Korean J. Chem. Eng., **31**(3), 485-489 (2014)
DOI: 10.1007/s11814-013-0232-3

pISSN: 0256-1115
eISSN: 1975-7220

Enhanced skin delivery and characterization of rutin-loaded ethosomes

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(*Received 31 July 2013 • accepted 30 October 2013*)

Abstract−We formulated rutin-loaded ethosomes and compared their vesicle size, loading efficiency, stability, and elasticity. In addition, an *in vitro* skin penetration experiment was performed. The results of the study indicated that ethosomes loaded with 0.005-0.03% rutin were stable during the four-week study period. Among them, those loaded with 0.03% rutin showed a vesicle size (190.20±14.57 nm) and loading efficiency (73.77%±0.01%). Therefore, the *in vitro* skin penetration experiment was performed using 0.03% rutin-loaded ethosome. Compared to an ethanolic solution of rutin (44.16%), rutin-loaded liposome (37.80%), and a distilled water (DW) solution of rutin (18.31%), rutin-loaded ethosome (93.31 μg; 61.30%) was superior in delivering rutin. Therefore, we suggest that ethosome loaded with 0.03% rutin enhance the skin penetration of rutin and may be used as a drug delivery system for natural cosmetic materials such as flavonoids.

Keywords: Ethosome, Rutin, Skin Penetration, Cosmetic Material, Elasticity

INTRODUCTION

The skin is exposed to various environmental stressors such as UV rays, pollution, and bacteria. These stressors promote aging by damaging skin components such as collagen, elastin, and DNA [1-4], consequently leading to the generation of reactive oxygen species (ROS). Although there are antioxidant barrier systems, enzymatic antioxidants, and nonenzymatic antioxidants in the human body, excessive production of ROS challenges these natural antioxidant defenses. Therefore, external supplementation of nonenzymatic antioxidants becomes essential. Typical nonenzymatic antioxidants such as vitamin E, vitamin C, and flavonoids can be obtained from plant sources. Flavonoids, polyphenolic, have multiple physiological properties including anti-inflammatory, antioxidant, and skin-whitening effects [5,6]. Most of the naturally occurring flavonoids exist as glycosides. Rutin, a glycoside of rutinose and quercetin, is a commonly found flavonoid. It has been implicated in blocking the generation of free radicals; in addition, it possesses free-radical-scavenging effects [7,8]. However, rutin has poor water solubility, which makes its formulation difficult. In addition, its absorption across the epidermis is limited. To address these issues and to effectively deliver rutin, an effective drug delivery system is essential. The stratum corneum, which is the outermost layer of the skin, plays a protective role to prevent the direct entry of substances into the skin [9]. The stratum corneum is made up of keratinocytes and intracellular lipids like brick and mortar. Since intracellular lipids exist as a bilayer, the structures of the drug delivery vesicles need to be similar to that of the lipid bilayer for effective delivery of active components into the skin. Therefore, liposomes were developed as transdermal drug delivery systems. Because liposomes consist of phospholipid biomaterials, they are very biofriendly and capable of delivering many active components into the skin. Because of this advantage, liposomes have been widely used in medicine and cosmetics. However, traditional liposomes have poor skin-penetration ability [10-12]. Recently, novel vesicular carriers have been developed in an attempt to resolve this problem. These novel drug delivery systems, including ethosomes and elastic liposomes, increase the membrane fluidity of the lipid bilayer of the skin to increase drug delivery across the narrow spaces of the keratinocytes [13-18]. Ethosomes are phospholipid vesicles containing a high concentration of ethanol. Ethanol is an efficient penetration enhancer that increases membrane flexibility by penetrating the gaps in the lipid membrane. Ethosome vesicles have been reported to be highly attracted to the skin and to possess higher membrane flexibility than that shown by liposomes [19,20,28].

Therefore, our objective was to formulate rutin-loaded ethosomes in order to evaluate their drug delivery across the skin. In addition, we examined the applicability of rutin-loaded ethosomes for delivering cosmetic materials.

MATERIALS AND METHODS

1. Materials

L-α-phosphatidylcholine from egg yolk (egg PC, ~60%) and rutin were obtained from Sigma (USA). Solvents such as ethanol and chloroform that were used of extra pure grade. Preparation of ethosomes required the use of a rotary evaporator (BUCHI, Switzerland) and a probe sonicator (BRANSON, USA). Ethosome diameter was measured using an ELS-Z (Otsuka, Japan). Skin penetration studies used a Franz diffusion cell kit (Permegear, USA) including a 9 mm Franz diffusion cell (receptor volume 5 mL) and V6A model stirrer. Calculation of ethosome loading efficiency and rutin skin penetration was performed using HPLC (high performance liquid chromatography, Shimadzu, Japan).

