Phenylethyl Resorcinol Loaded in Liposomal Cream Formulation for Cosmeceutical Application

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Authors’ contributions

This work was carried out in collaboration among all authors. Authors TA and PR designed the study, performed the statistical analysis and wrote the protocol. Author PR did experiment and wrote the first draft of the manuscript. Author SP managed the analyses protocol of studies. Author PR managed the literature search. All authors read and approved the final manuscript.

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ABSTRACT

Phenylethyl Resorcinol (PR) is a cosmeceutical skin lightening agent and the purpose of this study was to enhance its stability by using liposomal cream formulation which increases local efficacy and safety. Liposome formulation was prepared by modified ethanol injection method, and it contained soy phosphatidyl choline (SPC), cholesterol (CHO), Tween 80 (TW80) and deoxycholic acid (DA) mixed with 2% PR. The physicochemical properties, skin permeation as well as cellular study were evaluated in order to obtain the optimized formulation. The optimized liposome formulation composed of SPC:TW80:DA (84:16:2.5) and exhibited vesicle size, polydispersity index (PDI) and zeta potential of 286.4±8.04 nm, 0.317±0.03 and -39.20±3.85 mV, respectively. Entrapment efficiency (EE) of liposome formulation was 93.55±0.05%. The vesicle was spherical in shape and showed good physicochemical stability for 4 months. The skin permeation study demonstrated that liposome with a negative charge could result in a high PR skin deposition value of 1732.76±216.24 µg/cm² after 24 h. Cellular study showed that liposome formulation could inhibit melanin content in B16 melanoma cells and enhance cell viability in HaCaT keratinocyte cells. The

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optimized PR liposome was incorporated in cream and investigated physicochemical properties, stability and skin permeation. Liposomal PR cream showed a good stability and a superior result than PR cream in skin permeation parameters, as well as in tyrosinase inhibition.

Keywords: Phenylethyl resorcinol; liposomes; nano-vesicular system; liposomal cream; cosmeceutical.

1. INTRODUCTION

Skin pigmentation is a physiological defense against ultraviolet radiation (UVR) and it results in the production and distribution of melanin in epidermis [1,2]. UVR which has specific wavelengths can be divided into 3 types including UVA, UVB and UVC [3]. UVC (200-280 nm) is largely blocked by ozone layer and has little effect on the skin. UVB (280-320 nm) can penetrate into the epidermis and cause skin redness associated with sunburn. UVA (320-400 nm) can penetrate much deeper into the skin and can cause damage to the skin, such as photo aging, skin pigmentation and melasma [4]. Upon exposure to UVR, melanogenesis is increased by activation of the key enzyme tyrosinase, which stimulates melanin production. Typically, melanin can be found in 2 different types; yellow or red (pheomelanin) and brown or black (eumelanin) [5]. The production of melanin is undesirable and can become a serious aesthetic problem [6]. Therefore, inhibiting tyrosinase activity has generated much interest in several studies.

Phenylethyl resorcinol (4-(1-Phenylethyl) 1,3 Benzenediol, PR) is a lightening agent that can inhibit tyrosinase activity [7]. The results in previous studies of PR had reported that it can reduce tyrosinase activity approximately 22 times more effective than Kojic acid [8]. Moreover, PR is safe to use as skin lightening agent since it is non-toxic. There were no adverse effects such as oedema, erythema and eschar in rabbits after 72 h, when 0.5% PR solution was applied in a short-term exposure [9]. The clinical study on human subjects revealed that 0.5% of PR was more effective than 1% Kojic acid in skin lightening [10]. The recommended dose of PR for topical application is 0.1-0.5% [10]. Unfortunately, some drawbacks such as instability to light, poor solubility and local irritation can limit the topical use of PR. In order to overcome these limitations of topical application of PR, a delivery system was considered [11-13].

