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Research paper

Human skin penetration of the major components of Australian tea tree oil applied in its pure form and as a 20% solution in vitro

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Abstract

The safety of topical application of Australian tea tree Oil (TTO) is confounded by a lack of transdermal penetration data, which adequately informs opinions and recommendations. In this study we applied TTO in its pure form and as a 20% solution in ethanol in vitro to human epidermal membranes from three different donors, mounted in horizontal Franz-type diffusion cells, using normal 'in use' dosing conditions (10 mg/cm^2). In addition, we examined the effect of partially occluding the application site on the penetration of TTO components. Our data showed that only a small quantity of TTO components, 1.1–1.9% and 2–4% of the applied amount following application of a 20% TTO solution and pure TTO, respectively, penetrated into or through human epidermis. The largest TTO component penetrating the skin was terpinen-4-ol. Following partial occlusion of the application site, the penetration of terpinen-4-ol increased to approximately 7% of the applied TTO. Measurement of the rate of evaporation of tea tree oil from filter paper (7.4 mg/cm^2) showed that 98% of the oil evaporated in 4 hours. Overall, it is apparent that the penetration of TTO components through human skin is limited.

Keywords: Transdermal delivery; Essential oil; Risk assessment; Tea tree oil; Epidermal permeability

1. Introduction

The assessment of risk associated with the topical application of essential oils and natural products relies upon the availability of data regarding the penetration and disposition of individual components of these often complex mixtures both into and through human skin. In vitro permeation studies using excised human skin ARE one of the most common means by which transdermal absorption kinetics are able to be readily assessed for both quantification of drug delivery in pharmacological studies and the penetration of other chemical agents which may be associated with adverse effects for the assessment of the degree of risk following accidental or intentional application to the skin.

One essential oil of particular interest over recent times has been tea tree oil (TTO), derived from leaves of the Australian tea tree, *Melaleuca alternifolia*, by a steam distillation process [1]. TTO is popular as a topically applied product due to its reputed medicinal properties, in particular its well-known antimicrobial activity [2], and has been used topically to treat conditions such as acne, cold sores, dandruff, onychomycosis, oral candidiasis and tinea pedis [3]. Recently, due to variability in the chemical content of distilled oil products and as a means to regulate the quality of TTO, International Standard ISO 4730:2004 was introduced to set minimum and maximum concentrations for each of 15 main components of TTO to standardize products sourced from different regions or producers.

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Despite its traditional use by Australian Aborigines and centuries of application for the treatment of skin ailments, there is still ongoing debate surrounding the risk associated with absorption of chemical components following the topical application of TTO-containing products. The recent European Scientific Committee on Consumer Products (SCCP) opinion on TTO (December 2004) [4] stated that there are relevant gaps in regard to percutaneous absorption, together with other toxicological assessments, which together mean that the safe use of TTO as a cosmetic ingredient could not be assessed. The current lack of experimental evidence, combined with the lipophilic nature of many of the components of TTO, has led to the assumption that TTO is readily absorbed through human skin.

Skin reactions following application of TTO have an estimated prevalence of 0.5–4.8%, depending on the study [5,6], decreasing significantly for TTO formulations containing less than 25% oil [7]. The incidence of skin reactions to TTO is also reported to increase following photo-oxidation of TTO which leads to increased content of p-cymene and decreased concentration of α-terpinene, γ-terpinene and terpinolene [8].

Human skin penetration of the major component of TTO, terpinen-4-ol, has been demonstrated following the application of infinite doses of pure TTO and 5% TTO in semisolid, oil in water emulsion, white petrolatum and ambiphilic cream formulation bases [9]. However the application conditions used in these studies did not replicate that likely to be seen in a normal 'in use' application where application rates ranging from 2 to 10 mg/cm^2 can be reasonably expected. In addition, the receptor phase solution used in order to maintain the solubility of terpinen-4-ol in the study was 50% aqueous ethanol, a solvent system which is not considered physiological and has the potential to diffuse back into the membrane and affect the permeability characteristics of the stratum corneum barrier.

Our present study sought to address the lack of percutaneous penetration and disposition data of the components of TTO so that assumptions relating its absorption following normal consumer 'in use' application could be substantiated or proved otherwise. Determination of this penetration is therefore essential so that an accurate assessment of the potential risk from topical TTO formulations can be correctly evaluated and informed recommendations made by the relevant responsible bodies. We applied TTO as the neat oil and as a 20% solution in ethanol, chosen to represent a formulation likely to disrupt the barrier properties of the skin and potentially facilitate the transdermal delivery of TTO, to the skin of three different female donors under 'in use' application conditions and followed the penetration over a 24-h period. In addition to skin penetration, we quantified the retention of TTO components in the epidermis and examined the effect of partial occlusion on penetration rates following application of the pure oil.

