# The Effect of Component of Cream for Topical Delivery of Hesperetin

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**The aim of this study was to optimize hesperetin cream formulations by *in vitro* permeation study and evaluate topical whitening active effect and skin irritation by *in vivo* study. The results showed that the solubility of lipophilic compound of hesperetin was increased by short-chain alcohol including ethanol, glycerin, propylene glycol and polyethylene glycols 400 (PEG 400). PEG 400 showed strongest solubilized effect by increased 3400-fold. With the addition of 5% enhancers, it was found that menthol showed the most potent enhancing effect, followed by azone and depigmentation agents (linoleic acid and lecithin). Moreover, enhancers could shorten the lag time from 3.7 to 1 h. Combination of menthol, linoleic acid and lecithin of 2.5% had a higher permeation rate of 9.8 $\mu$g/cm$^2$/h and lower lag time 1 h, therefore the formulation was selected to process the skin whitening and irritation test. The results showed that a significantly topical photoprotective effect with acceptable skin irritation was obtained after hesperetin cream topical application when compared with that of the non-treatment group, indicating that the hesperetin cream may be used as an effective whitening agent.**

**Key words** hesperetin; cream; whitening effect; skin irritation

Exposure of human skin to ultraviolet (UV) radiation may cause skin damage such as erythema and pigmentation, and acceleration of skin aging.[1)] Hyperpigmentation including melasma, freckles and senile lentiginoses is caused by the over-production of melanin, a pigment in the human skin acting as a major defense mechanism against UV light.[2)] However, hyperpigmentation on faces is a high anxiety-producing symptom for people from the aspect of beauty appearance. Nowadays, skin whitening agents, particularly natural antioxidants such as flavonoids, are receiving increasing attention because such flavonoids possess potential antioxidant activity and are claimed to be free of toxicity and side effects.[3)] Recently, much research has been focused on the potential use of flavonoids for preventive oxidative skin damage.[4—6)]

For skin bleaching, the topical delivery system is the considered administration route. Nevertheless, the most difficult aspect of a transdermal delivery system is to overcome the barrier of stratum corneum against foreign substances. Use of penetration enhancers is valuable and important for improving drug permeation,[7—9)] but attention must be paid to the extensive damage to the skin caused by such enhancers, although the large increase in permeation rate is a positive. Therefore, optimal formulation design is important for topical application of pharmaceutical products.

Flavanone compounds such as hesperetin (and its glycoside hesperidin) are a kind of flavonoid and have been reported to possess a wide range of pharmacological properties such as being anti-inflammatory and possessing enzymes (including hyaluronidase and xanthine oxidase) inhibitors, antimicrobial activity, UV protecting activity, along with analgesic and antioxidant effects.[10)] Moreover, hesperidin is extremely safe and without side effects even during pregnancy.[10)] The molecular weight of hesperetin is about 302, which makes it a good candidate for topical application. Therefore, the aim of this study was to design an optimal hesperetin cream which is the most common dosage forms for topical application. The *in vitro* permeation study was used to evaluate the effect of the composite on permeability of the drug through rat skin. *In vivo* studies such as skin whitening and irritation tests were used to assess the clinical usability of hesperetin cream.

## Experimental

**Materials** The following reagents were used: hesperetin, naringenin, menthol, linoleic acid, azone, methyl paraben, stearic acid and PEG-30 dipolyhydroxystearate (Tokyo Chemical Industry, Japan), cetyl alcohol (Acros, Belgium), lecithin (Wako, Japan), propylene glycol (PG), polyethylene glycols 400 (PEG 400) (Merck Chemicals, U.S.A.). Plush blush® cream containing 1% ascorbyl magnesium phosphate (UNT, Taiwan). All other chemicals and solvents were of analytical reagent grade.

**Solubility Measurement** An excess of hesperetin was placed in sealed glass tubes containing 2 ml of solvent. The tubes were shaken occasionally on a vortex mixer and were maintained at room temperature for 24 h. The saturated solution was centrifuged and the supernatant was filtered through a 0.45 $\mu$m membrane. The concentration of drug in the saturated solution was determined by HPLC after appropriate dilution with the selected solvents.

