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Development and evaluation of the essential oil from *Magnolia fargesii* for enhancing the transdermal absorption of theophylline and cianidanol

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Abstract

To improve the skin permeation of theophylline and cianidanol ((+)-catechin), the essential oil of *Magnolia fargesii* was evaluated using in-vitro and in-vivo permeation techniques. Oxygenated monoterpenes and sesquiterpenes are the major components of *M. fargesii* essential oil. The in-vitro permeation of theophylline and cianidanol was significantly enhanced after treatment with *M. fargesii* essential oil. The essential oil increased the in-vivo skin deposition of cianidanol but not theophylline. On the other hand, in-vivo microdialysis showed a higher subcutaneous theophylline amount after essential oil treatment. In-vitro cell viability and prostaglandin E_2 release by skin keratinocytes indicated that there was low or negligible cytotoxicity by *M. fargesii* essential oil. The in-vivo skin tolerance study determined by transepidermal water loss and colorimetry confirmed that no irritation of the skin was detected when using *M. fargesii* essential oil.

Introduction

Tea (*Camellia sinensis*), next to water, is the most-often consumed beverage in the world. Theophylline and cianidanol ((+)-catechin) are naturally occurring compounds derived from tea. Theophylline is useful for treating asthma and recurrent apnoea. It is also a lipolytic agent for slimming purposes in some commercial products (Imbeault et al 2000). With oral administration, theophylline pharmacokinetics vary greatly among individuals, and several instances of gastric disturbance and first-pass metabolism have been reported (Murthy et al 2001). Green tea catechins are powerful antioxidants, a property which has been linked to the chemopreventive and anti-inflammatory activities of whole tea (Bedi & Shenefelt 2002). Cianidanol is one of the most widely distributed flavonoids found in green tea, red wine, and cocoa (Baba et al 2001). Previous studies have reported the oral bioavailability of cianidanol to be less than 5% (Baba et al 2001; Catterall et al 2003). Hence, a transdermal route may be suitable for theophylline and cianidanol to resolve the problems with their oral administration and to prolong the half-life of these drugs.

The skin has become an important portal for drug delivery for topical, regional, and systemic action. To achieve sufficient permeation of active substances into the skin, the barrier properties of the stratum corneum should be reduced using chemical enhancers (Touitou et al 2002). Considerable research is now in progress on the use of natural products as permeation enhancers. These enhancers include fatty acid extracts and essential oils (Gao & Singh 1998; Loftsson et al 1998; Fang et al 2003c).

Terpenes are major constituents of essential oils. The aim of this study was to develop a novel enhancer from the dry buds of *Magnolia fargesii*, which is rich in essential oils. The principal constituents of the essential oil from *M. fargesii* were identified by gas chromatography (GC)-mass spectrometry (MS). This study utilized in-vitro Franz cells to explore the influence of the essential oil on the skin permeation by theophylline and cianidanol. The amount of drug retained within the skin was also determined in-vitro and in-vivo. The sampling technique used was microdialysis as it has been shown to be minimally invasive, and directly supplies pharmacokinetic information on the target organ for cutaneous drug delivery (Kreilgaard 2002). The 3,-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay and

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prostaglandin E_2 (PGE_2) from human skin keratinocytes after treatment with essential oil were used to investigate the biologic responses of the skin. The irritant potential of the enhancer on the skin was assessed using in-vivo trans-epidermal water loss (TEWL) and colorimetry, using the Wistar rat as the animal model.

Materials and Methods

Materials

Theophylline, cianidanol, and 3,-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical (St Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were supplied by Biowest (France). PGE_2 kit (Correate-EIA) was from Assay Designs (Ann Arbor, MI). Dried buds of *M. fargesii* were obtained from Zi-Shuen Herbal Drugs (Taipei, Taiwan), 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

Dried buds of *M. fargesii* were coarsely cut and extracted using acetone three times. The acetone extracts were concentrated under reduced pressure. The condensed extract was subjected to open column chromatography on silica gel (70~230 mesh, Macherey-Nagel, Düren, Germany). A thin-layer chromatographic (TLC) technique was used to detect the eluted materials from the column. The column was eluted with chloroform, and 250 mL was collected in each flask to produce a fraction of extracted *M. fargesii* essential oil. The chloroform was evaporated by reduced pressure.