Liposome being a nanosized carrier, is an advantage for the topical and transdermal application of molecules, and liposomes can also increase the solubility and diffusion coefficient of molecules in the skin [14,15]. A conventional liposome composes of phospholipids and cholesterol [16]. They can carry both hydrophilic and lipophilic molecules and enhance skin permeation [17]. Besides cholesterol, edge activators such as surfactants and surface charge agents are used in the preparation of liposome formulations. The edge activators used in liposome preparations can modify or enhance the formulation in various ways such as reducing the size, increasing entrapment and also enhancing drug permeation [18]. Recently, Amnuaiikit et al. [12] developed a conventional liposome from 3% of phosphatidylcholine and 0.5% cholesterol, with 0.5% PR. Their conventional liposome had a big vesicle size and low EE. Thus, in this study, we developed a liposome formulation with edge activators to improve the physicochemical properties. Different types of liposomal compositions were prepared by modified injection method in order to find out the optimum liposome formulation of PR. The physicochemical properties including size, PDI, zeta potential and EE were determined. Skin permeation study was performed using Franz diffusion cell, PR residual and deposition on skin were also quantified. Moreover, a cellular study was carried out in order to determine the cytotoxicity of liposome formulation and to evaluate its efficacy in inhibiting the melanin content in cell lines. Lastly, the optimum liposome formulation was incorporated into dosage form as a cream and the physicochemical properties as well as skin permeation was evaluated.

2. MATERIALS AND METHODS

2.1 Materials

PR was purchased from Starchem Enterprises Limited (Nanjing, China). L-α-phosphatidylcholine from soy bean (SPC), cholesterol (CHO), Polysorbate 80 (Tween 80, TW80) and Deoxycholic acid (DA) were supplied from Sigma Aldrich (Missouri, USA). Triton X-100 was obtained from Loba chemical (Mumbai, India). Absolute ethanol, acetonitrile, methanol and Dimethyl Sulfoxide (DMSO) were purchased from Labscan Limited (Bangkok, Thailand).
L-DOPA, mushroom tyrosinase, Kojic acid was purchased from Sigma Aldrich. Ultrapure water (ELGA, UK) was used throughout the experiment. All chemicals were pharmaceutical grade and used as received.

2.2 Preparation of PR Liposomes

Liposome formulations were prepared by modified ethanol injection method [19]. Firstly, the lipid part including SPC, CHO, DA and PR was dissolved in ethanol, and further sonicated to dissolve them completely. Next, the water part including TW80 was dissolved in ultrapure water until it dissolved completely and then the water part was added into the lipid part. This resulting mixture was homogenized in a round bottom flask in a water bath. The mixture was then evaporated to remove the ethanol using a rotary vacuum evaporator, (Eyela Co., Japan) under reduced pressure at a temperature of 60°C until the liposome was obtained. The final concentration of PR was 2% (w/w). The physical appearance of all formulations was visually observed, and the formulations were stored at 25±1°C for further analysis.

2.3 Characterization of PR Liposomes

2.3.1 pH and Viscosity

pH values were measured using a digital pH meter (Mettler, Toledo, Switzerland) by directly putting the probe into the sample. The viscosity was determined using a viscometer (Model DV-III Ultra, Brookfield, USA) with spindle no.SC4-31, at a speed of 250 rpm, at 25°C [20].

2.3.2 Vesicle size, PDI and zeta potential

The mean vesicle size, PDI and zeta potential of freshly prepared formulation was measured by a Zeta Potential Analyzer (Model ZetaPALS, New York, USA). The sample (10 µL) was diluted with ultrapure water (4 mL) prior to the measurement. The zeta potential was measured by electro photometric light scattering using the capillary cell in the automatic mode of the same instrument [21]. All tests were carried out in triplicate at 25°C and the average values were calculated.

2.3.3 Entrapment Efficiency (EE)

The free PR was separated from liposome formulation by ultracentrifugation technique [12]. The liposome formulation (4 g) was accurately loaded in a centrifuge tube (Beckman Instrument, Inc., USA), and the tube was equipped in an SW60 Ti type rotor of an Ultracentrifuge (OptimaTM L-100XP, Beckman, USA). The liposome formulation was centrifuged at 40000 rpm at 4°C for 2 h. Free PR in the supernatant and the total amount of PR in the lipid formulation was assayed. Triton X-100: Methanol (1:1) was used in the measurement of total PR content. HPLC technique was used in the measurement of both free PR and total PR. %EE was calculated with the following equation:

\[ EE\% = \frac{(T-F)}{T} \times 100 \]

Where, T and F represent the amount of the total and free (non-encapsulated) PR, respectively.

2.4 Determination of PR Using HPLC

PR quantification was carried out on Agilent 1100 series system equipped with diode array detector (Agilent Technologies, Santa Clara, USA). The mixture of acetonitrile, methanol and ultrapure water (40:20:40, v/v) was used as mobile phase. Separation was achieved using a Hypersil BDS C18 column (250 x 4.6 mm, 5 µm) connected with a C18 pre-column (10 x 4 mm, 5 µm) (Thermo Fisher, Pennsylvania, USA). PR content was measured at a wavelength of 254 nm, with a flow rate of 0.8 mL/min and an injection volume of 20 µL [22].

