Liposome-Containing *Hibiscus sabdariffa* Calyx Extract Formulations with Increased Antioxidant Activity, Improved Dermal Penetration and Reduced Dermal Toxicity

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Hibiscus sabdariffa Linn, or Roselle, is a medicinal plant used extensively in traditional Thai medicine since ancient times. The extracts of Roselle calyces possess antioxidant activity and have potential for development as active ingredients in cosmetic products. However, the limitations of using Roselle extracts in cosmetics are its low skin permeation and dermal irritation. Liposome technology is an obvious approach that might overcome these problems. Liposome formulations of standardized Roselle extracts were developed with various lipid components. The formulation showing the highest entrapment efficiency was selected for stability, skin permeation and dermal irritability studies. The liposome formulation with the highest entrapment efficiency (83%) and smallest particle size (332 mm) was formulated with phosphatidylcholine from soybean (SPC): Tween 80: deoxycholic acid (DA); 84:16:2.5 weight ratio, total lipid of 200 g/mL and 10% w/v Roselle extract in final liposomal preparation. This liposome formulation was found to be stable after storage at 4°C, protected from light, for 2 months. The in vitro skin permeation studies, using freshly excised pig skin and modified Franz-diffusion cells, showed that the liposome formulation was able to considerably increased the rate of permeation of active compounds in Roselle extracts compared to the Roselle extract solution. The in vivo dermal irritability testing on rabbit skin showed that the liposome formulation dramatically decreased skin irritability compared to the unformulated extract. These results showed that the liposomes containing Roselle extracts had good stability, high entrapment efficacy, increased skin permeation and low skin irritation.

Keywords: *Liposome, Antioxidant, Skin irritation, Hibiscus sabdariffa, Roselle*

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Anti-aging research has elucidated the role of reactive oxygen species in the pathogenesis of photoaging. Reactive oxygen species (ROS), including superoxide anion, peroxide and singlet oxygen, are generated when human skin is exposed to ultraviolet light. These ROS mediate their deleterious effects by causing direct chemical alterations of DNA, cell membranes and proteins, including collagen[1]. Anti-aging products are the most requested class of cosmetics at this time by the public. A nationwide market survey conducted in the USA has shown that 63% of the respondents view the presence of the antioxidant vitamins E and C, in skin care and sun care products, as important factors in their purchasing decision. Furthermore, bio-ingredients such as herb extracts have a growing importance in modern skin care[2]. There are large number of botanical antioxidants, since all plants need protection if they are to survive from oxidative stress following UV exposure in the environments in which they grow. These protective chemical mechanisms have evolved over the millennia, providing interesting phytochemicals that can be extracted and incorporated into cosmetic products for the protection of human skin. Antioxidant botanicals quench singlet oxygen and reactive oxygen species such as superoxide anions, hydroxyl radicals, fatty peroxy radicals and

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hydroperoxides[3]. Most botanical antioxidants fall in one of these three classes: flavonoids, carotenoids and polyphenols. Such bioactive compounds are reputed to fight against a wide spectrum of aging signs, such as wrinkling, skin drying, photoaging and skin pigmentation. Free radicals are the first and most common cause of skin aging, in particular photoaging and the reduction of this free radical injury in the epidermis is the most important strategy for a modern skin care products[2]. Thailand has many botanical and medicinal plants containing antioxidants, such as *Curcuma longa* L[4] and *Hibiscus sabdariffa* L or Roselle[5]. Thailand and China are in fact the largest exporter of Roselle calyces in the world, extracts of which find various uses worldwide. Use of such antioxidant containing plants for the development of high-value products (such as cosmetics) is vital for the development of the country.

Hibiscus sabdariffa L or Roselle belongs to the Malvaceae family. Its extracts contain flavonoid constituents, including crysanthemin, delphinidin-3-O-sambubioside, myricetin, hibiscitrin[6] and gossypitrin[7]. Moreover, Roselle calyces also contain phenylpropanoid compounds, such as orthocoumaric acid, paracoumaric acid, ferulic acid[6] and polyphenolic constituents such as anthocyanin and protocatechuic acid[8-13].