Gas chromatography (GC)–mass spectrometry (MS) analysis

Essential oil extract (1 μL) was analysed by GC-MS using a Hewlett-Packard (HP) 6890 series gas chromatograph interfaced with an HP 5973 mass selective detector (MSD). An HP-MS 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.) coated with 5% phenylpoly(methylsiloxane) (0.25-μm phase thickness). The oven temperature was held at 50°C for 1 min, then increased from 50 to 220°C at a rate of 2°C min^{-1}, 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^{-1}. The injector temperature was maintained at 250°C. The percentage composition of the essential oils was computed from GC peak areas. Chromatographic peaks were checked for homogeneity with the aid of mass chromatograms of characteristic fragment ions. The NIST98 (NIST/EPA/NIH Mass Spectra Library) database was used for automatic identification of the GC peaks.

In-vitro skin permeation

In-vitro skin permeation experiments were carried out using a Franz diffusion cell. The protocol for the animal study was designed according to internationally recognized ethical guidelines and was approved by the Institutional Animal Care and Use Committee of Chang Gung University. The dorsal skin of a female Wistar rat (180–200 g) was shaved using electric clippers and then mounted on the receptor compartment with the subcutaneous side facing upwards into the donor compartment. The donor medium consisted of 1 mL vehicle containing 0.5% drugs with or without 3% *M. fargesii* essential oil. The receptor medium was 10 mL pH 7.4 citrate–phosphate buffer. A sink condition could be maintained during the experiments. The available diffusion area between cells was 1.54 cm^2. The stirring rate and temperature were maintained at 600 rev min^{-1} and 37°C, respectively. At appropriate intervals, a 300-μL sample of the receptor medium was withdrawn and immediately replaced with an equal volume of fresh buffer. The amount of drugs was determined by an HPLC method described below.

The amount of drugs 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 0.1 M HCl, and ground for 5 min with an electric stirrer. The resulting solution was centrifuged for 10 min at 10 000 rev min^{-1} and then filtered through a PVDF membrane (pore size = 0.45 μm, Millipore, USA). The supernatant was analysed by HPLC.

HPLC analytical method

The theophylline and cianidanol contents of the various samples were analysed with an HPLC system consisting of a Hitachi L-7110 pump, a Hitachi L-7200 sample processor, and a Hitachi L-7400 UV or L-7480 fluorescence detector. A 25-cm-long, 4-mm inner diameter stainless RP-18 column (Merck, Darmstadt, Germany) was used. The mobile phase for theophylline was 25:75 methanol:0.4% sodium acetate at a flow rate of 1 mL min^{-1}. The mobile phase for cianidanol was 10:90 acetonitrile:2.7% acetic acid at a flow rate of 1.2 mL min^{-1}. The UV detector was set at a wavelength of 269 nm for theophylline. For cianidanol, the fluorescence detector was set at 280 nm for excitation and 320 nm for emission.

In-vivo topical application

Rats were anaesthetized using 3 mL kg^{-1} urethane (25%) by an intraperitoneal route. The back fur of the rat was shaved. Two glass cylinders with an available area of 1.54 cm^2 were placed on the skin with glue (Instant Super Glue, Kokuyo, Japan). To each cylinder was added 2 mL 0.5% drug in 25% ethanol (EtOH)/pH 7.4 buffer with or without 3% *M. fargesii* essential oil. 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 for the in-vitro experiments.

In-vivo microdialysis

The microdialysis system comprised a CMA/100 microinjection pump (CMA, Stockholm, Sweden) and microdialysis probe. The dialysis probe for intradermal measurements (10 mm in length) was made of silica capillary tubes in a concentric design (Tsai & Liu 2004). Their tips were covered by a dialysis membrane (Spectrum Lab., 200-μm inner diameter with a cut-off at a nominal molecular weight of 13 000, Laguna Hills, CA), and all unions were cemented with epoxy. At least 24 h was allowed for the epoxy to dry. Rats were initially anaesthetized with 1 g mL^{-1} urethane and 0.1 g mL^{-1} chloralose (1 mL kg^{-1}, i.p.), and remained anaesthetized throughout the experiment. During the experiment a heating pad was used to maintain the body temperature of the rat at 37°C.