2.5 Stability of PR Liposomes

The selected PR liposome formulations were freshly prepared and stored at various temperatures of 4±1°C, 30±1°C and 45±1°C, under 75% RH in a constant climate chamber model HPP260 (Memmert, Schwabach, Germany). Samples were withdrawn in order to check their appearance, vesicle size, PDI as well as zeta potential. Total active content and %EE were also evaluated after 0, 1, 2, 3 and 4 months. The stability of PR liposome cream was determined by evaluating both physical appearance and PR content in 6 freeze thaw cycles (1 cycle; 4°C for 24 h and 45°C for 24 h).

2.6 Visualization of Liposome Formulation Using Field Emission Scanning Electron Microscopy (FESEM)

The optimum liposome formulation was examined by Gram's method using FESEM.
2.7 In vitro Skin Permeation

The experiments were carried out in a modified Franz diffusion cell (Model Hanson 57-6M, California, United States). The receptor chamber capacity was 11.4 mL and the available area was 1.77 cm² [9,12]. Pig ear skin was used as the skin model and mounted between the donor and receptor compartments to avoid bubble formation [24]. The receptor compartment was filled with phosphate buffer pH 7.4: propylene glycol (70:30; v/v) which allowed the sink condition to be maintained. After equilibration for 30 minutes on a water bath, 1 g of the selected formulation was applied onto the donor site. The receptor compartment was kept at 37°C, with the solution constantly stirred at 500 rpm throughout the experimental duration. At time points of 0.5, 1, 2, 4, 6, 8, 12 and 24 h, 1 mL of the receiving medium was withdrawn and immediately replaced with an equal volume of fresh receptor medium. The content of PR was analyzed by HPLC and permeation profile was calculated. The cumulative amount (Qt) of PR permeation per unit area was calculated from this equation:

\[ Q_t = (C_0 V + \sum_{i=1}^{n-1} C_i S) / A \]

Where \( Q_t \) is the PR concentration of the receiver solution at each sampling time, \( C_i \) is the PR concentration in the receiving medium at the \( i^{th} \) sampling time, \( V \) is the volume of the receptor compartment (11.4 mL), \( S \) is volume of sample withdrawn, and \( A \) is effective permeation area of the diffusion cell (1.77 cm²).

The \( Q_t \) values were plotted as function of time. PR flux (\( J_{ss} \), µg/cm²/h) and lag time (\( T_{lag} \)) were obtained from the slope and X-intercept of the linear portion of \( Q_t \) versus time plot by regression, respectively. The permeability coefficient (\( K_p \)) was calculated with the following formula:

\[ K_p = \frac{J_{ss}}{C_0} \]

Where \( C_0 \) represents the initial concentration of PR in the donor compartment.

2.7.1 Residual PR in the donor compartment

At the end of skin permeation study, the amount of formulation in the donor compartment was collected and gently cleaned (10 times) with a cotton pad soaked in 10 mL of methanol. The collected sample was then sonicated for 30 minutes until a homogenized solution was obtained. The obtained solution was centrifuged (Kubota Model 3740, Japan) at 4000 rpm at 4°C for 30 min. The PR residual was diluted with methanol (1:1) and then analyzed by HPLC [9].

2.7.2 Deposition of PR on the skin

The amount of PR deposited in the skin was quantified. Skin mounted on the diffusion cell was carefully taken off, then cut into small pieces. The tissue was further homogenized with 5 mL of methanol using a homogenizer (Model PT 1200E, Polytron, Switzerland) for 2 min. The resultant sample was sonicated for 30 minutes and centrifuged at 4000 rpm at 4°C for 30 min [9,12]. The PR content on the upper phase was determined by HPLC.