Tseng et al[10] reported that at the test concentration of 0.01 and 0.1 mg/mL, the protocatechuic acid isolated from Roselle calyx extracts scavenged about 58% and 82% of DPPH radicals in the solution, respectively. No toxicity was reported in rats after ingesting the aqueous extract of Roselle calyces in the dose range of 150-180 mg/kg per day, however, a higher human dose should be taken with caution, bearing in mind that it could affect the liver[14]. Roselle extracts were characterized by a very low toxicity with the LD_{50} of above 5,000 mg/kg in rats[15]. There was only one report suggested that excessive doses for relatively long period could have a deleterious effect on the testes of rats[15]. In Thailand, Roselle calyces are normally used for making cold drinks and jam and also used as traditional medicines, including as an astringent, antihypercholesterolemic, antidiabetic, diuretic, digestive, expectorant, stomachic, antihypertensive and for treatment of gallstones[15-17]. In addition, Roselle extract is one of several such plant extracts that have been used in cosmetics, such as skin toner/astringents and anti-aging skin care products. Although Roselle has GRAS status as a food ingredient, there is no clinical or scientific evidence for its recommendation in skin care products. Moreover, there is no information on what concentration levels in cosmetics that can safely be applied to human skin without skin irritation, efficacy studies are also lacking. Despite this lack of scientific data, a large number of cosmetic products are available worldwide containing Hibiscus calyx extract[18].

Since Roselle extracts contain several polar and acidic compounds, their chemical properties have hampered their use in skin products due to skin irritation and low skin permeation[19,20]. To improve these disadvantages, liposome technology was applied to formulate Roselle extracts such that they can be used in cosmetic products. Liposomes are widely used as carriers for a variety of chemical compounds and have application for topical drug delivery[21]. Liposomes not only enhance skin permeation, but also moisturize the skin. The composition and properties of liposomes play an important role in their interaction and possible penetration into the epidermis. Lipids, one class of components in liposome formulations, hydrate the skin, even in the absence of active ingredients. This is enough to improve skin elasticity and barrier function, which are among the main causes of skin aging[22]. In this study, various formulations of liposomes containing Roselle calyx extracts were prepared with different lipid compositions. The objectives of this study were to investigate the physical and chemical properties of the liposomes containing Roselle calyx extracts and evaluate their stability, antioxidant efficiency as well as dermal toxicity. The skin permeability and skin irritability of liposome formulations were compared with those of unformulated extract solutions, using *in vitro* and *in vivo* techniques.

Material and Method

Plant materials

Fresh calyces of *Hibiscus sabdariffa* L. (Roselle) were obtained from Amphur Jana, Songkhla province, Thailand in April 2004. The plant material was authenticated and a voucher specimen (No. SKP 1090819) has been deposited in the herbarium of the Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, Thailand.

Chemicals

Cholesterol (CHOL), phosphatidylcholine from dried egg yolk (EPC), phosphatidylcholine from soybean (SPC), Tween 80, stearylamine (SA), deoxycholic acid (DA), dicetyl phosphate (DP), a-tocopherol, butylate hydroxytoluene (BHT), 1,1-

Diphenyl-2-picrylhydrazyl (DPPH•), Triton X-100 were purchased from Sigma (St. Louis, MO, USA). Disodium salt dihydrate, chloroform, ethanol, potassium dihydrogen orthophosphate, sodium hydroxide were analytical reagent grade and were obtained from local commercial sources.

Preparation of dried extracts of Roselle calyces

Fresh Roselle fruits were washed with tap water three times, and the seeds were then removed to obtain fresh Roselle calyces. Fresh calyces of Roselle (5 kg) were boiled in water (30 L) for 15 minutes. The water extracts were filtered through a nylon cloth and then evaporated to dryness under vacuum at 40°C for 8-10 hours. This extract was placed in a vacuum desiccator for a further 2-3 days to ensure complete removal of moisture. The yield (calculated on the dried extract) was 4.1% w/w of the fresh Roselle calyces. The extract of Roselle was a dried powder with red color. Dried Roselle extract was packed in air-tight containers and kept in a vacuum desiccator at room temperature until required for further studies. The dried powdered extracts were determined for total phenolic and monomeric anthocyanin contents. In addition the LC-MS analysis of the extract was performed. Three active compounds, including cyanin-3-glucoside was identified by comparing with the authentic substances. These three compounds were also determined their content by HPLC, it found that cyanidin-3-glucoside was 2.39 mg/g.

