Essential Oils from Sweet Basil (*Ocimum basilicum*) as Novel Enhancers to Accelerate Transdermal Drug Delivery

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The purpose of this study was to evaluate the essential oils from sweet basil (*Ocimum basilicum*, OB) as skin permeation enhancers to promote the percutaneous absorption of drugs. The *in vitro* and *in vivo* irritancy of the essential oils was also examined. Terpenes with various carbon numbers (mono-, sesqui-, di-, and tri-) were identified in both the lower-polarity fraction (OB-1) and higher-polarity fraction (OB-2). *In vitro* skin permeation and deposition of indomethacin were significantly enhanced after treatment with OB essential oils. The enhancing effect of OB-1 was greater than that of OB-2 in the *in vitro* permeation and *in vivo* cutaneous microdialysis analyses as well as in the plasma concentration of indomethacin. On the other hand, the *in vivo* study showed that OB-2 had a greater ability to retain the drug within the skin than did OB-1. Enhancement of the skin permeation of drugs by OB essential oils might be mainly due to improvement in the partitioning of the drugs to the stratum corneum. Both *in vitro* cell cultures (keratinocytes and skin fibroblasts) and *in vivo* transepidermal water loss showed no or only negligible irritation to skin by OB essential oils.

Key words *Ocimum basilicum*; essential oil; transdermal delivery; enhancer

The delivery of drugs *via* skin routes has been extensively studied in the pharmaceutical field. Dermal and transdermal drug delivery is often limited by the poor permeability of the stratum corneum (SC) to drugs, which precludes their crossing the skin at therapeutic rates. The barrier properties of the SC can be reduced by the use of chemicals, which act as skin permeation enhancers.[1] Developing new enhancers and studying the mechanisms of permeation enhancement have gradually become a very active area of transdermal research. Natural products are receiving considerable interest as enhancers to improve drug permeation. These enhancers include fatty acid extracts and essential oils.[2—4]

Sweet basil (*Ocimum basilicum*, OB) is rich in essential oils that have been the subject of numerous chemical studies.[5] It has been grown by local people as a medicinal plant, culinary herb, and antimicrobial agent.[5,6] The aim of this study was to develop novel enhancers from the leaves of OB. Indomethacin and 5-fluorouracil (5-FU) were used as model drugs to examine the effect of the OB extract on their absorption by the skin. The principal constituents of essential oils from OB were identified by gas chromatography (GC)-mass spectrometry (MS). The present study utilized *in vitro* Franz cells to explore the influence of the OB extract on absorption of drugs by the skin. The amount of drug uptake within the skin reservoir was also determined *in vitro* and *in vivo*. Microdialysis is a sampling technique that has been shown to be minimally invasive and directly supplies pharmacokinetic information on the target organ for cutaneous drug delivery.[7,8] Hence the *in vivo* transdermal drug delivery by OB extracts was also evaluated using microdialysis as the sampling technique.

Since it is preferable in practical use to find a balance between the 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 OB essential oils. The release of the inflammatory mediator prostaglandin E_2 (PGE_2) from human keratinocytes and skin fibroblasts following treatment with OB essential oils was investigated to explore the biologic responses of the skin. The skin irritant potential of these oils was also assessed using transepidermal water loss (TEWL) *in vivo*.

MATERIALS AND METHODS

Materials Indomethacin, 1,8-cineole, dimethyl sulfoxide (DMSO), and phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). 5-Fluorouracil (5-FU) was supplied by Wako (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were purchased from Biowest (France). The prostaglandin E_2 (PGE_2) kit (Correlate-EIA) was from Assay Designs (Ann Arbor, MI, U.S.A.). The cellulose membrane (Spectra/Por 2, molecular weight cut-off of 12000—14000) was from Spectrum (Houston, TX, U.S.A.). Fresh leaves of OB were obtained from a local market and verified by Dr. Yu-Chi Hou, China Medical University, Taichung, Taiwan. A voucher specimen was deposited in the Graduate Institute of Natural Products, Chang Gung University, Taiwan.

Extraction Method Fresh leaves (911.32 g) of OB were coarsely cut and extracted three times using acetone. The acetone extracts were concentrated under reduced pressure. The residual water after the concentration procedure was partitioned by chloroform. The chloroform layer was subjected to open column chromatography on silica gel (70—230 mesh, Macherey-Nagel, Düren, Germany). The thin-layer chromatography (TLC) technique was used to detect the eluted materials from the column. The column was eluted with chloroform, and collected every 250 ml in each flask to produce two fractions (OB-1 and OB-2). The final amount of OB-1 and OB-2 fractions extracted from the leaves was 4.44 g and 3.19 g, respectively.

Gas Chromatography-Mass Spectrometry Analysis The essential oil extract of OB (100 ppm) was analyzed with GC-MS using a Hewlett-Packard (HP) 6890 series gas chromatograph interfaced to an HP 5973 mass-selective detector (MSD). An HP-mass ChemStation Data system was used for identifying the components. We used a Zebron-5 mass cross-linked fused-silica capillary column (30 m, 0.25 mm i.d.)