2.8 Determination of Anti-tyrosinase Activity

Tyrosinase inhibition was determined using the modified Dopachrome method with L-DOPA as a substrate and Kojic acid as positive control [25]. Assays were carried out in 96-well plates, and a microplate reader (BMG Labtech, Germany) was used to measure the absorbance at 492 nm. The PR solution obtained from the section 2.7.2 was diluted using DMSO, and used as the test sample. First, 20 µL of the test sample was mixed with 140 µL phosphate buffer (pH 6.8) in a 96-well plate. Then, 20 µL of mushroom tyrosinase (203.3 unit/mL) was added for pre-incubation for 10 mins at 25°C. Finally, 20 µL of 0.85 mM substrate (L-DOPA) was added to start the enzymatic reaction. The final volume in each well was 200 µL. The absorbance was measured to observe Dopachrome reaction for 20 minutes. The inhibition was achieved as follows:

\[ \% \text{tyrosinase inhibition} = \frac{[(A-B)-(C-D)]/(A-B)] \times 100}{\text{activity}} \]
Where A is the mixture with tyrosinase and without test sample, B is the mixture without tyrosinase and test sample, C is the mixture with tyrosinase and test sample and D is the mixture without tyrosinase and with test sample. All measurements were performed in triplicate.

2.9 Cell Culture

B16 melanoma cells and HaCaT keratinocyte cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) ATCC® supplemented with 10% fetal bovine serum and 1% antibiotics. The cells were maintained in a humidified atmosphere containing 5% CO₂ at 37°C.

2.9.1 Cytotoxicity assay in HaCaT keratinocyte cells

Briefly, the cells [(3x10^3) cells/well] were seeded in a 96 wells plate and incubated at 37°C, 5% CO₂ for 24 hours. The cells were then treated with or without tested liposome formulation at various testing formulation dilutions (1:2000, 1:1000, 1:500 and 1:250) for 48 hours. The cells were fixed with 40% w/v trichloroacetic acid and then incubated at 4°C for 1 hour. After removing the medium, the cells were rinsed with distilled water. The microplates were then dehydrated at room temperature, stained with 0.4% SRB solution, washed 4 times in 0.1% acetic acid and re-dehydrated at room temperature. The protein bound cells were lysed in 10 mm tris-buffer and the optical density was measured at 510 nm. Blank liposome was used as blank. The percentage of cell viability was calculated by the following equation:

\[
\text{% cell viability} = \frac{a}{b} \times 100
\]

Where a is the absorbance of sample and b is the absorbance of control.

2.9.2 Melanin content

The melanin content was measured by the method used by Limsuwan et al. [9] with modifications. B16 cells at the density of 5 x 10^5 cells/dish were placed in 60 mm dishes and treated with different test samples containing 10 µg of PR. After treatment, the cells were slowly washed twice with phosphate buffer solution and the cells were lysed in 200 µL of 1 N NaOH for 1 h at 95°C to solubilize the melanin. The absorbance was measured at 405 nm and melanin content was compared with control/untreated cells.

### Table 1. Formulation of cream base

| Ingredient list       | Rx 3 |
|-----------------------|------|
| Carbopol 934          | 0.75 |
| Cetomacrogol 1000     | 1.5  |
| Cetyl alcohol         | 1    |
| Rubber seed oil       | 5    |
| Isopropyl palmitate   | 2    |
| Tween 60              | 1.6  |
| Span 60               | 1    |
| Propylene glycol      | 1    |
| Glycerine             | 4    |
| Triethanolamine       | 0.75 |
| Vitamin E             | 0.5  |
| Phenoxyethanol        | 1    |
| Perfume               | qs   |
| Water to              | 100  |

2.10 Preparation of PR Liposomal Cream

Table 1 shows the composition of cream base. Cream base was prepared by emulsification method. Raw materials were accurately weighed and separated into 2 beakers, one for oil phase, and another for water phase. Next, both beakers were heated and when the temperature reached 75°C, the water phase was slowly added into oil phase with constant stirring. The mixture was continuously stirred until it congealed. The liposome formulation no. 15 (25% w/w) was added to the cream to obtain a concentration of 0.5% of PR at a temperature of 30±1°C.

2.10.1 Physical appearance and stability of PR liposomal cream

The physical appearance of liposomal cream was evaluated including color, odor, pH and viscosity. The stability of PR liposomal cream was evaluated under freeze thaw cycles for 6 cycles as described in section 2.5, and the PR content was determined by HPLC.

2.10.2 In vitro skin permeation of PR liposomal cream

In vitro skin permeation of PR liposomal cream was determined as described in section 2.7.

2.11 Statistic Analysis

All experiments were performed in triplicate and the results were expressed as the mean ± standard deviation (SD) or mean ± standard error of mean (SEM). Data analysis was carried out with analysis of variance software (ANOVA).
Multiple comparison of means (Post hoc) were used to substantiate statistical differences between groups, while one sample t-test was applied for comparison between two samples. Significance was tested at 0.05 level of probability (p).

