, catalysing these reactions through a change in the oxidative state of the copper atoms. Hydroxy groups could chalate the copper ions at the active site of the tyrosinase enzyme leading to inhibition of enzymatic activity [44]. In this study, the EPP was contained about 12% (w/w) of ellagic acid. Many researchers reported that the chemical composition of the pomegranate peel is rich in phenolic compounds and flovonoid compounds especially ellagic acid, punicalagin, punicalin, quercetin and kaemperol. It has been reported as possessing strong antityrosinase activity [5, 44]. This may be due to the synergistic effect of other compounds present

in the extract. These data further implicate EPP as a potential antityrosinase agent for used an excellent whitening agent for cosmetic [5].

Table 5 IC_{50} values of kojic acid, standard ellagic acid and EPP determined by Mushroom tyrosinase assay

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

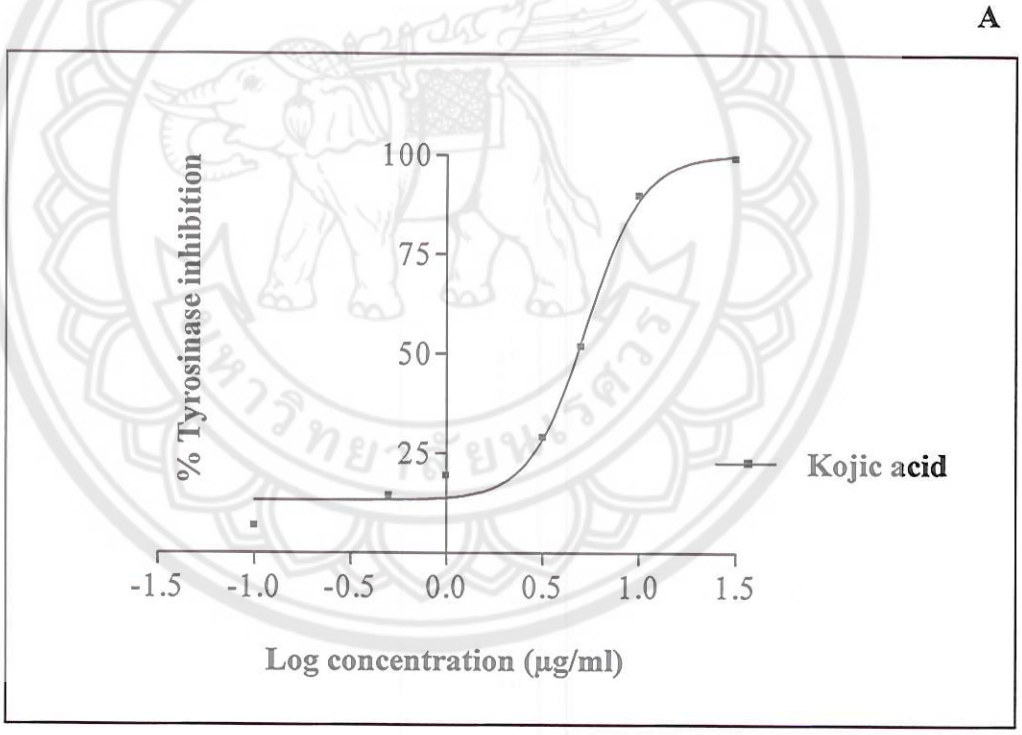

Figure 17 Tyrosinase inhibitory activities of (A) kojic acid, (B) standard ellagic acid and EPP determined by Mushroom tyrosinase assay

B

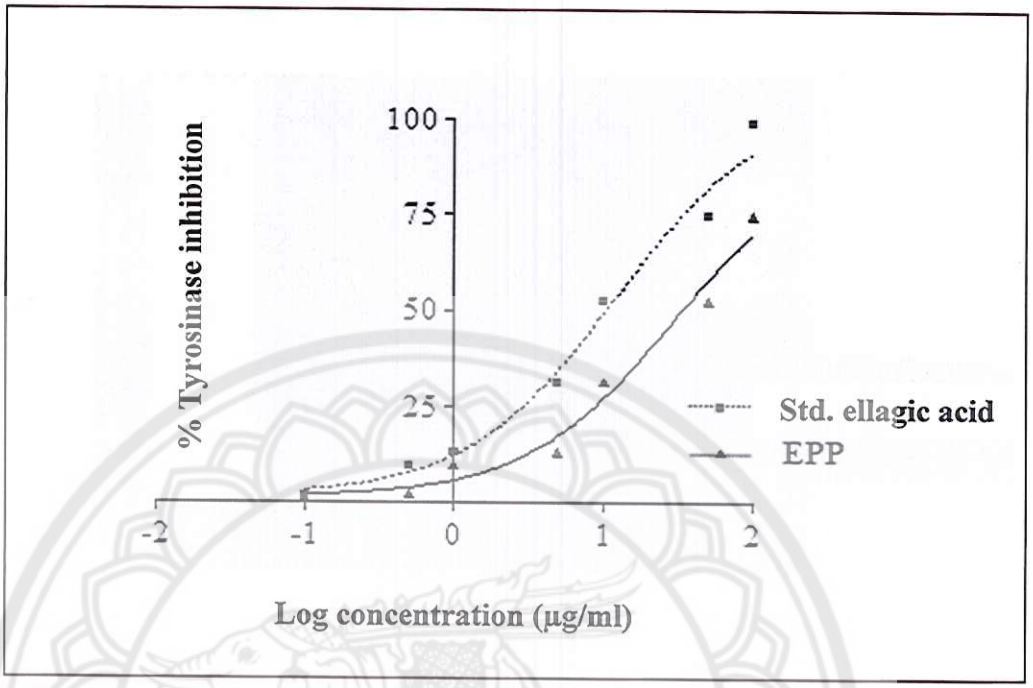

Figure 17 (CONT.)

Characterization of EPP loaded NLCs

1. Morphology

Lyophilized EPP loaded NLCs were successfully prepared by a warm microemulsion technique. SEM micrograph of EPP loaded NLCs showed that the particles were spherical in shape with smooth surfaces and uniformly distributed as shown in Figure 18. In addition, the variation in process parameters had no effects on the appearance of the particles. All formulations demonstrated a uniform spherical shape and a smooth surface.

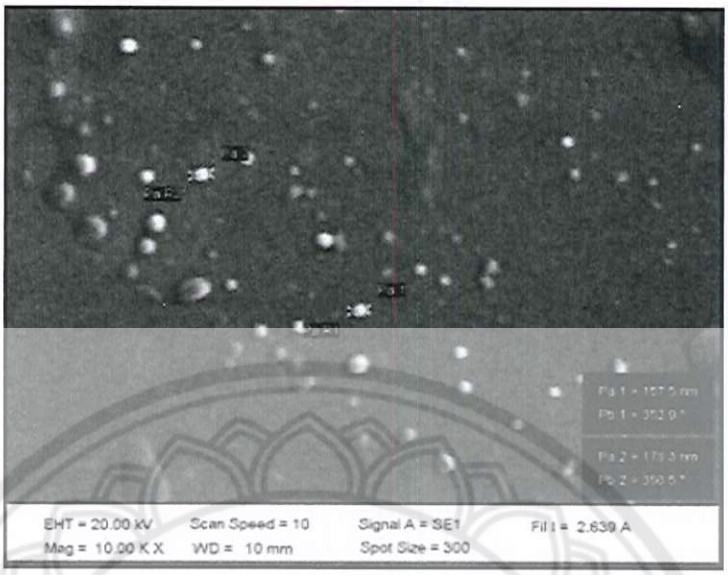

Figure 18 Scanning electron micrograph of EPP loaded NLCs consisting of lexol as oil, lipoid s75 as surfactant, solid lipid 25% (w/w), lipoid s75 7% (w/w) and lyophilized with 3% (w/v) mannitol

2. Mean particle size and size distribution

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 [45]. In this study, the mean particle size and size distribution of EPP loaded NLCs were investigated under different process parameters; types of oil, types of surfactant, the concentration of solid lipid and the concentration of surfactant. The results showed that the concentration of solid lipid and surfactant were critical parameters for the mean particle size and size distribution of nanoparticles.

