Transdermal Delivery of Tea Catechins and Theophylline Enhanced by Terpenes: a Mechanistic Study

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Using *in vitro* and *in vivo* techniques, terpenes were evaluated as enhancers to improve the skin permeation of therapeutically active agents derived from tea, including tea catechins and theophylline. The *in vitro* permeation was determined by Franz cells. The skin deposition and subcutaneous amounts of drugs sampled by microdialysis were evaluated *in vivo*. Terpenes varied in their activities of enhancing drug permeation. The oxygen-containing terpenes were effective enhancers of drug permeation, whereas the hydrocarbon terpenes were much less efficient. Oxygen-containing terpenes with a bicyclic structure had reduced enhancing activity. Terpenes enhanced tea catechin permeation to a much greater degree than they did theophylline. The isomers of (+)-catechin and (−)-epicatechin showed different permeation behaviors when incorporated with terpenes. In the *in vivo* status, terpenes promoted the skin uptake but not the subsequent subcutaneous concentration of (−)-epigallocatechin gallate (EGCG). Both increased skin/vehicle partitioning and lipid bilayer disruption of the stratum corneum (SC) contributed the enhancing mechanisms of terpenes for topically applied tea catechins and theophylline based on the experimental results from the partition coefficient and transepidermal water loss (TEWL). α-Terpineol was found to be the best enhancer for catechins and theophylline. The high enhancement by α-terpineol was due to macroscopic perturbation of the SC and the biological reaction in viable skin as evaluated by TEWL and colorimetry.

Key words tea catechin; theophylline; transdermal delivery; terpenes; microdialysis

Tea (*Camellia sinensis*) is the most-often consumed beverage in the world next to water. Tea catechins and theophylline are naturally occurring compounds derived from tea. Catechins are polyphenols and have been shown to function as chemopreventive and anticarcinogenic agents.[1)] With respect to the skin, catechins have been reported to be beneficial in treating UV-induced photodamage, basal cell carcinoma, melanoma, and sunburn.[2)] Theophylline is a commonly used drug for the treatment and prevention of asthma and recurrent apnea. It is also a lipolytic agent for slimming purposes.[3)] The oral bioavailability of catechins is known to be low with a bioavailability of less than 5%.[4,5)] Theophylline also undergoes first-pass metabolism on oral administration and has a short elimination half-life. A transdermal system would provide an alternative delivery mechanism for catechins and theophylline, thus giving consumers an option to formalize dosing.[6,7)]

The primary barrier to dermal and transdermal permeation is the outermost layer of the skin, the stratum corneum (SC). The barrier properties of the SC can be reduced by the use of enhancers. Terpenes, naturally occurring volatile oils, appear to be promising candidates for clinically acceptable enhancers.[8)] They are reported to have good toxicological profiles, high percutaneous enhancement abilities, and low cutaneous irritancy at low concentrations (1—5%).[9)] Although many studies have investigated the effect of various terpenes on drugs and the responses of various drugs enhanced by terpenes, there are few systemic investigations related to the effects of various terpenes on a variety of permeants.

The purpose of this study was to explore the enhancing effect of terpenes on theophylline and three catechins: (+)-catechin, (−)-epicatechin, and (−)-epigallocatechin gallate (EGCG). These terpenes, which are aromatic and aliphatic compounds, were each chosen to respectively represent a broad chemical class of hydrocarbons, alcohols, ketones, and epoxides (Fig. 1). This study utilized Franz cells to explore the influence of terpenes on the *in vitro* skin permeation of drugs. The amount of drug retained within the skin reservoir was also determined *in vitro* and *in vivo*. The *in vivo* transcutaneous drug amount was determined using a microdialysis technique. The mechanisms of the enhancing ability of terpenes were investigated in this study.

MATERIALS AND METHODS

Materials (+)-Catechin, (−)-epicatechin, EGCG, theophylline, and (+)-limonene were purchased from Sigma Chemical (St. Louis, MO, U.S.A.). Camphor, linalool, nerolidol, menthone, cymene, farnesol, fencone, and α-terpineol were supplied by Aldrich Chemical (Steinheim, Germany).

***In Vitro* Percutaneous Administration** *In vitro* skin permeation was carried out using a Franz diffusion assembly. The shaved back skin of female Wistar rats (180—200 g) or a cellulose membrane (SelluSep® T2, MW cutoff of 6000—8000, Membrane Filtration Products, U.S.A.) was mounted on the receptor compartment with the SC side facing upwards into the donor compartment. The donor medium was 1 ml of 25% ethanol/pH 7.4 buffer containing 3.44 mM drug with or without 3% terpenes. The receptor medium was 5 ml of pH 7.4 citrate-phosphate buffer. The available diffusion area between compartments was 0.785 cm^2. The stirring rate and temperature were kept at 600 rpm and 37 °C, respectively. At appropriate intervals, 300-μl aliquots of the receptor medium were withdrawn and immediately replaced with