**Preparation of Hesperetin Creams** The oleaginous phase including hesperetin 1%, stearic acid 2.5%, cetyl alcohol 4%, PEG-30 dipolyhydroxystearate 5% and hydrophobic enhancers (including azone, menthol, linoleic acid and lecithin) was melted together at about 75 °C. The potassium hydroxide 0.2%, methyl paraben 0.1% and cosolvent (PEG 400 of 5%, 10%, 20%) was dissolved in purified water at about 78 °C, and then added into the oleaginous phase by stirring, and temperature was maintained at 75 °C for 15 min. Then the mixture was removed from the heat and stirred at room temperature until the mixture congealed.

***In-Vitro* Skin Permeation Experiments** The permeability of hesperetin creams was determined using a modified Franz glass diffusion cell fitted with abdominal skin of excised Sprague-Dawley rat. The skin was mounted on the receptor compartment with the stratum corneum side facing upwards into the donor compartment and the dermal side facing downwards into the receptor compartment. The donor cell was filled with 1 g of hesperetin cream and occluded by parafilm. The receptor compartment was filled with 20 ml of pH 7.4 phosphate buffer containing 20% ethanol and 40% PEG 400 and its temperature was maintained at 37±0.5 °C by thermostatic water pump during the experiment. The effective diffusion area was 3.46 cm$^2$. Approximately 0.5 ml of the receptor medium was withdrawn at predetermined intervals and replaced immediately with an equal volume of receptor solution to maintain a constant volume. The sample withdrawn from the receptor compartment was then analyzed by HPLC method modified from previous study.[11)] A Merck Lichrocart® C18 column (125×4 mm i.d., particle size 3 $\mu$m) was used. The mobile phase was a mixture of 0.5% triethylamine (adjusted to pH 3.05 by acetic acid) and acetonitrile in the ratio of 75 : 25, at the flow rate of 1 ml/min. The UV detection was at 288 nm. The naringenin of 100 $\mu$g/ml was used as internal standard. The limit of detection was 0.02 $\mu$g/ml (signal-to-noise >4). Each data point represents the average of three determinations.

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End the permeation experiment, the applied drug concentration in the skin was also determined by a homogenization method. After wash, the skin was cut to small pieces and place into a glass tube containing 2 ml methanol in an ice bath. The sample was homogenized at 17800 rpm for 2 min, and then shaken horizontally for 30 min. The resulting solution was centrifuged for 10 min at 3100 ***g***. The supernatant was determined by HPLC.

***In Vivo*** **Study for Whitening Assessment** Seven male guinea pigs (body weight 500—700 g) were used in this study. The guinea pigs were housed separately and fed commercial chow and tap water *ad libitum*, and were acclimatized to a 12 h light and dark cycle. Hair was shaved from the dorsal skin of the guinea pigs with an electric shaver, and four slots (1.5 cm each) arranged on the exposed skin.

The dorsal skin of animals was exposed to UVB radiation (Spectronics Corp. XL-1000) three times a week (every other day) for two consecutive weeks. The total energy dose of UVB was 1 J/cm$^2$ per exposure. The animals were then left for an additional week to allow the UVB induced hyperpigmentation to stabilize. Test samples were then topically applied daily to the hyperpigmented areas (2 mg/cm$^2$) for 4 successive weeks. Both before exposure and at 7, 14, 21 and 29 d after exposure, the skin luminosity of each region was measured using a Chroma Meter (CR-200, Minolta Camera, Tokyo, Japan). $\Delta L^*$, the change in luminosity index $L^*$, was calculated as $\Delta L^*$=pre-exposure $L^*-L^*$ of reading on each measurement day after exposure. The effect of hesperetin cream on skin depigmentation was evaluated using $\Delta L^*$ as an index. The animals were cared for according to the guidelines for the Care and Use of Laboratory Animals of the Kaohsiung Medical University.