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2. Preparation of Ethosome

Ethosomes were prepared according to the thin-film hydration method [19-22]. Egg PC (2%, w/v) and rutin (0.005-0.05%, w/v) were dissolved in chloroform in a 50 mL round-bottomed flask. The mixture was evaporated in a rotary evaporator and solvent traces were removed. The film was hydrated with ethanolic solution (20% ethanol, v/v) at above the lipid transition temperature for 1 h. The vesicle suspension was dispersed using a probe sonicator at 30 min.

3. Preparation of Liposomes

Liposomes were prepared according to the thin-film hydration method [20-23]. Egg PC (2%, w/v) and rutin (0.03%, w/v) were dissolved in chloroform in a 50 mL round-bottomed flask. The mixture was evaporated in a rotary evaporator and solvent traces were removed. The film was hydrated with distilled water at above the lipid transition temperature for one hour. The vesicle suspension was dispersed using a probe sonicator at 30 min.

4. Measurement of Vesicle Size

Vesicle size in ethosomal and liposomal suspensions was measured with an ELS-Z instrument. Vesicles suspended in liquids are in Brownian motion due to random collisions with solvent molecules. The diffusion coefficient is inversely proportional to the vesicle size according to the Stokes-Einstein equation. Through dynamic light scattering, the fluctuations in the intensity of scattered light from vesicles in Brownian motion over can be measured. As vesicles in Brownian motion move about randomly, the scattered intensity fluctuations are random. The fluctuations of the scattered light were analyzed by using an autocorrelation function. The CONTIN method was used to resolve vesicle size distributions from measured autocorrelation functions. The CONTIN method takes into account the weighting of the distribution due to the use of discrete data points in the continuous distribution and then calculates different moments of the computed distribution.

5. Measurement of Loading Efficiency

Undissolved rutin was removed from a 1 mL volume of the ethosomal and liposomal suspension using a 1.2 μm filter (Minisart; CA, 26 mm). Vesicles were then degraded with 15 mL ethanol, and the ethanol was evaporated in a rotary evaporator. The rutin was then re-hydrated in 1 mL ethanol. To measure free rutin amount in the ethanolic solution or in the distilled water, the same amount of rutin was dissolved in the ethanolic solution or in the distilled water, and undissolved rutin was removed using a 1.2 μm filter. The samples were then measured by HPLC at 355 nm of the rutin maximum absorbance wavelength.

The following Eq. (1) was used to calculate the loading efficiency at ethosome.

$$\text{Loading efficiency } (\%) = \{(C_P - C_E)/C_0\} \times 100 \quad (1)$$

where, C_P is rutin passed through the 1.2 μm filter, C_E is free rutin in the 20% ethanolic solution, and C_0 is initially added rutin.

Eq. (2) was used to calculate the loading efficiency at liposome:

$$\text{Loading efficiency } (\%) = \{(C_P - C_D)/C_0\} \times 100 \quad (2)$$

where, C_P is rutin passed through the 1.2 μm filter, C_D is free rutin in the distilled water, and C_0 is initially added rutin.

6. Elasticity Measurement

The elasticity of vesicle membranes was determined by extrusion using a mini-extruder as described previously [23-25]. The ethosomal and liposomal suspensions were extruded through polycarbonate membranes of a definite pore size (80 nm) by applying a pressure of 0.2 MPa for one minute. Vesicle size was monitored before and after filtration by using ELS-Z. The elasticity of the vesicle membranes was expressed using the following formula (3):

$$E = J_{flux} \times (r_v/r_p)^2 \quad (3)$$

where, E is the elasticity of the vesicle membrane, J_{flux} is the amount of suspension that has passed through the membrane, r_v is the vesicle size (after extrusion), and r_p is the pore size of the barrier.