Antioxidant assay of Roselle extract by the DPPH [23]

The Roselle extract was dissolved in absolute ethanol at various concentrations, 100, 50, 10, 5 and 1 mg/mL. A aliquot (100 mL) of each concentration of the extract was mixed together with the DPPH solution (6 x 10^{-5} M in absolute ethanol) in 96 well-plate for 30 minutes at room temperature. The decolouration of DPPH was determined by measuring absorbance values at 520 nm using a UV spectrophotometer (Spectronic Genesys^{TM}5, Miltol Roy Company, USA). Butylated hydroxytoluene (BHT), with same concentration of the test sample, was utilized as a positive control. Each sample was analysed in triplicate. The free radical scavenging activity of each sample was determined corresponding to the intensity of quenching of the DPPH color. The results are expressed as the percentage inhibition, calculated according to the following Eq (1):

$$\% \text{ inhibition} = [(A_{control}-A_{sample})/A_{control}] \times 100 \quad (1)$$

Where; $A_{control}$ is absorbance of DPPH solution without sample solution, $A_{control}$ is absorbance of DPPH solution with sample solution.

The antioxidant activity was represented by the EC_{50} value; the effective concentration of sample requires scavenging the DPPH radical by 50%. The value of EC_{50} was obtained by the linear regression analysis of dose response curve plotting between % inhibition and concentration.

Assay of total anthocyanins in Roselle extracts by the pH differential method [24]

A stock solution (5 mg/mL) of Roselle extract was freshly prepared in water. Two diluted solutions (both 1 mg/mL) were prepared from the stock solution. The first one was diluted with 0.025 M potassium chloride buffer pH 1.0 and the other was diluted with 0.4 M sodium acetate buffer pH 4.5. After equilibrium for 15 minutes at room temperature, 200 mL of each diluted sample were transferred to a 96-well plate and the UV absorbance was measured at 520 and 700 nm. The absorbance of the sample was calculated according to Eq (2):

$$A = (A_{520nm}-A_{700nm})_{pH\ 1.0}-(A_{520nm}-A_{700nm})_{pH4.5} \quad (2)$$

Where; A is absorbance of diluted sample, A_{520nm} is absorbance of diluted sample at 520 nm and A_{700nm} is absorbance of diluted sample at 700 nm.

Total monomeric anthocyanins in dried Roselle extract was calculated as cyaniding-3-glucoside according to Eq (3):

Monomeric anthocyanin pigment (mg/g) =

$$\frac{[(A \times MW \times DF \times 1{,}000)/(\varepsilon)]}{W} \quad (3)$$

Where; A is absorbance of diluted sample, MW is molecular weight of cyaniding-3-glucoside (449.2 g/mole), DF is dilution factor, ε is the molar absorptivity of cyanididn-3-glucoside (26,900 mole/L) and W is weight of Roselle dried extract (g), respectively.

Preparation of liposome formulations

Liposome dispersion samples were prepared by a modified ethanol injection technique[25]. Liposomes containing Roselle extract (10% w/v) were formulated by optimizing types and ratios of lipid composition, as well as the total lipid contents. Each material was accurately weighed and placed into two separate vessels, one for the lipid phase and another for the water phase. The lipid phase consisted of a mixture of lipid, additive, surface charge and the extract in 95% ethanol (10 mL). The water phase was acetate buffer pH 5.5 (10 mL). The temperature of both phases was

controlled at 60°C before mixing. Subsequently, the ethanol was removed under a rotary evaporator at 40°C. Liposomes formed spontaneously after the ethanol was removed. After removal of ethanol under vacuum, the volume was measured and adjusted to 10 mL with acetate buffer (pH 5.5).

Determination of entrapment efficiency

The entrapment efficiency was measured after separation of the non-entrapped Roselle extract using a dialysis method. The dialysis membrane (molecular weight cut off 3,500 dalton) was soaked in distilled water for 30 minutes. The suspension of Roselle liposome (1 mL) was transferred into the dialysis membrane using a pipette and then the top and bottom of the dialysis bag were clamped with medicut. The dialysis bag containing liposomes was stirred in 500 mL of distilled water using a magnetic stirrer at 300 rpm for 16 hours. The concentration of free Roselle extract was then determined by analyzing the total anthocyanin content in the solution outside the dialysis bag. Subsequently, the total antocyanin content in the non-dialysed Roselle liposome formulation was analyzed after disrupting the lipid vesicles using Triton X-100 (10%, 1:1). The percentage entrapment efficiency was calculated according to Eq (4):

$$\% \text{ Roselle extract entrapment efficiency} = 100\,[1-(RE_F/RE_T)] \quad (4)$$

Where; RE_T is the total amount of anthocyanin in the liposome suspension and RE_F is the anthocyanin content in the 500 mL dialysis fluid (*i.e.* un-entrapped Roselle extract).

Determination of vesicle size

Diameters of the lipid vesicles were determined using photon correlation spectroscopy employing a Zetasizer (Malvern Instruments, Malver UK). Samples were diluted with distilled water which were previously filtered through 0.2 mm membrane filter to minimize interference from particulate matter.

