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Fatty acids in *Botryococcus braunii* accelerate topical delivery of flurbiprofen into and across skin

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Abstract

To improve the drug absorption into and across the skin, fatty acids extracted from *Botryococcus braunii* were evaluated using in vitro and in vivo techniques with Wistar rats as the animal model. Palmitic acid (C16:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3) were the major components in the *B. braunii* extract. Topical delivery of flurbiprofen was significantly enhanced after pretreatment with 3% *B. braunii* extract for 30 min in an in vitro Franz cell and in vivo pharmacokinetic studies. Pure unsaturated fatty acids were more-effective enhancers than the *B. braunii* extract. However, a greater irritant potential was also observed with those fatty acids than with the *B. braunii* extract according to the skin tolerance study as determined by transepidermal water loss (TEWL). Both human keratinocytes and skin fibroblasts showed a 1.5–2-fold increase in prostaglandin E_2 (PGE_2) release as compared to the control. The findings in this study indicate that the fatty acids in *B. braunii* may be useful enhancers for flurbiprofen delivery via the skin.

Keywords: *Botryococcus braunii*; Flurbiprofen; Topical delivery; Enhancers; Fatty acids

1. Introduction

Over the past two decades, the skin has become an important portal for drug delivery for topical, regional, and systemic actions. Dermal and transdermal drug delivery is often limited by the poor permeability of the skin to drugs, which precludes their crossing the skin at therapeutic rates. The primary barrier to dermal and transdermal permeation is the stratum corneum (SC), the outermost layer of the skin, which is comprised of keratin-rich cells embedded in multiple lipid bilayers (Barry, 1991). The barrier properties of the SC can often be reduced by the use of enhancers (Warner et al., 2001). Developing new skin permeation enhancers and studying the mechanisms of permeation enhancement have gradually become a very active area of transdermal research.

Fatty acids are a class of compounds frequently used to accelerate skin permeation. Oleic acid is a good example and has been shown to induce phase separation in the SC lipid domains, thereby reducing their barrier function (Moser et al., 2001). Various marine products

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such as fish oils are rich in unsaturated fatty acids, which have been used as skin permeation enhancers (Loftsson et al., 1995, 1998). The green microalga *Botryococcus braunii* is a freshwater species belonging to the Chlorophyceae and is known to possess unusually high levels of free fatty acids (Laureillard et al., 1988). This alga is distributed in oligotrophic and in some eutrophic fresh waters, brackish and saline ponds, lakes, and reservoirs throughout the world in a wide variety of climates (Aaronson et al., 1983). Hence *B. braunii* with its fatty acids may be suitable as permeation enhancers because of its broad distribution on all continents and its possible enhancement of drug absorption into the skin.

Flurbiprofen was selected as a model drug to examine the effect of *B. braunii* extract on its skin absorption. Flurbiprofen is used to treat gout, osteoarthritis, rheumatoid arthritis, and sunburn (Fang et al., 2003b). Flurbiprofen was one of the least-permeable drugs across skin among a series of lipophilic drugs (Morimoto et al., 1992). The principal constituents of fatty acids extracted from *B. braunii* were identified by HPLC in this study. We utilized in vitro Franz cells to explore the influence of *B. braunii* extract on the skin absorption of flurbiprofen. The amount of drug uptake within the skin reservoir was also determined in vitro and in vivo. Since it is preferable for practical use to find a balance between permeation enhancement and skin toxicity of a particular enhancer, a second aspect of this study was to examine the in vitro and in vivo irritancy profiles of the *B. braunii* extract. The skin irritant potential of the enhancers was assessed using transepidermal water loss (TEWL). The release of the inflammatory mediator, prostaglandin E_2 (PGE_2), from human keratinocytes and skin fibroblasts with treatment using *B. braunii* extract and fatty acids was investigated in order to explore the biologic responses of the skin under these enhancer treatments.

2. Materials and methods

2.1. Materials

Flurbiprofen, palmitic acid, oleic acid, linoleic acid, and linolenic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 2-Bromo-2-acetonaphthone and hexaoxacyclo octadecane were from Aldrich Co. (St. Louis, MO, USA). The carboxymethyl cellulose (CMC) sodium salt was purchased from Wako Chemical Co. (Japan). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were supplied by Biowest Co. (France). PGE_2 kit (Correate-EIA®) was from Assay Designs, Inc. (MI, USA). *Botryococcus braunii* used in this study was obtained from the Institute of Botany, Academia Sinica, Taiwan. All other chemicals and solvents were of analytical grade.