The method of theophylline administration was the same as the in-vivo topical application. The microdialysis probe was located within the intradermal area on the back and then perfused with Ringer solution at a flow rate of 0.85 μL min^{-1} using a microinjection pump. The tip of the microdialysis probe was implanted under the skin of a glass reservoir. The probe was connected to the microdialysis system and HPLC with an on-line injector (CMA 160) and injected every 20 min.

For in-vivo recovery, the microdialysis probe was inserted under anaesthesia as described above. Following a stabilization period of 2 h post-probe implantation, the perfusate (C_{perf}) and dialysate (C_{dial}) concentrations of theophylline were determined by HPLC. Ringer solution containing theophylline (0.1, 0.5, or 2 μg mL^{-1}) was perfused through the probe at a constant flow rate (0.85 μL min^{-1}) by an infusion pump (CMA/100). The relative in-vivo recovery (R_{dial}) of theophylline across the microdialysis probe was calculated by the following equation:

$$R_{dial} = (C_{perf} - C_{dial})/C_{perf} \quad (1)$$

The microdialysate recovery and concentration calculations were performed according to Tsai (2003) and Tsai & Liu (2004). Theophylline microdialysate concentrations (C_m) were converted to unbound concentration (C_u) as follows:

$$C_u = C_m/R_{dial} \quad (2)$$

Cell cultures

Human keratinocytes were obtained from the Department of Dermatology, Taipei Medical University (Taipei, Taiwan). Cells were maintained in DMEM supplemented with 10% heat-inactivated FCS and antibiotics (100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 2.5 μg mL^{-1} amphotericin B) 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^{-1} in 24-well Costar plates and cultured in DMEM containing 10% heat-inactivated FCS. Stock solutions of *M. fargesii* essential oil dissolved in ethanol were prepared. The final concentration of the stock solutions in the culture media was 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 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 determine the cell viability.

In-vivo skin tolerance study

For this evaluation, 1.5 mL 25% ethanol (EtOH)/pH 7.4 buffer containing 3% enhancer (*M. fargesii* essential oil or oleic acid) was uniformly spread over a sheet of nonwoven polyethylene cloth (3 × 3 cm, Johnson & Johnson, USA), which was then applied to the shaven back area of a rat. The polyethylene cloth was fixed with Tegaderm adhesive dressing (3M, St Paul, MN) and Fixomull stretch adhesive tape (Beiersdorf, Hamburg, Germany). After 24-h administration, the formulation was removed, and the application site was gently cleaned using a cotton wool swab. After withdrawal of the vehicle for 30 min, transepidermal water loss (TM210, Courage & Khazaka, Germany) and colorimetry (CD100, Yokogawa, Japan) were determined. 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 four days.

Statistical analysis

The statistical analysis of differences between different formulations in this study was performed using unpaired *t*-test. A 0.05 level of probability ($P < 0.05$) was taken as the level of significance. Analysis of variance test was used also. Post-hoc comparisons of the means of individual groups were performed using Tukey's Honestly Significant Difference test.

Results

Determination of the major constituents of *M. fargesii* essential oil

The essential oil of *M. fargesii* was identified in the GC analysis by means of the mass spectral fragmentation patterns. Profiles of the constituents in the essential oil are shown in Table 1. Four monoterpenes and two sesquiterpenes were identified from the essential oil and comprised 100% of the oil. All terpenes were found to be oxygenated compounds. The main constituent was farnesol (42.23%). Camphor (19.25%), eucalyptol (14.23%), and oplopanone (10.61%) showed high contents exceeding 10% in the essential oil.

In-vitro skin permeation

The effect of *M. fargesii* essential oil on the in-vitro skin permeation of theophylline and cianidanol across rat skin

Table 1 The major identified constituents of the *Magnolia fargesii* essential oil and their relative proportions

Retention time (min)	Compound	Classification	Area (%)
15.16	Eucalyptol	Monoterpene	14.23
19.52	*Cis*-β-Terpineol	Monoterpene	4.18
22.22	Camphor	Monoterpene	19.25
25.37	(+)-α-Terpineol	Monoterpene	9.50
57.57	Farnesol	Sesquiterpene	42.23
58.20	Oplopanone	Sesquiterpene	10.61

is shown in Figure 1. The cumulative amount–time profiles for various treatments were plotted. The slopes of the resulting linear plots were calculated, and the flux ($\mu g\,cm^{-2}\,h^{-1}$) was determined from the slope. Three percent essential oil in various ethanol/water vehicles (0%, 25%, or 50%) significantly increased ($P < 0.05$) the in-vitro permeation of theophylline and cianidanol as compared with the control. The increase in ethanol content generally increased the drug permeation. As shown in Figure 1, the low permeation of both drugs could be partly overcome by *M. fargesii* essential oil. As ethanol increased from 0% to 50% in the vehicle with the essential oil, the flux of theophylline increased as depicted in Figure 1A. A similar result was observed for cianidanol, although there was no significant difference ($P > 0.05$) between the ethanol concentrations of 25% and 50%.