2. Materials and methods

2.1. Tea tree oil (TTO) formulations

TTO used in the study, supplied by the Australian Tea Tree Industry Association, was analysed according to International Standard ISO 4730:2004 and shown to contain acceptable levels of each of the 15 defined TTO constituent components (Table 1). Table 1 also shows the physicochemical properties of TTO components, including water solubility at pH 7 and vapour pressures of individual components. In the epidermal penetration studies TTO was used as the pure oil (100%) and as a 20% solution in ethanol.

2.2. Human epidermal membrane penetration

Penetration and skin retention of TTO components were studied in vitro using female human epidermal membranes obtained with full consent following surgical abdominoplasty procedures and approved by the Human Research Ethics Committee of the Princess Alexandra Hospital. Epidermal membranes were prepared from full thickness tissue using the heat-separation technique, which involves immersion in water at 60 °C for 1 min and peeling of the epidermis from the underlying dermis. Membranes were then stored at −20 °C until use. Membranes were mounted stratum corneum side up on filter paper in Franz-type horizontal diffusion cells, exposed skin surface area approx. 1.3 cm^2 and receptor fluid volume approx. 3.5 mL. Membranes were left overnight with receptor phase chambers filled with PBS pH7.4 to allow hydration prior to the study. Phosphate-buffered saline at pH 7.4 containing 4% bovine serum albumin, to increase the solubility of lipophilic components, was used as the receptor phase medium throughout the study. Receptor phase was continuously stirred in the diffusion cells with magnetic fleas and maintained in a water bath at 35 °C in order to give a surface membrane surface temperature of approx. 32 °C. At $t = 0$ membranes were dosed with a finite dose, 10 μL/cm^2, of either the pure oil or 20% TTO solution using a positive displacement pipette and this was spread across the surface of the skin using a round-ended rod. Three different skin donors, $n = 6$ per donor, were used for application of the pure TTO and 20% formulation without occlusion and a further skin donor used to study application of the pure TTO together with occlusion of the top of the donor chamber (approx. 1 cm high) with a glass coverslip to reduce evaporation and loss of TTO components. Samples of receptor phase, 200 μL, were removed at 2, 4, 8, 12 and 24 h for non-occluded application and at 12 and 24 h for partially occluded donor chamber application. Prior to analysis of TTO components, the protein in the 200 μL receptor phase samples was removed by precipitation with 200 μL acetonitrile containing 0.01% thymol as internal standard, with samples vortexed, centrifuged and aliquots of supernatant removed for quantification by GC–MS as described below.

Table 1
Characteristics of the individual components of TTO

Component	Test oil[a] (%)	Test oil[b] (%)	Min–max levels (%)	MW[c]	Log P[c]	Water sol[c,d] (μg/mL)	V_p[c] (Torr)
Terpinen-4-ol	37.5	42.7	30–48	154	2.99	2200	0.05
γ-Terpinene	19.4	21.0	10–28	136	4.36	5.2	1.08
α-Terpinene	9.0	9.0	5–13	136	5.52	3.5	1.64
1,8-Cineole	4.5	2.5	Trace-15	154	2.82	560	1.65
Terpinolene	3.5	3.2	1.5-5	136	4.67	2.6	1.13
α-Terpineol	3.0	2.8	1.5–8	154	2.79	2500	0.03
α-Pinene	2.4	2.5	1–6	136	4.37	7.9	3.49
p-Cymene	2.4	3.9	0.5–8	134	4.02	13	1.65
Aromadendrene	1.4	1.3	Trace-3	204	6.41	0.014	0.02
δ-Cadinene	1.3	0.8	Trace-3	204	6.54	0.053	<0.01
Limonene	1.2	1.0	0.5–1.5	136	4.45	4.2	1.54
Ledene	1.0	0.6	Trace-3	204	6.45	0.013	0.02
Sabinene	0.7	0.1	Trace-3.5	136	4.13	13	2.63
Globulol	0.4	0.2	Trace-1	222	4.81	56	<0.01
Viridiflorol	0.3	0.4	Trace-1	222	4.81	56	<0.01

Percentage concentrations of 15 standard components of TTO in the test oils compared to recommended maximum and minimum levels defined in standard ISO 4730:2004, together with standard physicochemical properties of molecular weight (MW), Log octanol/water partition coefficient (Log P), solubility in water and vapour pressure (V_p).

[a] Dermal penetration study.
[b] Evaporation study.
[c] *Source*: SciFinder Scholar.
[d] Water solubility at pH 7.

2.3. Membrane integrity

Epidermal membrane integrity was determined prior to the study using electrical resistance. Phosphate-buffered saline was placed on both sides of the membrane mounted in the Franz diffusion cell and electrical resistance measured using silver/silver chloride electrodes (one placed in each chamber) using a multimeter. Membranes showing a resistance of less than 25 kΩ were removed from the study and replaced prior to TTO application. PBS was removed from the donor chamber with a plastic transfer pipette and the surface of the skin gently blotted dry with tissue paper before application of the TTO. Receptor chambers were also emptied of PBS and refilled with PBS containing 4% BSA at this time.

