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In vitro permeation and in vivo whitening effect of topical hesperetin microemulsion delivery system

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ABSTRACT

Hesperetin is one of the flavonoids and possess anti-inflammatory, UV-protecting and antioxidant effects. Permeation issues for topical delivery systems of such effects are occasionally problematic, and in view of the fact that microemulsions are potential carriers for transdermal delivery system, the objective of this study was to design an optimal microemulsion formulation by in vitro permeation study for hesperetin topical dosage form and determine its topical photoprotective effect and skin irritation by in vivo study. The hesperetin-loaded microemulsion showed an enhanced in vitro permeation compared to the aqueous and isopropyl myristate (IPM) suspension dosage form of hesperetin. In comparison, the effect of co-surfactant on the drug permeation capacity, propylene glycol showed highest permeation rate, followed by ethanol, glycerol and polyethylene glycol (PEG 400). Sunscreen agent padimate O, as a transdermal enhancer could increase the permeation rate of hesperetin. In case of in vivo study, the hesperetin-loaded microemulsion showed significant topical whitening effect and diminished skin irritation when compared with the non-treatment group, indicating that the hesperetin microemulsion could be used as an effective whitening agent.

1. Introduction

Oxidation is well known to be a major cause of material degradation. Recently, oxygen-reactive species have been recognized to be involved in several diseases, including cancer and atherosclerosis (Maxwell, 1995). Ageing may also be the result of the deleterious oxidation reactions which occur throughout cells and tissues (Maxwell, 1995). Nowadays natural antioxidants are receiving increasing attention; particularly, flavonoids have been reported to be efficient antioxidants by scavenging oxygen radicals, and have been used to treat various diseases (Braca et al., 2002; Hanasaki et al., 1994; Sosa et al., 2002). Moreover, flavonoids are claimed to be free of toxicity and side effects and, in particular, are harmless to the skin (Bonina et al., 1996). Recently, much research has been focused on the potential use of flavonoids for preventive oxidative skin damage (Bing-Rong et al., 2008; Huang et al., 2008b; Mortimer, 1997; Pacheco-Palencia et al., 2008; Schoemaker et al., 1995).

To prevent oxidative skin damage, the topical delivery system is the considered administration. Nevertheless, the most difficult aspect of a transdermal delivery system is to overcome the barrier of stratum corneum against foreign substances. The methods for improvement of drug permeation through the skin are to use chemical permeation enhancers and physical implements such as iontophoresis and ultrasound. Iontophoresis and ultrasound are not frequently used due to the requirement of qualified staff for their application. Microemulsion systems have received increasing attention during the past years, because of having several advantages such as ease of manufacturing, thermodynamic stability, enhanced drug solubilization and increased drug permeation rate (Laurence and Rees, 2000), therefore, they are useful as vehicles for topical delivery of drugs such as triptolide, apomorphine, estradiol, indomethacin and 8-methoxsalen (Baroli et al., 2000; Chen et al., 2004; Huang et al., 2008a; Paolino et al., 2002; Peira et al., 2001; Peltola et al., 2003).

In this study, hesperetin, one kind of flavonoid compounds with potential antityrosinase and antioxidant activity, has been demonstrated to have protective effect of skin damage (Dekker et al., 2005; Saija et al., 1998) was used as the model drug to prepare hesperetin microemulsions for topical whitening product after UV radiation. The permeability of the drug through rat skin in vitro study, skin whitening and irritation test in vivo studies, and stability of formu-

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lations were all evaluated for their clinical usability of whitening effect.

2. Materials and methods

2.1. Materials

Hesperetin, naringenin, padimate O, octisalate, sorbitan monolaurate (Span 20) and sorbitan monooleate (Span 80) were purchased from Tokyo Chemical Industry (Japan). Polyoxyethylene sorbitan monooleate (Tween 80) was acquired from Showa Corporation (Japan), lecithin was from Wako Pure Chemical (Japan). Isopropyl myristate (IPM), propylene glycol (PG), polyethylene glycols 400 (PEG) were purchased from Merck Chemicals (Germany). All other chemicals and solvents were of analytical reagent grade.

2.2. Partition coefficient measurement

The n-octanol/water partition coefficients of hesperetin were determined in various pH values of phosphate buffer solutions at room temperature. The various aqueous phases were buffered to pH 4.0, 5.0, 6.0, 7.0 and 8.0. Equal portions (2 mL) of buffer and n-octanol saturated with buffer were poured into a glass-stoppered centrifuge tube and shaken for 24 h. The aqueous phase was analyzed by HPLC. The partition coefficient was calculated according to the equation of hesperetin concentration in octanol phase/concentration in aqueous phase.