Table 6 Effect of the types of oil on the mean particle size and size distribution of lyophilized EPP loaded NLCs

Types of oil	Mean particle size (nm ± SD)	PI (Mean ± SD)
Lexol	169 ± 11	0.27 ± 0.01
Oleic acid	176 ± 3	0.26 ± 0.04
Olive oil	188 ± 2	0.34 ± 0.02
IPP	175 ± 5	0.26 ± 0.04
IPM	183 ± 7	0.27 ± 0.05

The effect of types of oil on the mean particle size and size distribution was chosen from lexol, oleic acid, olive oil, IPP and IPM while maintaining the lipoid s75 as surfactant, the solid lipid concentration at 25% (w/w) and the surfactant concentration at 7% (w/w). The results showed that the types of oil did not significantly influence the mean particle size and size distribution of EPP loaded NLCs (Table 6). The mean particle size and size distribution for all formulations were ~200 nm, 0.2-0.3, respectively. However, when using oleic acid and olive oil as surfactant, the high viscosity of warm microemulsion has occurred. According to these reasons, the method of warm microemulsion was dispersed in cold water difficult to be prepared and showed a low entrapment efficacy. Thus, using the lexol, IPP and IPM as oil were selected for the next study.

Table 7 Effect of the types of surfactant on the mean particle size and size distribution of lyophilized EPP loaded NLCs

Types of surfactant	Mean particle size (nm ± SD)	PI (Mean ± SD)
Lipoid s75	169 ± 11	0.27 ± 0.01
Span 80	197 ± 12	0.32 ± 0.05
Tween 80	225 ± 11	0.32 ± 0.04

The effect of types of surfactant on the mean particle size and size distribution was chosen from lipoid s75, span 80 and tween 80 while maintaining the lexol as oil, the solid lipid concentration at 25% (w/w) and the surfactant concentration at 7% (w/w). The results showed that the types of surfactant did not significantly influence the mean particle size and size distribution of EPP loaded NLCs (Table 7). The mean particle size and size distribution for all formulations were ~200 nm, 0.2-0.3, respectively.

Table 8 Effect of the solid lipid concentration on the mean particle size and size distribution of lyophilized EPP loaded NLCs

Solid lipid (%w/w)	Mean particle size (nm ± SD)	PI (Mean ± SD)
10	162 ± 6	0.27 ± 0.04
15	169 ± 2	0.28 ± 0.05
20	212 ± 8	0.27 ± 0.05
25	169 ± 11	0.27 ± 0.01
30	328 ± 15	0.32 ± 0.03

The effect of solid lipid concentration on the mean particle size and size distribution was evaluated by varying the concentration from 10 to 30% (w/w) while maintaining the type of oil as lexol, the type of surfactant as lipoid s75 and the surfactant concentration at 7% (w/w). The results showed that when increasing solid lipid concentration from 10% to 25% (w/w), the mean particle size and polydispersity

index did not significantly. The mean particle size and the polydispersity index were ~170 nm and 0.27, respectively. Nonetheless, the increase of the solid lipid concentration at 30% (w/w) resulted in a larger mean particle size, 328 nm and the broad size distribution (Table 8). Therefore, the concentration of solid lipid at 25% (w/w) was found to be and optimum concentration for the formulation of the NLCs.

The results revealed that as the solid lipid concentration was increased, the mean particle size and PI value increased. At 30% (w/w) of solid lipid concentration, the increasing in the mean particle size and PI value could be explained that insufficient amount of surfactant lead to aggregation of the particles. Moreover, the viscosity was increased at high lipid concentration lead to decrease the shear energy of homogenizer. According to these reasons, the larger particle size and broad size distribution were obtained. This finding was in agreement with Tiyaboonchai et al., 2007 who reported that increasing the solid lipid concentration resulted in larger mean particle sizes and broader size distributions [40].

Table 9 Effect of the surfactant concentration on the mean particle size and size distribution of lyophilized EPP loaded NLCs

Surfactant (%w/w)	Mean particle size (nm ± SD)	PI (Mean ± SD)
3	324 ± 8	0.34 ± 0.02
5	277 ± 5	0.27 ± 0.03
7	169 ± 11	0.27 ± 0.01

The effect of surfactant concentration on the mean particle size and size distribution was evaluated by varying the concentration from 3 to 7% (w/w) while maintaining the type of oil as lexol, the type of surfactant as lipoid s75 and the solid lipid concentration at 25% (w/w). As shown in Table 9, the formulation containing 3%, 5% to 7% (w/w) of surfactant can produced the particles with mean diameter of 324, 277 and 169 nm, respectively. The results revealed that as the surfactant amount was increased, the mean particle size and size distribution of NLCs tended to decrease.

Thus, using the concentration of surfactant at 7% (w/w) was selected for the next study.

The decreasing in the mean particle size and size distribution could be explained in term of function of surfactant. Surfactant can reduces the interfacial energy on the surface of nanoparticles that leads to decrease the particle size of nanoparticles.

3. Zeta potential value

The zeta potential value of EPP loaded NLCs were evaluated by electrophoretic mobility using Zeta potential analyzer. Zeta potential is a measurement of the electric charge at the surface of the particles indicating the physical stability of colloidal systems. The high zeta potential value indicates electrostatic long-term stability of aqueous dispersions. In general, particles could be stable when absolute value of zeta potential is above |-30| mV due to the electric repulsion between particles [46, 47]. Profiles of the zeta potential confirm the smaller size of SLNs and NLCs, since greater ionization at the interface tends to increase the electrostatic repulsion and prevent aggregation [12].

As shown in Table 10, the zeta potential studies of the nanoparticles showed that there were no significant differences in the resulting zeta potentials when varying the processing parameters. The zeta potential of all formulations was ~-34 mV. The results revealed that the zeta potential value showed negatively charge, suggesting the adsorption of anionic surfactant, the lipoid s75 and AOT, at the lipid nanoparticles surface. The negative zeta potential provides electrostatic repulsion to maintain the small size of these systems and allows predictions of the storage stability [12].

Table 10 Zeta potential value of EPP loaded NLCs different process parameters

Formulation	Zeta potential value (mV) (Mean ± SD)
Types of oil	
Lexol	-36.5 ± 2.1
Oleic acid	-33.4 ± 1.9
Olive oil	-33.9 ± 2.6
IPP	-36.8 ± 2.1
IPM	-35.9 ± 3.5
Types of surfactant	
Lipoid s75	-36.5 ± 2.1
Span 80	-33.1 ± 3.1
Tween 80	-34.1 ± 2.4
Solid lipid (% w/w)	
10	-35.4 ± 2.2
15	-35.4 ± 3.4
20	-32.6 ± 1.9
25	-36.5 ± 2.1
30	-34.3 ± 2.1
Surfactant (% w/w)	
3	-34.7 ± 1.9
5	-37.4 ± 1.8
7	-36.5 ± 2.1

Occlusive effect of EPP loaded NLCs

The occlusive property of NLCs was measured in term of occlusion factor (*F*). Here, the evaporation of water through a porcine ear skin was measured. Then, *F* value was calculated. An occlusion factor of 0 means no occlusive property compared to the cream base. The maximum occlusion factor is 100 [32]. Nanoparticles have been found to be 15-folds more occlusive than microparticles [35]. Film formation on the skin and subsequent occlusion effect was reported for lipid nanoparticles. Additionally, the extent of their occlusive properties depends on various factors, e.g. particle size and lipid concentration [32].

In this study the *F* values of NLCs dispersion was observed by varying the amount of solid lipid from 10 to 30% (w/w). As shown in Figure 19, the formulations containing 10, 15, 20, 25 and 30% (w/w) of solid lipid amount at 4 h gave the mean *F* values of 22.19, 30.02, 40.16, 47.69 and 49.41, respectively. The results revealed that as the solid lipid amount was increased, the mean *F* values increased. The formulation contained 25 and 30% (w/w) of solid lipid amount showed high occlusion and there was no significant difference ($p > 0.05$) between both formulations. This finding was in agreement with Wissing, S. et al., 2001 who reported that increasing the lipid amount gradually to 35% led to a gradual increase in occlusion factor [48]. Thus, at low amount of solid lipid, a film layer is not sufficient to fully cover the filter paper; the water can evaporate through the filter paper more easily than those formulated at higher amount of solid lipid. Therefore, low occlusive with *F* value was observed.

Regarding to the application time dependency of the occlusive, at 8 h, the mean *F* values of formulation containing 10, 15, 20, 25 and 30% (w/w) of solid lipid amount were 23.93, 32.62, 44.65, 57.61 and 53.86, respectively. The results showed that when increasing the application time from 4 to 8 h led to the slightly increase in the occlusion factor. The results showed no significant difference in the mean of occlusion factor observed at 4 and 8 h ($p > 0.05$), which the results was found that 4 h is the maximum occlusiveness.

