Original article

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DEVELOPMENT OF *PUERARIA MIRIFICA* EXTRACT-LOADED LIPID NANOPARTICLES FOR HAIR SPRAY

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ABSTRACT

Pueraria mirifica (PM) composed of active phytoestrogens has been used in Thai traditional medicine for rejuvenating qualities in aged women and men for nearly one hundred years. The application of PM formulation has been used for preventing hair loss. The aim of this study was to develop hair spray formulation using a lipid nanocarrier system. Solid lipid nanoparticles (SLN) of 3% cetyl palmitate and nanostructured lipid carriers (NLC) of 2% cetyl palmitate and 1% oleic acid were formulated for PM extract loading. The physicochemical characteristics of nanocarrier formulations were evaluated. The *in vitro* skin penetration and deposition study were performed using Franz diffusion cells through porcine skin. The visual appearance showed a good physical property at 1 to 10 % w/w of PM loaded-SLN formulations and 1 to 15 % w/w of PM loaded-NLC formulations. The particle sizes of SLN formulations were between 94 and 306nm, while NLC formulations were in the range of 66 to 250nm. All formulations exhibited a negative surface charge in the range of 20-30 mV. The entrapment efficiency (%EE) of 10% PM loaded-SLN was higher than that of other SLN formulations. 15% PM loaded-NLC exhibited a higher %EE and loading efficiency (%LE) than those of 10% PM loaded-NLC. However, the skin penetration results of PM were 10% PM loaded-SLN > PM extract > 15% PM loaded-NLC, while the amount of PM deposited in the skin by all formulations was similar. In conclusion, 10% PM loaded-SLN was the desirable carrier for PM delivery through skin, suggesting that the mechanism of SLN might occlude and transport the entrapped drug via the hair follicular pathway. Therefore, SLN might be a suitable hair spray formulation for dermal delivery of PM.

Keywords: *Pueraria mirifica*, lipid nanoparticles, skin penetration, hair spray

Introduction

White Kwao Krua (*Pueraria mirifica* (PM)) is a Thai medicinal plant which has provided rejuvenating qualities in elderly women and men for nearly one hundred years. At least 17 active phytoestrogens (plant substances with estrogen-like activity) were isolated using high performance liquid chromatography, when isoflavones were mainly found. The estrogenic activity of PM affects the reproductive organs, bones, cardiovascular diseases, and other climacteric related symptoms. Generally, the application of PM has been used for prevention of, or therapeutically for symptoms related to estrogen deficiency in menopausal women as well as in andropausal men.[1] Moreover, PM is used in traditional Thai medicine for preventing hair loss in a number of ways.[2] The hair spray formulation is extensively used for hair treatment because of its ability to target hair follicles and ease of use in daily life.

Lipid nanocarriers are colloidal dispersions composed of a dispersed lipid phase, which is stabilized by an emulsifier. The first generation of lipid nanocarriers consisted of solid lipid nanoparticles (SLNs) that were composed of high melting point lipid/s (including high melting point glycerides or waxes) as solid cores coated with surfactants,[3] whereas the second generation, composed of both solid and liquid lipids, is defined as a nanostructured lipid carrier (NLC). This colloidal system may increase the solubility and bioavailability of active pharmaceutical ingredients, protect entrapped compounds from outer influences such as light or oxygen, and facilitate drug targeting.[4] Therefore, these carriers are used as an alternative system to emulsions, liposomes, microparticles, and their polymeric counterparts, for various application routes due to a range of advantages such as feasibility of incorporation of lipophilic and hydrophilic drugs, improved physical stability, low cost compared to liposomes, and ease of scale-up and manufacture.[5,6] Moreover, SLNs and NLCs have been reported as potential transdermal carriers by being localized mainly in hair follicle openings and on the stratum corneum surface, where a particle size of less than or equal to 640 nm is appropriate for particulate formulations.[7]

The aim of this study was to develop hair spray formulations using lipid nanocarrier systems. SLNs and NLCs were formulated for the loading of various concentrations of PM extract. The physicochemical characteristics of the nanocarrier formulations, such as visual appearance, particle size, zeta potential, entrapment efficiency (%EE), and loading efficiency (%LE), were evaluated. Moreover, *in vitro* skin penetration and deposition studies were performed using a Franz diffusion cell system through porcine skin.