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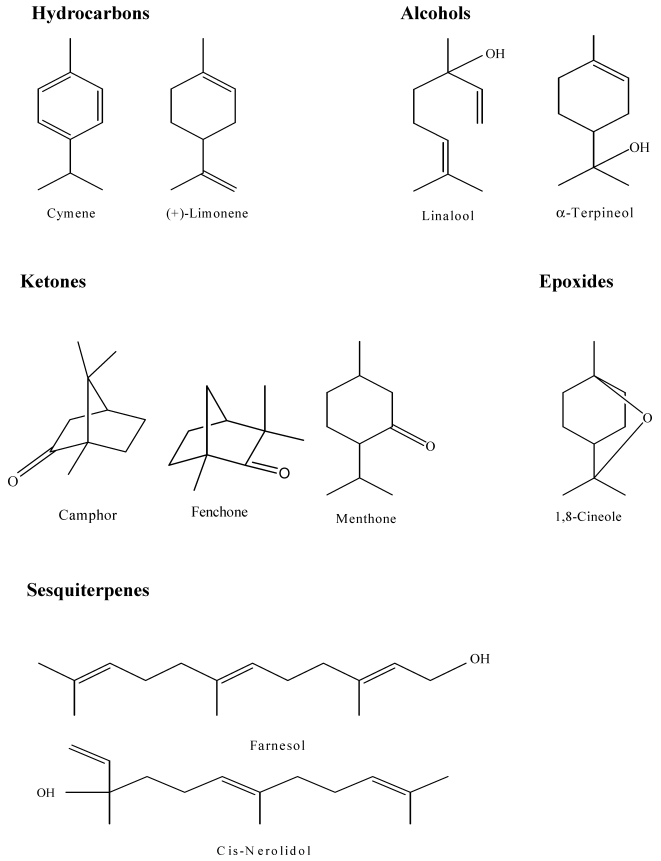

Fig. 1. Chemical Structures of the Evaluated Terpene Enhancers

an equal volume of fresh buffer. The amount of drug retained in the skin was determined at the end of the *in vitro* experiment (24 h) if necessary. The site of application on the skin was washed 10 times with a cotton cloth immersed in double-distilled water. A sample of skin was weighed, cut with scissors, positioned in a glass homogenizer containing 1 ml of 0.1 M HCl, and ground for 5 min with an electric stirrer. The resulting solution was centrifuged for 10 min at 10000 rpm and then filtered through a PVDF membrane (with a pore size of 0.45 μm, Millipore, U.S.A.). All samples in the *in vitro* experiment were analyzed by HPLC.[7,10]

***In Vivo* Percutaneous Administration** Female Wistar rats (180—200 g) were anesthetized using 3 ml/kg urethane (25%) *via* an intraperitoneal route. The back fur of the rat was shaved. A glass cylinder with an available area of 1.54 cm^2 was placed on the skin with glue (Instant Super Glue, Kokuyo, Japan). Two milliliters of 3.44 mM drug in 25% ethanol/pH 7.4 buffer with or without 3% terpenes was added to the cylinder. The application time of the vehicle was 6 h. The rat's body temperature was maintained at 37 °C with a heating pad during the experiment. The procedure for extraction of drug from the skin was the same as for the *in vitro* experiment.

The microdialysis system comprised a CMA/100 microinjection pump (CMA, Stockholm, Sweden) and microdialysis probe. The dialysis probe for the subcutaneous measurements (10 mm in length) was made of silica capillary tubes in a concentric design.[11] Their tips were covered by a dialysis membrane (Spectrum Laboratories, 200-μm inner diameter with a cutoff at a nominal molecular weight of 13000, Laguna Hills, CA, U.S.A.), and all unions were cemented with epoxy. At least 24 h was allowed for the epoxy to dry. The microdialysis probe was located within the subcutaneous area on the back and then perfused with Ringer solution at a flow rate of 0.85 μl/min by employing 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 anesthesia as described above. Following a stabilization period of 2 h after probe implantation, the perfusate (C_{perf}) and dialysate (C_{dial}) concentrations of the drug were determined by HPLC. The relative *in vivo* recovery (R_{dial}) of drug across the microdialysis probe was calculated by the following equation: $R_{dial}=(C_{perf}-C_{dial})/C_{perf}$. Drug microdialysate concentrations (C_m) were converted to unbound concentration (C_u) as follows: $C_u=C_m/R_{dial}$.

Skin/Vehicle Partitioning The back skin of a Wistar rat was positioned in a Franz cell. Neat terpenes (200 μl) were added to the donor side for 2 h. The treated skin was blotted with a cotton wool swab and then positioned in the wells of a 24-well culture plate (17 mm I.D.) with the SC side downward. One hundred microliters of the drug in 25% ethanol/pH 7.4 buffer was pipetted into the bottom of the well. After a 24-h incubation at 37 °C, the drug vehicle was withdrawn for analysis by HPLC. The skin/vehicle partition coefficient (PC) was calculated by the following equation:

$$PC=(C_{0\,h}-C_{24\,h})/C_{24\,h}$$

where $C_{0\,h}$ represents the total drug concentration in the vehicle at 0 h before the experiment and $C_{24\,h}$ represents the remaining drug concentration in the vehicle after the 24-h incubation.