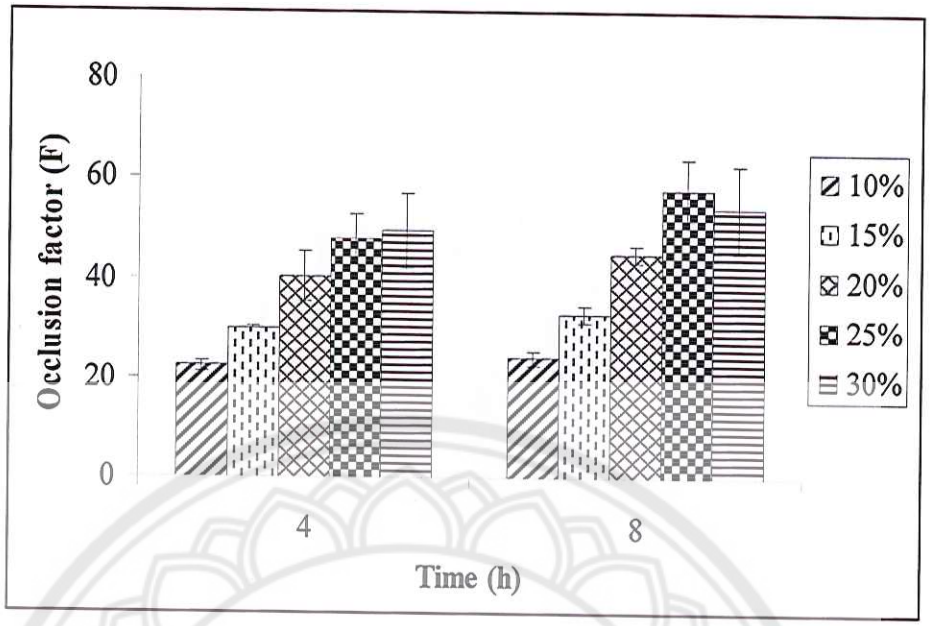

Figure 19 Occlusion factor (*F*) of NLCs dispersion at various times after application. Significant differences ($p < 0.05$ by one way ANOVA) are marked with asterisks

Determination of EPP incorporation efficacy

The EPP incorporation efficacy was determined by HPLC. The results suggested that, types of oil, types of surfactant, concentration of solid lipid and concentration of surfactant were critical factors determining the EPP incorporation efficacy. Most of the formulations studies demonstrated incorporation efficacy in the range of 40-90% (w/w).

As shown in Figure 20, Formulation with lexol showed the highest EPP incorporation efficacy, ~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 efficacy was decreased to ~40-50%. This might be due to the high viscosity of oleic acid and olive oil that leading to a significant loss of pomegranate peel extracts during the preparation process.

As shown in Figure 21, when varying the types of surfactant, formulation with lipoid s75 and span 80 showed the highest EPP incorporation efficacy, ~90%, while those formulated with tween 80 resulted in the lowest EPP incorporation efficacy, ~60%. 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 a high incorporation efficacy. This obsrevation was inagreement with Müller et al., 2000 who reported that the incorporation efficacy was proportional to the solubility of active substance in the lipid phase [49].

As shown in Figure 22, the EPP incorporation efficacy was increased when increasing the percentage of solid lipid. The highest incorporation efficacy of 90.75% was obtained with 25% (w/w) solid lipid. It can be explain by a high solid lipid concentration, more NLCs were produced. Thus, the EPP are incorporated in the nanoparticles leading to a high entrapment efficacy. Whereas, the EPP incorporation efficacy with 30% (w/w) solid lipid showed lower than with 25% (w/w) solid lipid. It could be explained that 25% (w/w) solid lipid at the optimum concentration. While 30% (w/w) solid lipid, maintaining the surfactant concentration at 7% (w/w) might be attributed to the insufficient amount of surfactant leading to a low entrapment efficacy.

As shown in Figure 23, the EPP incorporation efficacy was increased when increasing the percentage of surfactant. The highest incorporation efficacy of 90.75% was obtained with 7% (w/w) surfactant. These might be a result from a higher solubility of ellagic acid in surfactant with hydrophobic properties in leading to higher incorporation of ellagic acid into lipid component of microemulsion on the primary formed nanoparticles.

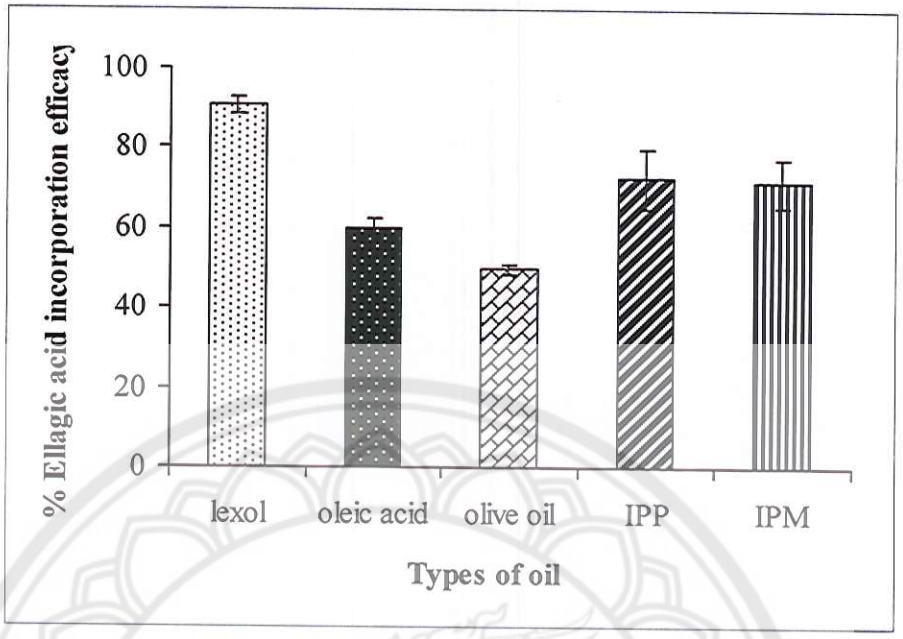

Figure 20 Incorporation efficacy of ellagic acid in NLCs with different types of oil

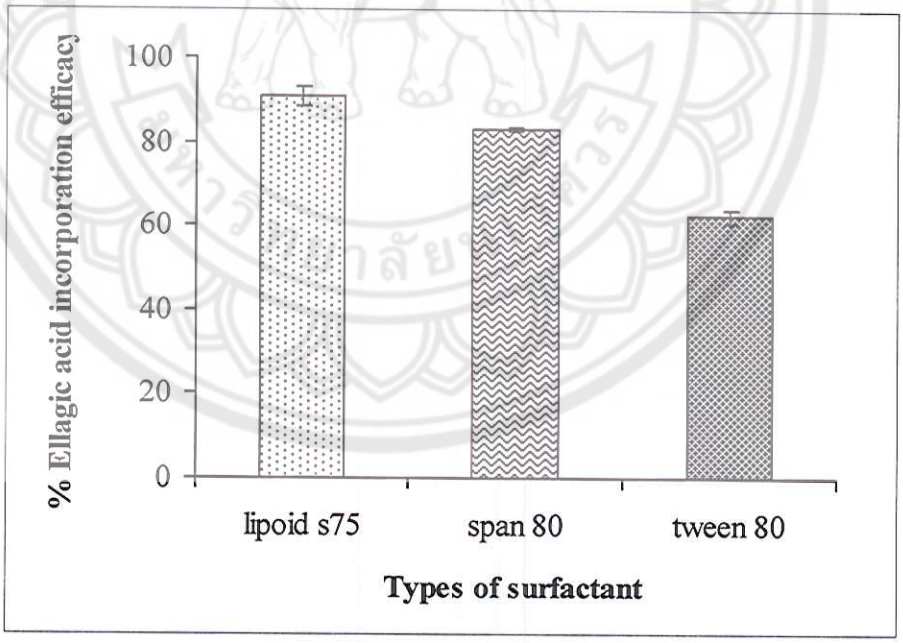

Figure 21 Incorporation efficacy of ellagic acid in NLCs with different types of surfactant