3. RESULTS AND DISCUSSION

3.1 Liposome Formulation and Characterization

The compositions and physical characteristics of liposome formulation are shown in Table 2. In this study 16 liposome formulations were prepared in different compositions. The physical appearance of liposome formulations showed desirable results including colloidal property and a yellowish-brown color. However, precipitation was observed in formulation no.1, 2, 3, 5, 9 and 13. This precipitation could be explained by the insufficiency of total lipid which could entrap PR in the vesicles. Since the PR is a lipophilic drug, it could be localized in the lipid bilayer or encapsulated in the liposome interior. Thus, increasing the total lipid content can result in a higher solubility of PR and no precipitation. The pH of liposome formulations is a considerable factor for topical applications. The pH of freshly prepared liposomes was slightly acidic, ranging from 5.25 to 5.89 which was optimal for use in topical applications, based on skin pH [20]. The viscosity of all liposome formulations was found to be in the range of 25.54±0.54 to 54±0.32 centipoise (cps). Thus, the pH level and the viscosity of prepared liposome were suitable for topical applications [14]. In case of size, it was found that all the liposome formulations were nanosized, ranging from 200.6 - 364.7 nm and in case of PDI, it was in the range of 0.193 - 0.484, indicating homogeneous population and narrow size distribution of vesicles [26]. Fang et al. [27] proposed that the inclusion of surfactant and surface charge could not reduce the size of vesicles. It was found that the presence of Tween 80 and deoxycholic acid in liposome formulations resulted in high vesicle sizes, because the effect of surfactant increased the volume of the lipid bilayers. Zeta potential is a key factor for determination of stability for colloidal dispersion. The liposome formulations had high negatively charged values ranging from -20.18 to -43.71 mV, due to the presence of surface charge in the formulations providing negative charge. These high zeta potential values (above -20 mV) increased the stability of vesicles, due to the electro static repulsion between the vesicles [19,20,28]. Although the zeta potential of some prepared liposomes was less than 30 mV, there was no precipitation. The EE of liposome formulation was high, and observed in the range of 80.47 to 97.79%. In this study, formulation no.15 prepared from SPC, Tween80, and DA showed the

| No. | Composition | Total lipid (μmol)/mL | Vesicle size (nm) | Zeta potential (mV) | Entrapment efficiency (%) |
|-----|-------------|-----------------------|------------------|--------------------|--------------------------|
| 1   | SPC:CHO     | 20                    | Precipitation    | Precipitation      | Precipitation            |
| 2   | (4:1)       | 40                    | Precipitation    | Precipitation      | Precipitation            |
| 3   |             | 60                    | Precipitation    | Precipitation      | Precipitation            |
| 4   |             | 80                    | 231.6±12.53      | -39.26±3.26        | 91.21±0.12               |
| 5   | SPC:TW80    | 20                    | Precipitation    | Precipitation      | Precipitation            |
| 6   | (84:16)     | 40                    | 207.7±0.69       | -25.89±2.20        | 93.56±0.11               |
| 7   |             | 60                    | 276.9±16.16      | -43.71±2.98        | 93.47±0.03               |
| 8   |             | 80                    | 364.7±12.72      | -29.81±4.56        | 80.47±0.44               |
| 9   | SPC:TW80:CHO| 20                    | Precipitation    | Precipitation      | Precipitation            |
| 10  | (4:1:1)     | 40                    | 200.6±7.65       | -20.18±4.66        | 85.54±0.18               |
| 11  |             | 60                    | 236.5±16.29      | -26.61±3.49        | 92.63±0.10               |
| 12  |             | 80                    | 260.6±12.04      | -40.08±4.40        | 92.78±0.20               |
| 13  | SPC:TW80:DA | 20                    | Precipitation    | Precipitation      | Precipitation            |
| 14  | (84:16:2.5) | 40                    | 224.0±8.81       | -27.55±3.40        | 89.17±0.05               |
| 15  |             | 60                    | 286.4±8.04       | -39.20±3.85        | 93.55±0.05               |
| 16  |             | 80                    | 354.7±25.06      | -32.14±2.65        | 97.79±0.05               |

Each data is represented as mean ± SD (n=3). 1 The ratio of liposome composition is molar ratio. 2 The ratio of liposome composition is weight ratio.
highest EE (93.55%). Amnuaikit et al. [12] reported that conventional liposome showed vesicle size, zeta potential and entrapment efficiency of 641.52±19.02 nm, -27.32±1.00 and 53.55±4.9%, respectively. However, compared to the above-mentioned study, liposome formulations in this study exhibited better results in terms of vesicle size, zeta potential and EE. For further studies, optimized liposome formulations were selected on the basis of vesicle size < 500 nm, PDI < 0.3, high zeta potential and high EE. Hence, we selected liposome formulation no.4, 7, 12 and 15 for the stability study.