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coated with 5%-phenylpoly(methylsiloxane) (0.25-μm phase thickness). The oven temperature was held at 50 °C for 1 min, increased from 50 to 220 °C at a rate of 2 °C/min, and then maintained at 220 °C for 5 min. The pressure of the helium inlet was set at 5.28 psi, with a linear velocity of 33 cm/s. The injector temperature was maintained at 250 °C.

The percentage composition of the essential oils was computed from the GC peak areas. Chromatographic peaks were checked for homogeneity with the aid of mass chromatograms with characteristic ion fragments. The NIST98 (NIST/EPA/NIH Mass Spectra Library) database was used for automatic identification of the GC peaks.

***In Vitro* Skin Permeation and Deposition** *In vitro* skin permeation experiments were carried out using a Franz diffusion cell. The dorsal skin of Wistar rats (180—200 g) was shaven using electric clippers and then mounted on the receptor compartment with the SC-side facing upward into the donor compartment. The donor medium was 1 ml of vehicle containing drugs. The receptor medium was 10 ml of 50% ethanol/pH 7.4 citrate-phosphate buffer for indomethacin or pH 7.4 buffer for 5-FU. The available diffusion area between cells was 1.539 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 using the HPLC method.[2,9]

The amount of indomethacin 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 10000 rpm. The supernatant was analyzed using HPLC.

***In Vivo* Skin Deposition** Wistar rats were anaesthetized with urethane 3 ml/kg (25%). The back fur of the rats was shaven. Two glass cylinders with an available area of 1.539 cm^2 were placed on the skin with glue (Instant Super Glue, Kokuyo, Japan). Two milliliters of 0.3% indomethacin in 25% ethanol (EtOH)/pH 7.4 buffer was added into each cylinder. The application times of the vehicle in the two cylinders were 6 and 12 h respectively. The procedure for extraction of drug from the skin was the same as that for the *in vitro* experiments.

***In Vivo* Microdialysis** The microdialysis system consisted of a CMA/102 microinjection pump (Carnegie Medicin, Sweden) equipped with a 1-ml Exmire microsyringe (Ito, Japan), which delivered pH 7.4 buffer at a flow rate of 1.5 μl/min as perfusate to the probe, and a microfraction collector (CMA/20, molecular weight cut-off of 20 kDa). Female Wistar rats (180—200 g) were anesthetized with an intraperitoneal injection of 25% urethane at a dose of 3 ml/kg. The back fur of the rats was shaven, and the skin was then incised over the dermis, followed by intradermal insertion of an introducer assembled by inserting a stainless steel needle into the tubing. After setting the tubing under the skin, the needle was withdrawn, followed by insertion of the probe, after which the tubing was removed. After probe implantation, a glass cylinder with an available diffusion area of 1.539 cm^2 was placed above the tip of the probe on the skin with glue. Two milliliters of 25% ethanol (EtOH)/pH 7.4 buffer containing 0.3% indomethacin was added into the cylinder. The samples of *in vivo* microdialysis were assayed using HPLC.

In the *in vivo* recovery of indomethacin across the microdialysis probe, a probe was inserted into the dermis as indicated above. The pH 7.4 buffer containing indomethacin 50 μg/ml was passed through the probe using an infusion pump. The dialysate samples were collected every 1 h for a total of 12 h, which was the same as the sampling time plots in the *in vivo* permeation study. The recovery ratio of indomethacin was calculated using the following equation:

$$\text{recovery (\%)} = 1 - (\text{effluent dialysate amount/influent dialysate amount})$$

This equation can be derived by assuming that drug recovery from the tissue to the perfusate is the same as the drug loss from the perfusate to the tissue across the probe membrane.[10]

***In Vivo* Plasma Concentration of Indomethacin** The concentration of indomethacin in plasma was also determined after *in vivo* topical administration of OB essential oils. The blood samples were withdrawn by heart puncture 6 h and 12 h after application. The plasma analytical procedure of indomethacin was the same as in our previous study.[11] Briefly, a 1-ml aliquot of plasma was pipetted along with 1 ml of citrate-phosphate buffer (pH 4). The mixture was shaken by vortex and extracted with 7 ml of ether–cyclohexane (8 : 2) by shaking for 20 min. After centrifugation, 5 ml of the supernatant was pipetted and evaporated. The residual was redissolved with the mobile phase for HPLC determination.

***In Vitro* PGE_2 Release** Human keratinocytes and human skin fibroblasts (Hs68) were obtained from the Department of Dermatology, Taipei Medical University (Taipei, Taiwan) and the Food Industry Research and Development Institute (Hsinchu, Taiwan), respectively. Cells were maintained in DMEM supplemented with 10% heat-inactivated FCS and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml, and amphotericin B 2.5 μg/ml) 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 DMSO for OB-1 and in EtOH for OB-2 were prepared. The final concentrations of the stock solutions in the culture media were 0.001% and 0.003% (v/v). PMA 1 μM in DMSO was used as a positive control. Cell cultures were exposed to test enhancers for 24 h for determination of PGE_2 levels. PGE_2 was measured in cell culture supernatant using an ELISA kit 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.