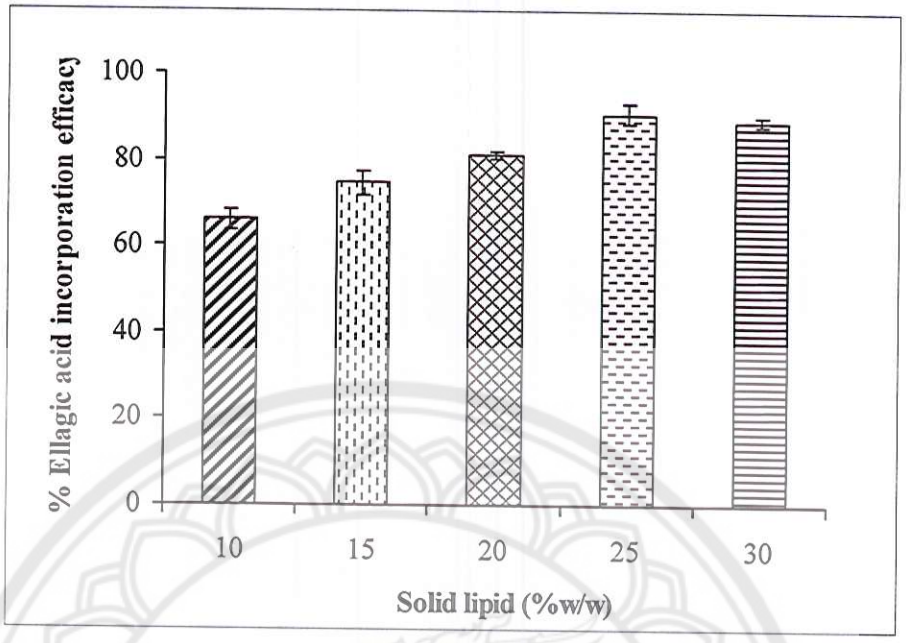

Figure 22 Incorporation efficacy of ellagic acid in NLCs with different solid lipid concentration (%w/w)

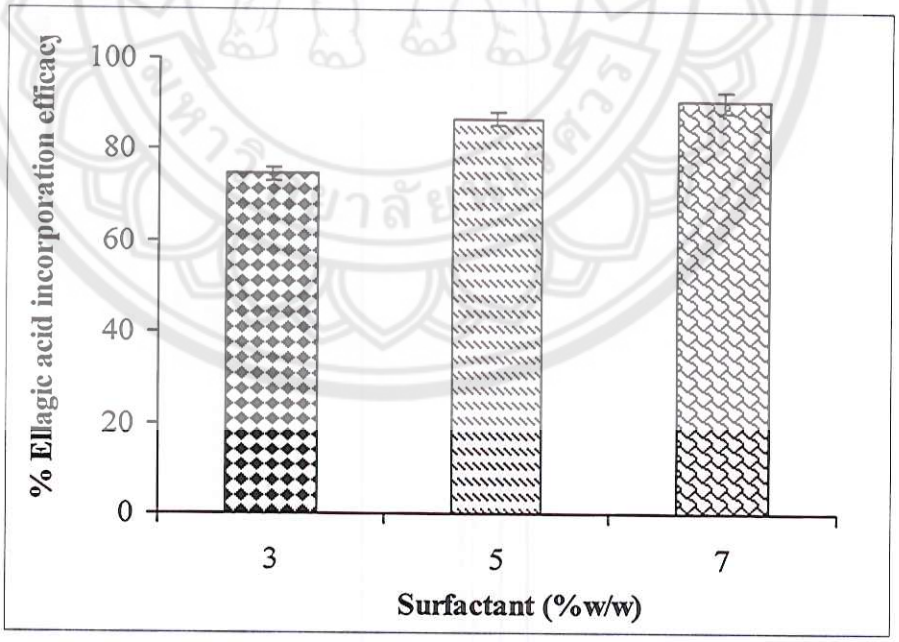

Figure 23 Incorporation efficacy of ellagic acid in NLCs with different surfactant concentration (%w/w)

***In vitro* release study**

The release profile of EPP from NLCs was evaluated by the vertical Franz diffusion cells with cellulose acetate membrane (pore size 0.22 μm). The percentages of EPP released from NLCs over varying time intervals were examined.

In order to clarify the release kinetic, the release profiles of zero-order, first-order and Higuchi's model were constructed. Zero-order was the linear correlation relationship, where the amount of active compound released was proportional to time. The linear correlation relationship, where logarithm of amount of active compound release was proportional to time, was called first-order. And the last linear correlation relationship was Higuchi's model, where the amount of active compound release was proportional to square root of time. The linear correlation (R^2) values of zero-order, first-order and Higuchi's model were shown in Table 11.

Table 11 Linear correlation value obtained from different kinetic models

Formulation	Linear correlation (R^2)		
	Zero-order	First-order	Higuchi's model
Types of oil			
Lexol	0.8384	0.8910	0.9515
IPP	0.8660	0.9146	0.9630
IPM	0.8902	0.9565	0.9836
Types of surfactant			
Lipoid s75	0.8384	0.8910	0.9515
Span 80	0.8429	0.8887	0.9620

This result was confirmed by the R^2 values, as shown in Table 11. The R^2 values calculated from Higuchi's model were higher than those calculated from zero-order and first-order respectively. Thus, the release kinetic of EPP loaded NLC was more fitted with the Higuchi's model than zero-order and first-order.

The release profiles of EPP form developed creams containing EPP loaded NLCs and EPP cream were shown in Figures 24-25, demonstrated a prolonged released characteristic following Higuchi's square root model. This not was surprise, as a numerous studies reported that SLNs or NLCs provided a controlled release system following Higuchi's square root model [50, 51]. All formulations indicated a biphasic pattern with a burst release during the first 1 h, followed by a prolong release up to 12 h. This finding was in agreement with Muller et al., 2012 who reported that burst release as well as sustained release has been reported for SLNs and NLCs dispersions [49]. For dermal application both features are of interest. Burst release might improve the penetration of active compounds. Sustained release becomes important for active ingredients that are irritating at high concentrations or to supply the skin over a prolonged period of time with an active compound (e.g. antimycotics) [35].

In this study, a more rapid released of cream containing EPP loaded NLCs was observed when compared with EPP cream. The effect of process parameters; types of oil and types of surfactant, on cumulative percent release of EPP were investigated. The results from *in vitro* release study showed that EPP cream potentially delivered the higher amount of ellagic acid as compared to cream containing EPP loaded NLCs. The result can be explained by the EPP cream can penetrate to the medium directly while the cream containing EPP loaded NLCs must released from the particles before penetrate to medium. The release behavior *in vitro* of cream containing EPP loaded NLCs with different types of oil were shown in Figure 24. The results demonstrated that the lexol-base formulation significantly difference higher than IPP and IPM-base formulation ($p < 0.05$) using one way ANOVA. This result may be explained by the effect of % entrapment of each formulation. Lexol-base formulation showed highest entrapment efficacy, thus it provided highest drug fluxes than IPP and IPM-base formulation.

Moreover, the *in vitro* release study with different types of surfactant was shown in Figure 25. As the results, percent cumulative release of EPP loaded NLCs with lipoid s75-based formulation was higher than span 80 based formulation by one way analysis of variance (ANOVA) analysis. Because lipoid s75 act as hydrophobic surfactant more than span 80 which could due to the ellagic acid are incorporated in the surfactant layer at the surface of the NLCs leading to enhance faster release and achieve a higher cumulative release. On the other hand, the very low percentage of ellagic acid released from NLCs might be explainable by the mode of ellagic acd incorporation in the particle matrix. If the active is enriched in the particle core (drug-enriched core model), release is prolonged [52].

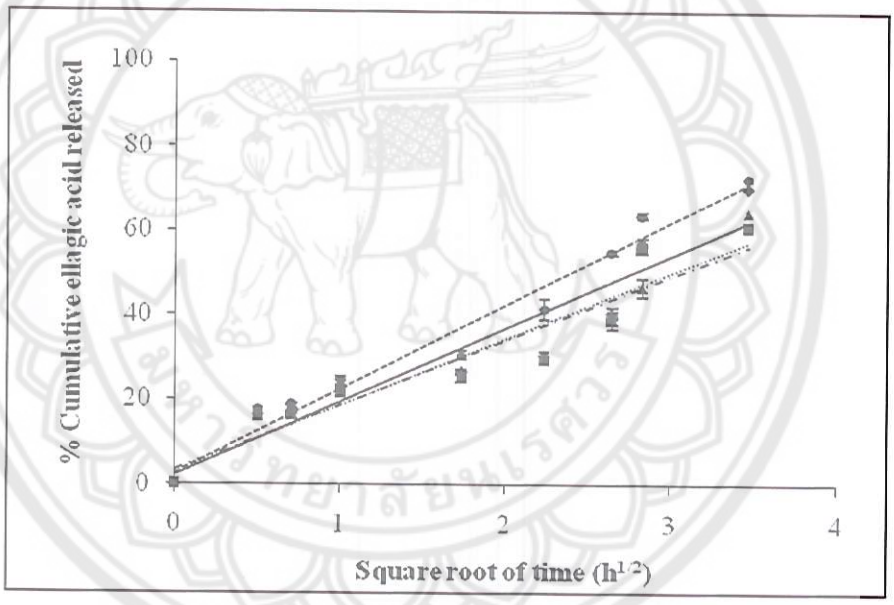

Figure 24 Release profile of cream containing EPP loaded NLCs with different types of oil (—◆— lexol, ···■··· IPP, –▲– IPM and --●-- PPE cream)