2.2. Extraction method

The *B. braunii* preparation (100 g) was extracted with 1 l of ethanol five times. The mixture was partitioned successively between H_2O (2 l) and *tert*-butylmethylether (4 l). The *tert*-butylmethylether layer was dried, and the residual was mixed with 90% ethanol. Then this mixture was subjected to the C18 solid-phase extraction (Strata® C18-E, Phenomenex, USA) using ethanol as the eluent at a flow rate of about 1 ml/min until no color was observed in the eluate. The collected eluate was concentrated under reduced pressure and then blended with 100 ml methanol. The methanol solution with *B. braunii* was fractioned on Sephadex® LH-20 gel permeation chromatography (Pharmacia Biotech, Sweden) at a flow rate of about 13 ml/min. Twenty milliliters of eluate was collected for every tube. The free fatty acids in the eluate were determined by TLC. The eluate in the tube in which the fatty acids occurred was collected together.

2.3. HPLC analysis

In the determination of fatty acids, the sample was dissolved in 0.5 ml acetonitrile. An excess amount of K_2CO_3 powder was added and then vortexed for 10 s. One-half a milliliter of a derivation agent (composed of 200 mg 2-bromo-2-acetonaphthone and 100 mg hexaoxacyclo octadecane in 20 ml acetonitrile) was added to the mixture and then vortexed for 1 min. The resultant mixture was sonicated at 55 °C for 1 h. The fatty acids were determined after 24 h. Samples were analyzed using a HPLC system consisting of a Hitachi L-7110 pump, a Hitachi L-7200 sample processor, and a Hitachi L-7400 UV detector. A 25-cm-long, 4-mm inner diameter C18 column (LichroCart® 250-4, Merck, Germany) was used. The mobile phase con-

sisted of 7% double-distilled water and 93% methanol at a flow rate of 1 ml/min. The wavelength of the UV detector was set at 247 nm.

The HPLC method for flurbiprofen was reported in our previous investigation (Fang et al., 2003a,b).

2.4. In vitro skin permeation

For preparing the CMC hydrogels as the drug vehicle, a 3% (w/v) concentration of polymer was added into about half of the total amount (47.1%) of pH 7 citrate–phosphate buffer (0.06 M), after which the mixture was stirred continuously for 1 h. After 24 h, the residual pH 7 buffer (47.1%) and 0.8% (w/v) flurbiprofen were added into the mixture with continuous stirring for 5 min. In vitro skin permeation experiments were carried out using a Franz diffusion cell. The dorsal skin of Wistar rats (150–180 g) was shaved using an electric clipper and then mounted on the receptor compartment with the SC-side facing upwards into the donor compartment. The 3% *B. braunii* extract or fatty acids in 25% propylene glycol (PG)/pH 7.4 buffer solution (1 ml) was used to pretreat the skin for 30 min. The applied area was then gently blotted with cotton cloth to remove the residual solution. One gram of flurbiprofen hydrogel was then applied to the treated skin. The receptor medium was 10 ml of pH 7.4 citrate–phosphate buffer. The available diffusion area between cells was 0.785 cm^2. The stirring rate and temperature were maintained at 600 rpm and 37 °C, respectively. At 1, 2, 4, 6, 8, 10, and 12 h after application, a 300-μl aliquot of the receptor medium was withdrawn and immediately replaced with an equal volume of fresh buffer. The amount of drugs was determined by the HPLC method.

The amount of flurbiprofen retained in the skin was determined at the end of the in vitro permeation experiment (12 h). The skin was washed 10 times using a cotton cloth immersed in methanol. A sample of skin was weighed, cut with scissors, positioned in a glass homogenizer containing 1 ml of methanol, and ground for 5 min with an electric stirrer. The resulting solution was centrifuged for 10 min at 10,000 rpm. The supernatant was analyzed by HPLC.

2.5. In vivo topical application

An accurately weighed 0.4-ml amount of 25% PG/pH 7.4 buffer solution containing 3% enhancer was spread uniformly over a sheet of non-woven polyethylene cloth (2.5 cm × 2.5 cm, Johnson & Johnson, USA), which was then applied to the shaved back area of a rat. After 30 min, the cloth was removed and the treated skin area was swabbed clean with cotton. Four-tenths of a gram of hydrogel in the polyethylene cloth (2.5 cm × 2.5 cm) was administered to the enhancer-treated site. The polyethylene cloth was fixed with Tegaderm® adhesive dressing (St. Paul, MN, USA) and Fixomull® stretch adhesive tape (Beiersdorf AG, Hamburg, Germany). Four pieces of cloth containing hydrogels with application durations of 2, 4, 6, and 8 h, respectively, were applied to each rat. Then the rat was sacrificed by ether inhalation, and the treated skin was excised. The procedure for extraction of drug from the skin was the same as that for the in vitro experiments.

2.6. In vivo TEWL determination

The application method of the enhancer solution was the same as that for the in vivo topical application. After a 24-h administration, the hydrogel was removed, and the application site was gently cleaned using a cotton wool swab. After withdrawal of the enhancer solution for 30 min, values of TEWL were determined. TEWL was measured quantitatively using a Tewameter® (TM300, Courage & Khazaka, Germany). An adjacent untreated site was used as the baseline standard for each determination. The TEWL was examined once a day for 4 days.