2.3. Preparation of hesperetin microemulsions

The oil and aqueous phase were separately prepared. Two kinds of mixture of surfactant: Tween 80/Span 80 = 3/2 (MS1) and Tween 80/Span 20 = 3/2 (MS2) were used. The oil phase consisted of oil and mixture surfactants of MS1 or MS2, while the aqueous phase consisted of double-distilled water and co-surfactants such as ethanol, PG, glycerol and PEG 400. The aqueous phase was added to the oily phase and shaken by a vortex for 2 min at room temperature. The clear o/w microemulsions were obtained. Hesperetin of 1% and transdermal enhancers (padimate O and octisalate) at different levels were dissolved in the final microemulsion formulations.

2.4. Microemulsion characterization

The average particle sizes of hesperetin microemulsions were determined by photo-correlation spectroscopy by laser light scattering (Zetasizer 3000HSA, Malvern, UK) using a helium–neon laser with a λ of 633 nm. Samples were loaded into 1 cm^2 cylindrical cuvettes and placed in a thermostated scattering chamber. Light scattering was monitored at a fixed angle of 90° and fixed temperature of 25 °C.

The electrical conductivity of the microemulsions was measured by a handheld conductivity meter (WTW Cond 315i, SUNTEX) at 25 ± 2 °C.

2.5. In vitro skin permeation study

The permeability of hesperetin microemulsions 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 mL of hesperetin microemulsion and occluded by paraffin. 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 (Kanaze et al., 2004). A Merck Lichrocart® C18 column (125 mm × 4 mm I.D., particle size 3 μm) 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. Naringenin of 100 μg/mL was used as internal standard. The limit of detection was 0.02 μg/mL (signal-to-noise > 4). Each data point represented the average of three determinations.

At the end of the in vitro permeation experiment, the residual applied drug level 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 17,800 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.

2.6. Whitening assay of in vivo study

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 Corporation XL-1000) three times a week (every other day) for 2 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 (Maeda and Naganuma, 1998; Shigeta et al., 2004). 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 days after exposure, the skin luminosity of each region was measured using a Chroma Meter (CR-200, Minolta Camera, Tokyo, Japan). ΔL^*, the change in luminosity index L^*, was calculated as ΔL^* = pre-exposure $L^* - L^*$ of reading on each measurement day after exposure. The effect of hesperetin microemulsion on skin depigmentation was evaluated using ΔL^* 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.

2.7. Skin irritation evaluation

At the same specific time interval 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 (Fang et al., 1997; Huang et al., 2004). Δa^*, the change in a^*, the balance between red (100) and green (−100) was used as index for erythema degree of skin. Δa^* = pre-exposure $a^* - a^*$of reading on each measurement day after exposure.

2.8. Stability of microemulsions

Ten milliliters of hesperetin microemulsions were preserved in light-resistant glass and stored at room temperature. The chemical and physical stability of hesperetin microemulsions was studied

Table 1
The partition coefficient of hesperetin in n-octanol and various pH values of phosphate buffer.

Medium	log *P*
Distilled water	2.26 ± 0.09
Phosphate buffer pH 4	2.03 ± 0.30
Phosphate buffer pH 5	2.14 ± 0.02
Phosphate buffer pH 6	2.15 ± 0.14
Phosphate buffer pH 7	2.16 ± 0.26
Phosphate buffer pH 8	1.77 ± 0.03

via clarity and phase separation observation, droplet size determination and HPLC analysis.

3. Results and discussion

3.1. Partition coefficient

As shown in Table 1, the logarithm value of partition coefficient of hesperetin in various mediums and n-octanol ranged from 1.77 to 2.26, showing that hesperetin possesses appropriate lipophilicity for skin permeation (Zhao et al., 2008) and the pH value had no effect on the partition coefficient.