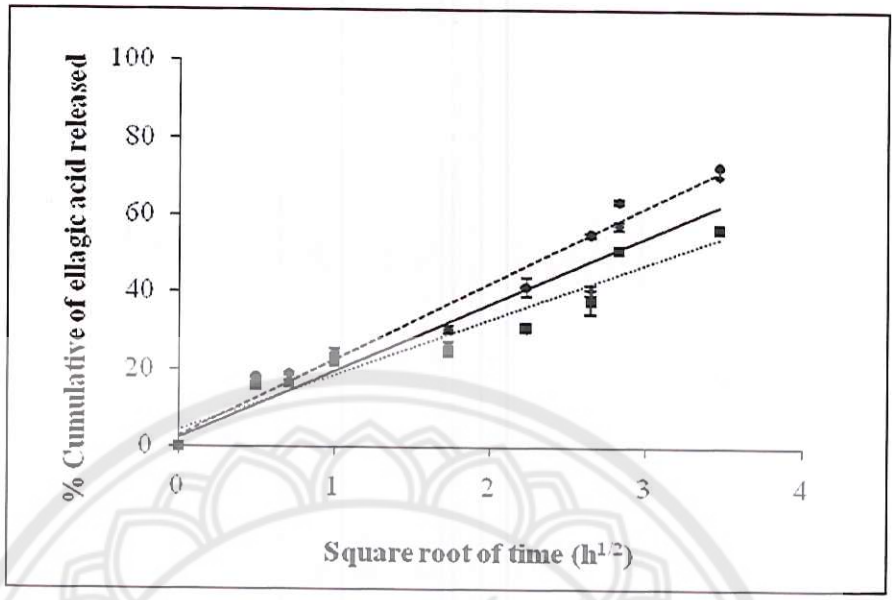

Figure 25 Release profile of cream containing EPP loaded NLCs with different types of surfactant (—◆— lipoid s75, ···■··· span 80 and -·●·- PPE cream)

***Ex vivo* permeation study**

Ex vivo penetration study were carried out on intact and barrier impaired porcine ear epidermis in vertical Franz diffusion cells varying the NLCs lipid component, duration of application, and the presence of occlusive conditions in order to evaluate how NLCs affect the drug substance penetration profile into and across the skin.

In this study, cream containing EPP loaded NLCs with different types of oil and types of surfactant were determined for *ex vivo* permeation study compared to the EPP cream. The amount of ellagic acid was quantified in the suitable acceptor medium after applying for 12 h. Due to lipophilic characteristic of ellagic acid, ethanol and acetate buffer in the ratio of 1:1 was used as the acceptor medium in order to achieve sink conditions. All formulations showed that there were longer lag time (Figure 26-27). The lag time may be affected by ellagic acid exhibit poorly water soluble and partition at a slower rate from the porcine ear epidermis to the acceptor medium. This effect indicates that ellagic acid need time to penetrate through skin and interact with stratum corneum (SC) lipid. The top most skin layer (SC) possesses an excellent

barrier, which limits a number of drug molecules for transdermal delivery [53, 54]. A relative burst of drug permeation was found in the initial 3 h, after which the drug slowly permeated with a linear relationship. The cumulative permeated amount of ellagic acid from cream containing EPP loaded NLCs was significantly higher than that from EPP cream at 3 h ($p < 0.05$). However, from the *in vitro* drug release studies, the cumulative amount of ellagic acid released from cream containing EPP loaded NLCs was lower than that of EPP cream. This means that not only the high amount of ellagic acid released from carriers but also occlusiveness can be pointed out as the major keys to promote and subsequently enhance the drug penetration into the deep skin. When applying lipid nanoparticles onto the skin, a film layer will be formed, having a surface area, the dimensions of the air channels will be much smaller in the former; thus, the hydrodynamic evaporation of water will decrease. A consequence of the occlusive properties, NLC may also be the increase skin hydration and increase in skin penetration of active ingredients. Skin occlusion can increase the stratum corneum hydration and therefore influence percutanious absorption [35, 55].

In addition, as shown in Figure 26 these results demonstrated that the varying the types of oil, lexol-base formulation showed the highest cumulative permeated amount (~0.63 μg/cm^2) at 3 h ($p < 0.05$). The effect of types of oil may be explained by the effect of % entrapment of ellagic acid. In this work, the lexol-base formulation showed the highest incorporation efficacy of ellagic acid when compared with IPP and IPM-base formulation. This finding was in agreement with Buraphacheep et al., 2012 who reported that the amount of ellagic acid loaded noisome on permeation study may be explain by the effect of percent entrapment [56]. The higher drug load of the nanoparticle matrix would result in an increase driving force for partitioning of the drug from the nanoparticle matrix into the startum corneum after adhesion of the nanoparticles onto the skin surface. In this regard, the localization of the drug in the nanoparticles (in the matrix or in the interface) seems to be of special importance [57].

As shown in Figure 27 when varying the types of surfactant, lipoid s75-base formulation showed the highest cumulative permeated amount (~0.63 μg/cm^2) at 3 h ($p < 0.05$). However, no significant were observed in the total amounts of ellagic acid after applying the formulations for 12 h ($p > 0.05$). This result may be explained by the appearance of surfactant is related to the solid state of the lipid matrix. Lipoid s75 is

solid and span 80 is liquid. Thus, lipid matrix of lipoid s75-base formulation possessed more crystallinity than that of span 80-base formulation resulting in the higher occlusion effect. This finding was in agreement with the previous work, NLCs system was developed to effectively enhance drug penetration by using nile red, a lipophilic fluorescence dye, as a model agent. *Ex vivo* penetration of nile red loaded NLCs was assessed using porcine ear and mounted in vertical Franz diffusion cells. The penetration and distribution of nile red in epidermis and dermis of porcine skin were studied using cryosection and fluorescence microscopy. The results showed that, the skin treated with nile red loaded NLCs showed higher amount of dye in the viable epidermis as compared to the control experiment (nile red solution). In addition, nile red loaded NLCs formulated from different types of oil showed similar skin permeability. On the contrary, nile red loaded NLCs formulated from different types of surfactant showed different skin permeation. That was the skin treated with lipoid s75- base formulation had higher amount of dye to all skin layer as compared to that treated with span 80-base formulation [58]. These results can be explained by the occlusive properties of NLCs and crystallinity of the lipid matrix. By, the higher occlusion factor observed for NLC is related the solid state of the matrix which can avoid the evaporation of water. This finding was in agreement with Wissing et al., 2001 who reported that crystalline lipid nanoparticles are required in order to obtain a high occlusion factor [48].

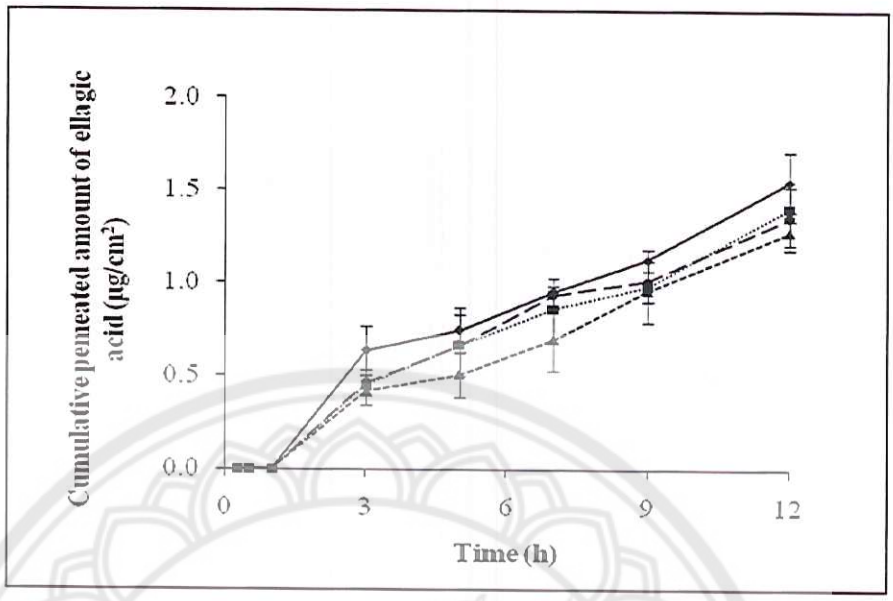

Figure 26 Cumulative permeated amount per unit area (µg/cm^2) of cream containing EPP loaded NLCs with different types of oil (···■··· lexol, ····■···IPP, ---▲--- IPM and --●-- EPP cream)