2.7. Cell cultures

Human keratinocytes and human skin fibroblasts (Hs68) were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). Cells were maintained in DMEM supplemented with 10% heat-inactivated FCS and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) in a humidified incubator at 37 °C and 5% CO_2. For further experiments, cells were seeded at a density of 10^5 cells/ml in 24-well Costar plates and cultured in DMEM containing 10% heat-inactivated FCS.

Stock solutions of enhancers dissolved in PG were prepared. The final concentration of the stock solution in the culture medium was 0.001 and 0.003% (v/v). Cell cultures were exposed to test enhancers for 24 h

for determination of PGE_2 levels. PGE_2 was measured in cell culture supernatant by use of commercially available ELISA according to the manufacturer's instructions. Assays using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were performed to ensure that treatments with these enhancers had no effect on cell viability.

2.8. SEM examination

Excised skin samples were cut into appropriate-sized cubes and immediately fixed at 4 °C in 2% paraformaldehyde and 2.5% glutaldehyde in 0.2 M cacodylate buffer (pH 7.4) overnight. Then they were washed three times with 0.2 M cacodylate and 7% sucrose buffer for 15 min, post-fixed with 2% osmium tetroxide for 1 h, washed three times as above, and immersed in 0.5% aqueous uranyl acetate for 30 min. Specimens were then dehydrated in graded concentrations of ethanol, transferred to isoamyl acetate, and critical-point dried using liquid CO_2. The dried specimens were affixed with gold–palladium in an ion coater and examined with an SEM (Hitachi S-2400, Japan). All histopathologic analyses were performed in a blinded fashion.

3. Results and discussion

3.1. Determination and quantification of fatty acids from B. braunii

The free fatty acids from *B. braunii* extract were identified by means of HPLC. Profiles of the fatty acids are shown in Table 1. Three unsaturated fatty acids and only one saturated fatty acids were identified from the extract representing 55.41% of the total. The predominant component from the *B. braunii* extract was oleic acid, followed by palmitic acid, linolenic acid, and linoleic acid. These compounds are typical for the fatty acid composition of *B. braunii* according to previous research (Ahlgren et al., 1992). Besides free fatty acids, it was inferred that the remainder (44.95%) of the *B. braunii* extract was also composed of lipids. The other components may have included hydrocarbons, carotenoids, and chlorophyll (Yamaguchi et al., 1987). The *B. braunii* extract showed a brownish-red color which may indicate the existence of pigments such as carotenoids and chlorophyll which commonly occur in *B. braunii*.

Table 1
Fatty acid constituents in *Botryococcus braunii* extract and their relative proportion amount percentage

Fatty acid	Amount (%)
Palmatic acid (C16:0)	19.51 ± 0.04
Oleic acid (C18:1)	22.92 ± 0.80
Linoleic acid (C18:2)	1.21 ± 0.01
Linolenic acid (C18:3)	11.77 ± 0.3
Total amount	55.41 ± 0.29

Each value represents the mean ± S.D. ($n = 3$).

3.2. In vitro skin permeation

Because the *B. braunii* extract was composed of four kinds of fatty acids, the enhancement action on drug permeation of each of the major components was separately determined. A mixture of the four fatty acids simulating the *B. braunii* extract was also prepared based on the composition ratios in Table 1, corresponding to the 55.41% extract. The skin permeation of flurbiprofen from hydrogels after pretreatment of a series of enhancers was thus investigated. The pretreatment method avoided cosolvent effects on the thermodynamic activities of the model drug. This method can also verify whether or not the enhancers act directly on the skin structure. The cumulative amount–time profiles for flurbiprofen permeation were plotted as shown in Fig. 1. The slopes of the resulting linear plots were calculated, and the flux ($\mu g/cm^2/h$) was determined from the slope as depicted in Table 2. Previous studies have shown that the enhancing effects of fatty acids are greatest with PG vehicles (Yamada et al., 1987; Gwak and Chun, 2002). Hence, 25% PG in pH 7.4 buffer was used as the pretreatment vehicle for enhancers. The PG vehicle itself slightly but significantly (*t*-test, $P < 0.05$) increased both flurbiprofen flux and skin deposition (Table 2). It is proposed that PG appears to dissolve α-keratin and occupy hydrogen-bonding sites, thus reducing the barrier properties of skin (Loftsson et al., 1998; Gwak and Chun, 2002). PG has two possible effects in the vehicle (Loftsson et al., 1998). It can act as a skin permeation enhancer on its own, and it can increase the concentration of dissolved fatty acids in the pretreatment vehicle, both of which