To further examine the effect of *M. fargesii* essential oil on drug flux, pretreatment with 3% essential oil in 25% ethanol/pH 6 buffer vehicle for 1 h followed by application of pH 6 buffer with the model drugs was performed for in-vitro skin permeation. As shown in Figure 1, the flux of drugs was significantly higher ($P < 0.05$) for *M. fargesii*-pretreated skin than for 25% ethanol-pretreated skin. This phenomenon was more significant for cianidanol than for theophylline. The drug within the skin was determined at the end of the in-vitro pretreatment study (12 h). *M. fargesii* essential oil did not significantly increase the theophylline skin content value ($P > 0.05$) relative to the control (151.56 ± 57.90 vs $192.81 \pm 34.81\,ng\,mg^{-1}$). On the other hand, it was clear that the essential oil-pretreatment formulation resulted in a higher level of cianidanol within the skin (18.58 ± 5.28 vs $6.12 \pm 2.11\,ng\,mg^{-1}$).

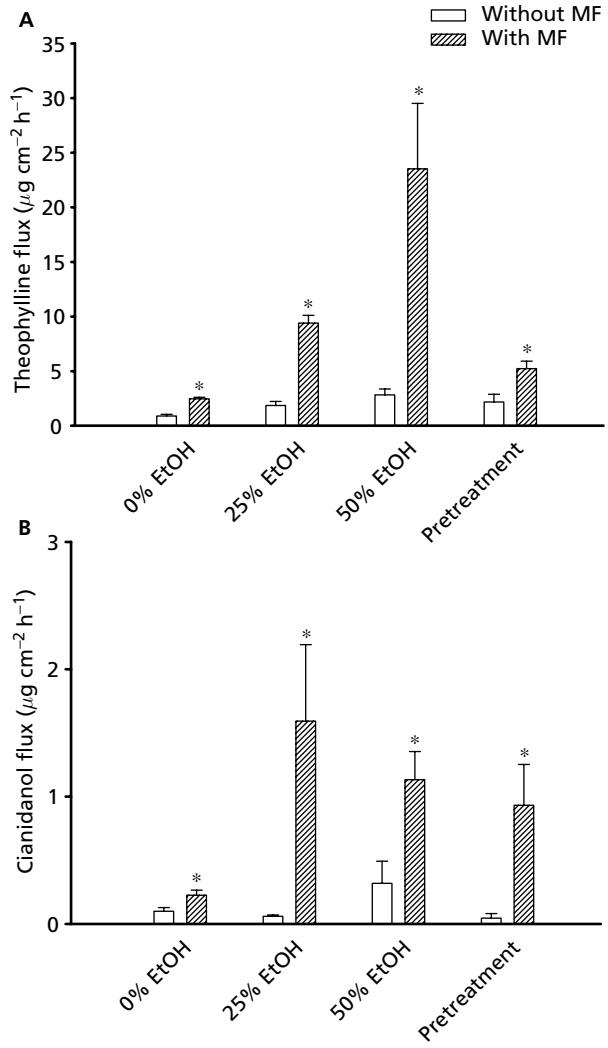

Figure 1 In-vitro flux of theophylline (A) and cianidanol (B) from vehicle with various ethanol contents across Wistar rat skin in the absence or presence of 3% *M. fargesii* essential oil. All data represent the mean ± s.d. of four experiments. $*P < 0.05$ (*t*-test) compared with the control group (with *M. fargesii* vs without *M. fargesii*). MF, *Magnolia fargesii*.

In-vivo skin permeation

The in-vivo pharmacokinetics of theophylline and cianidanol located within the skin were evaluated by treatment with *M. fargesii* essential oil. The essential oil was either incorporated into the 25% ethanol vehicle with the model drugs or used to pretreat the skin before administration of the drug. As shown in Figure 2A, treatment with *M. fargesii* essential oil did not produce higher deposition of theophylline within the skin after either 6- or 12-h administration. As shown in Figure 2B, the amount of cianidanol in skin when incorporating 3% essential oil in the vehicle was higher than the control ($P < 0.05$) after 12-h administration. A greater enhancement of skin uptake of cianidanol was observed by the pretreatment method.