7. *In Vitro* Skin Penetration Studies

Skin penetration studies were used to determine the effect of increasing skin penetration of rutin in the ethosomal delivery system. Outbred albino ICR mice (8 weeks old, female) were used. Mouse skin, including epidermis and dermis, was taken from the dorsal surface. Experiments were run in Franz diffusion cells with a receptor compartment volume of 5 mL. The receptor compartment contained a receptor phase (HCO-60 : ethanol : phosphate buffered saline (PBS) =2 : 20 : 78 [w/w/w%]), and the skin was fixed between the donor and the receptor phase of the stratum corneum side facing upward into the donor compartment. The temperature was maintained at 37±1 °C. Samples were applied to the skin in the donor compartment. Samples were withdrawn through the sampling port of the diffusion cell at 2, 4, 8, 12, and 24 h. The receptor phase was immediately replenished with an equal volume of fresh receptor phase. The rutin in withdrawn samples was analyzed by HPLC at 355 nm. The amount of rutin retained in skin was determined at the end of the *in vitro* penetration experiment (24 h). The skin was washed three times with PBS. The remaining skin was analyzed for rutin quantity in the stratum corneum by tape stripping [26,27]. After tape stripping, the skin was cut into small sections with scissors. The tape and skin were added to 10 mL ethanol. The solution was evaporated in a rotary evaporator using a hydrated receptor phase. The rutin quantity was then analyzed by HPLC at 355 nm.

8. HPLC Assay

The amount of rutin was determined by HPLC assay using 2% acetic acid in water : 0.5% acetic acid in 50% acetonitrile (90 : 10-40 : 60, gradient [v/v%]) mixture as mobile phase and was delivered at a flow rate of 1 mL/min. 20 μL injection was eluted in C18 column (4.6×250 mm, Shim-pack VP-ODS, Shimadzu) at room temperature. The column eluent was monitored at 355 nm using SPD-M20A diode array detector (Shimadzu). Rutin peaks were separated with a retention time of 16 min.

9. Statistical Analysis

All reported data are presented as mean±S.E.M. Statistical significance was determined by Student's *t*-test.

RESULTS

1. Vesicle Sizes of Rutin-loaded Ethosomes

Ethosomes containing 0.005-0.05% of rutin were prepared. They were stable and monodisperse vesicles until a loading dose of 0.03% of rutin was used (data not shown). Vesicle sizes were determined; large rutin-loaded ethosomes were 189-225 nm, whereas empty ethosomes were 163.3±2.0 nm. Vesicular size increased inversely with rutin concentration (Fig. 1). In a previous study, the vesicle size of 0.03% quercetin-loaded ethosome was found to be 126.8 nm, which

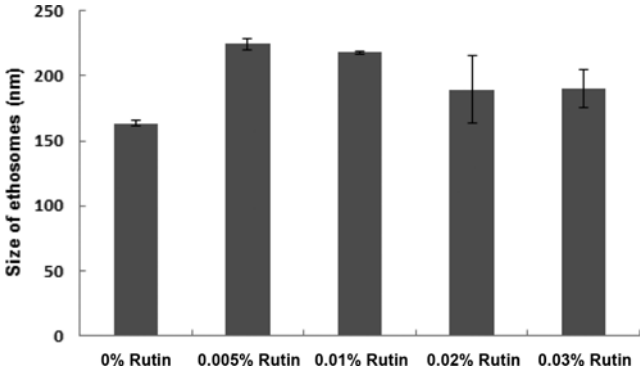

Fig. 1. Vesicle size of ethosomes with varying concentrations of rutin in a system composed of 2% egg PC and 20% ethanol.

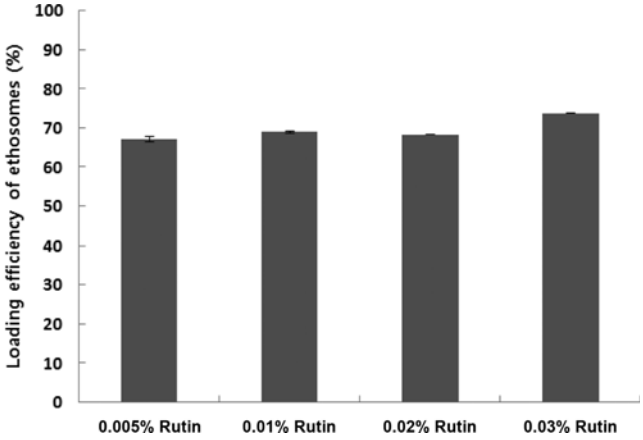

Fig. 2. Loading efficiency of ethosomes according to the concentration of rutin.