***In Vivo*** **Study for Skin Irritation Evaluation** At the same specific time period of whitening assessment, the erythema color and the transepidermal water loss (TEWL) of each region was measured using a Chroma Meter (CR-200, Minolta Camera, Tokyo, Japan) and an evaporimeter (Tewameter TM210, Koln, German), respectively.[12,13] $\Delta a^*$, the change in $a^*$, the balance between red (100) and green ($-100$) was used as the index for erythema degree of the skin.

$\Delta a^*$=pre-exposure $a^*-a^*$ of reading on each measurement day after exposure

**Data Analysis** *In vitro* study, the cumulative amount of the drug permeation through rat skin was plotted as a function of time and a linear regression analysis was used to determine the permeation rate (flux) of the drug. The lag time was defined as the first time of detected drug.

Statistical comparison between data were made using analysis of variance (ANOVA) analysis. Sub-group comparison were made using the Newman–Keuls multiple comparisons.

## Results and Discussion

**Solubility** The solubility of hesperetin in water, various pH values of buffers and short chain alcohol is shown in Fig. 1. The solubility of hesperetin in water was 8.03 $\mu$g/ml, indicated it was a lipophilic compound. The result was consistent with previous reports which indicated most flavonoids are highly lipophilic substances and consequently difficult to prepare pharmaceutical products from.[3,14] For obtaining a suitable solvent to prepare the topical application dosage forms, the effect of pH value of medium and cosolvent on the solubility was evaluated. As presented in Fig. 1, the solubility was increased about 8-fold from 7.98 to 54.61 $\mu$g/ml when the pH value of medium increased from 4 to 8. Although increase in pH could increase the solubility of hesperetin, the solubility of the drug at pH 8 was inadequate in providing the needed amount of drug for preparing a topical preparation (1%). Moreover, any topical applier with higher pH value (pH $>$8) is not suitable for (human) skin. Therefore, some of the most common cosolvents such as ethanol, propylene glycol, glycerin and PEG 400 are used to solubilize drugs in pharmaceutical preparations. As shown in Fig. 1, the solubility of hesperetin in ethanol, propylene glycol, glycerin and PEG 400 was about 19.1, 7.9, 5.4 and 27.7 mg/ml, respectively, indicated these short chain alkanols can significantly increase the solubility of hesperetin. Moreover, PEG 400 had the most potential solubilized effect by an increase of about 3400-fold.

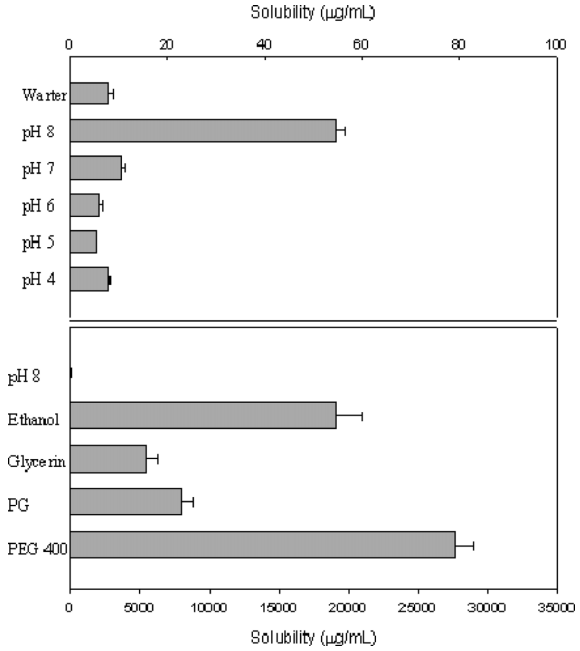


Fig. 1. Solubility of Hesperetin in Different Medium

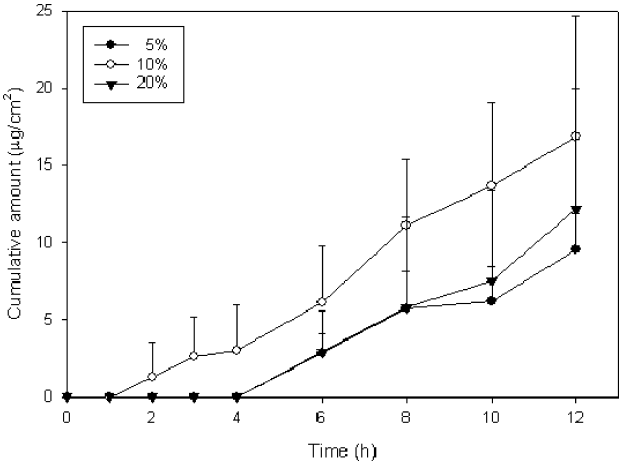


Fig. 2. *In Vitro* Permeation–Time Profile of Hesperetin Creams with Different Levels of PEG 400 Incorporated