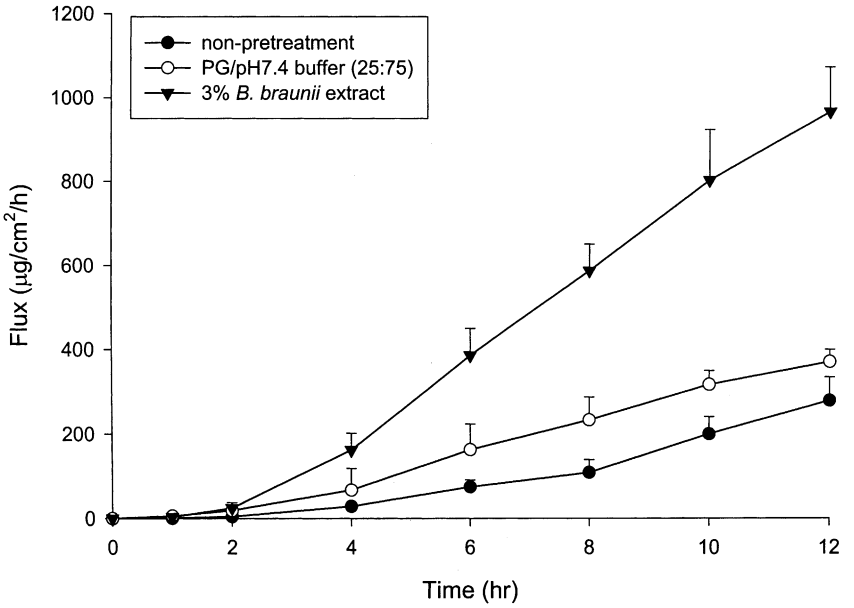

Fig. 1. Cumulative amount of flurbiprofen detected in the receptor vs. time following in vitro topical delivery across excised rat skin after pretreatment with *Botryococcus braunii* extract. All data represent the means of four experiments ± S.D.

may enhance the drug permeation into and across skin.

Pretreatment with the 3% *B. braunii* extract increased the permeation of flurbiprofen by 2.6-fold as compared to the control. Disruption of the skin structure and increment of drug partitioning into the SC may be the possible effects of fatty acids in the *B. braunii* extract. As compared to the enhancing effects of the four fatty acids which occur in the *B. braunii* extract, a significantly larger flux (*t*-test, $P < 0.05$) was obtained from the unsaturated fatty acids than from the saturated fatty acid (Table 2). Moreover, there were no statistically significant differences (ANOVA test, $P > 0.05$) among the drug fluxes after pretreatment using the three unsaturated fatty acids. The same trend of enhancement was observed in the skin uptake of flurbiprofen. The acyl chains of the SC lipids of normal skin are predominantly saturated C16 or longer chains, and they are tightly packed together. As the literature suggests (Aungst, 1989), unsaturated fatty acids are more disruptive of these skin lipids than are saturated fatty acids.

Table 2
Effect of enhancer pretreatment on flurbiprofen flux and amount in the skin after topical application in vitro

Formulation	Flux (μg/cm²/h)	Amount in skin at 12 h (×10² μg/mg)
Non-pretreatment	22.78 ± 3.85	31.42 ± 5.73
PG/buffer (25:75)	33.33 ± 2.29	41.98 ± 5.15
3% *B. braunii* extract	86.43 ± 10.25*	80.94 ± 16.15*
3% linolenic acid	154.81 ± 13.59*	73.88 ± 13.96*
3% linoleic acid	148.43 ± 35.34*	71.77 ± 3.37*
3% palmitic acid	47.14 ± 6.68	50.40 ± 7.83
3% oleic acid	125.75 ± 15.82*	68.71 ± 6.27*
Simulated extract	115.00 ± 20.50*	76.79 ± 16.90*

Each value represents the mean ± S.D. ($n = 4$–5).
* $P < 0.05$, higher than the PG/pH 7.4 buffer (25:75) pretreatment group.

In order to elucidate the importance of fatty acids in the *B. braunii* extract to drug permeation, the difference in flurbiprofen absorption between pretreatment with natural and simulated *B. braunii* extract was compared. The flux and skin depot of flurbiprofen by the simulated fatty acids mixture approximated those of the *B. braunii* extract. This may indicate that free fatty acids in *B. braunii*, but not other compounds such as hydrocarbons, carotenoids, and chlorophyll are the predominant components enhancing the permeation of flurbiprofen into and across skin.

3.3. In vivo skin deposition

Although attempts are currently being made to develop a generally accepted standardized methodology for in vitro testing, a large extrapolation still needs to be made from the in vitro performance to use in an in vivo setting (Finnin and Morgan, 1999). To ascertain the enhancing effect of *B. braunii* extract in an in vivo status, the in vivo pharmacokinetics of flurbiprofen located within the skin were evaluated. As shown in Fig. 2, almost all curves leveled off after a 2-h application of hydrogels. This suggests that hydrogels can act as a carrier, making it possible to maintain an almost constant drug concentration in the skin. The controlled release of flurbiprofen from the hydrogels could thus be achieved. The drug amount in skin with pretreatment using the *B. braunii* extract showed slightly higher values than the control group, although a significant difference (*t*-test, $P < 0.05$) was only observed at 6 h. Skin deposition at 4, 6, and 8 h after application of hydrogels with linolenic acid pretreatment were significantly higher (*t*-test, $P < 0.05$) as compared to the control. It is clear that the oleic acid-pretreatment group resulted in the highest levels of skin depot at all durations. The mechanism of oleic acid as a permeation enhancer is attributed to the partitioning rate and skin disturbance it causes (Gwak and Chun, 2002). The surface layers of the skin rapidly equilibrate with oleic acid. Hence the 30-min pretreatment in this study was sufficient for oleic acid to exhibit higher enhancement than the other enhancers.