Stability study

Liposome formulations were evaluated for their entrapment efficiency and physical properties, such as color and appearance, after storing at 4°C for 2 months, comparing to values for the freshly prepared liposome formulations.

In vitro skin permeation study

Newborn pigs that had died of natural causes shortly after birth were obtained fresh from a local pig farm (Songkhla, Thailand). Full thickness flank skins of newborn pigs weighing 1.4-1.8 kg were used. The epidermal hair at the flank area was clipped with an electric hair clipper as close as possible to the skin without causing any damage and the skin carefully excised with a scalpel. The subcutaneous fat and underlying tissues were carefully removed from the dermal surface[26]. Studies were performed in a modified Franz-diffusion cell. Liposomes (2.5 g) were put in the donor compartment on top of the skin. The receptor compartment was filled with 12 mL acetate buffer pH 5.5, thermoregulated with a water bath at 37°C and magnetically stirred at 400 rpm, with effective skin area 0.79 cm^2. Samples (1 mL) were withdrawn at 0.5, 1, 2, 3, 4, 8, 10 and 12 hours. An equal volume of fresh acetate buffer was immediately added to the receptor solution after each sampling. Concentration of Roselle extract in acetate buffer of the receptor compartment was determined by measuring the antioxidant activity using the DPPH assay. Aliquot of each sample (100 μg/ml) were subjected to this assay exactly as described before for the assay of Roselle extract. The % inhibition of each sample was calculated as previously described following Eq (1).

In vivo skin irritation testing

Skin allergy and irritation test was determined follow in the test guideline (TG) No. 404 of OECD guidelines for testing of chemicals (2001)[27]. White rabbits (New Zealand) were used for this testing. The animal ethical committee of Prince of Sonkla University approved this study. The epidermal hair at the back area was removed in the range of 10 x 10 cm^2. A 0.5 mL of sample (10% w/v of either liposome or un-formulated Roselle solution) was poured on the gauze patch size 2.5 x 2.5 cm^2 and applied on the hairless area of the back of rabbit and the gauze patch was covered with transpore adhesive tape for 4 hours. Distilled water 0.5 ml was used as control. The sample patch was removed after 4 hours and the skin area of application was cleaned. If any irritation of skin was observed, the level of erythema and oedema at any time (1, 24, 48 and 72 hours) was recorded using guideline given in Table 1.

Statistical analysis

Results are expressed as the mean ± SD of at least three experiments. Analysis of variance (ANOVA) was used to test the statistical significance of difference among groups. Statistical significance in the difference of the mean was determined by Dunnet's method or

Table 1. Level of erythema and oedema formation-Guideline for recording skin allergy and irritation

Erythema and eschar formation	Level	Oedema formation	Level
No erythema	0	No oedema	0
Very-slight erythema (barely perceptible)	1	Very slight oedema (barely perceptible)	1
Well-defined erythema	2	Slight oedema (edges of area well-defined by definite raising)	2
Moderate to severe erythema	3	Moderated oedema (raised approximately 1 mm)	3

Guideline (TG) No. 404 of OECD for testing of chemical (2001)[27]

Table 2. Particle size and % entrapment efficiency of liposomes of various compositions and various total amount of lipid

Compositions	Total amount of lipid (mmol/ml)	Particle size (nm) Mean $\pm$ SD (n = 3)	% entrapment (n = 3)
SPC: CHOL(7:2, molar ratio)	20	583 $\pm$ 176	6 $\pm$ 0.2
	100	757 $\pm$ 405	27 $\pm$ 2.5
	200	586 $\pm$ 158	76 $\pm$ 4.7
EPC: Tween 80(84:16, weight ratio)	20	251 $\pm$ 40	6 $\pm$ 0.4
	100	218 $\pm$ 61	18 $\pm$ 0.3
	200	327 $\pm$ 78	29 $\pm$ 3.6
SPC: Tween 80(84:16, weight ratio)	20	392 $\pm$ 66	6 $\pm$ 0.6
	100	303 $\pm$ 51	11 $\pm$ 0.5
	200	473 $\pm$ 120	43 $\pm$ 3.4

Roselle extract 10% w/v was used in all formulations of liposome

Student's t-test.

