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In Vitro Skin Permeation Enhancement of Asiaticoside by Liquid Crystal Vesicles

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Egg phosphatidylcholine (Egg PC) liposomal suspensions of Centella asiatica (C. asiatica) extract were prepared by a sonication method. On transmission electron microphotographs (TEM), multi-lamellar vesicles coexisted with small uni-lamellar ones. The size on TEM fell within the range of size distribution, obtained by a dynamic light scattering technique. In vitro *permeation through hairless mouse skin was investigated using a diffusion cell. Asiaticoside, one of biological active ingredients of C. asiatica extract, was detected in the receptor cell. As controls, suspensions of C. asiatica extract in phosphate buffered saline (pH 7.4), 30% ethanol solution and absolute ethanol were investigated for the* in vitro *permeation of asiaticoside. The egg PC liposomes turned out to enhance the permeation of asiaticoside, compared with the control vehicles. Egg PC liposomes could be a candidate vehicles for the use as a permeation enhancer of asiaticoside.*

Keywords: asiaticoside; *Centella asiatica*; *in vitro* permeation; liquid crystal vesicles

1. INTRODUCTION

Centella asiatica (*C. asiatica*) has been used as a medicinal herb for hundreds of years [1]. The herb is known to be effective for treating symptoms of anxiety, skin disease and gastrointestinal disorders [2]. It is also reported to have wound healing [3], anticancer [4] and

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antioxidant properties [5]. The biologically-active ingredients of *C. asiatica* are pentacyclic triterpenes, mainly asiaticoside, madecassoside, asiatic acid and madecossic acid [6,7]. Among them, asiaticoside is the most abundant triterpene glycoside. It promotes the secretion of antioxidant in the wound healing process and has been used for the treatment of wounds [8]. When the extract is used for the treatments of skin disease and wound healing, the selection of an adequate vehicle would be important to enhance the bioavailability of biologically active ingredients. If an ingredient is soluble in a vehicle, it has an affinity with the vehicle. As a consequence, the ingredient would hardly transfer from the vehicle into skin. Accordingly, a vehicle should be chosen so that the thermodynamic activity of an agent is high [9,10]. Another strategy for efficient dermal delivery is nanoencapsulation. Nanoparticulate drug carriers, such as liposomes, lipid vesicle, micelle, and nanocapsules, have been extensively studied for the dermal delivery or the transdermal delivery. They were found to be useful in the dermal delivery of hydrocortisone (an anti-inflammable agent) [11], amphotericin B (antifungals) [12] and retinoids [13]. Liposomes, especially, have attracted many scientists' interest for their application as dermal or transdermal drug carriers. Since liposomes are composed of phospholipids which are major component of mammalian cell membrane, they are known to interact with skin [14]. Little study has been done to enhance the dermal delivery of *C. asiatica* extract using liposomes. In this study, the extract from *C. asiatica* was obtained by methanol extraction, and the liposomal suspensions of *C. asiatica* extract were prepared by a sonication method. In parallel, suspensions of the extract in phosphate buffered saline (PBS, 7.4), 30% ethanol solution, and absolute ethanol. The *in vitro* skin permeability of asiaticoside in liposomal suspension was compared with those of asiaticoside in the other three suspensions to investigate whether liposome enhance the skin permeability of asiaticoside.

2. EXPERIMENTAL

2.1. Materials

Egg phosphatidylcholine (egg PC) were purchased from Sigma (St. Louis, MO). *C. asiatica* was obtained from a local vendor in Jeju island (South Korea). Asiaticoside was provided by Chromodex (California, USA). Phosphotungstic acid for a negative staining the liquid crytalline bilayers of egg PC liposomes were purchased from Aldrich Chemical co. (Milwaukee, USA). All other reagents were in analytical grade.

2.2. Animals

Female hairless mice (type SKH) were obtained from Orient Bio (Seongnam, Korea). They were housed in suspended wire mesh cages in a room illuminated from 09:00 to 21:00 hr and kept 20–25°C, with a rodent diet and water ad libitum.

2.3. Methods

2.3.1. *Preparation of Methanol Extracts of C. asiatica*

The fresh herb of *C. asiatica* was washed with distilled water and it was air dried in the dark. The herb (100 g) was ground in a power form and then added (1 L) ethanol and kept at 60°C for 12 hrs. After that the flask was transferred to an ultrasonic bath (Asia ultrasonic, Seoul, Korea) for 1 hr at 40 KHz. The extract was filtered and concentrated by vacuum evaporator and lyophilized in freeze dryer.