To apply the microdialysis technique for obtaining the extracellular concentration of analytes, knowledge of the fractional recovery of the solute is a prerequisite. The relative recovery of theophylline was $36.6 \pm 3.1\%$ in the rat ($n = 4$), whereas cianidanol showed a low recovery of $< 5\%$. Hence in this study only theophylline was used as a model drug for in-vivo microdialysis. The in-vivo permeation of theophylline by *M. fargesii* essential oil was determined over a 6-h period. Figure 3 shows the results of microdialysis for topical theophylline delivery with 3% essential oil in the 25% ethanol vehicle. The theophylline concentration in the dialysate was calibrated using the

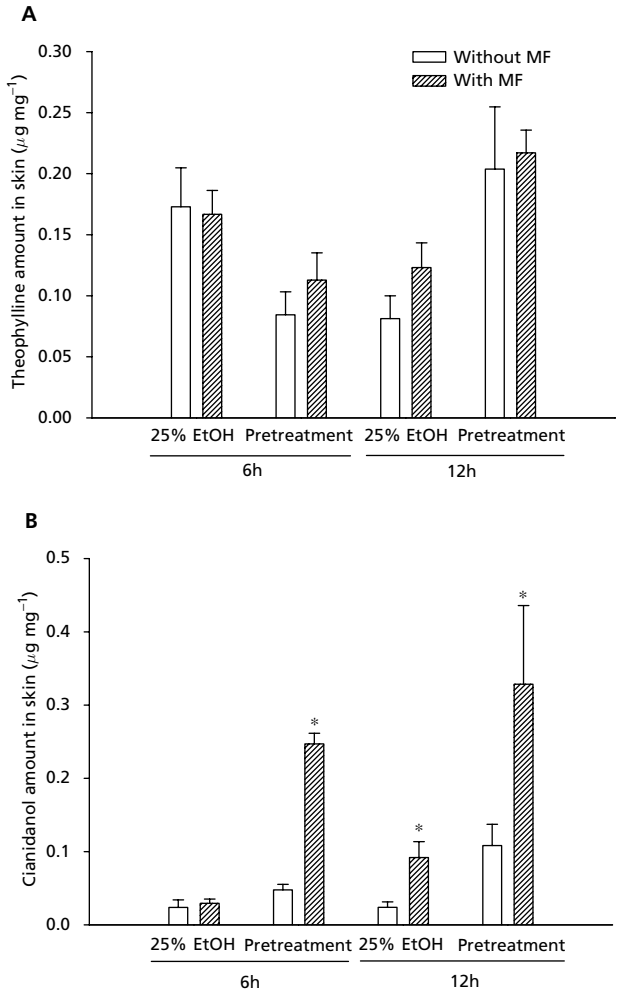

Figure 2 In-vivo skin permeation of theophylline (A) and cianidanol (B) uptake within skin after topical application of 25% EtOH/pH 6 buffer with or without 3% *M. fargesii* essential oil. All data are presented as the mean ± s.d. of six experiments. $*P < 0.05$ (*t*-test) compared with the control group (with *M. fargesii* vs without *M. fargesii*). MF, *Magnolia fargesii*.

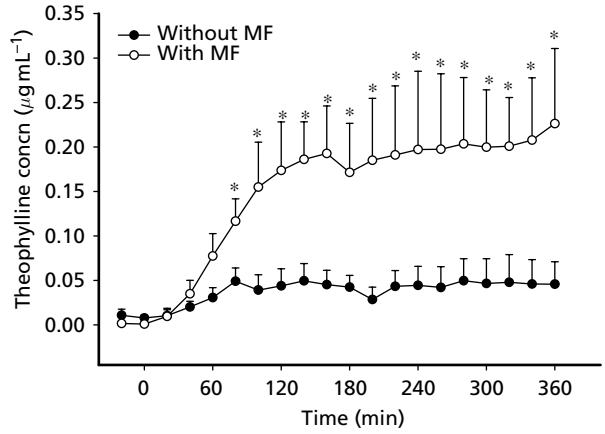

Figure 3 Theophylline concentration in dialysate collected from the subcutaneous region in the in-vivo microdialysis in the absence or presence of 3% *M. fargesii* essential oil. All data are presented as the mean ± s.d. of six experiments. $*P < 0.05$ (*t*-test) compared with the control group (with *M. fargesii* vs without *M. fargesii*) at the same time plots. MF, *Magnolia fargesii*.