***In Vivo* TEWL Evaluation** For TEWL evaluation, 1.5 ml of 25% ethanol (EtOH)/pH 7.4 buffer containing enhancer was uniformly spread over a sheet of nonwoven polyethylene cloth (3×3 cm, Johnson & Johnson, U.S.A.), which was then applied to the shaven back area of a rat. The polyethylene cloth was fixed with Tegaderm adhesive dressing (St. Paul, MN, U.S.A.) and Fixomull stretch adhesive tape (Beiersdorf AG, Hamburg, Germany). After 24-h administra-

tion, the hydrogel was removed, and the application site was gently cleaned using a cotton wool swab. After withdrawal of the hydrogel for 30 min, TEWL and colorimetry 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. After the measurements, the application site was again covered with a polyethylene cloth of Tegaderm and Fixomull. Recovery from the skin irritation was examined once a day for 3 d.

RESULTS AND DISCUSSION

Determination of the Major Constituents from OB Two fractions of OB, OB-1 and OB-2, were identified in GC analysis by means of the mass spectral fragmentation patterns. The profiles of the essential oils extracted from OB are summarized in Table 1. The major components from OB were terpenes with various carbon numbers. Two sesquiterpenes, one triterpene, and one aromatic ether comprised 100% of the OB-1 fraction. The predominant component from OB-1 was estragol (76.72%), followed by squalene, α-bergamotene, and θ-muurolene. Most of the components in OB-1 were found to be hydrocarbon constituents, and only estragol was an oxygenated constituent. Estragol is the compound responsible for the aniseed-like aroma and sweet taste of OB.[5] Squalene is a highly unsaturated hydrocarbon which can be used as an agent to minimize skin irritation caused by enhancers.[12]

The OB-2 fraction had a higher polarity than OB-1. Five identified compounds made up 88.32% of OB-2. Unlike OB-1, most of the components in OB-2 were oxygenated terpenes, except for (+)-epi-bicyclosesquiphellandrene which is a hydrocarbon sesquiterpene. The compound with the highest content in OB-2 was phytol (52.27%). Linalool was also identified in the OB extract by other investigators.[5,13] Estragol in OB-2 was also observed in the OB-1 fraction. However, the content of estragol was much lower in OB-2 than in OB-1.

***In Vitro* Skin Permeation and Deposition** Incorporation of the cosolvent in the donor vehicle is important for the solubility consideration of essential oils in the vehicle. A solvent such as EtOH in combination with an enhancer accumulates in the tissue and increases the partitioning of the drug due to the greater affinity of the drug for the solvent.[14] The influence of EtOH as a cosolvent on indomethacin permeation was thus initially investigated. The cumulative amount-time profiles for *in vitro* indomethacin permeation were plotted. The slopes of the resulting linear plots were calculated, and the flux (μg/cm^2/h) was determined from the slope. As shown in Table 2, the presence of EtOH influenced the indomethacin flux. Incorporation of 25% EtOH in pH 7.4 buffer significantly increased (*t*-test, $p<0.05$) the indomethacin flux. However, the transport of indomethacin was less with 50% EtOH than with 25% EtOH. No significant difference (ANOVA test, $p>0.05$) was observed in the skin uptake of indomethacin from various EtOH proportions. Indomethacin, being highly lipophilic, is probably transported through a nonpolar pathway such as intercellular lipids of the SC. When EtOH greater than 50% was used, the solute and concomitant EtOH fluxes began to decrease, probably due to the dehydrating effect of EtOH on the skin.[3,15] Another explanation for these findings can be offered based on the thermodynamic activity of indomethacin. The flux is proportional to the gradient of thermodynamic activity. The solubility of indomethacin was less in 25% EtOH than in 50% EtOH. At a constant drug concentration, drug activity is reduced as its solubility in a solvent is increased.[3,16]

The effects of OB essential oils on the percutaneous absorption of indomethacin from the 25% EtOH/pH 7.4 buffer vehicle are shown in Fig. 1. Both OB-1 and OB-2 enhanced the skin permeation of indomethacin as compared with the control. Previous investigations have suggested that linalool, which occurs in OB-2, enhances transdermal drug delivery. τ-Anethole shows an enhancing effect on the transdermal transport of azidothymidine.[17] The chemical structure of τ-anethole is very similar to that of estragol, which occurs in OB-1, although the double bonds are in different positions. Table 2 summarizes the concentration effects of OB essential oils on the skin absorption of indomethacin. OB-1 and OB-2 had different enhancement-promoting effects. In the case of OB-1, all three concentrations (1%, 3%, and 5%) showed enhancing activity to accelerate indomethacin permeation. No significant difference (*t*-test, $p>0.05$) was observed between the drug flux of 3% and 5% OB-1. Another observation was