2.3.2. *Suspension Preparation of Methanol Extracts of C. asiatica*

The liposomal suspension of *C. asiatica* extract was prepared as follows. 20 mg of egg PC in chloroform (100 mg/ml) was mixed with 2 mg of *C. asiatica* extract in methanol (1 mg/ml) in a 50 ml-round bottom flask. The solvents were evaporated in a reduced pressure to obtain the mixed dry thin film of egg PC and *C. asiatica* extract. And then, the dry film was dispersed into 2 ml of PBS (pH 7.4), and were sonicated for 15 min with a bath-type sonicator (Sonics & Materials). The concentration of *C. asiatica* extract was adjusted to 1 mg/ml. In parallel, *C. asiatica* extract were suspended in 30 % ethanol solution, absolute ethanol, or PBS (pH 7.4), following the same procedure as in preparing the liposomal suspension, and they are used as controls for the experiment of *in vitro* skin permeation.

2.4. Transmission Electron Microscopy

The suspensions of liposomes were negatively stained with freshly prepared phosphotungstic acid solution (2%, pH 6.8). The stained liposomal suspension was transferred onto a formvar/carbon coated grid (200 mesh) and it was air-dried at room temperature. The electron microphotographs were taken on an electron microscope (LEO-912AB OMEGA, LEO, Germany) with magnification of 100,000.

2.5. Dynamic Light Scattering

The size distributions of liposomal suspensions were obtained using a particle size analyzer (ZetaPlus 90, Brookhaven Instrument Co., USA). The content of liposome in the suspension was adjusted to 0.03%.

2.6. *In vitro* Permeation

Female hairless mice (type SKH) aged 6 weeks were sacrificed by cervical dislocation. The dorsal skin of each hairless mouse was excised and the adhering fat and other visceral tissue were removed. The skins were mounted onto Franz diffusion cells (0.636 cm^2 surface area) equipped with 5 ml receptor compartment. PBS (pH 7.4) was used as the receptor content, thermostated to 37°C under stirring. 200 μl, of *C. asiatica* extract suspensions were applied onto the skins and then the receptor solutions, 300 μl, were assayed for asiaticoside using HPLC at the predetermined time. The asiaticoside assay was performed in a liquid chromatograph (M600E, M7725i/Waters, 996PDA) equipped with a UV detector (0.05 AFUS). A reversed phase column (4.4 × 250 mm, C18 5 μm, XBridge™) was eluted with a mobile phase (acetonitrile/10 mM sodium phosphate, 1/1(v/v) at a flow rate of 0.5 ml/min and a sample of 10 μl was injected. The detection wave length was 210 nm.

3. RESULTS AND DISCUSSION

3.1. HPLC

Panel A in Figure 1 shows elution profile of asiaticoside reagent (1 mg/ml). The most intensive peak was observed around retention time of 17.8 min and it corresponds to asiaticoside. The other peaks would come from impurities contained in the reagent. A calibration curve was determined by diluting the reagent with distilled water and the formula was $A = 5898.9C - 98$, where A is the peak area and C is concentration in μg/ml. Panel B shows elution profile of methanol extract. When compared with the elution profile of asiaticoside reagent, the peak around 17.12 min in panel B could be ascribed to asiaticoside. Accordingly, it is assured that methanol extract contains asiaticoside.

3.2. Transmission Electron Microscopy

Figure 2 shows transmission electron microphotographs of liposomal suspension of *C. asiatica* extract. Multi-lamellar vesicles (MLVs) were observed along with small unilamellar vesicles (SUVs) (panel A). Panel B is the photo taken by focusing on SUVs. The diameter of MLVs was hundreds of nanometer and that of SUVs was less than 50 nm. By a sonication method, in general, SUVs are obtained and the liposomal suspension is homogeneous in terms of size [15]. The