Results

Roselle was extracted by boiling their fresh calyxes in water for 15 minutes and then dried under vacuum at 40°C for 8-10 hours. The dried extracts of Roselle were attractively red in color with a typical odor of Roselle. The yield (based on the dried extract) was 4.1% by weight of fresh calyces. The extract had antioxidant activity (EC_{50}) at the concentration of 8.45 $\pm$ 0.35 mg/mL which was similar to antioxidant activity of the standard compound, BHT (EC_{50} = 9.14 $\pm$ 0.42 mg/mL). The amount of total monomeric anthocyanin pigments, calculated as cyaniding-3-glucoside in mg/g of dried Roselle extract was found to be 3.46 $\pm$ 0.12 (n = 6). A linear relationship between the monomeric anthocyanin content in Roselle extract and its DPPH activity was observed with the correlation coefficient (r^2) of 0.9525 as shown in Fig. 1.

Roselle extract (10% w/v) was incorporated in the formulations of liposome with various ratios of lipid composition and total lipid contents. All formulations were determined for their physicochemical properties including particle size and entrapment efficiency. The differences of lipid components, or ratios of phospholipids and cholesterol, or surfactants gave liposomes with different entrapment efficacy. Moreover, as the total lipid contents were increased, the entrapment efficacy of liposomes increased (Table 2). At the total lipid level of 200 mmole/mL, the liposome composed of SPC gave higher entrapment efficiency (76%) than those composed of EPC. Therefore, the liposome formulations of SPC were selected for further studied on the effect of surface charged (Table 3). All formulations of liposome containing Roselle extract appeared as the pink colloidal suspensions with the lipid vesicular size in range of 0.218-1.234 mm (Table 2 and 3). The formulations containing Tween 80 afforded liposomes with greater ease, and gave smaller vesicular size than those containing cholesterol because the latter formulations were more viscous. The uses of both positive and negative surface charge agents were able

Table 3. Particle size and % entrapment efficiency of liposomes, which gave entrapment efficiency of more than 50%

Compositions	Ratio		Particle size (nm) mean ± SD (n = 3)	% entrapment (n = 3)
	Molar	Weight		
SPC: CHOL	6:1	-	784 ± 168	67 ± 2.2
SPC: CHOL	4:1	-	1,243 ± 154	61 ± 5.7
SPC: CHOL: DP	4:1: 0.25	-	673 ± 84	65 ± 9.4
SPC: CHOL: DA	4:1: 0.25	-	729 ± 52	71 ± 12.4
SPC: CHOL: DA	7:1: 4	-	834 ± 47	87 ± 0.7
SPC: Tween 80	-	40:5	364 ± 22	50 ± 2.5
SPC: Tween 80: SA	-	40:5:1	412 ± 16	58 ± 4.4
SPC: Tween 80: DA		84:16:2.5	332 ± 17	83 ± 8.0

Roselle extract 10% w/v was used in all formulations of liposome

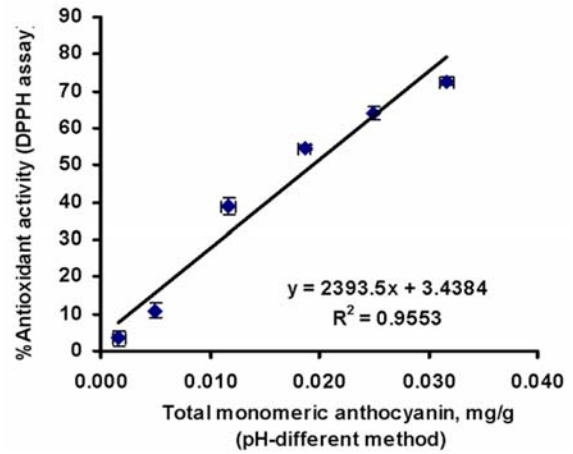

Fig. 1 Correlation between total monomeric anthocyanins (mg/g) determined by pH-differential method and its antioxidant activity determined by DPPH radical scavenging assay. The plotted data are means ± SD (n = 3). Solid line is a linear regression fit to the data

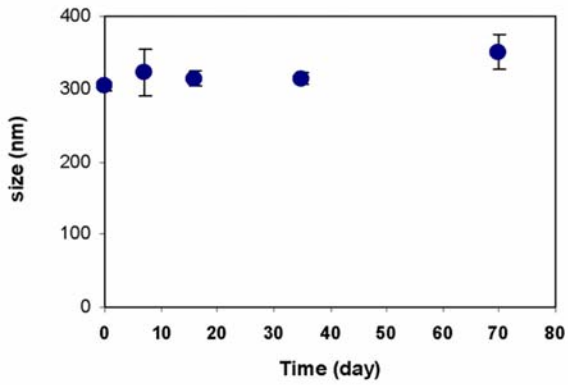

Fig. 2 Change in particle size (●) in liposomal preparation containing Roselle extract when stored at 4°C, protected from light for 2 months