***In Vivo* Transepidermal Water Loss (TEWL) and Colorimetry** Four milliliters of vehicle with or without terpenes was spread uniformly over a sheet of non-woven polyethylene cloth (2.5×2.5 cm, Johnson & Johnson, U.S.A.), which was then applied to the shaved back area of a rat. The polyethylene cloth was fixed with Tegaderm® adhesive dressing (3M, U.S.A.) and Fixomull® stretch adhesive tape (Beiersdorf AG, Germany). After 24 h, the cloth was removed, and the treated skin area was swabbed clean with a cotton wool swab. After withdrawal of the vehicle for 30 min, TEWL and colorimetry were determined. TEWL was measured quantitatively using a Tewameter® (TM300, Courage & Khazaka, Germany). The TEWL was automatically calculated and expressed in g/m^2/h. A spectrocolorimeter (CD100, Yokogawa Electrical, Japan) was used to measure the skin erythema (a*) caused by the terpenes. An adjacent untreated site was used as a baseline standard for each determination. The temperature and relative humidity in the laboratory were kept at 26 °C and 55% respectively.

Statistical Analysis The statistical analysis of differences between different formulations in this study was performed using the unpaired *t*-test. A 0.05 level of probability ($p<0.05$) was taken as the level of significance. An analysis of variance (ANOVA) test was also used.

Table 1. The Flux, Skin Deposition, and Enhancement Ratio of (+)-Catechin across Skin after *in Vitro* Permeation for 24 h Duration

Classification	Terpenes	Flux (nmol/cm^2/h)	ER_{Flux} *a)*	Skin deposition (nmol/mg)	$ER_{Deposition}$ *b)*
—	Control	0.58±0.18	—	0.48±0.22	—
Hydrocarbon	Cymene	39.86±13.23	68.72	1.94±0.79	4.04
Hydrocarbon	(+)-Limonene	15.77±4.51	27.19	1.55±0.42	3.23
Alcohol	Linalool	162.23±36.70	279.71	2.51±1.63	5.23
Alcohol	α-Terpineol	217.50±13.55	375.00	5.08±0.55	10.58
Ketone	Camphor	9.58±1.20	16.52	1.01±0.32	2.10
Ketone	Fenchone	77.26±3.91	133.21	0.96±0.21	2.00
Ketone	Menthone	216.24±5.40	372.83	2.67±0.80	5.56
Epoxide	1,8-Cineole	129.69±15.70	223.60	2.38±0.65	4.96
Sesquiterpene	Farnesol	81.89±10.44	141.19	2.36±0.37	4.92
Sesquiterpene	Nerolidol	31.72±7.17	54.69	1.71±0.78	3.56

a) The enhancement ratio (ER_{Flux}) was the (+)-catechin flux with terpene treatment/(+)-catechin flux of control group. *b*) The enhancement ratio ($ER_{Deposition}$) was the (+)-catechin deposition in skin with terpene treatment/(+)-catechin deposition in skin of control group. All the values of terpenes (flux and skin deposition) were significantly higher than the control ($p<0.05$). Each value represents the mean±S.D. ($n=4$).

Table 2. The Flux (nmol/cm^2/h) of (+)-Catechin, (−)-Epicatechin, EGCG, and Theophylline across Skin after *in Vitro* Permeation for 24 h Duration

Terpenes	(+)-Catechin	(−)-Epicatechin	EGCG	Theophylline
None (Control)	0.58±0.18	0.62±0.12	0	11.38±5.68
α-Terpineol	217.50±13.55	214.19±31.05	33.11±6.53	233.673±30.56
Linalool	162.23±36.70	158.85±15.90	9.16±2.86	247.38±54.92
1,8-Cineole	129.69±15.70	173.78±11.31	16.52±2.41	210.98±7.36
Nerolidol	31.72±7.17	52.57±10.68	0.28±0.08	177.89±8.79

All the flux values of terpenes were significantly higher than the control ($p<0.05$). Each value represents the mean±S.D. ($n=4$).

RESULTS

***In Vitro* Percutaneous Administration** The effect of terpenes on the *in vitro* percutaneous absorption of (+)-catechin through rat skin is shown in Table 1. The slopes of the resulting linear plots were calculated, and the flux (nmol/cm^2/h) was determined from the slope. All the evaluated terpenes had significant effects on the (+)-catechin delivery relative to the control. The enhancement activities are expressed as the enhancement ratio (ER) which is the ratio of the flux with enhancers to that obtained with the control. Transdermal transport of (+)-catechin was enhanced between 16- and 375-fold with the terpenes compared to transport by the control (Table 1). The rank order of enhancement was α-terpineol≥menthone>linalool>1,8-cineole>farnesol≥fenchone>cymene≥nerolidol>(+)-limonene>camphor. α-Terpineol and menthone provided the best enhancement activity for (+)-catechin. They increased the drug flux by approximately 370-fold relative to the control, followed by linalool, an acyclic terpene alcohol, with an ER of 280.