There was no significant difference (*t*-test, $P > 0.05$) between the skin deposition achieved with the simulated fatty acids mixture and the *B. braunii* extract during an 8-h application (Fig. 2). This result confirms the above discussion in the in vitro experiment that fatty acids play an important role in enhancing drug absorption. The in vivo pharmacokinetic profiles were generally correlated with the in vitro permeation, which showed a trend of oleic acid = linolenic acid > *B. braunii* extract = simulated mixture > control.

3.4. In vivo TEWL determination

The proposed mechanisms involving promotion of drug permeation by enhancers which may cause skin responses to irritation are: (i) induction of inflamma-

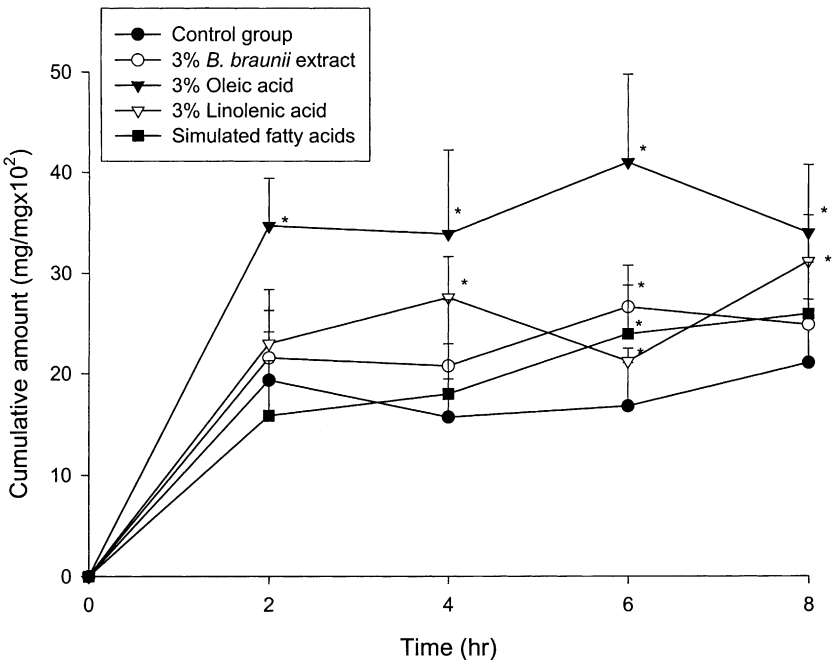

Fig. 2. Kinetics of flurbiprofen uptake within skin after pretreatment with enhancers. All data represent the means of five experiments ± S.D.

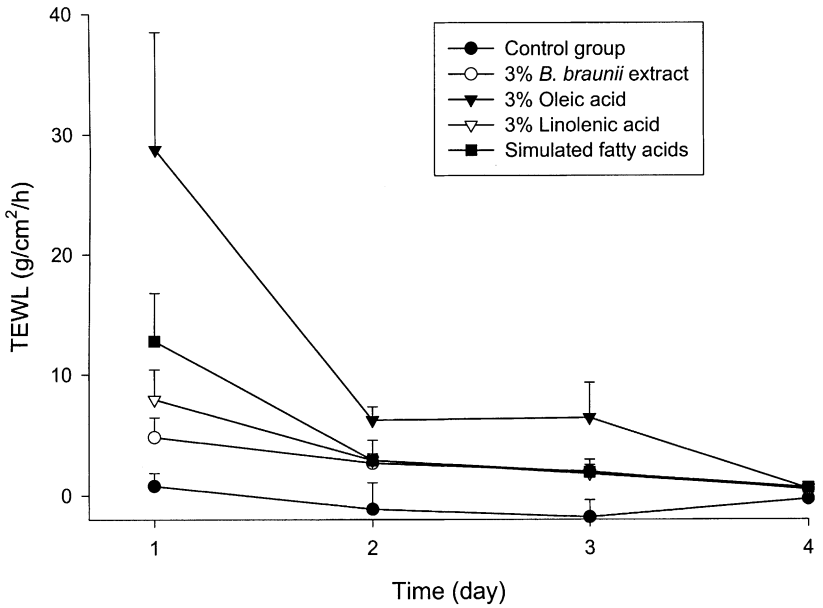

Fig. 3. Kinetics of TEWL for 4 days after topical application with or without enhancers for 24 h. All data represent the means of six experiments ± S.D.