to increase the entrapment efficiency of liposomes. The liposome containing SPC: CHOL (4:1) had 61% entrapment while the formulations with added DP or DA (0.25 molar ratio) had entrapment efficiency of 65% or 71%, respectively (Table 3). It was found that the most suitable liposome formulation with the highest entrapment efficacy (83%) and smallest particle size (332 mm) was composed of SPC: Tween 80: DA (84: 16: 2.5) by weight ratios. This formulation was therefore selected for further studies of its stability, skin permeation and skin irritation. The stability test of liposomes was performed for 2 months at 4°C under light protection. Its entrapment efficiency and particle size were evaluated during storage, and compared to the data for freshly prepared liposomes (Fig. 2 and 3). Fig. 2 depicts that, no significant change in the particle size of liposomes was observed after one month storage. However, after 2 months, the particle size of liposomes was slightly larger (~13%) due to the coalescence effects. Fig. 3 illustrates that entrapment efficiency of the Roselle liposome decreased about 10% after 2 months under the testing conditions.

The *in vitro* skin permeation study of the Roselle liposomes was evaluated, comparing the data to the results from an identical study antioxidant constituent in aqueous solutions of the extract. The studies were performed by measuring the antioxidant activity of the fluid in the receptor compartment of the modified Franz-diffusion cell at various time points from 0.5 to 12 hours. The active ingredients from the extract

were clearly transported through the skin and accumulated in the receptor compartment. The results, shown in Fig 4, indicate that the receptor fluid of the liposome formulation had antioxidant activity about two times higher than that of the diffusion studies using aqueous solution. This clearly indicates that the liposome formulation is able to deliver more of the active ingredients present in Roselle extracts through the skin.

The total monomeric anthocyanin contents of the Roselle liposome formulation and the water solution of Roselle extract was examined. It was found that the total anthocyanin content of the Roselle extract solution was 0.865 ± 0.03 mg, whereas that in the liposome formulation was 0.718 ± 0.02 mg. In the skin permeation study, although the percent antioxidant activity of the receptor fluid could not truly measure the amount of Roselle extract transported through the skin, it showed the efficiency of liposomes as a skin delivery system for active constituents in Roselle extract. The antioxidant activity of Roselle extracts was related to the total anthocyanin content via the linear correlation shown in Fig. 1. The liposome formulation had an advantage not only in enhancing skin permeation of the active constituents in Roselle extract, but also prolonging the antioxidant activity (Table 4 and Fig. 4). In addition, liposome formulation decreased the skin irritation of Roselle extract as shown in Table 5A and 5B. Skins of three rabbits were observed to show irritation with the score level 1

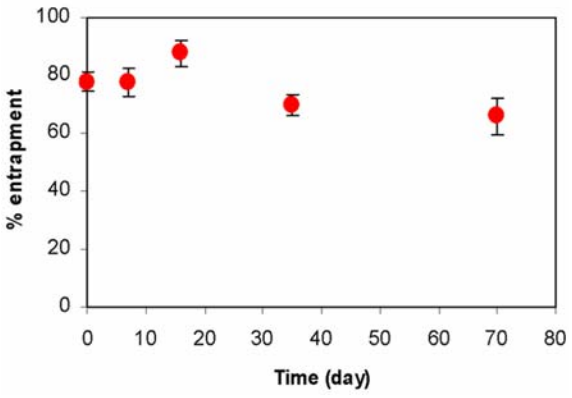

Fig. 3 Change in entrapment efficiency (●) in liposomal preparation containing Roselle extract when stored at 4°C, protected from light for 2 months

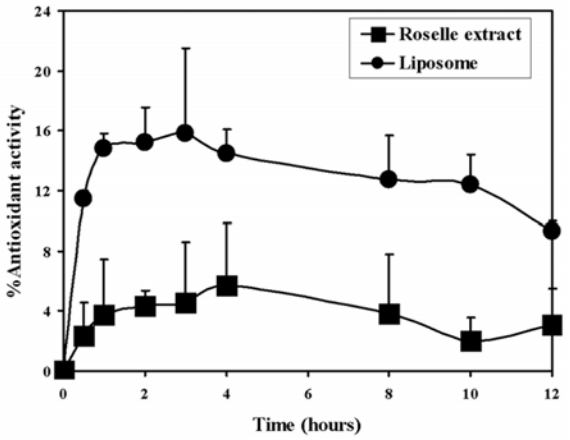

Fig. 4 Antioxidant activity of liposomal preparation containing Roselle extract (●) and aqueous solution of Roselle extract (■) after transport through pig skin at various time points from 0.5 to 12 hours using Franz-diffusion cell.