The uptake of (+)-catechin within the skin after a 24-h application was also determined as shown in Table 1. The enhancement trend of the various terpenes for skin deposition was generally the same with that for the flux. The highest (+)-catechin skin content was observed with α-terpineol, followed by menthone and linalool. Of all terpenes tested, fenchone and camphor exhibited the lowest $ER_{Deposition}$ of around 2. Enhancement of the skin's drug uptake capacity by terpenes was relatively lower than that of drug flux across the skin.

Effects of Selected Terpenes on the Permeation of Catechins and Theophylline In order to examine the effect of terpenes on various compounds derived from tea, four terpenes with different enhancing abilities for (+)-catechin permeation were selected for treatment (Fig. 2). α-Terpineol, linalool, 1,8-cineole, and nerolidol represent alcohol, acyclic, epoxide, and sesquiterpene structures, respectively. Although both menthone and α-terpineol showed the highest enhancement of (+)-catechin absorption, menthone was excluded from further experiments because of its toxicity.[12] (+)-Catechin and (−)-epicatechin are isomers of each other. As shown in Table 2, (−)-epicatechin exhibited a comparable flux with (+)-catechin in the control (ethanol–water system). The terpenes increased the (−)-epicatechin permeation in the order of α-terpineol>1,8-cineole>linalool>nerolidol.

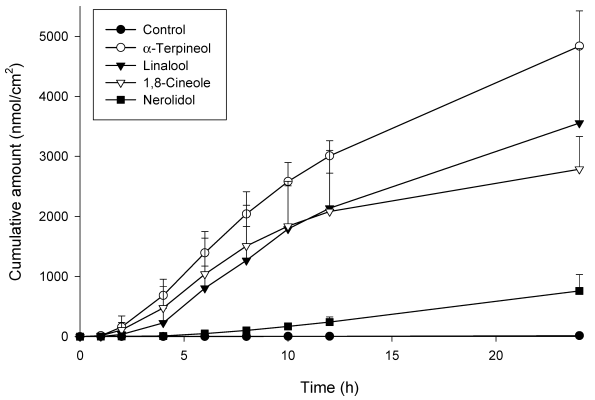

Fig. 2. *In Vitro* Cumulative Amount–Time Profiles of Transdermal (+)-Catechin Delivery from a 25% Ethanol/pH 7.4 Buffer with 3% Terpene Enhancers

All data are presented as the mean±S.D. of four experiments.

EGCG contains a galloyl group at the 3 position which is absent from (+)-catechin and (−)-epicatechin. No EGCG molecules permeated across the skin within 24 h in the control group (Table 2). The incorporation of terpenes elicited the permeation of EGCG across the skin. The rank order of

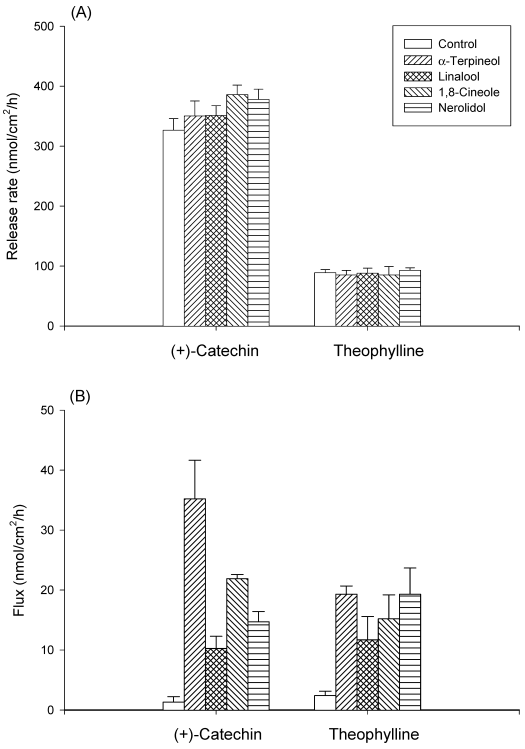

Fig. 3. Release Rate (A) and Flux (B) of (+)-Catechin and Theophylline from a 25% Ethanol/pH 7.4 Buffer with 3% Terpene Enhancers across a Cellulose Membrane and Rat Skin

All data are presented as the mean±S.D. of four experiments.

the effectiveness of the terpenes was α-terpineol>1,8-cineole>linalool>nerolidol. It can be observed that α-terpineol showed the best enhancement of drug permeation for all of the tea catechins examined herein.

Theophylline permeation by the control was relatively higher ($p<0.05$) than that for the tea catechins (Table 2). Although terpenes can promote theophylline flux across the skin, the enhancement was significantly lower than those of the catechins as shown in Table 2. There was no significant difference ($p<0.05$) among the theophylline fluxes enhanced by α-terpineol, linalool, and 1,8-cineole.