Table 2 Cell viability and PGE_2 production by human keratinocytes in culture medium after treatment with ethanol and 0.003% *M. fargesii* essential oil/ethanol

Formulation	Basal (non-treatment)	Ethanol	*M. fargesii* essential oil/ethanol
Cell viability (%)	100	92.44 ± 6.98	86.07 ± 6.74
PGE_2 production (fold of basal)	1	0.93 ± 0.15	0.88 ± 0.36

All data are presented as the mean ± s.d. of four experiments.

recovery value for all profiles. There was no or negligible permeation of theophylline after application of the formulation without *M. fargesii* essential oil. Incorporation of essential oil greatly increased the permeated amount of subcutaneous theophylline. The pharmacokinetics of theophylline in the subcutaneous region were significantly higher ($P < 0.05$) in the essential oil group than in the control after 60-min administration.

Cell viability and PGE_2 release

Cell viability and PGE_2 release by cultured human keratinocytes stimulated by *M. fargesii* essential oil were investigated. Due to solubility considerations, the essential oil had to be dissolved in ethanol to treat the cells. As shown in Table 2, the cell viability according to the MTT assay indicated that there were no significant differences ($P > 0.05$) between the levels of the essential oil group and the control. The same phenomenon was observed for PGE_2 release.

In-vivo skin tolerance study

The skin of Wistar rats was treated with *M. fargesii* essential oil for 24 h to evaluate skin tolerance to the essential oil. As shown in Figure 4A, the ΔTEWL (TEWL value of treated site minus the TEWL value of an adjacent untreated site) determined over four days was evaluated. The curve of *M. fargesii* essential oil approximated that of the control (25% ethanol/pH 6 buffer vehicle). On the other hand, oleic acid demonstrated a significant increase ($P < 0.05$) in the extent of water loss from the stratum corneum relative to the control during four days post-enhancer removal. Levels of Δa* (a* value of treated site minus the a* value of an adjacent untreated site) by treatments with and without *M. fargesii* essential oil were similar during the four days as depicted in Figure 4B.