Materials and methods

Materials

Cetyl palmitate was purchased from SABO SpA (Levate, Italy). D-limonene was obtained from Sigma-Aldrich, MO, USA. Polysorbate 80 (Tween 80) was from the NOF Corporation (Osaka, Japan). Oleic acid was purchased from Fluka Chemic AG, Seelze, Germany. Lecithin was purchased from Acros, New Jersey, USA. Polysorbate 20 (Tween 20) was purchased from Ajax Finechem, Auckland, New Zealand. The PM ethanolic extract (brown color solution) was a gift from Kaewmungkorn Co. Ltd., Ratchaburi, Thailand.

Preparation of lipid nanocarriers

Lipid nanocarrier formulations were prepared using the *de-novo* emulsification method.[8] The composition of the NLC and SLN formulations is shown in Table 1. Various amounts of PM extract (1, 2.5, 5, 10, 15, and 20 % w/w) were added to the oil phase. Briefly, the oil and aqueous phases were separately heated to 65 $\pm$ 5 °C. The aqueous phase was added to the oil phase while being stirred at 14,000 rpm for 5 min with a magnetic stirrer. The emulsion was probe-sonicated for 15 min to reduce

the particle size and then filtered through a 0.45-μm membrane filter to remove any precipitate matter.

Visual characterization

To detect the presence of solid particles in all SLN and NLC formulations, the clarity and opaqueness were visually observed.

Particle size and zeta potential analysis

Each formulation was diluted to a 1:20 ratio with distilled water and measured for particle size and zeta potential, using a dynamic light scattering (DLS) particle size analyzer (Zetasizer Nano-ZS, Malvern Instrument, Worcestershire, UK) with a 4 mW He-Ne laser at a scattering angle of 173°. All measurements were performed in triplicate.

Entrapment efficiency and loading efficiency

The lipid nanocarriers were disrupted by adding isopropyl alcohol at a ratio of 1:1 and centrifuged at 14,000 rpm for 15 minutes. The PM content in the supernatant was determined using HPLC analysis.

The drug entrapment efficiency (%EE) and loading efficiency (%LE) were calculated using the following equations (1 and 2) respectively:

$$\%EE = \frac{C}{C_i} \times 100 \quad (1)$$

$$\%LE = \frac{C}{\text{Lipid composition}} \times 100 \quad (2)$$

where C is the concentration of PM extract in the formulation, and C_i is the initial concentration of PM added.

In vitro skin penetration and deposition study

Abdominal porcine skin was taken from intrapartum stillborn animals from a farm in Ratchaburi province. Subcutaneous fat was carefully removed using medical scissors and surgical blades to obtain skin thickness approximately of 0.6-0.7 mm. Samples were frozen at -20 °C until use.

Table 1 Compositions of PM-loaded lipid nanocarrier formulations

Compositions (% w/w)		Formulations	
		SLN	NLC
Oil phase	Oleic acid	-	1
	Cetyl palmitate	3	2
	Lecithin	0.12	0.12
	D-limonene	1	1
	BHT	0.002	0.002
Aqueous phase	Polysorbate 80	0.8	0.8
	Polysorbate 20	2	2
	Phosphate buffer saline (PBS; pH7.4) qs.	100	100

A skin penetration study was performed using Franz diffusion cells. The receptor compartment of the cell was filled with 6 mL of PBS at 32°C while being stirred at 500 rpm. Approximately 1 mL of formulation was applied to the skin in the donor compartment and all formulations were diluted to obtain an amount of PM of 0.1 g. Samples (0.5 mL) were taken from the receiver compartment at 48 h. The skin was cut into small pieces, digested in 5 mL of PBS with a probe sonicator for 20 min, and then centrifuged for 10 min at 12,000 rpm. The extracts of PM-accumulated in the skin and receiver medium were analyzed using HPLC. Each sample was performed in triplicate.