Table 1. Major Identified Constituents of *Ocimum basilicum* (OB) Essential Oils and Their Relative Proportions

R_t (min)	Compound	Classification	Area (%) of OB-1	Area (%) of OB-2
26.14	*d*-Linalool	Monoterpene	—	22.10
33.01	Estragol	Aromatic ether	76.72	2.49
48.67	α-Bergamotene	Sesquiterpene	8.30	—
53.29	θ-Muurolene	Sesquiterpene	3.40	—
53.33	Butylated hydroxytoluene	Sesquiterpene	—	2.86
60.51	(+)-*Epi*-bicyclo sesquiphellandrene	Sesquiterpene	—	8.60
84.14	Phytol	Diterpene	—	52.27
123.1	Squalene	Triterpene	11.58	—

Table 2. Effect of OB Enhancers and Vehicles on Indomethacin Permeation across Skin and Artificial Membrane *in Vitro*

Formulation	Flux (μg/cm^2/h)	Drug amount in skin (μg/mg)$\times 10^2$
pH 7.4 buffer	1.61±0.28	31.51±19.44
25% EtOH/pH 7.4 buffer	8.35±1.77	32.07±16.31
50% EtOH/pH 7.4 buffer	2.51±0.34	24.86±14.02
1% OB-1 in 25% EtOH	34.28±2.98	90.02±18.33
3% OB-1 in 25% EtOH	110.34±8.98	112.64±2.08
5% OB-1 in 25% EtOH	115.37±14.04	55.32±8.02
1% OB-2 in 25% EtOH	8.89±3.99	28.16±4.82
3% OB-2 in 25% EtOH	50.25±6.00	52.02±13.05
5% OB-2 in 25% EtOH	78.32±7.01	50.75±13.03
3% cineole in 25% EtOH	55.33±8.01	41.44±8.40
Pretreatment 25% EtOH	12.36±1.53	24.32±3.56
Pretreatment 3% OB-1	94.14±9.19	61.78±5.92
Pretreatment 3% OB-2	62.37±12.21	51.09±9.93
25% EtOH across membrane	170.24±10.49	—
3% OB-1 across membrane	140.50±22.59	—
3% OB-2 across membrane	160.33±17.76	—

Indomethacin concentration in vehicles was 0.3%. Each value represents the mean±S.D. ($n=4$).

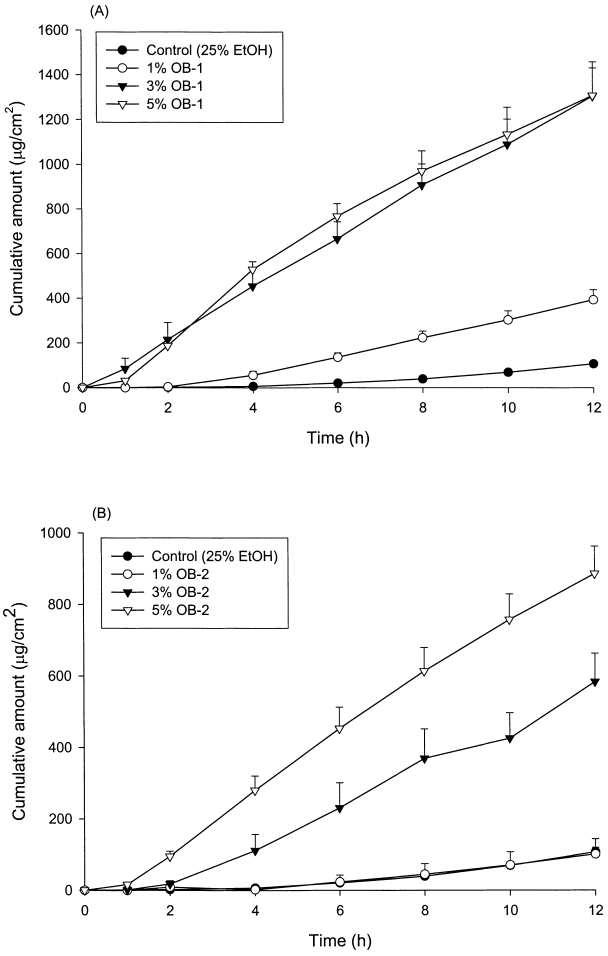

Fig. 1. Cumulative Amount of Indomethacin Detected in the Receptor *versus* Time Following *in Vitro* Topical Delivery across Excised Rat Skin with Different Concentrations of OB-1 (A) and OB-2 (B)

All data represent the mean of four experiments±S.D.

that 5% OB-1 did not increase the skin deposition of indomethacin relative to the control.