(−)-Catechin and Theophylline Permeation across the Cellulose Membrane and Terpene-Pretreated Skin To clarify the mechanism of the skin disruption, the release of (+)-catechin and theophylline across the cellulose membrane, through which the drugs can freely traverse, was studied (Fig. 3). The incorporation of terpenes did not increase the (+)-catechin and theophyline release ($p>0.05$). The amounts of drug released by the various terpenes were comparable ($p>0.05$).

To further examine the effect of terpenes on drug flux, pretreatment with 3% terpenes in an ethanol-buffer vehicle for 1 h followed by application of drug to the donor compartment was performed for *in vitro* skin permeation. Pretreatment with terpenes significantly enhanced (+)-catechin and theophylline permeation as compared to the control (Fig. 3). Linalool pretreatment showed lower enhancement ($p<0.05$) of (+)-catechin permeation than did 1,8-cineole. Pretreatment of the skin by the four terpenes produced similar levels of theophylline permeation enhancement ($p>0.05$).

Skin/Vehicle Partitioning The partition coefficient of (+)-catechin and theophylline in the skin and vehicle is shown in Table 3. The incorporation of all terpenes increased the partitioning of drugs into the skin. α-Terpineol, linalool, and nerolidol gave similar partition coefficients for (+)-catechin ($p>0.05$), whereas the partitioning by 1,8-cineole was lower ($p<0.05$). For theophylline, linalool and nerolidol showed similar partitioning values, which were higher ($p<0.05$) than those of α-terpineol and 1,8-cineole.

Table 3. Skin/Vehicle Partition Coefficient of (+)-Catechin and Theophylline by Treating Skin in 3% Terpenes

Terpenes	(+)-Catechin	Theophylline
None (Control)	2.51±0.36	0.23±0.18
α-Terpineol	9.43±3.30	0.84±0.09
Linalool	8.40±2.00	1.80±0.92
1,8-Cineole	4.90±1.25	0.70±0.32
Nerolidol	9.94±1.82	1.65±0.43

All the partition coefficient values of terpenes were significantly higher than the control ($p<0.05$). Each value represents the mean±S.D. ($n=4$).

Table 4. *In Vivo* TEWL and Erythema of Rat Skin after Treatment of 3% Terpenes for 24 h

Terpenes	ΔTEWL (g/m²/h)	Δa* (arbitrary unit)
None (Control)	2.66±0.65	−1.06±0.73
α-Terpineol	4.82±1.21[a)]	1.95±0.22[a)]
Linalool	2.57±1.02	−2.79±0.97
1,8-Cineole	2.62±0.43	−1.30±0.27
Nerolidol	3.06±1.07	−0.18±0.05

a) The value significantly higher than the control ($p<0.05$). Each value represents the mean±S.D. ($n=6$).

***In Vivo* TEWL and Colorimetry** As shown in Table 4, the value of ΔTEWL (TEWL value of the treated site minus the TEWL value of an adjacent untreated site) was determined after 24-h treatment with terpenes. The ΔTEWL of the control was 2.66 g/m²/h. This may have been due to the effect of ethanol on the skin which extracted some of the lipids within the SC. Treatments with linalool, 1,8-cineole, and nerolidol produced no enhancement of ΔTEWL ($p>0.05$). α-Terpineol demonstrated a significant increase ($p<0.05$) in the extent of water loss from the SC relative to the control. Similar to the results of ΔTEWL, α-terpineol showed a higher Δa* value than did the other terpenes (Table 4).

***In Vivo* Percutaneous Administration** The *in vivo* pharmacokinetics of the catechins and theophylline were evaluated by microdialysis. 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 recoveries of (+)-catechin, (−)-epicatechin, and theophylline in Ringer's solution were 36.8±6.7%, 31.9±4.7%, and 36.5±1.8% respectively. Because of the lipophilic characteristic of EGCG, 25% ethanol was added to Ringer's solution as the eluent to obtain a recovery of 26.5±1.3%. Figure 4 shows the results of microdialysis for transdermal drug delivery with 3% α-terpineol in the 25% ethanol vehicle. The drug concentration in the dialysate was calibrated using the recovery for all profiles. There was no permeation of tea catechins after application without α-terpineol. Theophylline was able to passively