HPLC analysis

PM concentration was analyzed using HPLC (Agilent 1100 Series HPLC value system, Agilent Technologies, Waldbronn, Germany). A C18 reverse-phase column (Luna® 5 µm, 150 x 4.60 mm, Phenomenex, USA) was used. The UV detector was set at 254 nm. The mobile phase was a gradient of acetonitrile: 1.5% acetic acid in ratios of 0:100, 5:95, 10:90, 15:85, 20:80, 25:75, 30:70, 35:65, 40:60, and 45:55 for 50 min. The injection volume was 20 µL and the flow rate was 1 mL/min at ambient temperature. Standard solutions in concentrations of 0.5, 1, 2.5, and 5% w/v of PM extract in ethanol were prepared. Pueraria is one of the main components in PM extract, and was therefore used as a marker at a retention time of 16 min.[9]

Statistical Analysis

All measurements were collected in triplicate. Values are expressed as the mean ± standard deviation (S.D.). Significance was analyzed using one-way ANOVA. The significance level was set at $p<0.05$.

Results

Physicochemical characterization of lipid nanocarriers

The physical appearances of SLN and NLC formulations were observed. SLN formulations showed a white and yellow clear solution at PM concentrations of 1 to 10% w/w. Increasing PM extract concentrations, such as 10% and 20% in SLN formulation, exhibited phase separation. For NLC formulations, 1 to 15% w/w of PM extract were successfully formulated and showed a white and yellow clear solution. However, NLC 20% showed a separate layer in the way as SLN 15%. Concentrations higher than 15% of PM extract were not therefore able to form a lipid nanocarrier.

The particle sizes and surface charges of SLN and NLC formulations were evaluated as shown in Figure 1. At PM concentrations of 1%, 2.5%, and 5%, both SLN and NLC formulations had significantly smaller particle sizes than 10% and 15% PM-loaded nanocarriers ($p < 0.05$). The particle sizes of NLC formulations were smaller than SLN formulations at the same concentrations of PM extract. All formulations demonstrated a narrow size distribution (polydispersity index < 0.3). The zeta potentials of all formulations showed negative surface charges. SLN formulations exhibited charges between -20 mV to -25 mV, while those of NLC formulations were between -18 mV to -29 mV.

The entrapment efficiency and loading efficiency of PM-loaded lipid nanocarriers are shown in Figure 2. SLN 10% and SLN 15% presented the significantly highest %EE and %LE ($p<0.05$). Although SLN 15% had the highest %LE, the physical appearance of SLN 15% was not homogeneous, showing separate layers of precipitate and wax on the surface at room temperature. The incorporation of 10% w/w PM into SLN (SLN 10%) thus provided good physicochemical properties. For NLC formulations, NLC 1% and NLC 15% showed the significantly highest %EE and %LE ($p<0.05$), respectively. Thus NLC 15% had the highest %LE with good appearance.

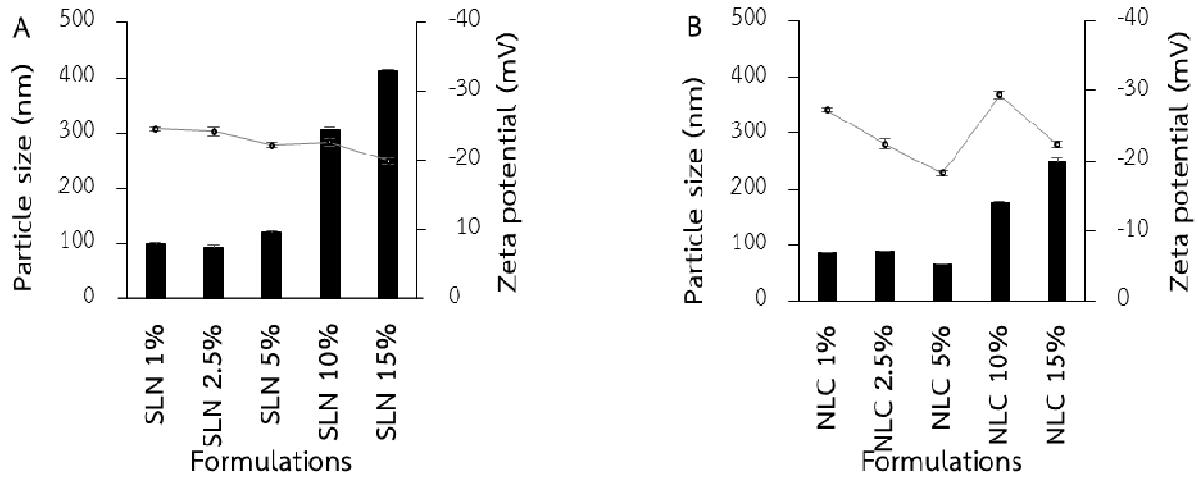

Figure 1 Effect of initial amount of PM on the particle size (■) and zeta potential (○) of PM-loaded SLN (A) and NLC (B). Each value represents the mean ± S.D. (n=3).