### 3.2 Stability of Liposomes

Stability study of selected liposome formulations was carried out by monitoring the physical and chemical properties of the formulations for 4 months, in 3 storage conditions. The physical appearance of liposome formulation was milky and colloidal, and remained unchanged at 4°C and 30°C for over 4 months. The stability results showed that a change in vesicle sizes of liposomes on storage were observed, which showed a decrease of vesicle sizes in all formulations. This result can be due to the bilayer degradation [29]. However, PDI value indicated that the liposome was narrow (<0.3) and uniform in diameter. In case of zeta potential, it was found to be above 20 mV in all formulations, indicating that the liposome formulation had good stability [30]. Interestingly, the active content and EE only showed a slight change over the 4 months storage period. The active content of formulation no.12 (99.42%) reduced to 84.96% when stored at 4°C, whereas the active content of formulation no.4 (98.92%) and high EE. These results indicated that the optimum liposome formulations were stable at 4 and 30°C, because of the high negative net charge, leading to charge stabilization which prevents the formation of vesicle aggregates. A slight change in EE value was observed in all the formulations when stored at 4°C. It can be concluded that all these liposome formulations had favorable physicochemical stability with no sedimentation.

### 3.3 Skin Permeation Study

The skin permeation of liposome formulations in pig ear skin was determined and PR solution (2% PR in caprylic/capric triglyceride) was used as control. The total amount of PR applied in each compartment was 20 mg/mL. Permeation profile (Fig. 1) showed that all liposome formulations showed a higher cumulative amount of PR than PR solution over 24 hours. Throughout the experiment period, liposome formulation no.15 showed the highest cumulative amount of PR (1215.72±133.12 µg/cm²) and high flux (52.82±6.22 µg/cm²/h) due to its composition, which enhanced PR solubility (Table 3). It was observed that the addition of additives in the formulation caused an increase in Jₚ, indicating the rapid achievement of the steady state in skin permeation, with the shortest Tₚₐₚ of 1.91±0.13 h. Similar results were obtained in others studies by the use of deoxycholic acid and Tween 80, which made the vesicles in the phospholipid bilayer greatly elastic, thus enhancing skin permeation [31,32]. Table 4 shows the amount of PR in 3 compartments including donor, pig ear skin and receptor compartment. The results revealed that most of the PR remained in the donor compartment due to the barrier property of the outer layer of skin [15]. The lowest amount of PR in donor compartment was in liposome formulation no. 4, which indicated that it had high deposition in pig ear skin compared to other formulations after 24 h and the PR content deposited was 2641.42±326.54 µg/cm² or 13.21±0.94% (p<0.05) (Fig. 2). In formulation no. 7, 12 and 15, the amount of PR deposited was 1692.60±68.85, 1301.46±180.74 and 1732.76±216.24 µg/cm², respectively. Ashtikar et al. [16] mentioned that cholesterol could increase the rigidity of vesicles, which can result in a change in the permeation behavior of molecules through the skin.

### 3.4 Anti-tyrosinase Activity

The result of tyrosinase activity (Table 5) revealed that all liposome formulations exhibited tyrosinase inhibition activity above 90%, which was higher than PR solution (89.54%) and kojic acid (76.22%) (p<0.05). Even though the result of PR deposited on skin (Fig. 2) showed that formulation no.4 had the highest PR deposition,
Fig. 1. *In vitro* skin permeation of PR liposome formulation no. 4, 7, 12, 15 and PR solution across pig ear skin

(n = 3, mean ± SEM)

Fig. 2. PR deposition results in pig ear skin and receptor compartment

*Each bar is represented as mean ± SEM (n=3). * to *** means - in the same color bar and the different number of asterisks indicate significant difference of values (p<0.05)*