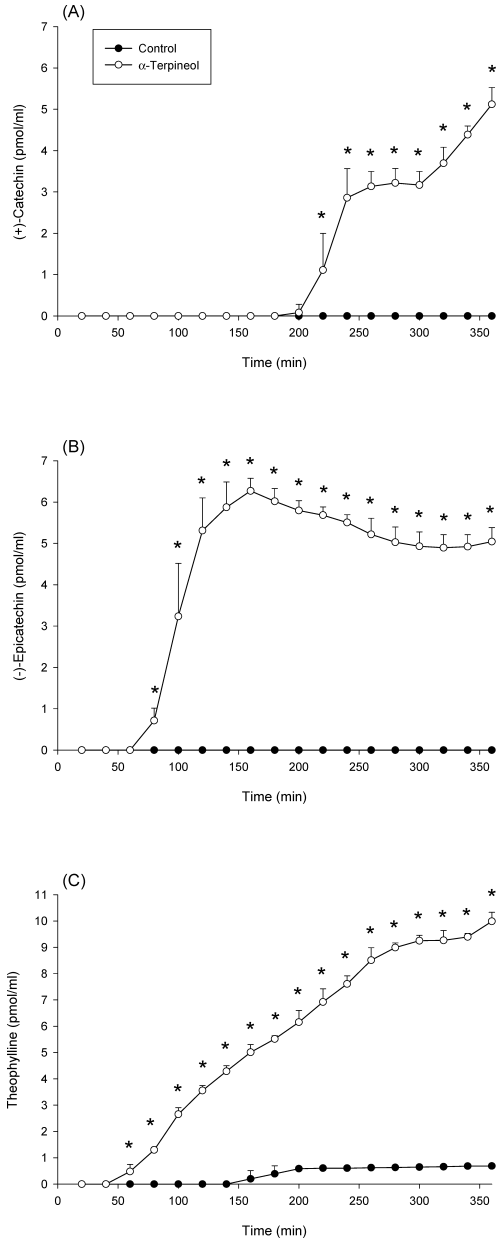

Fig. 4. (+)-Catechin (A), (−)-Epicatechin (B), and Theophylline (C) Concentrations in Dialysate Collected from the Subcutaneous Region with *in Vivo* Microdialysis in the Absence or Presence of 3% α-Terpineol

All data are presented as the mean±S.D. of six experiments. An asterisk (*) indicates a value significantly higher (*t*-test, $p<0.05$) than that of the control group (with α-terpineol *vs.* without α-terpineol) at the same time points.

move through the skin to the subcutaneous region. Incorporation of α-terpineol greatly increased the permeated amount of subcutaneous drugs except for EGCG. EGCG also showed no increase in permeation for 6 h after α-terpineol treatment.

The subcutaneous theophylline concentration gradually increased during 6 h in the presence of α-terpineol. The *in vivo* topical application of drugs located within the skin was evaluated and is depicted in Table 5. The *in vivo* skin uptake of (−)-epicatechin was relatively higher ($p<0.05$) than that of (+)-catechin in the absence of α-terpineol. However, the data among different drugs cannot be directly compared because of the different recovery rates for extracting various drugs from the skin. α-Terpineol promoted the skin deposition of all drugs examined. A pronounced effect of this enhancement was observed for EGCG.

Table 5. Skin Deposition (nmol/mg) of (+)-Catechin, (−)-Epicatechin, EGCG, and Theophylline after *in Vivo* Application for 6 h Duration

Drug	Control	α-Terpineol
(+)-Catechin	0.11±0.03	0.46±0.16
(−)-Epicatechin	0.26±0.09	0.59±0.18
EGCG	0.003±0.001	0.55±0.14
Theophylline	0.11±0.08	0.37±0.13

All the skin deposition values of terpenes were significantly higher than the control ($p<0.05$). Each value represents the mean±S.D. ($n=6$).

DISCUSSION

Previous studies have shown that terpenes in combination with ethanol increase the permeation of hydrophilic drugs.[13,14] Hence 25% ethanol in pH 7.4 buffer was used as the vehicle for the enhancers. In general, hydrophilic terpenes containing functional moieties with oxide were effective in enhancing skin permeation. These compounds have structures suitable for disrupting the lipid packing of the SC because of the presence of definitive hydrocarbon tails in addition to a polar head group.[8,15] Farnesol and nerolidol, two aliphatic sesquiterpene alcohols, produced mildly enhanced activity. This may suggest that the oxygen-containing monoterpenes were more effective than oxygen-containing sesquiterpenes at enhancing drug delivery. The hydrocarbon terpenes such as cymene and (+)-limonene were less-effective enhancers. This result is in agreement with previous findings that limonene cannot perturb the SC lipid bilayers because it does not contain a free group (–OH or =O) to break up the tight network.[16]

Of the terpenes, hydrocarbons are the most lipophilic. The solubility of cymene and (+)-limonene in the ethanol–water system was lower than that of the others, leading to cymene and (+)-limonene having incomplete strength for acting on the skin structure. Camphor exhibited the lowest ER_{flux} of only 16. Fenchone and camphor showed the least enhancement among the oxygen-containing monoterpenes. This may be related to their bicyclic (bridge) structure which differs from that of the other terpenes. This assumption should be further explored to confirm the real mechanisms producing fenchone and camphor's poor results.