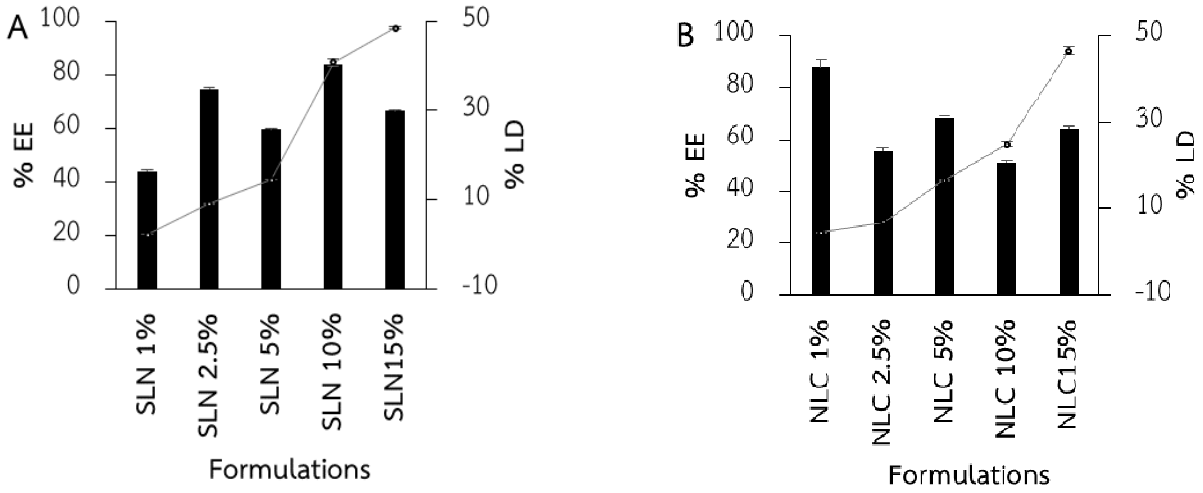

Figure 2 Effect of initial amount of PM on the %EE (■) and %LE (○) of PM-loaded SLN (A) and NLC (B). Each value represents the mean ± S.D. (n=3).

For the *in vitro* skin penetration study at 48 h (Figure 3), the amount of PM deposited in the skin of all formulations was the same. The amount of PM which penetrated through the skin into the receiver compartment was in the order: SLN 10% > PM extract > NLC 15%. The total amount of PM in, and penetrating through the skin were 14.90, 7.93, and 5.40% of the initial amount in the donor compartment for SLN 10%, PM extract and NLC 15% respectively. However, the amount of PM in the receiver compartment of SLN 10% and PM extract was higher than in the skin.