Table 3. *In vitro* skin permeation parameters of PR following topical application of liposome formulations and PR solution

| Formulations     | Jss (µg/cm²/h) | Kp (x10⁻³ cm/h) | Tlag (h) | Qt (µg/cm²) |
|------------------|----------------|-----------------|----------|-------------|
| Liposome no. 4   | 38.94±3.66a    | 1.95±0.18ab     | 2.26±0.12b | 902.24±82.63a |
| Liposome no. 7   | 37.20±3.21ab   | 1.86±0.16ab     | 2.39±0.32b | 856.80±66.62ab |
| Liposome no. 12  | 38.71±3.02ab   | 1.94±0.15ab     | 1.75±0.03c | 911.06±84.97a |
| Liposome no. 15  | 52.82±6.22a    | 2.64±0.31a      | 1.91±0.13b | 1215.72±133.12a |
| PR solution      | 20.58±1.85ab   | 1.69±0.15b      | 1.03±0.09b | 476.61±34.80b |

*Each data is represented as mean ± SEM (n=3). Jss, steady state flux; Kp, permeability coefficient; Tlag, lag time; Qt, cumulative amount; a-c: means in the same column with different superscript letter differ significantly (p<0.05)*
B16 melanoma cells are well established for liposome formulations. Liposome formulations showed toxicity to the cells with a 1:2000 dilution using SRB assay. Viability of HaCaT cells treated with liposome formulations, cytotoxicity test was performed on HaCaT keratinocyte cells. In order to determine the cytotoxicity of liposome formulations, a receiver operating characteristic (ROC) curve of liposome formulations and PR solution was determined.

Tyrosinase inhibition values of all the formulations were more or less the same. These findings once again confirmed the previous report by Limswan et al. [9], who mentioned that PR in delivery systems provided high tyrosinase inhibition activity over 80%, leading to skin whitening behavior.

**Table 4. The percent recovery of PR compartment of the diffusion cell following application of liposome formulations and PR solution**

| Formulations   | Amount recovery (% of applied dose) |
|----------------|-------------------------------------|
|                | Donor | Pig ear skin | Receptor | Total         |
| Liposome no.4  | 72.57±2.33<sup>a</sup> | 13.21±0.94<sup>b</sup> | 7.75±0.71<sup>a</sup> | 93.65±3.99<sup>a</sup> |
| Liposome no.7  | 72.68±1.26<sup>a</sup> | 8.46±0.34<sup>b</sup> | 7.45±0.56<sup>a,b</sup> | 88.59±2.17<sup>a</sup> |
| Liposome no.12 | 77.36±4.23<sup>a</sup> | 6.51±0.90<sup>b</sup> | 7.92±0.66<sup>a</sup> | 91.78±5.80<sup>a</sup> |
| Liposome no.15 | 74.35±2.32<sup>a</sup> | 8.66±1.08<sup>b</sup> | 10.56±1.15<sup>a</sup> | 93.57±4.56<sup>a</sup> |
| PR solution   | 81.15±5.39<sup>a</sup> | 2.66±0.30<sup>c</sup> | 4.13±0.31<sup>b</sup> | 87.55±6.00<sup>a</sup> |

Data are expressed as % of the total PR in the applied dose (20 mg/mL) (mean ± SEM, n=3), a-b: means in the same column with different superscript letter differ significantly (p<0.05)

Further to this cellular study, all formulations were tested in B16 melanoma cells by treating the cells with various formulations at a non-toxic dilution (1:2000). Fig. 4 shows the remaining melanin content in the formations after the experiment. Compared to the control which was 100%, the content of melanin remaining in formulation no. 4, 7, 12 and 15 were 41.01, 43.30, 41.39 and 43.72% respectively. From these results, formulation no. 15 was selected for further study.

**3.7 Morphology of Liposomes**

Based on the physicochemical properties, skin permeation study and cellular study, we selected liposome formulation no. 15 as the optimum formulation. Liposome formulation no. 15 composed of SPC:Tween80:DA (84:16:2.5 weight ratio) and this formulation was characterized by vesicle size of 237±12.92 nm, PDI of 0.316±0.018, zeta potential of -26.33±0.55 mV and EE of 92.05±0.07%. The physical appearance was milky-yellowish and coloidal, and the FESEM image is shown in Fig. 5. In the figure we can clearly see that the liposome has a closed spherical shape.