3.2. Characteristics of microemulsion

The physico-chemical parameters of hesperetin microemulsions were measured and are listed in Tables 2 and 3. The droplet size of microemulsion was small with all the formulations having a mean size between 101.5 and 233.0 nm. The electrical conductivity of IPM (oil phase) and doubled water were 0 and 0.6 μS/cm respectively. As shown in Table 2, the electrical conductivity of all drug-loading microemulsions ranged from 0.5 to 87.2 μS/cm. The electrical conductivity of microemulsions was significantly higher than the oil phase. Moreover, electrical conductivity of microemulsions increased by the increase in the level of aqueous phase or the polarity of co-surfactant. The increase in electrical conductivity might be due to the increase in dissociation of surfactant as a function of water content (Baker et al., 1984). These results were in accordance with a previous study (Sintov and Shapiro, 2004) and indicated that the microemulsions were oil-in-water type. The solubility of hesperetin in IPM and water were 385.4 and 8.03 μg/mL respectively. All microemulsion formulations containing 1% hesperetin in this study were clear and transparent solutions; no precipitate was observed, indicated that microemulsion can increase the solubility of the drug (Teichmann et al., 2007).

Microemulsion stored at room temperature for 2 months showed no change in clarity, phase behavior and particle size (data not shown). The concentration of hesperetin in microemulsion was above 98.0 ± 1.5%, showing there was no degradation.

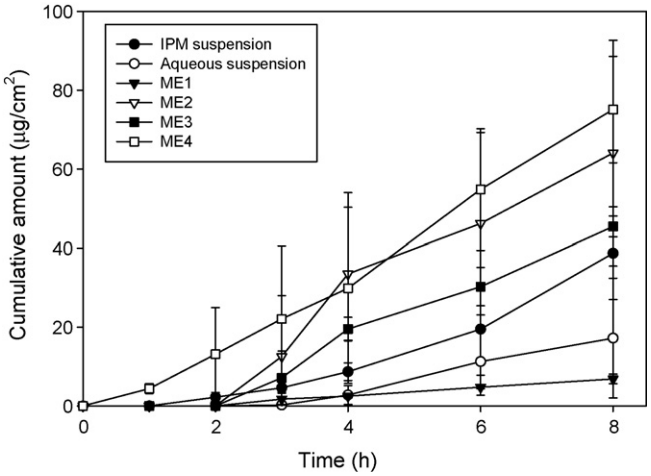

Fig. 1. In vitro permeation-time profile of hesperetin microemulsion with different compositions.

3.3. In vitro skin permeation study

The permeation parameters of the tested ME formulations with various compositions are presented in Table 2. The permeation profiles of hesperetin through rat skins are shown in Fig. 1. A steady increase of hesperetin in the receptor chambers with time was observed. The permeation profiles on microemulsions followed zero order release kinetics. As shown in Table 2, the permeation rates of hesperetin from aqueous and IPM suspension were 3.51 and 6.77 μg/(cm² h), respectively. The flux in IPM was significantly higher than in the aqueous solution. This result might be due to the fact that the lipophilic compound, hesperetin was more dissolved in IPM (demonstrated above) which led high concentration gradients towards the skin (Goldberg-Cettina et al., 1995). Moreover, IPM is an effective permeation enhancer, hence causing an extensive permeation (Goldberg-Cettina et al., 1995).

The flux in different compositions of microemulsions ranged from 1.04 to 10.89 μg/(cm² h) and lag time (the first of drug detection) ranged from 1 to 3.3 h, indicating the drug permeation characters through rat skin was significantly affected by the composition of microemulsion formulation. Moreover, most of the microemulsion formulations provided a higher permeation rate and shorter lag time than that of hesperetin in IPM and water, showing that the test microemulsion formulations had a potent enhancement effect for hesperetin transdermal delivery system. These results were consistent with previous studies that demonstrated microemulsion systems have favourable solubilization and transdermal transport behaviour, because the combined effect of hydrophilic and lipophilic components of the microemulsion enhances the activity in the whole system. Moreover, microemul-

Table 2
The characteristics and permeation parameters of different compositions of hesperetin microemulsion formulations.

Code	MS1	MS2	IPM	A	Flux (μg/(cm² h))	LT (h)	Residual amount in skin (μg/cm²)	Particle size (nm)	EC (μs/cm)
ME1	0.6	–	0.3	0.1	1.04 ± 0.07	3.3	31.90 ± 7.02	195.3 ± 7.7	0.8
ME2	0.4	–	0.1	0.5	7.94 ± 4.15	1.7	33.34 ± 5.05	169.6 ± 0.4	69.3
ME3	0.4	–	0.3	0.3	6.43 ± 0.21	2.0	35.56 ± 16.97	101.5 ± 0.7	32.0
ME4	–	0.4	0.1	0.5	10.89 ± 2.00	1.0	59.09 ± 11.93	168.8 ± 3.3	87.2
Water suspension					3.51 ± 3.00	3.7	25.93 ± 7.39		
IPM suspension					6.77 ± 2.38	2.0	20.09 ± 3.43		

MS1: mixture surfactant (Tween80/Span80 3:2, HLB = 10.72).
MS2: mixture surfactant (Tween80/Span20 3:2, HLB = 12.44).
A: aqueous phase containing ethanol as co-surfactant.
IPM: isopropyl myristate (oil phase).
EC: electrical conductivity.