A low concentration (1%) of OB-2 exhibited no significant enhancement (Table 2); however, the enhancing activity gradually increased followed by an increase in OB-2 concentrations in the vehicle. Skin deposition of indomethacin did not significantly differ (ANOVA test, $p>0.05$) between the OB-2 group and the control. Drug permeation enhancement by OB-1 was generally greater than that by OB-2, indicating an efficient enhancement by essential oils with a lower polarity. Although 25% EtOH was added to the vehicle, the donor of the 25% EtOH/pH 7.4 buffer showed a higher polarity for the lipophilic drug and essential oils. Hence the solubility of OB-2 in the vehicle was lower than that of OB-1. The thermodynamic activity of OB-1 was thus thought to have already attained a maximum level in this condition. On the other hand, OB-2 could be dissolved in the vehicle, suggesting that the thermodynamic activity of OB-2 was lower than the maximum level.

One commonly used monoterpene enhancer, 1,8-cineole, was used to promote skin absorption of indomethacin for comparison with OB essential oils. As shown in Table 2, both the flux and skin deposition of indomethacin enhanced by 3% 1,8-cineole attained the level of enhancement by 3% OB-2.

Table 3. Effect of OB Enhancers and Vehicles on 5-FU Permeation across Skin *in Vitro*

Formulation	Flux (μg/cm^2/h)
pH 7.4 buffer	5.0±2.972
25% EtOH/pH 7.4 buffer	10.13±2.46
50% EtOH/pH 7.4 buffer	9.55±2.14
3% OB-1 in 25% EtOH	13.13±1.53
3% OB-2 in 25% EtOH	23.45±2.59

5-FU concentration in vehicles was 0.5%. Each value represents the mean±S.D. ($n=4$).

To examine further the effect of OB extracts on drug permeation, 1-h pretreatment with enhancers on the skin was performed. The pretreatment method avoided cosolvent effects on the thermodynamic activities of the model drugs. This method can also verify whether the enhancers act directly on the skin structure. The skin was pretreated with 3% OB-1 or OB-2 in 25% EtOH for 1 h, followed by *in vitro* permeation with indomethacin. Pretreatment with the essential oils significantly enhanced the permeation and skin reservoir of indomethacin (t-test, $p<0.05$) as compared with the control (Table 2), suggesting that these extracts played some roles in the skin. Disruption of the skin morphology and increment of drug partitioning into the SC may be the possible effects of OB essential oils.

With respect to drug permeation across the skin by enhancers, the increase in drug solubility by the incorporation of the enhancer may accelerate drug permeation. To clarify the mechanism of the role of solubility, the release rate of indomethacin from the vehicle across a cellulose membrane was studied. As shown in Table 2, OB-1 and OB-2 did not increase the release rate of indomethacin across the membrane. This may indicate that OB essential oils do not increase drug permeation by the solubility mechanism. This result also confirmed the mechanism by which OB essential oils predominantly act on skin to promote the skin absorption of indomethacin.

A hydrophilic drug, 5-FU, was used as a permeation molecule in comparison with indomethacin. As shown in Table 3, the addition of EtOH significantly increased (t-test, $p<0.05$) the permeation of 5-FU. There was no significant difference (t-test, $p>0.05$) between 5-FU fluxes when using the 25% and 50% EtOH vehicles. OB-1 or OB-2 3% was selected as the vehicle for 5-FU permeation. The enhancing activity of 5-FU by OB essential oils was lower than that of the lipophilic indomethacin. This result was contrary to those of other investigations which indicated that terpene enhancers were more effective in enhancing the permeation of hydrophilic drugs than that of lipophilic drugs.[18—20] The partitioning of the OB essential oils between the SC and the vehicle appeared to be important for this effect. The essential oils better escape from the vehicles and produce a stronger affinity to the lipophilic SC. The partitioning of the essential oils to the SC may have reduced the polarity of the SC, which resulted in facilitating the permeation of lipophilic indomethacin into the skin.

***In Vivo* Skin Deposition** Although attempts are currently being made to develop a generally accepted, standardized methodology for *in vitro* testing, a large extrapolation

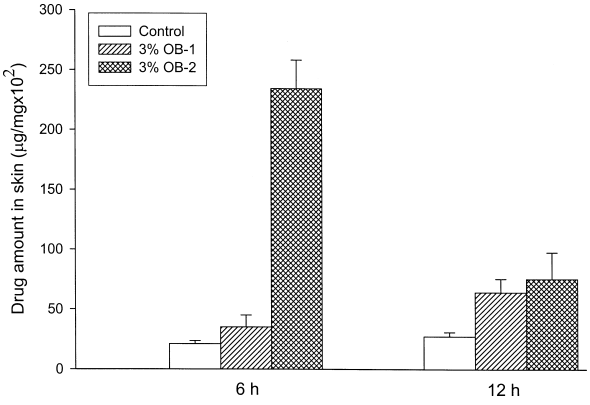

Fig 2. Kinetics of Indomethacin Uptake within Skin after Topical Application of 25% EtOH/pH 7.4 Buffer with or without OB Essential Oils