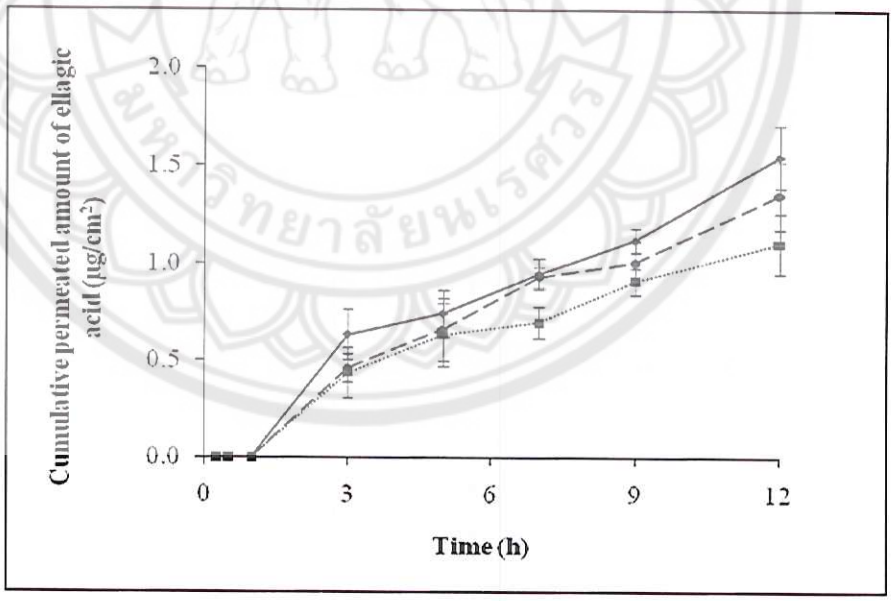

Figure 27 Cumulative permeated amount per unit area (µg/cm^2) of cream containing EPP loaded NLCs with different types of surfactant (—◆— lipoid s75, ···■··· span 80 and --●-- EPP cream)

Stability studies of ellagic acid rich pomegranate peel extract loaded NLCs

1. Stability studies of lyophilized EPP loaded NLCs

The stability studies of lyophilized EPP loaded NLCs were investigated under different process parameters; types of oil were chosen from lexol, IPP and IPM and types of surfactant were chosen from lipoid s75 and span 80 while maintaining the solid lipid concentration at 25% (w/w) and the surfactant concentration at 7% (w/w). The lyophilized EPP loaded NLCs demonstrated physical and chemical stabilities for 3 and 6 months storage in desiccators at room temperature in the absence of day light. The mean particle size, size distribution and zeta potential for all formulations were ~200 nm, 0.2-0.3 and ~-34 mV, respectively

In physical stability, the change of mean particle size, PI value and zeta potential value of lyophilized EPP loaded NLCs with different types of oil and types of surfactant were evaluated and the results were showed in Table 12-13. After 3 and 6 months, the mean particle size and PI value of all formulation tended to increase but still remained in the nanosize rang with narrow size distribution. The mean particle size and size distribution for all formulations were ~300 nm and 0.2-0.3, respectively. While, the zeta potential value of all formulation showed that there were no significant differences. The zeta potential of all formulations was ~-34 mV. In addition, types of oil and types of surfactant had no effect on the mean particle size, PI value and zeta potential value of lyophilized EPP loaded NLCs.

Table 12 The mean particle size and polydispersity index of lyophilized EPP loaded NLCs; freshly prepared 3 and 6 months in desiccators at room temperature in the absence of day light

Formulation	Mean particle size (nm ± SD)			PI (Mean ± SD)		
	Initial	3 months	6 months	Initial	3 months	6 months
Types of oil						
Lexol	169 ± 11	224 ± 9	273 ± 18	0.27 ± 0.01	0.24 ± 0.03	0.26 ± 0.03
IPP	175 ± 5	264 ± 11	281 ± 6	0.26 ± 0.04	0.29 ± 0.02	0.21 ± 0.01
IPM	183 ± 7	248± 5	260 ± 8	0.27 ± 0.05	0.24 ± 0.03	0.27 ± 0.06
Types of surfactant						
Lipoid s75	169 ± 11	224 ± 9	273 ± 18	0.27 ± 0.01	0.24 ± 0.03	0.26 ± 0.03
Span 80	225 ± 11	271 ± 6	269 ± 5	0.32 ± 0.04	0.28 ± 0.04	0.24 ± 0.02

Table 13 The zeta potential of lyophilized EPP loaded NLCs; freshly prepared 3 and 6 months in desiccators at room temperature in the absence of day light

Formulation	Zeta potential (mV) (Mean ± SD)		
	Initial	3 months	6 months
Types of oil			
Lexol	-36.5 ± 2.1	-34.56 ± 2.5	-31.51 ± 1.16
IPP	-36.8 ± 2.1	-31.45 ± 3.0	-34.25 ± 2.64
IPM	-35.9 ± 3.5	-33.62 ± 2.4	-32.46 ± 1.33
Types of surfactant			
Lipoid s75	-36.5 ± 2.1	-34.56 ± 2.5	-31.51 ± 1.16
Span 80	-33.1 ± 3.1	-33.5 ± 1.7	-35.65 ± 3.12

In chemical stability, the percentage of remaining ellagic acid was evaluated by HPLC method. The percentages of remaining ellagic acid were shown in Figure 28-29. From the results, there was no significant difference ($p > 0.05$) in percentages of remaining ellagic acid after 3 and 6 months. In addition, types of oil and types of surfactant had no effect on the percentage of remaining ellagic acid.

These results show that lyophilized EPP loaded NLCs have the same ellagic acid content as the initial preparation with a 95% confidence level. The mean particle size was slightly increased as the initial preparation. These findings are in agreement with the results of other researchers suggesting that the transformation of SLNs into a dry powder can prevent the aggregation of nanoparticles and improve the stability of light and oxygen sensitive substances [36, 49].

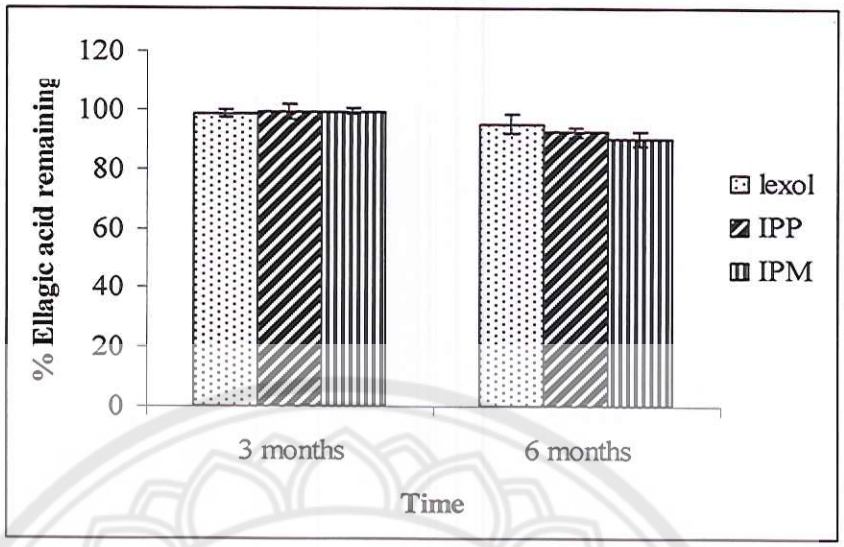

Figure 28 Percentage of ellagic acid remaining of lyophilized EPP loaded NLCs with different types of oil after 3 and 6 months storage in desiccators at room temperature. Significant differences ($p < 0.05$ by one way ANOVA) are marked with asterisks