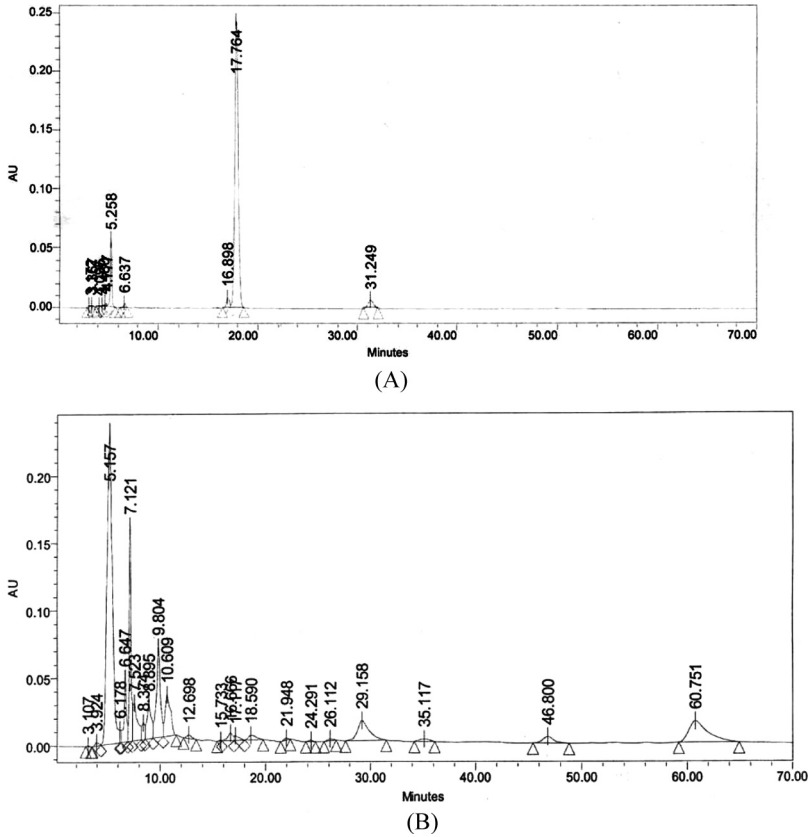

FIGURE 1 Elution profile of asiaticoside (A) and methanol extract of *C. asiatica* (B) in a high performance liquid chromatography.

liposomes on the photo, however, were inhomogeneous. This is possibly because a mild sonic energy was used in our sonication process to avoid the degradation of biologically active ingredients of the plant extracts.

3.3. Dynamic Light Scattering

Figure 3 shows the size distributions of liposomal suspension of *C. asiatica* extract. Two populations were observed. The small sized-population was less than 50 nm and it was thought to be SUVs. The population of 100 nm–300 nm would be MLVs. According to the electron microphotographs (Fig. 2), SUVs and MLVs were observed. SUVs are thought to be responsible for the small-sized populations

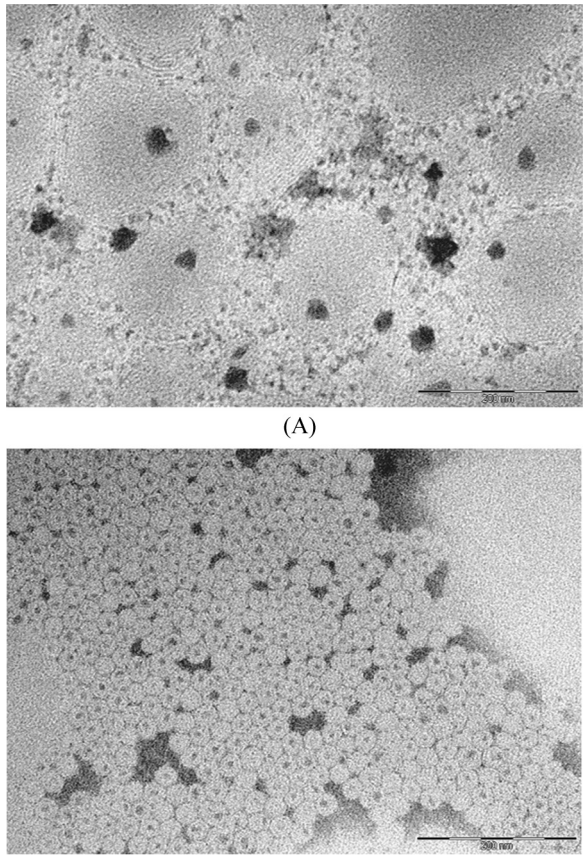

FIGURE 2 Transmission electron microphotographs of egg PC liposomal suspension of *C. asiatica* extract. Panel (A) shows MLVs coexisting with SUVs, and Panel (B) is a photo focusing on SUVs. Bars in panel (A) and panel (B) represent 200 nm.

and MLVs could give a rise to the larger populations. In fact, it was reported that the size of SUVs is tens of nm, and that of MLVs is hundreds to thousands of nm [16,19].