Table 3
The characteristics and permeation parameters of hesperetin microemulsion with different co-surfactants.

Co-surfactant	Flux (μg/(cm² h))	Q_{8h} (μg/cm²)	LT (h)	Residual amount in skin (μg/cm²)	Particle size (nm)	EC (μs/cm)
Ethanol	10.89 ± 2.00	75.1 ± 13.5	1.0	59.09 ± 11.93	168.8 ± 3.3	87.2
Glycerol	8.41 ± 3.06	60.0 ± 37.9	1.7	43.76 ± 6.66	233.0 ± 5.7	10.7
PG	30.43 ± 8.38	171.1 ± 50.0	1.0	57.30 ± 3.17	158.8 ± 5.3	4.2
PEG 400	0.86 ± 0.37	9.2 ± 3.3	2.3	61.50 ± 2.37	168.3 ± 0.4	0.5

The composite of microemulsion was Tween 80/Span 20 (3/2): IPM: aqueous phase containing co-surfactant.

sions are able to reduce the interface tension between skin and vehicle because of its close contact to the skin lipid, and thus result in a faster permeation (Schmalfuß et al., 1997; Teichmann et al., 2007). However, it is important to design an appropriate microemulsion formulation with higher permeation rate for the hesperetin transdermal delivery.

In comparison of the effect of composition of microemulsions on hesperetin permeation (Table 2 and Fig. 1), it was found that the permeation rate was significantly increased by decreasing the level of surfactant ($p < 0.05$; ME1 and ME3). A possible reason for this phenomenon was that the progress of emulsification might be compromised by viscous liquid crystalline gel forming at the surfactant–water interface at high surfactant concentration, thus leading to the decrease of drug diffusion through the double layer microemulsion to the receptor chamber (Zidan et al., 2007). The other possibility was the decrease of thermodynamic activity of the drug in microemulsion with higher concentration of surfactants (Rhee et al., 2001). The thermodynamic activity of drug in the formulation is a significant driving force for the release and permeation of the drug into skin (Walters et al., 1998). The thermodynamic driving force for release reflects the relative activities of the drug in different phases (Delgado-Charro et al., 1997), since the drug can be released from the internal phase to external phase and then from the external phase to the skin; the relative activities may monitor the skin permeation flux. Further, the flux was slightly decreased (from 7.94 to 6.43 μg/(cm² h)) and lag time was trifling lengthened (from 1.7 to 2.0 h) by increasing the oily phase of amount from 10% to 30% (ME2 and ME3). A previous study reported that the increase in the concentration of the internal phase of an emulsion causes a gradual rise in the viscosity of the system (Halbaut et al., 1996). In comparison to the effect of HLB value of mixture surfactant, it can be seen that the flux was increased from 7.94 to 10.89 μg/(cm² h) and the lag time was shorten from 1.7 to 1 h, via increase of HLB value of surfactant from 10.72 to 12.44 (ME2 and ME4). This phenomenon might be attributed to the fact that a surfactant with higher HLB value could facilitate continuous lipophilic drug distribution and enhance the permeation.

The residual applied drug level in the skin of these microemulsions was from 31.90 to 59.09 μg/cm² (Table 2). There was no significantly linear correlation ($r = 0.747$, $p < 0.05$) between the skin content and flux. But it was found that the microemulsions showed higher skin content of drug when compared to the control groups of water suspension and IPM suspension.