tory mediator release in epidermal cells in the presence or absence of cytotoxicity, and (ii) direct physicochemical interaction with the SC eliciting corrosive damage. Hence a series of experiments for evaluating the safety of these enhancers was conducted in vitro and in vivo in the following sections. TEWL was performed to assess SC damage, and a good correlation between chemical damage to the skin barrier and an increase in TEWL was demonstrated (Fang et al., 2002). The 3% enhancers in the 25% PG/pH 7.4 buffer solution were used to treat skin for 24 h to examine the tolerance of the skin to enhancers. As shown in Fig. 3, the TEWL (TEWL value of treated site minus the TEWL value of an adjacent untreated site) determined over 4 days was evaluated. The PG/pH 7.4 buffer vehicle did not increase the TEWL values over 4 days, indicating that PG itself caused no skin irritation as determined by TEWL.

The treatment of enhancers generally induced ascendant values of TEWL at the beginning, then the values gradually dropped to the baseline as the skin recovered to its normal status (Fig. 3). The TEWL level showed a trend of oleic acid > simulated mixture > linolenic acid > *B. braunii* extract > control. There was a slight or negligible increase in TEWL with the *B. braunii* extract which may indicate the good tolerance of the skin to the *B. braunii* extract. However, the simulated mixture showed detectable skin disruption based on TEWL. This may indicate that the components other than fatty acids in the *B. braunii* extract produced a buffer effect to reduce the skin irritation caused by the fatty acids. Further investigation is needed to elucidate this mechanism. The fatty acids, especially oleic acid demonstrated a significant increase in the extent of water loss from the SC relative to the control. There was no correlation between the permeation enhancement and the irritation index for these enhancers. This may indicate that the degree of SC barrier disruption was not necessarily correlated to the efficiency of the enhancement. Other mechanisms such as increasing the partition coefficient of flurbiprofen to the SC may have also governed the permeation enhancement by these fatty acids (Chi et al., 1995; Fang et al., 2003a,b).

3.5. *In vitro* PGE_2 *release*

Local inflammatory cells first attack foreign toxins in the early stage. Then local inflammatory mediators, such as bradykinin, serotonin, and prostaglandins

are released. The presence of human keratinocytes and skin fibroblasts made in response to inflammation is a potential means of evaluating irritation (Lawrence, 1997). The PGE_2 release by cultured human keratinocytes and skin fibroblasts stimulated by enhancers was investigated. Because of solubility considerations, the fatty acids should be dissolved in PG to treat cells. As shown in Fig. 4A, PG itself moderately increased (*t*-test, $P < 0.05$) the PGE_2 level. It was found that PG dissolved α-keratin and irritated the epidermal structure, and so it is classified as a moderate irritant (Takeuchi et al., 1995). The same phenomenon was observed in skin fibroblasts (Fig. 4B). When keratinocytes were incubated in the presence of enhancers for 24 h with various concentrations in the culture medium, the release of PGE_2 was significantly increased (*t*-test, $P < 0.05$) as compared to the basal group (Fig. 4A). In the lower concentration (0.001%) of enhancers, neither the *B. braunii* extract nor the simulated mixture increased the PGE_2 level as compared to the PG group. On the other hand, the higher-concentration (0.003%) *B. braunii* extract and simulated mixture induced significant increases (*t*-test, $P < 0.05$) in the release of PGE_2. Both oleic acid and linolenic acid showed similar profiles of PGE_2 release from keratinocytes regardless of the concentration used. Another observation was that the discrepancy in PGE_2 release between the lower and higher concentrations of enhancer was not large.

Fig. 4B illustrates the PGE_2 release profiles generated by human skin fibroblasts following treatment with a series of enhancers. All enhancers tested showed higher levels of PGE_2 release as compared to the basal and PG groups. Various types and doses of enhancers did not affect the expression of fibroblasts at the PGE_2 level. An approximately two-fold increase in PGE_2 was observed for all enhancers. Enhancers can induce inflammation, and mediators can cause some chemical, structural, and compositional alterations of cutaneous lipids and proteins, thus enhancing skin permeability (Zhao and Singh, 2000).

3.6. SEM examination of the skin surface

The skin is a multilayered organ and anatomically has many histological layers. In this study, the influence of 3% enhancers on the anatomical structure is discussed with the aid of SEM findings. Fig. 5A shows the SEM appearance of normal rat skin. The keratinocytes are smooth-looking and intimately connected to each other. Several stumps of remaining hairs can also be noted. After treatment with the 3% *B. braunii* extract for 30 min, most keratinocytes had retained their shapes and cohesion with neighboring cells as observed in Fig. 5B. Only a few keratinocytes were detached from the skin surface; subtle signs of dehydration (a wrinkled surface) were also noted. A similar appearance was observed with the simulated mixture-treated skin for a 30-min application (Fig. 5C). This may indicate that only mild changes were detected for the SC structure after treatment with the *B. braunii* extract for a short duration. The alteration of keratinocytes may be related to the increased PGE_2 release by cultured keratinocytes when incubated with the *B. braunii* extract or simulated mixture. Oleic acid and linolenic acid also showed similar effects on the SC as with *B. braunii* extract-treated skin (data not shown).