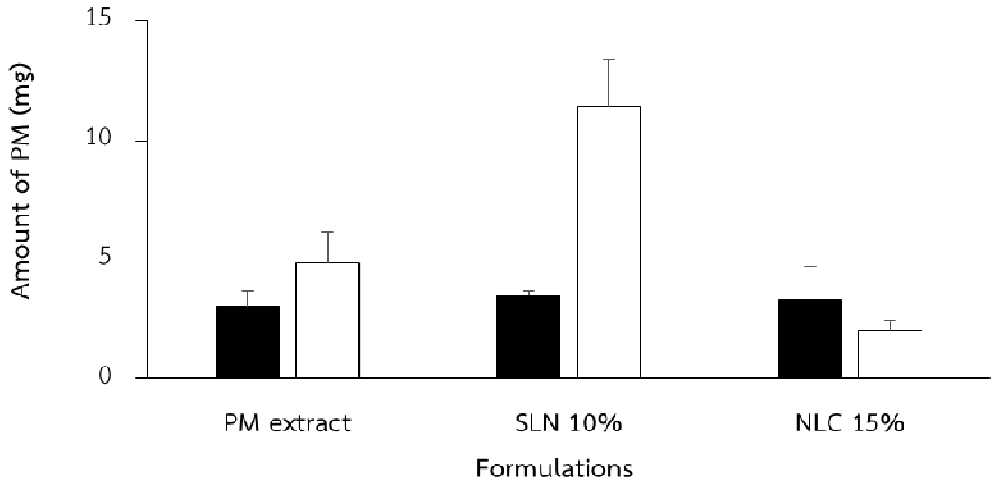

Figure 3 Comparison of PM deposits in the skin (■) and receiver compartment (□) from PM extract, SLN 10%, and NLC 15% after 48 h *in vitro* skin penetration. Each value represents the mean ± S.D. (n=2).

Discussion

The physicochemical properties of lipid nanocarriers depended on the amount of PM extract in the formulations. At low concentrations of PM extract in the formulations (not more than 5% w/w), both SLN and NLC could form a homogeneous formulation with a small particle size. Increasing the amount of PM extract in the formulations increased the particle size and a separate layer in the formulations was found. This suggested that lipid compounds were solubilized in the lipid phase of the nanocarriers and entrapped in the particles. The excessive amount of PM extract was therefore unsuccessfully incorporated into the limited amount of the lipid phase of lipid nanocarriers. However, the incorporation ability of PM into NLC was higher than that of SLN, indicating that the drug is more soluble in liquid lipids than solid lipids. The incorporation of liquid lipids into solid lipids leads to reduced crystallinity and increased imperfections in the crystal lattice, resulting in increased space to accommodate drug molecules and increased drug loading efficiency.[10,11] Moreover, NLC exhibited smaller sizes than SLN when prepared using the same procedure, surfactant types and concentrations, and total lipid amount.[12] This suggested that the incorporation of oil into the solid matrix provided better emulsification of the formulation.[13]

For the skin penetration study, SLN 10% proved to be the most effective carrier to deliver PM through the skin. The particle size of SLN 10% was 306 nm, which might penetrate through the hair follicle pathway. Hair follicles have been reported to be an important pathway for skin invasion of particles of medium size. Patzelt et al. (2010) reported that increasing the particle sizes of PLGA-particles led to a significantly deeper penetration into the porcine hair follicles (122 nm < 230 nm < 300 nm < 470 nm < 643 nm).[14] In addition, the solid structures of the lipid particles were more lipophilic and enabled close contact with the stratum corneum, leading to the formation of a thin film on the skin and creating an occlusive effect to increase skin hydration.[15-18] The occlusion factor depended on the sample volume, particle size, crystallinity, lipid concentration, and type of colloidal system.[19] Teeranachaideekul et al. (2007) reported that an increase in liquid lipid content in Nile red loaded NLC led to a decrease in the occlusion effect, relating to the decrease in the penetrated amount of Nile red in the skin.[20] Therefore, the skin

permeability of PM-load NLC was lower than SLN, indicating that solid lipids have an effect on the skin permeability of PM.

D-limonene is a chemical penetration enhancer, which might modify the organization of the stratum corneum and enhance drug delivery through the skin.[21] However, no intact SLN or NLC penetrated into the skin, so both D-limonene and the encapsulated drug have to be released from the nanoparticles into the skin.[22] In this study, D-limonene might not have been released from the lipid particles to the skin surface, because the lipophilic compound was contained in the immobilization of active elements by the solid particle structure resulting in increased chemical protection, less leakage, and sustained release.[23] Moreover, D-limonene might have increased the PM solubility in the liquid lipids of the NLC, leading to a reduction of the release of PM and d-limonene into the skin.

Conclusion

In this study, two types of lipid nanocarriers were successfully formulated for the dermal drug delivery of PM, with SLN exhibiting higher skin permeability of PM than PM extract and NLC. This suggested that a formulation containing a greater amount of solid lipids would lead to greater skin penetration. The mechanism of SLN might occlude and transport the entrapped drug via the hair follicular pathway, therefore SLN proved to be a potential carrier of hair spray formulation for the dermal delivery of PM.

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