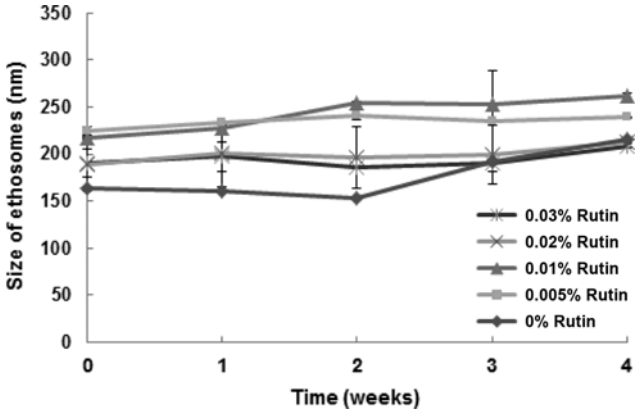

Fig. 3. Changes in the vesicle sizes of ethosomes with varying rutin concentrations during the 4-week study period.

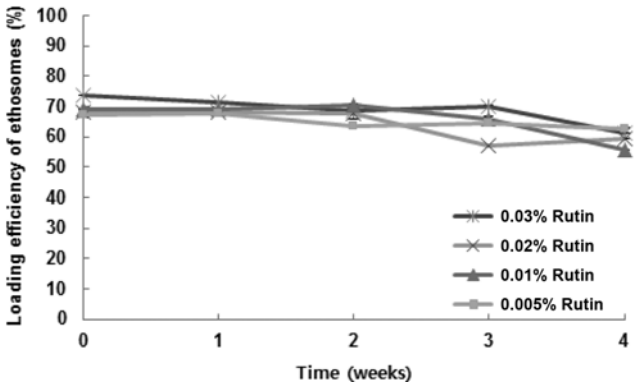

Fig. 4. Changes in the loading efficiency of ethosomes with varying concentrations of rutin.

is 63 nm smaller than that of the 0.03% rutin-loaded ethosome [28]. These results point to the increased vesicular size of glycoside-loaded ethosomes.

2. Loading Efficiency of Rutin-loaded Ethosomes

The loading efficiency of 0.005-0.03% rutin-loaded ethosomes was measured (Fig. 2). The mean loading efficiency was found to be above 65%, which indicates a correlation between loading dose of rutin and loading efficiency. The loading efficiency of 0.03% rutin-loaded ethosome was the highest (73.77%±0.01%). When the loading efficiency of 0.03% rutin-loaded ethosome was compared to that of 0.03% quercetin-loaded ethosome (57.61%), the 0.03% rutin-loaded ethosome were found to have larger vesicle sizes and higher loading efficiency [28].

3. Stability of Rutin-loaded Ethosomes

To determine the stability of rutin-loaded ethosomes, we visually inspected the ethosomes and measured vesicle size and loading efficiency. The vesicle sizes of 0.005-0.03% rutin-loaded ethosomes increased to 6.52-20.10% (Fig. 3) during the four-week study period, whereas the loading efficiency decreased in the range of 6.4-19.3% (Fig. 4). Therefore, we attributed the four-week stability of ethosomes to the continued integrity of their lipid bilayer, relatively equality in vesicle size, and slow release of rutin [29].

4. Characterization of Rutin-loaded Ethosomes and Liposomes

Based on data on vesicle size, loading efficiency, and four-week stability, the optimal concentration of rutin was determined to be 0.03%. Therefore, this formulation was used in *in vitro* skin penetration experiments. First, 0.03% rutin-loaded ethosome were formulated. Then, they were compared with 0.03% rutin-loaded liposome for vesicle size, loading efficiency, and elasticity (Table 1). The vesicle size and loading efficiency of 0.03% rutin-loaded liposome were respectively two-fold smaller and 12% higher compared to the corresponding values for 0.03% rutin-loaded ethosome. However, elasticity of 0.03% rutin-loaded ethosome was 2.61 times higher than that of 0.03% rutin-loaded liposome. These physical characteristics were considered attributable to the ethanol present within the ethosomes; ethanol increases vesicle size and elasticity.