In order to distinguish the tolerance of the skin to these enhancers, a longer treatment period (6 h) was also used to examine the SEM images of rat skin. The skin surface of the *B. braunii* extract-treated group was more disrupted with a 6-h treatment than a 30-min treatment (Fig. 5D). The skin surface was studded with fragments of keratinocytes detached from squamae, especially near the hair follicle openings. The most severe alteration was shown in the simulated mixture-treated skin with a 6-h application (Fig. 5E). Denatured and necrotic materials covered most of the skin surface, suggesting the extraction of intercellular lipid bilayers in the SC by the simulated mixture. The contours of keratinocytes were totally absent. This result may confirm the above discussion of the TEWL determination that the 45% remainder in the *B. braunii* extract may exhibit a buffer effect to reduce the irritation caused by the fatty acids.

In summary, the efficacy and safety of the *B. braunii* extract were systemically evaluated using a series of in vitro and in vivo methods. Both in vitro and in vivo permeation studies showed that the *B. braunii* extract effectively promotes drug absorption into and across the skin. The in vivo TEWL determination and SEM images demonstrated the low irritancy of the *B. braunii* extract. Since *B. braunii* is spread widely all over the world, and furthermore, the extraction method is easy to reproduce, it can serve as a cheap and safe source

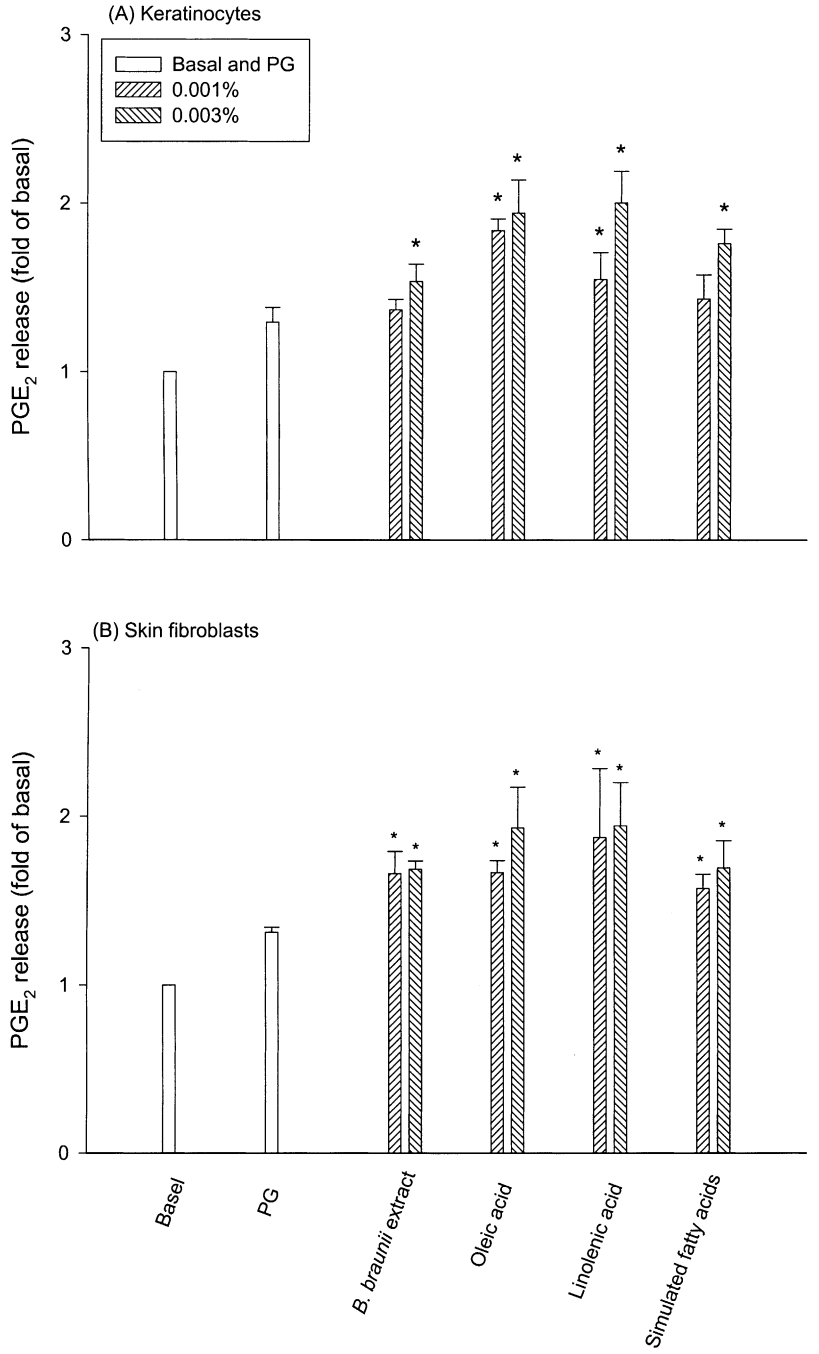

Fig. 4. PGE_2 production by human keratinocytes (A) and human skin fibroblasts (Hs68) (B) in cultured medium after treatment with enhancers at various concentrations. All data represent the means of four experiments ± S.D.