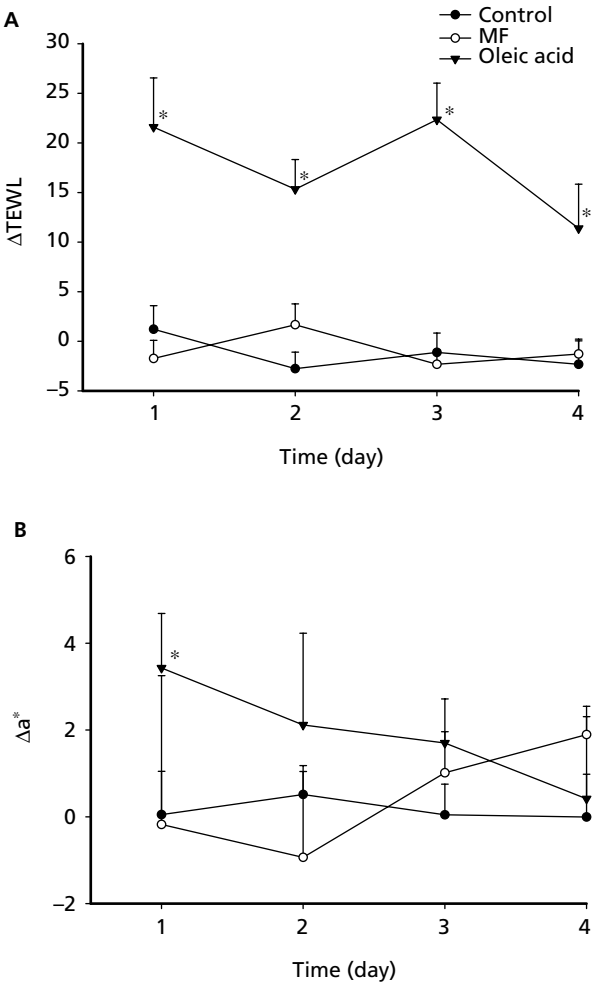

Figure 4 In-vivo skin tolerance study determined by ΔTEWL (A) and Δa* (B) for four days after topical application of 25% EtOH/pH 6 buffer with or without 3% enhancers (*M. fargesii* essential oil or oleic acid) for 24 h. All data are presented as the mean ± s.d. of six experiments. $*P < 0.05$ (analysis of variance test) compared with the control group. ΔTEWL, the TEWL value of treated site minus the value of an adjacent untreated site; Δa*, the a* value of treated site minus the value of an adjacent untreated site; MF, *Magnolia fargesii*.

Discussion

In the in-vitro skin permeation study, an increase in ethanol content in the donor vehicle generally resulted in an increase in the amount of drug which permeated through the skin. Since theophylline and cianidanol were completely soluble in all vehicles used, the increase of flux may have been due to structural modification of the skin, not to an increase in drug solubility (Fang et al 2003a). The permeation of theophylline was generally higher than that of cianidanol, which may have been due to the more hydrophilic characteristics of cianidanol, rendering it difficult for cianidanol to pass across the lipophilic layers of the stratum corneum. With respect to drug permeation across the skin from the vehicle, a drug should first diffuse out from the vehicle to the skin surface. To clarify the mechanisms of the skin absorption of theophylline and cianidanol, the release of model drugs across a cellulose membrane (Cellu-Sep T2, MW cut-off: 6000∼8000), through which the drugs can freely traverse, was studied. The fluxes of theophylline and cianidanol across the cellulose membrane were comparable (123.49 ± 3.41 vs $119.60 \pm 6.71\ \mu g\,cm^{-2}\,h^{-1}$, respectively) and greatly lower ($P < 0.05$) than those across rat skin. This may indicate that the stratum corneum presents a significant barrier for the transport of model drugs, especially for cianidanol.

As shown in Figure 1, this barrier property of the stratum corneum could be partly overcome by addition of *M. fargesii* essential oil. As ethanol increased from 0% to 50%, the flux of theophylline and cianidanol generally increased. This may have been due to the increase in ethanol content, possibly further enhancing the solubility of the essential oil in the vehicle (Arellano et al 1996), thus promoting the transdermal delivery of both drugs. The pretreatment method avoided a cosolvent effect on the thermodynamic activities of the drug or enhancer. This method can also verify whether the enhancer acts directly on the skin structure (Fang et al 2003b). As shown in Figure 1, the flux of drugs was significantly higher ($P < 0.05$) for essential oil-pretreated skin than for 25% ethanol-pretreated skin. Hence the effect of essential oil on skin structure modification may have contributed to enhancement of the drug flux (Cornwell et al 1996; Zhao & Singh 1998). This phenomenon was more significant for cianidanol than for theophylline, since the stratum corneum presents a strict barrier for more-hydrophilic drugs such as cianidanol.

As a function of solubility, skin structural modification, and the partition behaviour between vehicle and skin, many mechanisms have been found by which the enhancer affects the skin permeation of drugs (Zhao & Singh 2000; Fang et al 2003a). The possibility of a partitioning effect can be confirmed by determining the drug reservoir in the skin. It is clear that the essential oil-pretreatment formulation resulted in a higher level of cianidanol within the skin. However, this trend was not observed for theophylline. This suggested that disruption of the skin morphology and an increment of drug partitioning into the stratum corneum were the possible effects of *M. fargesii* essential oil on cianidanol permeation, whereas the partitioning effect of the essential oil was not apparent for theophylline. Some compounds in the essential oil such as camphor, eucalyptol, and α-terpineol have been reported to show potent enhancing effects on drug permeation (Zhao & Singh 1998; Vaddi et al 2002; Narishetty & Panchagnula 2004). All compounds in the *M. fargesii* essential oil were oxygen-containing terpenes. It appears that oxygenated terpenes preferentially form hydrogen bonds with ceramide head groups, which break the interlamellar hydrogen bonding network of the lipid bilayer, thus disrupting the barrier property of the stratum corneum (Narishetty & Panchagnula 2004).