Table 1. Physical characteristics of ethosome loaded with 0.03% rutin

	Vesicle size (nm)	Loading efficiency (%)	Elasticity
0.03% Rutin-loaded ethosome	190.20±14.57	73.77±0.01	9.75±0.30
0.03% Rutin-loaded liposome	92.40±2.28	82.62±0.34	3.74±0.29

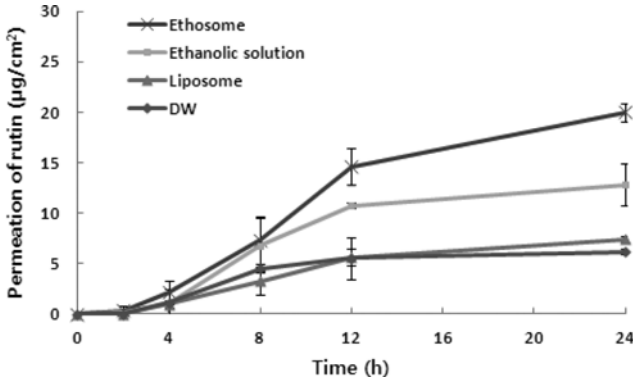

Fig. 5. Comparative cumulative transdermal delivery of rutin from ethosome, liposome, ethanolic solution, and distilled water (DW) over 24 h into the mouse dorsal skin.

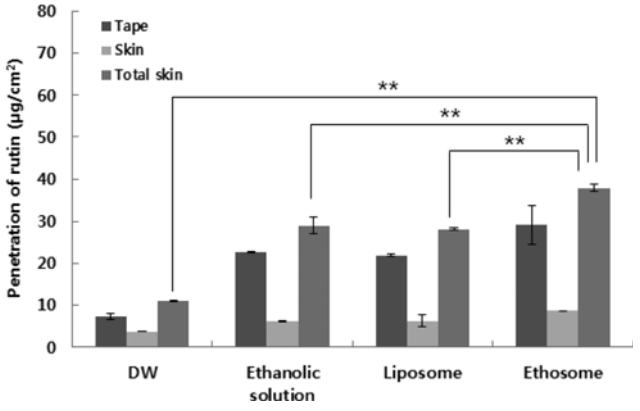

Fig. 6. Deposotion of rutin from ethosome, liposome, ethanolic solution, and distilled water (DW) into the mouse dorsal skin after 24 h (tape: stratum corneum; skin: dermis+epidermis without stratum corneum). *p<0.01.

5. *In Vitro* Skin Penetration Studies

Ethosome containing 0.03% rutin were used for the skin penetration studies. The three controls used in the study were 0.03% rutin-loaded liposome, 0.03% rutin hydrated in ethanolic solution, and 0.03% rutin hydrated in distilled water (DW). The *in vitro* skin penetration studies were performed to determine the 24 h penetration of rutin into the skin, which is shown in Fig. 5. A time-dependent increase in rutin penetration across the skin was observed. After 24 h, the skin permeation of rutin was the highest with ethosome; rapid absorption was seen between 0 h and 12 h. Deposition of rutin across the stratum corneum (divided tape) and the dermis and was measured (Fig. 6). In addition, the transdermal permeation of rutin was evaluated. The amount of rutin used for the evaluation of penetration characteristics was 93.31 μg/cm^2 in every system. The amount of rutin deposited in the stratum corneum increased in the following order: ethosome (31.22%; 29.13±4.48 μg/cm^2)>ethanolic solution (24.31%; 22.68±0.26 μg/cm^2)>liposome (23.45%; 21.88±0.33 μg/cm^2)>DW solution (7.81%; 7.29±0.70 μg/cm^2). The amount of rutin deposited in the epidermal and the dermal layers (excluding the stratum corneum) increased in the following order: ethosome (9.30%; 8.68±0.03 μg/cm^2)>liposome (6.78%; 6.33±1.34 μg/cm^2)>ethanolic solution (6.57%; 6.13±0.14 μg/cm^2)>DW solution (4.05%; 3.78±0.13 μg/cm^2). The transdermal penetration of rutin at 24 h increased in the following order: ethosome (21.43%; 20.00±0.88 μg/cm^2)>ethanolic solution (13.76%; 12.84±2.04 μg/cm^2)>liposome (7.96%; 7.43±0.24 μg/cm^2)>DW solution (6.64%; 6.20±0.23 μg/cm^2). Penetration of rutin was high except in the epidermal and the dermal layers of the skin; therefore, ethanol can be thought of as an efficient penetration enhancer that promotes drug delivery across the stratum corneum. In addition, we evaluated the total penetration of each of the formulations (Fig. 7). The total penetration of rutin was shown to be the highest for ethosome (61.30%; 57.81±4.12 μg/cm^2), followed by the ethanolic solution (44.16%; 41.64±4.80 μg/cm^2), liposome (37.80%; 35.64±2.97 μg/cm^2), and DW solution (18.31%; 17.27±2.05 μg/cm^2). In a previous study, the drug delivery rate of ethosome containing quercetin was 36.9%, which is 1.66 times lower than that seen with rutin-loaded ethosome [28]. These results indicate that ethosomes effectively deliver flavonoidal glycosides, which show poor absorption across the epidermis. Despite ethosomes being smaller and having lower loading efficiency, the amount of rutin delivered by the ethosome was much higher than that delivered by liposome. Such an effect is attributable to ethanol present in the formulation and to the higher elasticity of ethosome compared to liposome.