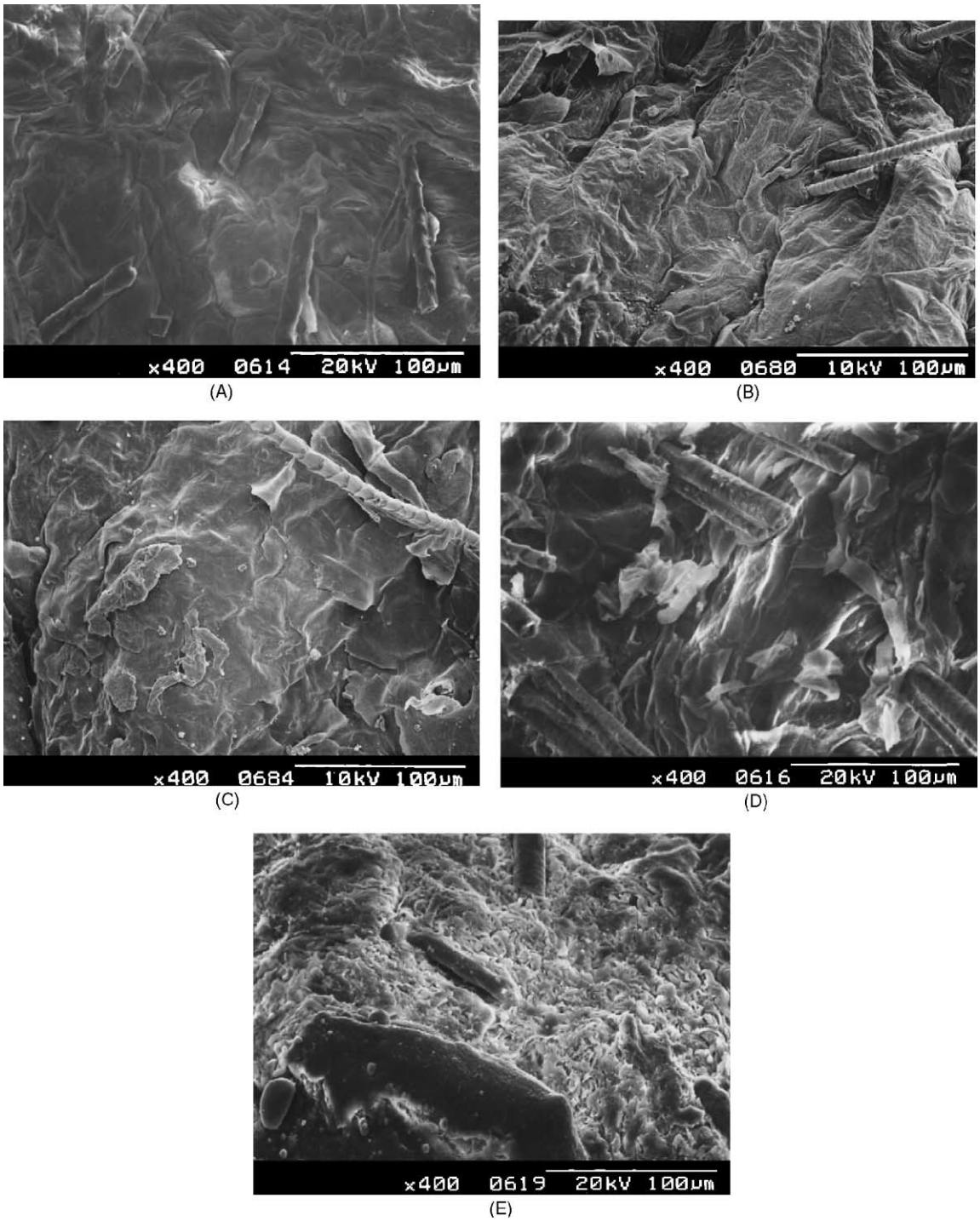

Fig. 5. SEM images of nude mouse skin after treatment with: (A) no enhancers; (B) 3% *Botryococcus braunii* extract for 30 min; (C) 3% simulated mixture for 30 min; (D) 3% *B. braunii* extract for 6 h; and (E) 3% simulated mixture for 6 h.

of an effective accelerator for the skin absorption of drugs as compared to the pure fatty acids commercially available.

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