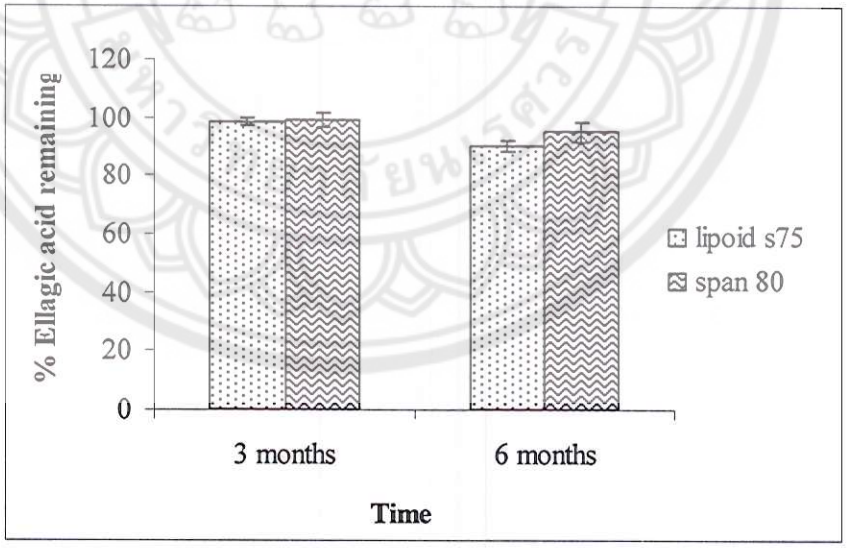

Figure 29 Percentage of ellagic acid remaining of lyophilized EPP loaded NLCs with different types of surfactant after 3 and 6 months storage in desiccators at room temperature. Significant differences ($p < 0.05$ by one way ANOVA) are marked with asterisks

2. Stability studies of cream containing EPP loaded NLCs

Cream containing EPP loaded NLCs and EPP cream were obtained by dispersing lyophilized EPP loaded NLCs and non-encapsulated EPP in cream base, respectively. The final product contained 0.05% ellagic acid. The stability of each formulation was studied under stress condition employing 6 heat-cool cycles and normal condition. The stability of cream containing EPP loaded NLCs and EPP cream in term of physical and chemical properties were studied.

2.1 Under stress conditions (6 heat-cool cycles)

In this study, different formulation of cream containing EPP loaded NLCs and EPP cream were subjected to 6 heat-cool cycles and evaluated the physical and chemical stability.

2.1.1 Physical stability of cream containing EPP loaded NLCs

The physical stability of cream containing EPP loaded NLCs and EPP cream were studied in terms of viscosity and phase separation. The viscosities of different formulations of cream containing EPP loaded NLCs and EPP cream were shown in Table 14.

Table 14 The viscosity of cream containing EPP loaded NLCs and EPP cream, formulated with different types of oil and types of surfactant, after storage under stress condition employing 6 heat-cool cycles in the absence of day light

Formulation	Viscosity (cps) (Mean ± SD)	
	Initial	After heat-cool cycle
Types of oil		
Lexol	1654 ± 23	1613 ± 16
IPP	1865 ± 12	1850 ± 21
IPM	1630 ± 16	1604 ± 26
Types of surfactant		
Lipoid s75	1654 ± 23	1613 ± 16
Span 80	1735 ± 28	1713 ± 19
EPP cream	1683 ± 15	1653 ± 15

As shown in Table 14, the results revealed that the viscosity of freshly prepared formulations was independent on the formulation factors. After storage under stress condition, the viscosity of all formulations showed no significant difference ($p > 0.05$) using paired t-test when compared to those freshly preparation. In addition, no phase separation was observed in all formulations.

These results indicated that the formulation factors had minor effect on the physical stabilities of the tested products under stress condition.

2.1.2 Chemical stability of cream containing EPP loaded NLCs

The chemical stability of cream containing EPP loaded NLCs and EPP cream were studied in terms of pH value and percentage of ellagic acid remaining. The pH values of cream containing EPP loaded NLCs and EPP cream after storage under stress condition were shown in Table 15.

Table 15 pH value of cream containing EPP loaded NLCs and EPP cream, formulated with different types of oil and types of surfactant, after storage under stress condition employing 6 heat-cool cycles in the absence of day light

Formulation	pH value (Mean ± SD)	
	Initial	After heat cool cycle
Types of oil		
Lexol	7.34 ± 0.10	7.51 ± 0.05
IPP	7.35 ± 0.05	7.18 ± 0.02
IPM	7.30 ± 0.06	7.04 ± 0.06
Types of surfactant		
Lipoid s75	7.34 ± 0.10	7.51 ± 0.05
Span 80	7.30 ± 0.03	7.00 ± 0.09
EPP cream	7.28 ± 0.07	7.16 ± 0.06

As shown in Table 15, the results revealed that the pH value of freshly prepared formulations was independent on the formulation factors. After storage under stress condition, the pH value of all formulations showed no significant difference ($p > 0.05$) using paired t-test analysis when compared to those freshly preparation.

The percentage of remaining ellagic acid with different types of oil and types of surfactant after stress condition employing 6 heat-cool cycles were shown in Figure 30-31, respectively. The percentage of remaining ellagic acid in NLCs were greater than that EPP cream, significant different ($p < 0.05$) using paired t-test analysis. The result showed that 75% of ellagic acid remaining in EPP cream while 80-85% of ellagic acid remaining in cream containing EPP loaded NLCs.

Thus, under stress condition employing 6 heat-cool cycles, in absence of day light, cream containing EPP loaded NLCs show better chemical stability than EPP cream. The formulation factors had minor effect on the chemical stabilities of the tested products under stress condition.

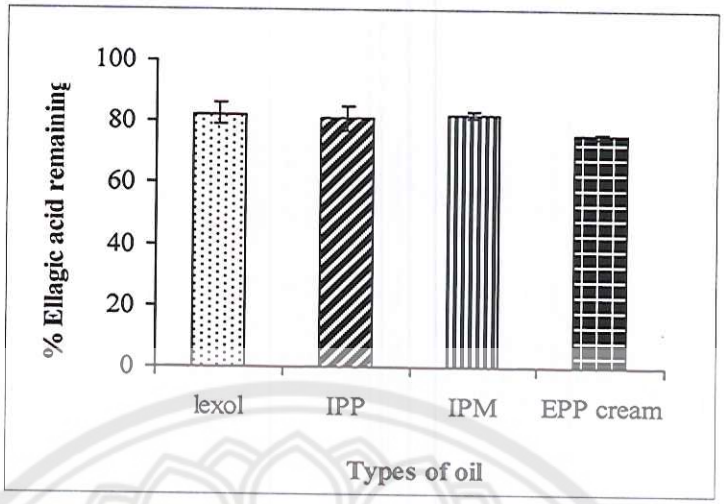

Figure 30 Percentage of ellagic acid remaining of cream containing EPP loaded NLCs and EPP cream with different types of oil after storage under stress condition employing 6 heat-cool cycles in the absence of day light. Significant differences ($p < 0.05$ by one way ANOVA) are marked with asterisks

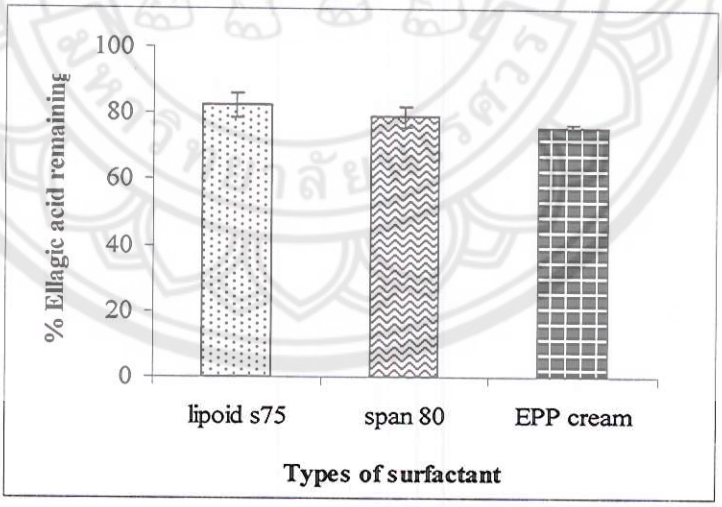

Figure 31 Percentage of ellagic acid remaining of cream containing EPP loaded NLCs and EPP cream with different types of surfactant after storage under stress condition employing 6 heat-cool cycles in the absence of day light. Significant differences ($p < 0.05$ by one way ANOVA) are marked with asterisks

2.2 Under normal conditions

To assess the effect of light on physical and chemical stability of different formulations of cream containing EPP loaded NLCs and EPP cream; they were stored in the absence and presence of day light at room temperature over a period of 3 and 6 months.

2.2.1 Physical stability of cream containing EPP loaded NLCs

The physical stability of cream containing EPP loaded NLCs and EPP cream were studied in terms of viscosity and phase separation. The viscosities of different formulations of cream containing EPP loaded NLCs and EPP cream were shown in Table 16.