***In-Vitro*** **Skin Permeation Experiments** Cosolvents can increase the solubility of a drug in formulation, and then modify the permeation of a variety of such drugs through the skin barrier by changing the thermodynamic activity of the drug in the formulation. As shown in Fig. 2, with addition of PEG 400 from 5 to 20%, the 12 h cumulative amount and lag time were 9.6$\pm$2.3 $\mu$g/cm$^2$ and 6 h for 5% added; 16.9$\pm$7.8 $\mu$g/cm$^2$ and 3.7 h for 10% added; 12.2$\pm$7.7 $\mu$g/cm$^2$ and 6 h for 20% added, respectively. It was found that the level of PEG 400 had no significant effect on the 12 h cumulative amount, but the lag time was shortened by 10% of PEG 400 added. Therefore, 10% PEG 400 was incorporated into the following formulations.

Addition of enhancers is one of the most commonly used

methods for increasing skin permeation of drug. The effects of an enhancer on the permeation of a drug usually depend upon the physicochemical characteristics of permeant as well as the enhancer molecule. Menthol and azone are two popular enhancers for topical use. Lecithin and linoleic acids are fatty acids and used in cosmetic products as depigmenting agents.[15—18] Both of them have also permeation enhancement effect for drugs such as piroxicam and ketoprofen.[6,19—21] These four enhancers were used to improve the permeation of hesperetin in this study. As shown in Fig. 3, it can be seen that the 12 h cumulative amount of hesperetin cream with 5% enhancers of azone, menthol, lecithin and linoleic acid were 104.6±29.9, 157.6±32.8, 83.1±30.0 and 79.0±20.8 $\mu$g/cm$^2$, respectively. Menthol showed the most potent enhancing effect; the 12 h cumulative amount increased about 9.3-fold when compared with the formulation without enhancer. Earlier reports[7,8] indicated that the enhancing effect of menthol was mainly due to an increase in diffusion, and it was speculated that the menthol might increase the partition of lipophilic drug. Azone has been shown to be effective in enhancing the permeability of many compounds through the stratum corneum.[19,22—24] The mechanism of action of azone is suggested to influence the lipid fluidizing and to alter the keratin structure on stratum corneum lipids.[25] In this study, the 12 h cumulative amount for the control formulation was increased about 6.2 folds with incorporation of 5% azone. Lecithin and linoleic acids had moderate enhancement effect of about 4.5-fold increase in drug permeation. This mechanism of action might be attributed to fatty acid ability to change the skin lipid fluidity, thus leading to an enhanced permeation absorption of drugs.[6,19—21] Furthermore, enhancers may have the advantage of a shorter lag time after topical application. The lag time of cream with 5% enhancers of azone, menthol, lecithin and linoleic acid were 1 h, 1 h, 1 h and 2.0 h, respectively, showing that incorporated enhancers could shorten the lag time when compared with the formulation without enhancer (3.7 h). The residual drug concentration in the skin showed slightly higher in formulation with higher flux (Table 1).