3.4. *In vitro* Permeation

Figure 4 shows the effect of vehicles on the amount of asiaticoside transported across hairless mouse skins. The flux was proportional to time elapse, and the value at 18 hr ranged from 1.8 to 4.6 mg/cm^2/hr,

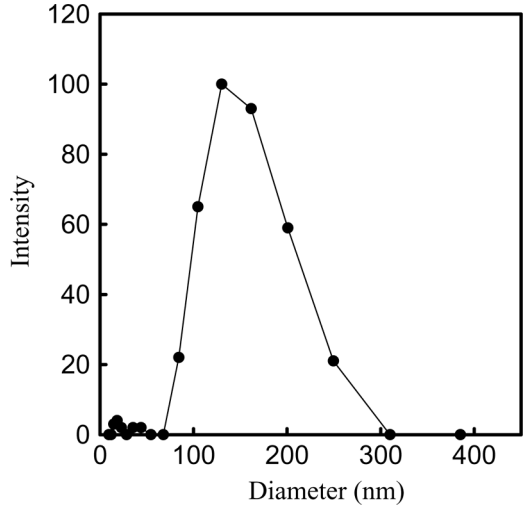

FIGURE 3 Size distributions of egg PC liposomal suspension of *C. asiatica* extract.

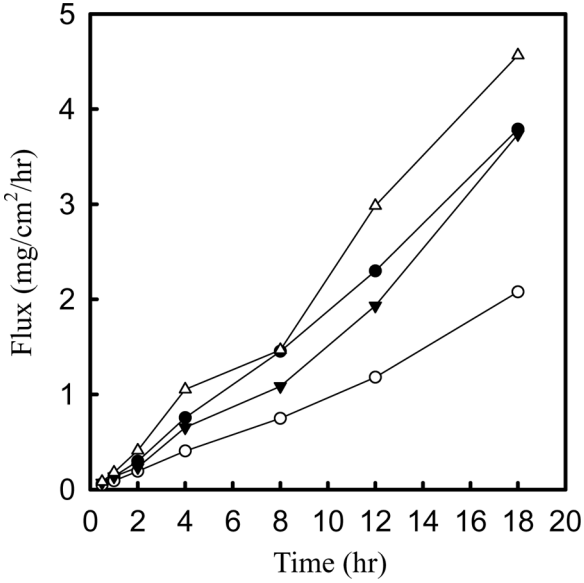

FIGURE 4 Effect of vehicles on amount of asiaticoside transported across hairless mouse skin. △: Liposomal suspension; ●: Suspension in PBS; ▼: Suspension in absolute ethanol; ◯: Suspension in 30% ethanol solution.

depending on the vehicles. The most effective vehicle for the skin permeation was liposome. The liposome is composed of phospholipids, which is a major component of biological membrane. It was reported that liposomes interact with skin and they penetrate into epidermis and even into dermis [17]. And, it has been known that nanoparticles less than 50 nm can penetrate into skin through a space between coenocytes [18]. In addition, the phase transition temperature of egg PC liposomes is below 0°C and thus the membrane is in the liquid crystal state [19]. In this circumstance, liposomes are flexible in their shape and take amoeba-like motion. Therefore, even thought the size is hundreds of nm like MLVs, liposomes could fit their shape into paracellular space. These may account for why liposome enhanced the *in vitro* permeation of asiaticoside. On the other hand, PBS was more effective in the permeation enhancement than 30% ethanol solution and absolute ethanol were. When ethanol is included in vehicle (this is the case of ethanol solution and absolute ethanol), two conflicting factors might be involved in the skin permeation. One is the thermodynamic activity of asiaticoside in the alcoholic vehicles and the other is the permeation-enhancing effect of ethanol. Asiaticoside is readily soluble in ethanol so that the thermodynamic activity of asiaticoside in the alcoholic vehicles would be much lower than that of asiaticoside in aqueous phase. As a result, asiaticoside would have a high tendency to stay in the alcoholic vehicles and it would hardly penetrate into skin. This may account for why permeability is lower when ethanol is included in the vehicles of asiaticoside. Although ethanol is reported to play a role as a skin permeation enhancer [20], the effect of the thermodynamic activity might be more dominant than the permeation-enhancing effect. Therefore, ethanol is likely to be poor enhancer for asiaticoside.

4. CONCLUSIONS

In summary, four kinds of suspensions of *C. asiatica* extract, namely, egg PC liposomal suspension, suspensions in PBS, 30% ethanol solution, and absolute ethanol, were prepared by a sonication method. According to the results of the *in vitro* skin permeation study, the liposome turned out to enhance the permeation of asiaticoside when compared with the permeation of asiaticoside in PBS. On the contrary, 30% ethanol solution and absolute ethanol were poor vehicles in terms of the skin permeation of asiaticoside. Egg PC liposomes could be clinically used as a permeation enhancer of asiaticoside.

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