Table 4. Antioxidant activity of liposomes containing Roselle extract, and aqueous solution of Roselle extract after transport through pig skin using Franz-diffusion cell

Time (hours)	Liposomes containing Roselle extract (10% w/v) (% antioxidant activity)	Aqueous solution of Roselle extract (10% w/v) (% antioxidant activity)
0	0	0
0.5	11.14 ± 0.6	2.2 ± 2.4
1	14.9 ± 1.0	3.7 ± 3.8
2	15.2 ± 2.4	4.4 ± 1.0
3	15.8 ± 5.7	4.5 ± 4.5
4	14.5 ± 1.6	4.2 ± 5.7
8	12.0 ± 4.0	3.8 ± 4.0
10	14.3 ± 5.1	2.0 ± 1.6
12	8.8 ± 0.9	3.1 ± 2.4

All results are means ± SD n = 3 or greater

(slightly erythema). In two of these rabbits, the irritation disappeared within 1 hour after applying the liposome formulation, and in the third, it disappeared in 6 days. None of the rabbits had oedema formation on skin applied with the liposome formulation, whereas the skin of all rabbits applied with the 2% w/v Roselle extract showed well defined erythema and very slightly oedema after one hour. Moreover, the aqueous extract solution had still showed an effect on rabbit skin (very slightly erythema) right until the end of observation period (14 days).

Discussion

The water extract of *Hisbiscus sabdariffa* Linn (Roselle) is a strong free radical scavenger. It shows similar activity to BHT in the DPPH radical scavenging assay. With the EC_{50} value of 8.45 ± 0.35 mg/mL, Roselle extract can be considerate as one of the important sources for natural antioxidants. The active components of Roselle extract are polyphenolic compounds especially anthocyanin[8-13]. High linear correlation is found between the antioxidant capacity of Roselle extract and the anthocyanin contents. This result indicates that anthocyanins are one of major class of components that contribute to antioxidant capacity in Roselle extracts. Since anthocyanin is only stable in acidic solutions, the extract should be formulated in an acidic vehicle, but this acid solution may cause skin irritation and allergy. In addition, due to the hydrophilic nature of the constituents in Roselle extracts, they may not be able to penetrate into the lipophilic structure of the stratum corneum, an important protective barrier on the outer surface of the skin. Therefore, liposome technology was applied to overcome these problems. In this study, the liposomes have been prepared by the modified ethanol injection technique to avoid the use of organic solvents such chloroform which are no longer permitted in cosmetic products. The entrapment efficiency of liposome depends on the ratio and total amount of the lipid components. In fact, surface charge, vesicle type and size of liposomes can be affected by the structural lipid components as well as the process

Table 5A. Scoring time values of rabbit skin irritation when applied with liposomes containing Roselle extract (equivalent to 0.05 g dry extract)

Rabbit No.	Scoring time (hr)							
	1		24		48		72	
	Erythema	Oedema	Erythema	Oedema	Erythema	Oedema	Erythema	Oedema
103	1	0	0	0	0	0	0	0
104	1	0	1	0	1	0	*1	0
105	1	0	0	0	0	0	0	0

* Disappeared on day 6 of observation period

Table 5B. Scoring time values of rabbit skin irritation when applied with Roselle extract (equivalent to 0.05g dry extract)

Rabbit No.	Scoring time (hr)							
	1		24		48		72	
	Erythema	Oedema	Erythema	Oedema	Erythema	Oedema	Erythema	Oedema
103	2	1	2	1	1	0	*1	0
104	2	1	2	0	1	0	*1	0
105	2	1	2	0	2	0	*1	0

*Slight erythema existed till the end of observation period (14 days)

of preparation[28]. It is found in our study that the most suitable formulation for the Roselle liposome composes of SPC: Tween 80: DA (84: 16: 2.5 weight ratio, 200 mmole/mL of total lipid).

Since the Roselle extract consists of several highly polar phenolic compounds, the feasibility of liposomes as a transport delivery system for such hydrophilic substances into the skin was examined. For this purpose, *in vitro* skin permeation studies were performed using modified Franz diffusion cells and pig skin as the skin model. The antioxidant activity of the Roselle extract chemical constituents delivered by the liposome formulation into the receptor cell was tested to reflect the skin permeation of the antioxidant constituents. The results of these studies have revealed that the antioxidant activity of the Roselle extract components delivered by the liposomes was higher than that penetrated from its aqueous solutions. These results imply significant transdermal transportation of the hydroplilic Roselle extract constituents by the liposome formulations.