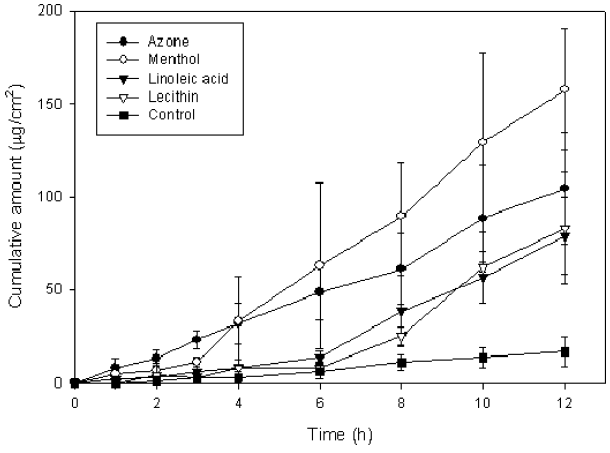


Fig. 3. *In Vitro* Permeation–Time Profile of Hesperetin Creams with Different Type of Enhancers through Rat Skin ($n$=3)

In general, enhancers cause extensive damage to the skin along with the large increase in permeation enhancement. Therefore, a combination of enhancers may be able to create a synergistic effect and mitigate the irritation caused by formulation applied by decreasing the addition level of enhancers. Previous studies[15—18] reported that lecithin has an inhibitory effect on melanization, and linoleic acid can promote degradation of tyrosinase and skin turnover acceleration of melanin pigment, so they are used in cosmetic products as depigmenting agents and have benefit on photoprotective effect. Furthermore, lecithin and linoleic acid showed moderate enhancement effect for hesperetin in this study. Therefore, the enhancement effect of combination of menthol, lecithin and linoleic acid was evaluated. As shown in Table 1, at total amount of 5% enhancers incorporated, the enhancer alone showed higher enhancement effect than that of their combination. The enhancer combination with menthol showed higher enhancement effect. Combination of linoleic acid and lecithin showed lower permeation rate and longer lag time. This might be attributed to the different mechanisms of menthol and fatty acid, resulting in larger enhancement effect. However, the combination of menthol, linoleic acid and lecithin at each of 2.5% showed the higher flux, cumulative amount and residual drug concentration in skin, and shorter lag time. Therefore, this formulation was used to determine the photoprotective effect.

***In Vivo* Study for Whitening Assessment** The quantitative evaluation of whitening was done by determining the changing level of luminosity index $L^*$ after 4 weeks of daily topical application of samples. As shown in Fig. 4, the lightness of skin increased time-dependently after UV exposure. In comparison of the topical application of cream with and without hesperetin and non-treatment, the hyperpigmentation was lightened more effectively when cream with hesperetin was applied, particularly after 21 d of application, which indicated that hesperetin had depigmentation effect. The change level of $L^*$ by blank cream (without drug but containing lecithin and linoleic acid of 2.5%) application was higher than that of non-treatment, although no significant difference was found in this study. The result was similar to previous research studies[15—18] that pointed out linoleic acid and lecithin

Table 1. The Composite and Permeation Parameters of Hesperetin Formulations with Enhancer Alone and Their Combination

| Enhancer | | | Cumulative amount ($\mu$g/cm$^2$) | Flux ($\mu$g/cm$^2$/h) | Lag time (h) | Residual amount in skin ($\mu$g/cm$^2$) |
|---|---|---|---|---|---|---|
| Menthol | Linoleic acid | Lecithin | | | | |
| 2.5 | 2.5 | 2.5 | 100.2±21.0 | 9.38±1.98 | 1.0 | 17.88±5.35 |
| 0.0 | 2.5 | 2.5 | 17.2±2.9 | 1.51±0.26 | 3.3 | 12.36±2.98 |
| 2.5 | 0.0 | 2.5 | 43.7±11.0 | 3.72±0.93 | 2.3 | 14.18±4.17 |
| 2.5 | 2.5 | 0.0 | 52.7±16.9 | 4.95±1.91 | 2.3 | 15.91±9.41 |
| 5.0 | 0.0 | 0.0 | 157.6±32.8 | 14.74±5.68 | 1.0 | 19.21±6.75 |
| 0.0 | 5.0 | 0.0 | 79.0±20.8 | 7.04±2.05 | 2.0 | 16.33±6.37 |
| 0.0 | 0.0 | 5.0 | 83.1±30.0 | 7.41±1.30 | 1.0 | 15.78±7.23 |