According to earlier reports (Trotta et al., 1999; Wu et al., 2001), the co-surfactant can lower the interfacial tension of the surfactant file in microemulsions, resulting in a more flexible and dynamic layer. The drug in this energy-rich system can diffuse across the flexible interfacial surfactant film between the phases; a thermodynamic process that increases partitioning and diffusion into the stratum corneum. Therefore, the influence of various co-surfactants on permeation absorption and skin content of hesperetin was also evaluated in this study. Various short chain alcohols instead of ethanol were used to prepare the microemulsion. As shown in Table 3, there was no significantly difference ($p < 0.05$) in the microemulsions with different co-surfactants. With the addition of various co-surfactants, PG showed highest permeation rate, followed by ethanol, glycerin and PEG 400. According to our previous study, the solubility of hesperetin was 7.9 mg/mL in PG, 27.7 mg/mL in PEG, 5.4 mg/mL in glycerol and 19.1 mg/mL in ethanol. It can be seen that the microemulsion with co-surfactant of lower drug solubilization capacity demonstrated higher permeation rate. A possible reason for this phenomenon was the increase of thermodynamic activity of the drug in microemulsion form which lead to enhancement of the permeation rate (Rhee et al., 2001). For an exception, glycerol had lower solubility of the drug and showed lower permeation rate. This might be due to its higher viscosity, and therefore impede the diffusion rate.

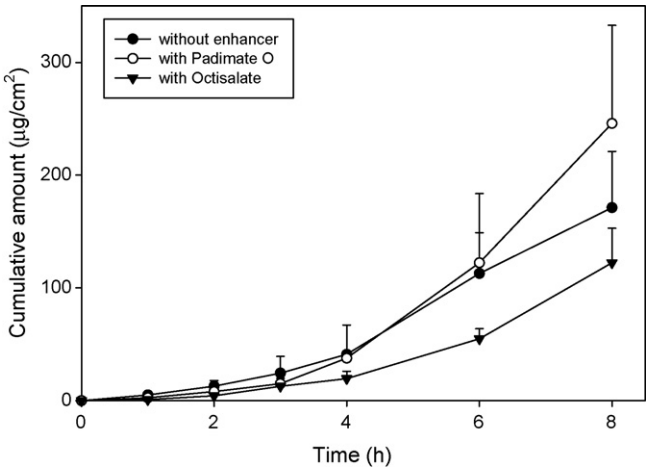

Fig. 2. In vitro permeation-time profile of hesperetin microemulsion with and without transdermal enhancers.

To maximize the permeation rate of the microemulsion, transdermal enhancers were incorporated. Padimate O and octisalate have been reported to improve the transdermal permeability of various compounds (Nicolazzo et al., 2004, 2005). Furthermore, they have been extensively used as safe and effective sunscreens in topical concentrations up to 5% (v/v) for octisalate and 8% (v/v) for

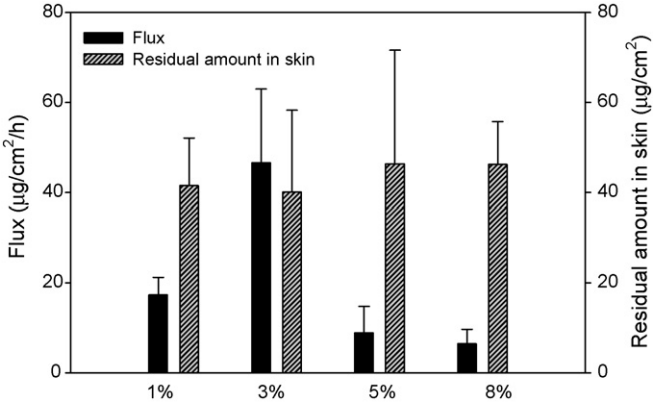

Fig. 3. The effect of concentration of padimate O on permeation rate and residual amount in skin of hesperetin microemulsion.

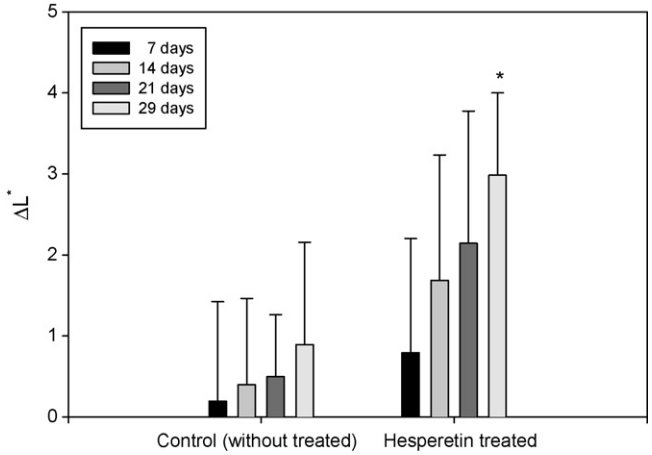

Fig. 4. Whitening effect of hesperetin microemulsion on UV induced hyperpigmentation.