The skin permeability of the active antioxidants in Roselle extract containing liposomes can be explained by the size of liposomes. A small particle size might assist the deep penetration and distribution of the drugs[29]. The intercellular space between corneocytes of the skin is about 0.1 mm[30]. Nevertheless, the liposome particles with larger size than the intercellular space can also be absorbed, since the elasticity of liposomal membrane allows the extrusion of liposomes through this gap[29]. In addition, the incorporation of surfactant into the liposomes could also enhance the skin permeation due to the mechanism of additive effect with possible synergism (surfactants or ethanol with phosphatidylcholine)[31,32]. The *in vitro* skin permeation of antioxidant components of the Roselle liposomal preparations supported the evidence that liposomes could delivery the active components of the Roselle extracts into the skin. The optimum miscibility of liposomes with the lipid bilayer of stratum cornoun might facilitate the release of active compound from formulation and transport of active compound through skin[28].

The Roselle extract aqueous solution is red in color and highly acidic (pH 2.0), it causes high skin irritation and is therefore unsuitable for skin care products. By entrapping the extract or its consti tuents inside the vesicles, liposomes can remarkably reduce the skin irritation of the aqueous extract and make it more suitable for cosmetic use. Additionally, the liposome formulations help skin maintain a good state of health due to their moisturizing effects.

Conclusion

Oxidative stress plays an important role in the biological events leading to the signs of aging in the skin. Therefore, a recent concept for anti-aging for skin care products is topical antioxidant application. Roselle calyx extracts contain strong natural antioxidants. However, inappropriate properties of these extracts, such as hydrophilic components, low skin permeation, and skin irritation decrease their value in cosmetic products. Liposome-containing Roselle extracts should have potential for development as ingredients in cosmetic products. However, the long term stability of these liposomal formulations is still a challenge; the possibility for further overcoming the stability problem by developing proliposomes or dried-liposome formulations are currently being examined.

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ไลโบโซมสารสกัดกระเจี๊ยบแดงเพิ่มฤทธิ์ต้านอนุมูลอิสระเพิ่มการดูดซึมผิว และลดการระคายเคืองผิว

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กระเจี๊ยบแดงเป็นพืชที่ใช้เป็นยามานานแล้วสารสกัดจากกลีบเลี้ยงทีฤทธิ์ต้านอนุมูลอิสระ และสามารถจะพัฒนาเป็นสารที่ใส่ในเครื่องสำอางอย่างไรก็ตาม มีข้อจำกัดในการซึมผ่านผิว และมีการระคายเคืองผิวเทคนิคไลโบโซมสามารถแก้ปัญหานี้ได้ดีสูตรไลโบโซมของสารสกัดกระเจี๊ยบแดง ถูกพัฒนาขึ้นในลักษณะเป็น ของเหลวซึ่งมีประสิทธิภาพในการที่สารสกัดถูกบรรจุในโลโบโซมได้มากที่สุด มีความคงตัว สามารถซึมผ่านผิวได้ และลดการระคายเคืองของผิวสูตรนี้ สามารถกักเก็บสารสกัดได้ถึง 83% มีขนาดอนุภาคเล็กที่สุดคือ 333 ไมโครเมตร สูตรประกอบด้วย ฟอสฟาทิดิลโคลีน จากถั่วเหลือง ทวีน 80 และ ดีออกซีโคลิคในอัตาส่วน 84: 16: 2.5 ปริมาณไลปิดเป็น 200 กรัมต่อมิลลิลิตรสามารถกักสารสกัดกระเจี๊ยบแดงได้ร้อยละ 10 สูตรนี้ มีความคงตัวหลังจากเก็บที่ 4 องศาเซลเซียสภาวะป้องกันแสงในเวลา 2 เดือนเมื่อทดสอบผิวหนังหมู และทดสอบการซึมผ่านแสดงการซึมผ่านของสาระสำคัญในสารสกัดได้เพิ่มขึ้น เมื่อทดสอบการระคายเคืองต่อผิวกระต่าย เปรียบเทียบกับสารสกัดกระเจี๊ยบแดงที่ไม่มีไลโบโซมพบว่าสามารถลดการระคายเคืองได้ ผลการทดลองนี้สรุปได้ว่าไลโบโซมกระเจี๊ยบแดงมีความคงตัวดีมีประสิทธิภาพในการกักเก็บสารสกัดได้สูงสามารถซึมผ่านผิวได้ดี และมีความระคายเคืองต่ำ