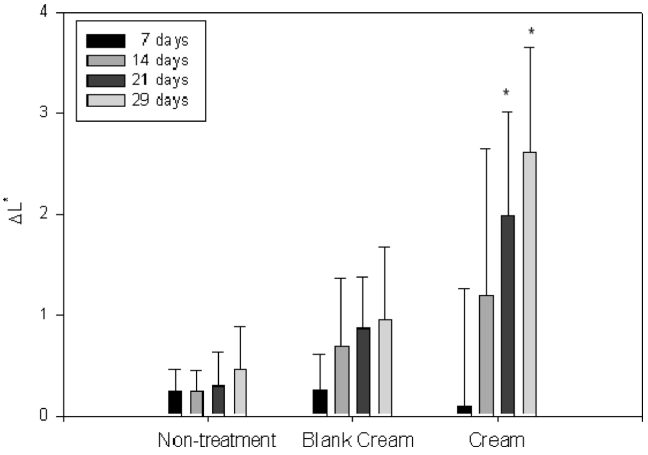


Fig. 4. Whitening Effect of Hesperetin Cream on UV Induced Hyperpigmentation

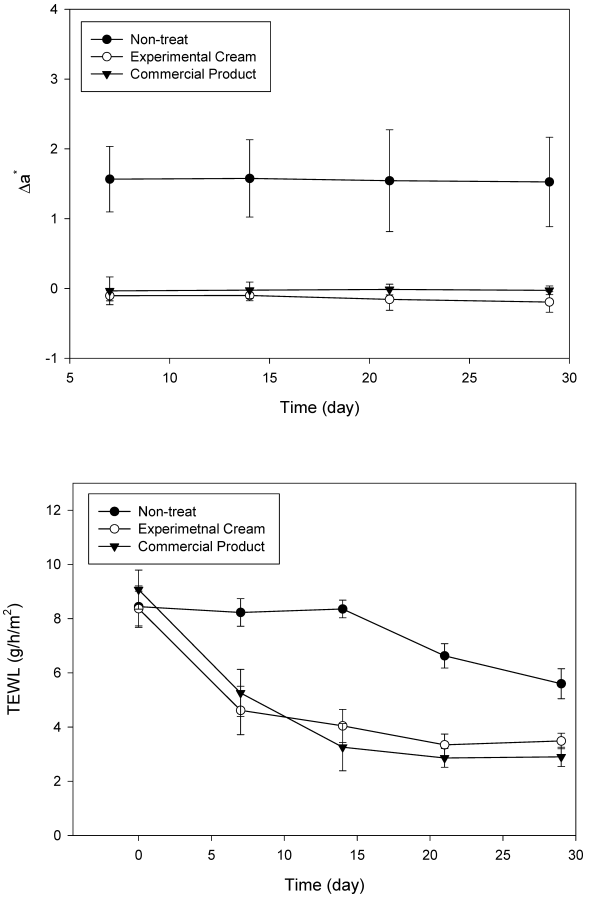


Fig. 5. Irritation Effect of Hesperetin Cream after Topical Application by Determining the Color Differences of Redness and Transepidermal Water Loss (TEWL)

have depigmentation effect, but the basal formulation influences their whitening effect.[8)]

***In Vivo* Study for Skin Irritation Evaluation** The quantitative evaluation of irritation was done by determining the changing level of index "*a**" and TEWL after 4 weeks of daily topical application of samples. As shown in Fig. 5, the change of "*a**" and TEWL after cream was applied was lower than that of non-treatment indicating that hesperetin cream had an inhibitory effect on irritation. This might be attributed to the anti-inflammatory effect of flavonoids.[26)] In additional, a commercial product (Plush blush® cream containing 1% ascorbyl magnesium phosphate) was subjected to this study for another control group. As shown in Fig. 5, the change of "*a**" and TEWL after experimental formulation was similar to the commercial product, demonstrated that the skin irritation caused by the hesperetin cream was acceptable for clinical use.

## Conclusion

Menthol, azone, linoleic acid and lecithin showed potential enhancement effect for hesperetin cream through rat skin. Hesperetin cream of 1% with combination enhancers of menthol, linoleic acid and lecithin of 2.5% possessed topical photoprotective effect with acceptable skin irritation by means of *in vivo* studies.

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