padimate O; hence, with the addition of screen agents in hesperetin, the microemulsion might yield a synergistic benefit on the photoprotective effect. Therefore, 3% of padimate O and octisalate were loaded into the hesperetin microemulsion and the enhancement effect of drug permeation was evaluated. As shown in Fig. 2, the flux was 21.99 ± 9.66 μg/(cm^2 h) by octisalate incorporated, showing no effect on the permeation rate of hesperetin. On the other hand, the flux was 46.56 μg/(cm^2 h) with an enhancement ratio of about 1.5 when padimate O was incorporated. In comparison to the effect of concentration of padimate O on the permeation rate, it was found that the flux of hesperetin was reduced as the padimate O concentration was increased to 5% and 8% (Fig. 3). This might be owing to the microemulsion becoming more lipophilic via higher levels of padimate O added, obstructing the release of hesperetin from the microemulsion. However, the microemulsion formulation with 3% padimate O was selected to process the skin whitening and irritation study.

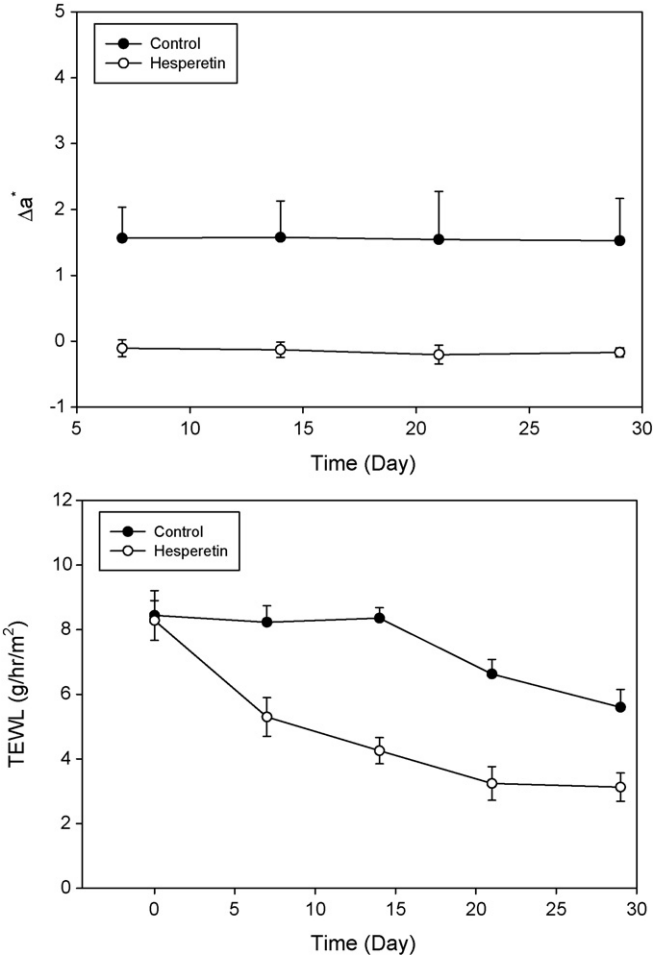

Fig. 5. Assessment of skin irritation of hesperetin microemulsion by determining the color differences of redness and transepidermal water loss (TEWL).

3.4. 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, skin lightness increased time-dependently after UV exposure. After topical application of hesperetin microemulsion, the hyperpigmentation was lightened when compared to the control group, particularly after 29 days of application which indicated that hesperetin had a depigmentation effect.

3.5. Skin irritation evaluation

The quantitative evaluation of irritation was done by determining the changing levels 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 hesperetin microemulsion applied was lower than that of control (non-treatment), indicating that hesperetin microemulsion had an inhibition irritation effect. This might be attributed to the anti-inflammatory effect of flavonoids (Garg et al., 2001; Nagao et al., 1999).

4. Conclusion

The permeation rate of drug was markedly affected by the composition of microemulsion formulations including the ratio of surfactant/aqueous/IPM, HLB value of surfactant and type of co-surfactant. A sunscreen agent (padimate O) as a permeation enhancer could increase the in vitro permeation rate. The hesperetin microemulsion incorporating PG and padimate O of 3% showed significant skin whitening effect.

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