Because of their lipophilic properties, terpenes can easily partition into the SC, thereby disrupting the lipid structure and increasing the fluidity of lipids.[17] Many studies have reported the effect of various terpenes on the permeation enhancement of both hydrophilic and lipophilic drugs. Since the model drugs used in this study were linked to hydrophilic permeants, the application of terpenes with hydrophilic drugs of other investigations was compared with ours. Cornwell and Barry[18] reported that the enhancing effect of terpenes on 5-fluorouracil decreased in the order of 1,8-cineole>nerolidol>α-terpineol>(+)-limonene. El-Kattan *et al.*[19] showed an increasing trend of nerolidol>(+)-limonene>fenchone for nicardipine permeation enhancement. Xiong *et al.*[20] showed better enhancement by nerolidol than by 1,8-cineole for low-MW heparin permeation. Magnusson *et al.*[21] indicated that 1,8-cineole increased the transdermal permeation

of a tripeptide thyrotropin-releasing hormone to a greater level than did menthone. The permeation of the polar steroid, hydrocortisone, showed an increasing trend by terpenes in the order of nerolidol>(+)-limonene>cymene>1,8-cineole>α-terpineol>menthone>fenchone.[9] Panchagnula and colleagues[16] reported that the enhancement of zidovudine permeation by terpenes is in the order of 1,8-cineole>menthone>α-terpineol. Our results somewhat differ from these obtained by other investigators. A certain trend of the enhancing activity of various terpenes cannot be found for these hydrophilic permeants. It is noteworthy that there are discrepancies in vehicles, terpene concentrations, treatment durations, and skin barriers among these studies. The effects of some terpenes may have been veiled by the insufficient dose or application time. It is important to establish enhancing mechanisms of terpenes for each drug individually, as they cannot be reliably fitted to other drugs.

Among the terpenes tested, fenchone and camphor exhibited the lowest $ER_{Deposition}$ values of only 2. This may again suggest the unfavorable effect of the bicyclic structure for enhancing (+)-catechin absorption. The enhancement of the skin's drug capacity by terpenes was relatively lower than that of drug flux across the skin. Saturation of the limited space of the skin reservoir for the drug may explain this phenomenon.

The flux of (−)-epicatechin was comparable ($p>0.05$) to that of (+)-catechin from the control (without terpenes). This result indicates that differences in steric confirmation might not influence skin permeation. α-Terpineol and linalool provided comparable enhancements for both isomers. However, (−)-epicatechin showed higher flux than did (+)-catechin in the presence of 1,8-cineole and nerolidol. This may indicate the permeation behaviors of both isomers were changed when treated with 1,8-cineole and nerolidol. This may have been due to 1,8-cineole and nerolidol producing selective absorption of (+)-catechin over (−)-epicatechin.

Based on a broad survey of clinically used allergens, pharmacological agents, and topical drugs, 500 Da was proposed as the limit for transdermal delivery.[22] Since EGCG has a MW of 458 Da, it is near the upper limit of size for molecules to pass through the skin. The presence of gallic acid esters in the structure of EGCG is responsible for its high lipophilicity. The *n*-octanol/water partitioning coefficient indicates the greater lipophilicity of EGCG (16.0) compared to (−)-epicatechin (1.4).[23] EGCG possibly strongly locates on the surface of the lipid bilayers within the SC. It is thought that EGCG cannot permeate across the skin due to its retention in the skin.

Drugs that permeate most easily are small molecules of moderate lipophilicity.[24] The MW of theophylline is only 180. Theophylline is weakly acidic in nature, with a pK_a value of 8.7.[25] Although theophylline is a model polar drug, most of the molecules are in a un-ionized form at the pH of the donor vehicle. On the other hand, catechins basically possess negative charges at pH 7.4.[10] There is also good evidence that the SC is much more permeable to neutral molecules than to the salts of weak acids or bases.[26] The enhancement of theophylline permeation by terpenes was significantly lower than that of catechins. This may have been due to terpenes enhancing the ionized form of the drugs to a much greater extent than the un-ionized form. The enhancing activities for theophylline by the four terpenes were approximately equal. This again demonstrates that no general rule exists for the enhancing trend of terpenes for all permeants.

Enhancers have been categorized into those that increase the thermodynamic activity of permeants and those that disrupt the lipid packing of the SC. The permeation of polar drugs, like theophylline, across the skin can be enhanced simply by increasing their thermodynamic activity in the donor solution, and this is qualitatively predictable by regular solution theory.[27] With respect to drug permeation across the skin from the vehicle, a drug should first diffuse out of the vehicle to the skin surface. The release rates of (+)-catechin and theophylline were significantly higher ($p<0.05$) than their fluxes across the skin. This indicates that a significant barrier by the SC exists for the transport of both drugs. The drug release rates with and without various terpenes were comparable ($p>0.05$). This result may indicate that the addition of terpenes did not change the diffusivity of those drugs within the donor vehicle. Hence the four terpenes may have predominantly acted on the skin to manifest their enhancing ability on drug permeation.