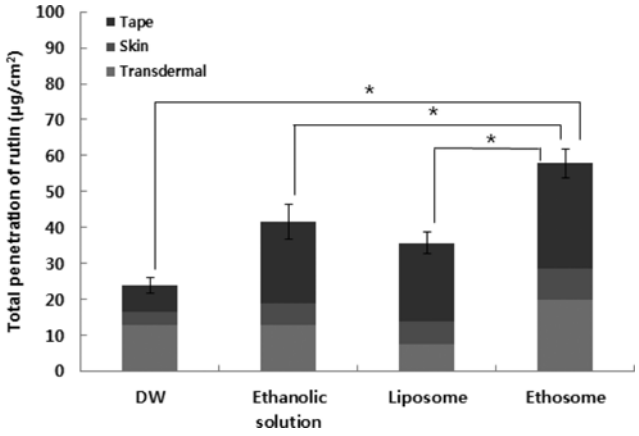

Fig. 7. Total penetration of rutin from ethosome, liposome, ethanolic solution, and distilled water (DW) into the mouse dorsal skin after 24 h (tape: stratum corneum; skin: dermis+epidermis without stratum corneum; transdermal: permeation through the skin). **p<0.05.

DISCUSSION

In our previous study, we evaluated quercetin-loaded ethosomes and compared the drug delivery characteristics of quercetin-loaded ethosomes with those of rutin-loaded ethosomes. Ethosomes were stable until rutin concentration reached 0.03%, whereas ethosomes containing quercetin were stable up to a concentration of 0.04%. The vesicle size of rutin-loaded ethosome was 190.2 nm and that of quercetin-loaded ethosome was 126.77 nm. The rationale behind these results would be that rutin is highly polar compared to quercetin, and therefore occupies a hydrophilic position within the ethosomes. The loading efficiency of rutin-loaded ethosome (73.77%) is higher than that of quercetin-loaded ethosome (57.61%); this is attributable to the higher solubility of quercetin in 20% ethanol. Simi-

larly, the penetration ability of rutin-loaded ethosome was greater than that of quercetin-loaded ethosome (61.3% vs. 36.9%). Most of the quercetin-loaded ethosome were deposited in the stratum corneum (23.5%), whereas rutin-loaded ethosome penetrated into the deep layers of the skin (epidermis and the dermis). Therefore, we confirmed that ethosomes are effective carriers of rutin into the skin [28].

CONCLUSIONS

Rutin, a well-known antioxidant, was formulated with ethosomes for its effective delivery across the skin. Rutin-loaded ethosomes formed monodisperse vesicles until the rutin concentration was 0.03% and were stable for four weeks. Among them, ethosome that had the highest loading efficiency (73.77±0.01%) was chosen for further study. Rutin-loaded ethosome were compared with 0.03% rutin-loaded liposome. Although the vesicle size and loading efficiency of liposome were higher than those of ethosome, ethosome was highly elastic compared to liposome. In the *in vitro* skin penetration experiment, the amount of rutin delivered into the skin was 61.30% (57.81 ±4.12 μg/cm^2) of the total amount (93.31 μg/cm^2) formulated. Skin penetration of rutin was the highest with ethosome, followed by liposome (44.16%), ethanolic solution (37.80%) and distilled water (18.31%). Therefore, we confirm ethosomes to be an effective formulation for the delivery of rutin into the skin. Furthermore, ethosomes can be used for the delivery of antioxidant cosmetics such as flavonoids.

ACKNOWLEDGEMENT

This study was supported by a grant of the Korean Healthcare technology R&D Project, Ministry of Health & Welfare, Republic of Korea (Grant No.: A103017).

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