Each value represents the mean±S.D. All data represent the mean of five experiments±S.D.

still needs to be made from the *in vitro* performance to use in an *in vivo* setting.[21] To ascertain the enhancing effect of OB extracts *in vivo*, the *in vivo* pharmacokinetics of indomethacin sampled by skin deposition and microdialysis were evaluated. As shown in Fig. 2, a greater enhancement in skin deposition by OB-2 compared to that by OB-1 was found with a 6-h application. This result differed greatly from the results of *in vitro* experiments, which showed a greater enhancement of skin uptake by OB-1. The discrepancy may have been due to the different conditions *in vitro* and *in vivo*. The *in vitro* permeation study lacked elimination routes in terms of the vascular system and viable metabolizing enzymes, and there were alterations in the SC structure due to water uptake.

Both OB-1 and OB-2 enhanced the skin deposition of the drug after 12 h of application (*t*-test, $p<0.05$). The skin deposition of indomethacin by OB-2 decreased to the level of the OB-1 group at 12 h of application (*t*-test, $p>0.05$), indicating that the permanence of the enhancing activity of OB-2 is limited. This may be because OB-2 is more hydrophilic than OB-1. Hence OB-2 may no longer exist in the lipophilic SC layer, reducing the effective duration of enhancement by OB-2. With respect to drug permeation across the skin, a drug should first diffuse or partition into the skin, then be transported to the subdermal region. To verify the mechanisms of OB essential oils, *in vivo* microdialysis was used to monitor the amount of indomethacin permeated subcutaneously.

***In Vivo* Microdialysis** Cutaneous microdialysis is an *in vivo* sampling technique for measuring a solution in the extracellular fluids of the dermis. To apply this technique for obtaining extracellular concentrations of analytes, knowledge of the fractional recovery of the solute is a prerequisite. The relative recovery of indomethacin gradually decreased from 90.66 to 51.92% over time from 1 to 12 h. The observed decrease and fluctuation in the relative recovery can likely be attributed to variations in blood flow and clotting of the fiber membrane. It is generally acknowledged that implantation of a probe increases the cutaneous blood perfusion, skin thickness, and histamine release, which slowly subside during the subsequent hours.[8,22] The *in vivo* permeation of indomethacin by OB essential oils was determined over a period of 12 h. Figure 3 shows the *in vivo* microdialysis results

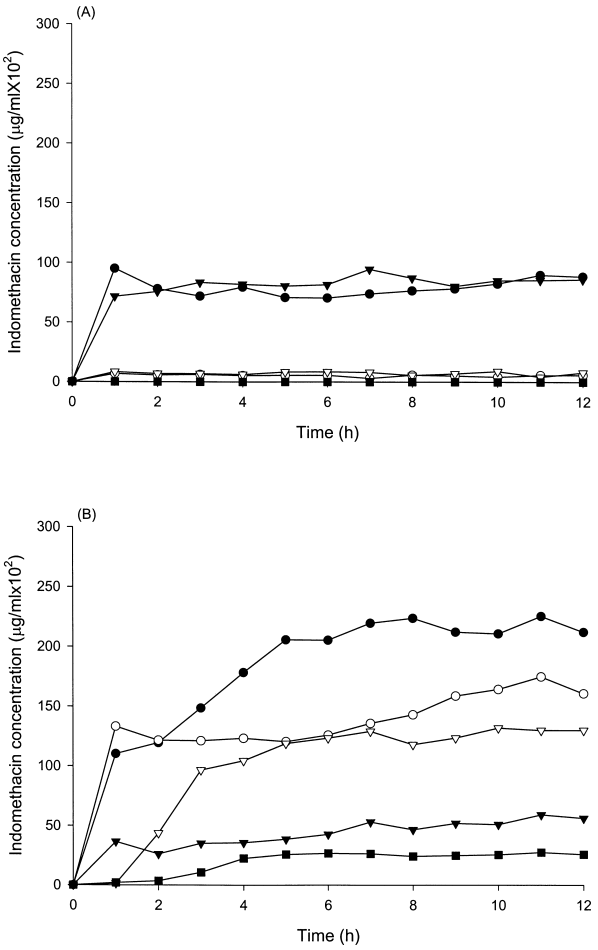

Fig. 3. Indomethacin Concentration in Dialysate Collected from the Subcutaneous Region in the *in Vivo* Microdialysis in the Absence (A) or Presence (B) of OB-1

The data represent the values of five individuals.

for topical indomethacin delivery. The indomethacin concentration in the dialysate was calibrated with the recovery value for all formulations. It was noted that a relatively large variability was always found in the permeation profiles of our microdialysis data. A relative large variability is often found in cutaneous drug delivery determined by the microdialysis technique.[7,8] The main variability may be attributable to individual differences in skin barrier function, but also to differences in the elimination rate and volume of distribution. The probe implantation depth and relative recovery may also have contributed to the variability.