In the in-vivo skin permeation study, treatment with *M. fargesii* essential oil did not produce higher deposition

of theophylline within the skin after either 6- or 12-h administration. This result was similar to that of in-vitro permeation. The limited space in the skin reservoir for the drug to become saturated may explain this result. The amount of cianidanol in skin when incorporating 3% essential oil in the vehicle showed a higher value than the control ($P < 0.05$) after 12-h administration. A greater enhancement of skin uptake of cianidanol was observed by the pretreatment method, confirming the high partitioning of cianidanol from the vehicle to the skin.

After uptake into the skin, the drug further diffused across the skin to the subcutaneous region. Cutaneous microdialysis is an in-vivo sampling technique for measuring a solution in the extracellular fluids under the dermis. The result of microdialysis for theophylline permeation suggested that although *M. fargesii* essential oil could not enhance the skin deposition of theophylline, a higher subcutaneous amount of theophylline was achieved with this essential oil.

The skin is a viable organ with a variety of biological functions, such as protection, metabolism, and sensation. A skin permeation enhancer may trigger some biological responses beyond the physicochemical interactions with the lipids and/or proteins of the skin (Xu & Chien 1991). As a result, the biological responses of the skin to an enhancer may modify the skin permeation of drugs. The results of cell cultures may suggest that the enhancing effect of the permeation of theophylline and cianidanol mainly resulted from the mechanism of physicochemical interactions with skin but not biological responses. The presence of human keratinocytes produced in response to cell viability and inflammation is also a potential means of evaluating irritation to the skin (Lawrence 1997). It is proposed that *M. fargesii* essential oil is safe when administered topically because it produced no cytotoxicity to the skin.

TEWL was performed to assess damage of the stratum corneum, and a good correlation between chemical damage to the skin barrier and an increase in TEWL has been demonstrated by Fang et al (2003a, c). The colorimeter records colour reflectance three-dimensionally (L*, a*, and b*), as recommended by CIE (Commission Internationale de l'Eclairage). The luminance (L*) gives the relative brightness from black to white. The a* represents the balance between red and green and the b* represents the balance between yellow and blue (Fang et al 1997). The total difference of skin colour can be expressed as ΔE^*, $\Delta E^* = ((\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2)^{1/2}$. The enhancers such as *M. fargesii* essential oil and oleic acid exhibited non-significant changes ($P > 0.05$) in skin colour after determination of ΔL^* and Δb^*. Hence we only showed the value of Δa^* for comparison of different formulations. The a*-coordinates of colorimetry have been demonstrated to correlate well with inflammatory reactions of the skin (Serup & Agner 1990). Oleic acid, a commonly used enhancer, was used as a positive control in the skin tolerance study because of its verified irritation of skin (Boelsma et al 1996; Fang et al 2003c). The curve of *M. fargesii* essential oil approximated that of the control (25% ethanol/pH 6 buffer vehicle), indicating that the essential oil produced limited perturbation of the structure of the stratum corneum. According to the previous in-vitro permeation data, however, the effect of *M. fargesii* essential oil on skin structure modification may have contributed to enhancement of the drug permeation. It may be concluded that *M. fargesii* essential oil could cause the perturbation of the structure of the stratum corneum. However, this effect was still insufficient to produce the significant change of TEWL. Levels of Δa^* with treatments of *M. fargesii* essential oil and the 25% ethanol/pH 6 buffer vehicle were similar over the four days as depicted in Figure 4b. This result indicated that the skin showed good tolerance to *M. fargesii* essential oil itself. The colorimetric determinations reflected mainly erythema and vasodilation triggered by inflammatory reactions. This result coincided with the profiles of in-vitro keratinocyte cultures which showed no increase in PGE_2 release after *M. fargesii* essential oil treatment.

In summary, the efficacy and safety of *M. fargesii* essential oil were systemically evaluated using a series of in-vitro and in-vivo methods. Oxygenated monoterpenes and sesquiterpenes were the main components of this essential oil. Farnesol comprised the largest portion of this essential oil, at over 40% of the total components. In-vitro and in-vivo skin permeation studies showed that *M. fargesii* essential oil could effectively promote theophylline and cianidanol absorption across and/or into the skin. Alteration of the skin structure and the increase in the skin-vehicle partitioning by *M. fargesii* essential oil might be the mechanisms of this enhancement. Results of the in-vitro MTT assay and PGE_2 release indicated the safety of *M. fargesii* essential oil to skin keratinocytes. The in-vivo skin tolerance study by TEWL and colorimetry also confirmed its safety.

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