Table 16 The viscosity of cream containing EPP loaded NLCs and EPP cream, formulated with different types of oil and types of surfactant, after storage under normal condition in the absence and presence of day light after 3 and 6 months

Formulation	Viscosity (cps) (Mean ± SD)				
		Absence day light		Presence day light	
	Initial	3 months	6 months	3 months	6 months
Types of oil					
Lexol	1754 ± 15	1745 ± 13	1745 ± 13	1705 ± 23	1726 ± 23
IPP	1655 ± 19	1701 ± 24	1701 ± 24	1671 ± 11	1698 ± 11
IPM	1698 ± 26	1736 ± 14	1736 ± 14	1632 ± 18	1716 ± 18
Types of surfactant					
Lipoid s75	1754 ± 15	1745 ± 13	1745 ± 13	1705 ± 23	1726 ± 23
Span 80	1725 ± 16	1703 ± 17	1703 ± 17	1706 ± 17	1706 ± 17
EPP cream	1695 ± 14	1713 ± 20	1713 ± 20	1679 ± 19	1679 ± 19

As shown in Table 16, after 3 and 6 months storage under normal condition, the viscosity of different formulations of cream containing EPP loaded NLCs and EPP cream showed no significant difference ($p > 0.05$) by one way ANOVA. These results suggested that the formulation factors had no effect on the viscosity of cream after 6 months storage at room temperature. In addition, no phase separation was observed in all formulations.

The above results suggested that under normal condition cream containing EPP loaded NLCs show better physical stability than cream containing non-encapsulated EPP.

2.2.2 Chemical stability of cream containing EPP loaded NLCs

The chemical stability of cream containing EPP loaded NLCs and EPP cream were studied in terms of pH value and percentage of ellagic acid remaining. The pH values of cream containing EPP loaded NLCs and EPP cream after storage under stress condition were shown in Table 17.

Table 17 pH value of cream containing EPP loaded NLCs and EPP cream, formulated with different types of oil and types of surfactant, after storage under normal condition in the absence and presence of day light after 3 and 6 months

Formulation	pH value (Mean ± SD)				
	Initial	Absence day light		Presence day light	
		3 months	6 months	3 months	6 months
Types of oil					
Lexol	7.34 ± 0.10	7.21 ± 0.03	7.12 ± 0.04	7.51 ± 0.05	7.13 ±0.09
IPP	7.35 ± 0.05	7.08 ± 0.05	7.03 ± 0.07	7.18 ± 0.02	7.08 ± 0.02
IPM	7.30 ± 0.06	7.11 ± 0.01	7.26 ± 0.02	7.04 ± 0.06	7.08 ± 0.01
Types of surfactant					
Lipoid s75	7.34 ± 0.10	7.21 ± 0.03	7.12 ± 0.04	7.51 ± 0.05	7.13 ±0.09
Span 80	7.30 ± 0.03	7.04 ± 0.04	7.38 ± 0.04	7.00 ± 0.09	7.09 ± 0.02
PPE cream	7.28 ± 0.07	7.03 ± 0.06	7.30 ± 0.06	7.16 ± 0.06	7.13 ± 0.04

As shown in Table 17, after 3 and 6 months storage in the absence and presence of day light, the pH values of all formulations after preparation, 3 and 6 months storage had no significant difference ($p > 0.05$) using paired t-test analysis. The results showed pH stable in both case of the absence and presence of day light.

The percentages of remaining ellagic acid in cream containing EPP loaded NLCs and EPP cream after 3 and 6 months storage at room temperature in the absence and presence of day light were shown in Figure 32-33. The effects of formulation factor, the types of oil and types of surfactant, on percentage of ellagic acid remaining were investigated.

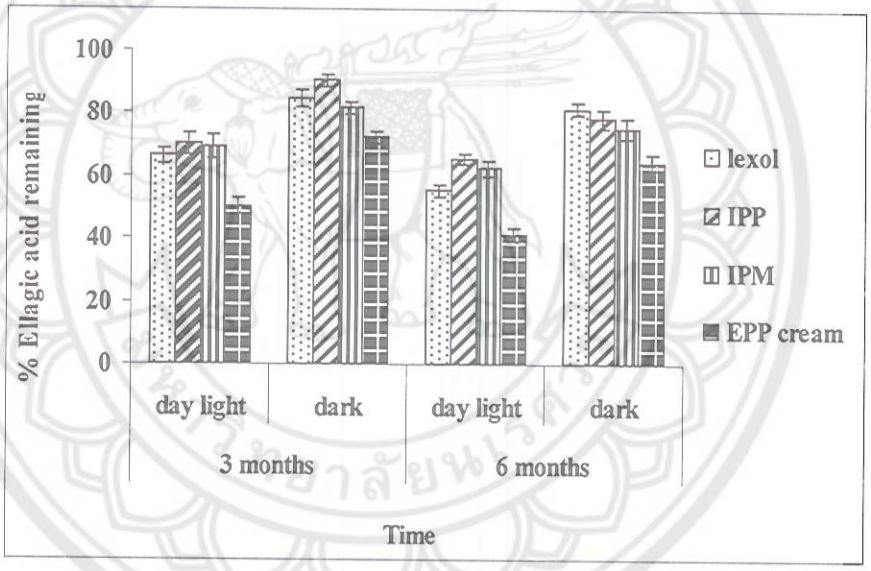

Figure 32 Percentage of ellagic acid remaining of cream containing EPP loaded NLCs and EPP cream with different types of oil after storage at room temperature in the absence and presence of day light. Significant differences ($p < 0.05$ by one way ANOVA) are marked with asterisks

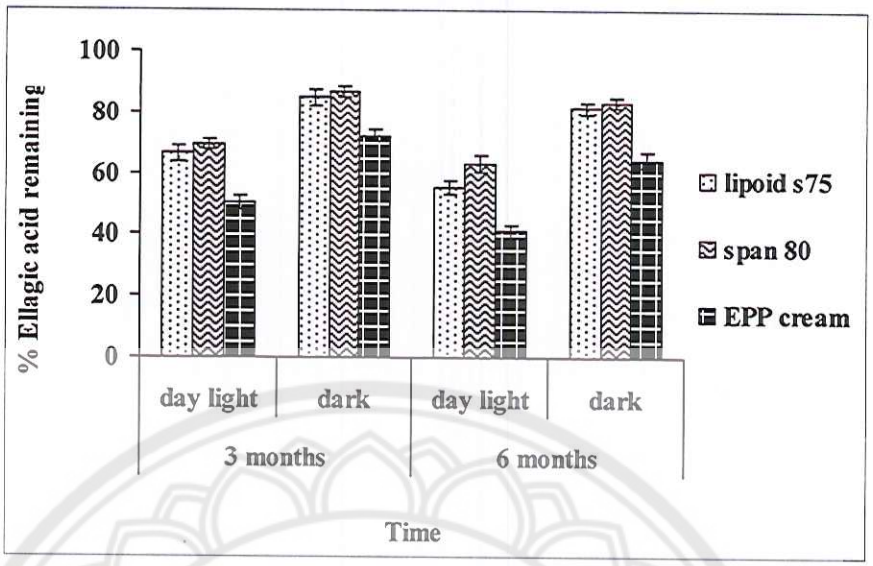

Figure 33 Percentage of ellagic acid remaining of cream containing EPP loaded NLCs and EPP cream with different types of surfactant after storage at room temperature in the absence and presence of day light. Significant differences ($p < 0.05$ by one way ANOVA) are marked with asterisks

The results demonstrated that light showed critical effect on the chemical stability of ellagic acid. The results revealed that after storage in the presence of day light for 3 months, the percentage of remaining ellagic acid was decrease and more decrease after 6 months storage. After 6 months, the percentage of the remaining ellagic acid of cream containing EPP loaded NLCs and EPP cream were about 80% and 65%, respectively. In the presence of day light, the percentage of the remaining ellagic acid of cream containing EPP loaded NLCs and EPP cream were ~ 65 and 40%, respectively. The degradation of the ellagic acid during storage in the cream base in the presence of day light was a result of the ellagic acid released from NLCs into the cream base. This amount of degraded ellagic acid was roughly consistent with burst release observed in the *in vitro* release studies. Thus, the findings indicate that NLCs could improve ellagic acid stability against light. In addition, the formulation factors, types of oil and types of surfactant had no effect on the chemical

stability of cream containing EPP loaded NLCs after 3 and 6 months storage at room temperature

These results strongly confirm that NLCs significant improve the ellagic acid stability against light and oxidation reaction during storage when incorporated in cream.