The amount of indomethacin in the subcutaneous region were generally higher in the OB-1 group than in the controls (Fig. 3). Surprisingly, there was no subcutaneous indomethacin concentration in the OB-2 group during the 12-h application in any individual. The trend of *in vivo* microdialysis was the same as that in the *in vitro* results in that the effect of OB-1 was greater than that of OB-2 on indomethacin permeation. The absence of indomethacin in the dialysate after treatment with OB-2 may have been due to the greater ability of OB-2 to retain the drug within the skin reservoir as indicated by *in vivo* skin deposition.

***In Vivo* Plasma Concentration of Indomethacin** After uptake into the skin and subcutaneous region, the drug further diffuses to the circulatory system. Hence the plasma

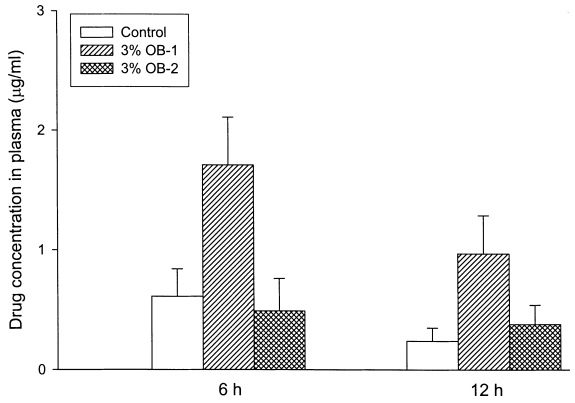

Fig. 4. Kinetics of Indomethacin in Plasma after Topical Application of 25% EtOH/pH 7.4 Buffer with or without OB Essential Oils

Each value represents the mean±S.D. All data represent the means of four experiments±S.D.

concentration of indomethacin at 6 h and 12 h after topical application was determined, as shown in Fig. 4. The level of drug showed a trend of OB-1>control group=OB-2. The metabolism of indomethacin in the blood circulation contributed to the lower drug level at 12 h than at 6 h. This may suggest that subcutaneous indomethacin could further transfer to the circulation after treatment with OB-1. On the other hand, treatment with OB-2 did not increase the plasma level of the drug as compared with the controls. Since OB-2 retains indomethacin molecules within the skin reservoir based on the *in vivo* skin deposition data, resulting in low levels of indomethacin in the subcutaneous region and the circulation. However, the lower concentration of indomethacin within the skin at 12 h than at 6 h may indicate the possible distribution of the drug over time (Fig. 2). Other regions such as adipose tissue or muscle may be the possible reservoir for topical indomethacin when applied with OB-2.

***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 produced in response to inflammation is a potential means of evaluating irritation.[23] PMA is a well-documented activator of protein kinase C. Since the potency of PMA as a skin irritant has been described in the literature,[24] PMA 1 μM was used as a positive control in this study. As shown in Fig. 5, when keratinocytes and fibroblasts were incubated in the presence of PMA for 24 h, the release of PGE_2 was markedly increased compared with the basal group.

Because of solubility considerations, OB-1 and OB-2 should be respectively dissolved in EtOH and DMSO, respectively to treat cells. Figure 5A illustrates the PGE_2 release profiles generated by OB-1 and OB-2 at various concentrations in the culture medium. EtOH or DMSO itself caused no irritation to keratinocytes. Neither OB-1 nor OB-2 at either concentration increased the PGE_2 level as compared with the control group. This may indicate the negligible toxicity of OB essential oils to keratinocytes in the SC. As shown in Fig. 5B, DMSO itself moderately increased (*t*-test, $p<0.05$) the PGE_2 level in human skin fibroblasts. It was found in a previous investigation that DMSO can induce some inflammatory responses in skin cells.[25] There was no