**3.8 Formulation of PR Liposomal Cream**

The physicochemical property and quantity of PR in cream before and after the stability test is shown in Table 6. No significant change in physical appearance (color, odor and phase separation) was found in PR liposomal cream, before and after stability test. Moreover, there was no change in pH and viscosity of the formulation during the period of investigation. The amount of PR content in formulation was determined. The mean recovery assay for each formulation was in the range of 70 - 130% (Table 6), which is acceptable according to ICH guidelines [33].
Fig. 3. The effect of liposome formulations and blank on cell viability against HaCaT keratinocyte cells

Cell viability of control was 100%. The data is represented as mean ± SD (n=3)

Fig. 4. The effect of liposome formulations on melanin production in B16 melanoma cells. Cells were treated with liposome formulations (at the dilution 1:2000)

Each value is represented as mean ± SD (n=3); *p<0.05 compared with the control

Fig. 5. Physical appearance and the FESEM image of liposome formulation no. 15
Table 6. Physical appearance and measurement of PR concentration of PR liposomal cream before and after stability test

| PR liposome cream | pH | Viscosity (cps) | Actual concentration (mg/mL) | Measured concentration (mg/mL) | % Recovery |
|-------------------|----|----------------|-----------------------------|--------------------------------|------------|
| Before FT         | 6.65±0.01 | 28326±111 | 5                           | 4.76±0.05                      | 95.14±1.00 |
| After FT          | 6.54±0.02 | 27467±185 | 5                           | 4.61±0.04                      | 92.28±0.71 |

*Each data is represented as mean ± SD (n=3). FT mean freeze thaw*

Table 7. Skin permeation study of PR liposome cream and PR cream in 24 h

| Formulations        | Jss (µg/cm²/h) | Kp (x10⁻³ cm/h) | Tlag (h) |
|---------------------|----------------|-----------------|----------|
| PR liposome cream   | 4.79±0.14      | 1.11±0.03       | 3.57±0.33 |
| PR cream            | 2.90±0.13      | 0.67±0.33       | 5.07±0.44 |

*Data are represented as mean ± SD, *p<0.05

Fig. 6. *In vitro* deposition of PR in pig ear skin and receptor compartment after testing PR liposome cream and PR solution cream

Each bar is represented as mean ± SEM (n=3)

3.9 Skin Permeation Study of PR Liposomal Cream

PR liposome cream was further evaluated for its efficiency in terms of permeation through the pig ear skin, compared to the PR cream. Various skin permeation parameters, including Qt, Jss, Kp and Tlag over a period of 24 h were calculated and presented in Table 7. It showed that Qt of PR liposomal cream was 1.45 times greater than PR cream. The Jss, Kp and Tlag values shown by PR liposomal cream were found to be 4.79±0.14 µg/cm²/h, 1.11±0.03 x10⁻³ cm/h and 3.57±0.33 h respectively, which were significantly (p<0.05) higher than the PR cream. From the above result, we can clearly see that liposome caused a significant increase in skin permeation parameters. Furthermore, as shown in Fig. 6 the PR deposition in pig ear skin was not significantly different (p>0.05) between PR liposomal cream and PR cream. This result maybe because, the PR in the PR cream is in free state, whereas, the PR in the PR liposome cream is entrapped within the vesicle. However, the result of tyrosinase activity showed significantly different (p<0.05) values between PR liposomal cream and PR cream and the values were 88.30±3.64 and 81.11±3.76%, respectively. This may be because the PR liposome can deliver the PR to the desired site in the skin more efficiently than PR cream.

4. CONCLUSION

In this study, liposome formulations were prepared by modified ethanol injection method.
The optimum liposome formulation had a nanosized vesicle, low value of PDI, high zeta potential, high EE, and it was spherical in shape. After storage in various conditions, the optimum liposome formulation was found to be stable at 4 and 30°C, where the physicochemical properties, EE and total PR content remained unchanged. In vitro skin permeation study showed that the optimum liposome formulation provided the best results in skin permeation parameters, and also, the tyrosinase inhibition was over 90%. The cellular studies indicated that, the optimum liposome formulation displayed a noticeable reduction in melanin content on B16 melanoma cells and formulation was safe to use according to the results of cell viability of HaCaT keratinocyte cells. The liposomal cream had also good physicochemical properties and stability, and showed superior results in skin permeation and tyrosinase activity than PR cream. From all these results obtained in this study, it was concluded that liposomal cream is a great formulation of PR for cosmeceutical applications.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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