Pretreatment with terpenes promoted (+)-catechin and theophylline permeation as compared to the control, suggesting that these terpenes play some role in the skin. Contrary to the coexistence of the permeants and terpenes, linalool pretreatment showed lower enhancement ($p<0.05$) of (+)-catechin permeation than did 1,8-cineole. This may have been due to the insufficient pretreatment duration of 1 h for linalool. As depicted in Fig. 2, (+)-catechin permeation by linalool was lower than that by 1,8-cineole during the first 10 h of application. The drug permeation by linalool increased over that by 1,8-cineole after 10 h. This suggests that different kinetics of different terpenes have variable actions on the skin.

According to the results of the drug release and pretreatment experiments, it was confirmed that terpenes mainly act on the skin itself to promote the skin permeation of catechins and theophylline. Both disruption of the skin structure and increases of the drug partitioning into the SC are possible mechanisms for terpene enhancers based on the lipid-protein-partitioning theory proposed by Barry.[28] Skin/vehicle partitioning is an index of the relative affinity of the drug for the vehicle and the skin. It plays a significant role in establishing the drug flux. It is noteworthy that although nerolidol only showed limited enhancement of drug permeation, it produced the maximum partition coefficient of drugs into the SC. The effective promoting activity of nerolidol for skin partitioning may be attributable to its amphiphilic and long alkyl chain structure that is suitable for alignment within the lipid lamellae of the SC and for changing the SC environment.[9,18,19] The partitioning mechanism may have contributed a predominant effect to nerolidol enhancement. The trend of the partition coefficients of the four terpenes differed from those of drug permeation enhancement. Other factors such as disruption of the SC lipid bilayers should be elucidated for these terpenes.

To ascertain the enhancing effect of terpenes in an *in vivo* status, *in vivo* bioengineering methods and pharmacokinetics were evaluated. Enhancement of drug permeation in the skin results from two effects: (1) physicochemical interactions

which alter the chemical structure or composition of lipids and/or proteins in the SC, and (2) the biological or physiopathological responses of the skin's defensive function.[27] Both effects can be determined macroscopically by *in vivo* bioengineering methods such as TEWL and colorimetry. TEWL is performed to assess SC disruption, and a good correlation between chemical damage to the skin barrier and an increase in TEWL has been demonstrated.[7,29] α-Terpineol demonstrated a significant increase in ΔTEWL. It may be concluded that α-terpineol produced the highest perturbation of the lipid bilayers in the SC. It has been shown that the complete removal of lipids from the SC leads to a 100-fold increase in water permeability.[30] The safety of α-terpineol can be accepted because of its limited increase in ΔTEWL. The no or negligible increase of ΔTEWL by linalool, 1,8-cineole, and nerolidol does not mean that there is no lipid-disrupting effect because TEWL is a macroscopic method.

Enhancers may trigger some biological responses, beyond the physicochemical interactions with lipids and/or proteins of the skin. As a result, the biological responses of the skin to enhancers may modify drug permeation.[27] The a*-coordinate of colorimetry (erythema) has been demonstrated to correlate well with inflammatory interactions of the skin.[29] Colorimetric determinations mainly reflect erythema and vasodilation in the subepidermal vascular plexus and papillary loops.[31] This greatly differs from TEWL, which mainly reflects the physicochemical properties of the SC layers. Both lipid disruption in the SC and biological responses in viable skin may have contributed to the greatest enhancement of drug permeation by α-terpineol.

It is notable for the *in vivo* microdialysis that the permeation of (−)-epicatechin into the subcutaneous region was faster than that of (+)-catechin in the presence of α-terpineol ($p<0.05$). The subcutaneous (−)-epicatechin concentration reached a steady state after a 2.5-h application. This may indicate that topically applied isomers exhibited different *in vivo* pharmacokinetics when incorporating terpene enhancers.

CONCLUSIONS

The efficacies of terpenes on skin permeation of tea catechins and theophylline were systemically evaluated using a series of *in vitro* and *in vivo* methods. It was found that terpenes with particular structures produced particular enhancing responses. Oxygen-containing terpenes generally exhibited higher enhancing activity for drug permeation, except the terpenes with bicyclic structures which showed poor enhancement. Based on the experimental results, the enhancement of drug permeation by terpenes is due to the increased partitioning into the SC and perturbation of the lipid bilayers. α-Terpineol showed the highest enhancing activity among all terpenes tested. The increased TEWL and a* values of the skin confirm this greatest enhancement of drug permeation. These results are linked to the molecular mechanisms cited by previous investigations that skin disruption is attributed to the preferential hydrogen bonding of oxygen-containing terpenes with ceramide head groups thereby breaking the lateral/transverse hydrogen bond network of the lipid bilayers.[16,32] *In vivo* microdialysis indicated the different pharmacokinetic behaviors of topically applied (+)-catechin and (−)-epicatechin after treatment with α-terpineol. α-Terpineol did not increase the *in vivo* subcutaneous EGCG. However, the *in vivo* skin deposition of EGCG was greatly increased by α-terpineol. The effective terpenes for the percutaneous absorption of various drugs are not the same, and drug permeation through the skin may be closely related to the physicochemical characteristics of the drugs, the terpenes, and the vehicle in which they are formulated.

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