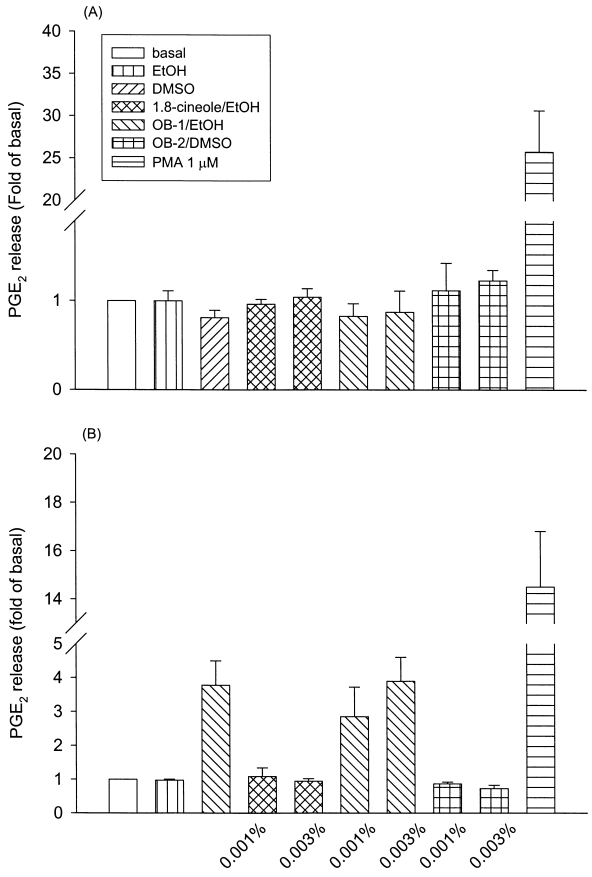

Fig. 5. PGE_2 Production by Human Keratinocytes (A) and Human Skin Fibroblasts (Hs68) (B) in Culture Medium after Treatment with Enhancers at Various Concentrations

All data represent the means of four experiments±S.D.

difference (*t*-test, $p>0.05$) between the OB-1 group and DMSO group, indicating that no inflammation was observed after OB-1 incubation. The essential oil of OB-2 also produced no statistically significant increase (*t*-test, $p>0.05$) in extracellular PGE_2 levels at the concentrations tested.

***In Vivo* TEWL Evaluation** The *in vivo* macroscopic barrier properties of skin were investigated by measuring TEWL. One of the SC barrier functions in homeostasis is limiting water loss. An increase in water permeability of the skin may correspond to an increase in skin injury by a topically applied compound.[26] The 3% enhancers in the 25% EtOH/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. 6, TEWL (the TEWL value of the treated site minus the TEWL value of an adjacent untreated site) determined over 3 d was evaluated. The EtOH/pH 7.4 buffer vehicle (control) did not increase TEWL over 3 d, indicating that EtOH itself caused no skin irritation as determined by TEWL.

Treatment with OB essential oils for 24 h produced no enhancement of TEWL. Treatment with 1,8-cineole induced ascendant values of TEWL initially, and then the values gradually decreased to nearly the baseline by day 3 as the skin recovered to its normal status. Previous investigations support the speculation that monoterpene enhancers mainly increase drug permeation in the skin by disrupting the highly ordered intercellular lipid structure of the SC.[20,27] Furthermore, this

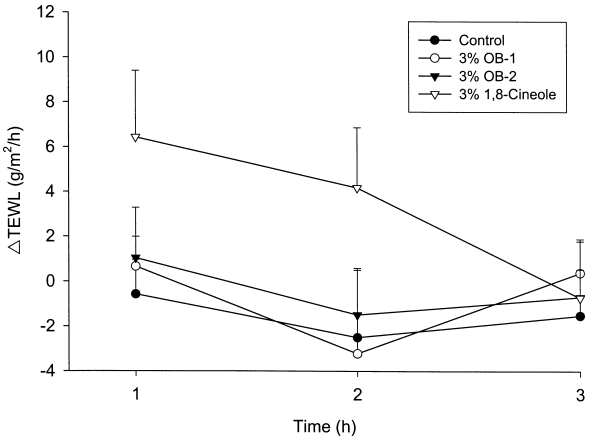

Fig. 6. Kinetics of TEWL during 3 d after Topical Application of 25% EtOH/pH 7.4 Buffer with or without Enhancers for 24 h

All data represent the means of six experiments±S.D.

interaction with SC lipids is reversible. Lipid extraction by enhancers always causes higher TEWL values in comparison with the control.[28] The reduction in skin resistance as a permeation barrier by disruption of tightly packed lipid regions of the SC is one possible mechanism to accelerate drug transport. Another mechanism is increased skin–vehicle partitioning of the drug. The results of TEWL may indicate that the enhancing mechanism of 1,8-cineole could mainly be attributed to disruption of the lipid bilayer of the SC. No or only a negligible increase in TEWL by OB essential oils may indicate limited disruption of the intercellular routes by drug transport. Hence the mechanism of OB-1 and OB-2 as permeation enhancers may be attributed mainly to a partitioning effect.

CONCLUSIONS

The efficacy and safety of OB essential oils were systemically assessed using a series of *in vitro* and *in vivo* methods. The predominant compounds of OB-1 and OB-2 were estragol and phytol, respectively. Both OB-1 and OB-2 effectively accelerated indomethacin permeation across the skin. The permeation enhancement of OB-1 was higher than that of OB-2. Increased skin–vehicle partitioning by OB essential oils may be the main mechanism of this enhancement. The negligible indomethacin concentration in the microdialysis dialysate after treatment with OB-2 may have been due to the potent ability of OB-2 to retain the drug within the skin reservoir. Some discrepancies were noted between the *in vitro* and *in vivo* results, suggesting that a correlation between *in vitro* and *in vivo* studies should not be assumed beforehand. The *in vivo* TEWL determination demonstrated the lower irritancy of OB extracts compared with 1,8-cineole. The *in vitro* PGE_2 release of cultured keratinocytes and fibroblasts also showed the nonirritancy of OB essential oils.

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