2.4. Membrane retention and surface recovery

At the end of each study (24 h), the surface of the skin was cleaned with methanol soaked swabs and stripped once with sticky tape to remove any non-penetrated material. Swabs and tapes were collected and extracted into 2 mL acetonitrile containing 0.1% thymol as an internal standard. Epidermal membranes were removed from the diffusion cells and extracted into 200 μL acetonitrile, containing 0.1% thymol as an internal standard, for quantification of TTO components.

2.5. GC–MS analysis of tea tree oil components

Component identifications were performed on a Hewlett Packard 6890 series GC/MS fitted with an HP5-MS 29.5 m × 0.25 mm, 0.25 μm film thickness, FSOT column with helium (36 cm/s) as carrier gas, injection port (split 1:50) at 250 °C, mass selective detector (HP 5973) at 250 °C (source) and 150 °C (quad) with transfer line 280 °C and ion source filament voltage of 70 eV. Retention indices were measured with respect to *n*-alkane standards on the HP5-MS column. Component identifications were made on the basis of mass spectral fragmentation, retention time comparison with authentic constituents and mass spectral and retention matching with commercial (NIST, Wiley and Adams) and in-house libraries. Reference standards: 1,8-cineole (99.7%) Ajax, Unilab, terpinen-4-ol (96%) Aldrich, α-terpineol (98%) Aldrich. Tea tree oil was analysed according to: ISO 4730:2004: Oil of Melaleuca, terpinen-4-ol type (Tea tree oil). The limit of quantification for tea tree oil components in the GC assay was 1 μg/mL.

2.6. Evaporation rates

In an attempt to understand the potential volatility of TTO, fresh tea tree oil of known chemical composition (Table 1) was added dropwise (either one [1.4 mg/cm^2] or five [7.4 mg/cm^2] drops) to pre-weighed Whatman No. 1 filter papers (4.25 cm diameter) contained in glass Petri dishes (4.5 cm i.d.). The dish, paper and oil were stored in an oven at 30 °C and re-weighed after 0, 1/2, 3/4, 1, 2, 4, 8, 12 and 24 h or until evaporation ceased. Six replicates and a blank were weighed each time.

2.7. Data analysis

All statistical analysis was performed using Analysis of Variance (ANOVA) and Tukey's post-hoc testing with

Minitab software v13.32, significance was taken at $p < 0.05$.

3. Results

3.1. Solubility of TTO components in receptor phase

The solubility of the major TTO components in 4% bovine serum albumin receptor phase was determined to be 4.080 mg/mL for terpinen-4-ol (Log *P* 2.99), 6.066 mg/mL for α-terpineol (Log *P* 2.79) and 3.165 mg/mL for 1,8 cineole (Log *P* 2.82). Receptor phase concentrations determined in the study were all significantly below 10% of this solubility.

4. Epidermal penetration of TTO components into receptor phase

4.1. Non-occluded application using three different skin donors

Terpinen-4-ol and α-terpineol were found to penetrate epidermal membranes and could be quantified in the receptor phase in samples from each of the three different skin donors (Fig. 1 and Table 2). No other TTO components could be readily detected in the receptor phase samples at any time point following application of either the pure oil or 20% TTO formulation. The three donors showed varying penetration of terpinen-4-ol (140-310 μg/cm^{2}or 3.6–8.0% of the applied amount) and α-terpineol (14-33 μg/cm^2 or 3.6–8.4% of the applied amount) over the 24-h period following application of the pure oil (Fig. 1 and Table 2). Following application of the 20% TTO formulation, amounts of terpinen-4-ol in the receptor phase after 24 h were found to represent 18-33 μg/cm^2 or 1.1–1.9% of the applied amount of TTO however, no α-terpineol could be detected in these receptor phase samples (Table 2).

Statistical differences in the penetration rate of terpinen-4-ol and α-terpineol were found for the three donor skins at all time points following application of the pure oil, with one skin much more permeable than the other two ($p < 0.05$). However, following application of the 20% TTO formulation, the more permeable skin only showed significant differences in the penetration of terpinen-4-ol to the other two donors in the 4–12 h time period ($p < 0.05$).

4.2. Partially occluded application using one skin donor

Following partial occlusion of the donor chamber and application of the pure oil formulation, terpinen-4-ol, α-terpineol and 1,8-cineole could be detected in the receptor phase, the levels at 24 h can be seen in Table 2. At 12 h the average absorption of terpinen-4-ol and α-terpineol following application of pure TTO was 289.72 ± 117.76 and 22.77 ± 9.76, respectively, or approximately 54% and 51% of the amount absorbed at 24 h. All other TTO components were still below the detection limit of the GC-MS assay in these samples. Compared to non-occluded application conditions, at the 24 h time point the mean amount of terpinen-4-ol detected in the receptor phase increased approximately 2.5-fold and amounts of α-terpineol in the receptor phase increased approximately 2-fold (Table 2). However, individual diffusion cells did show increases of up to twice this magnitude.

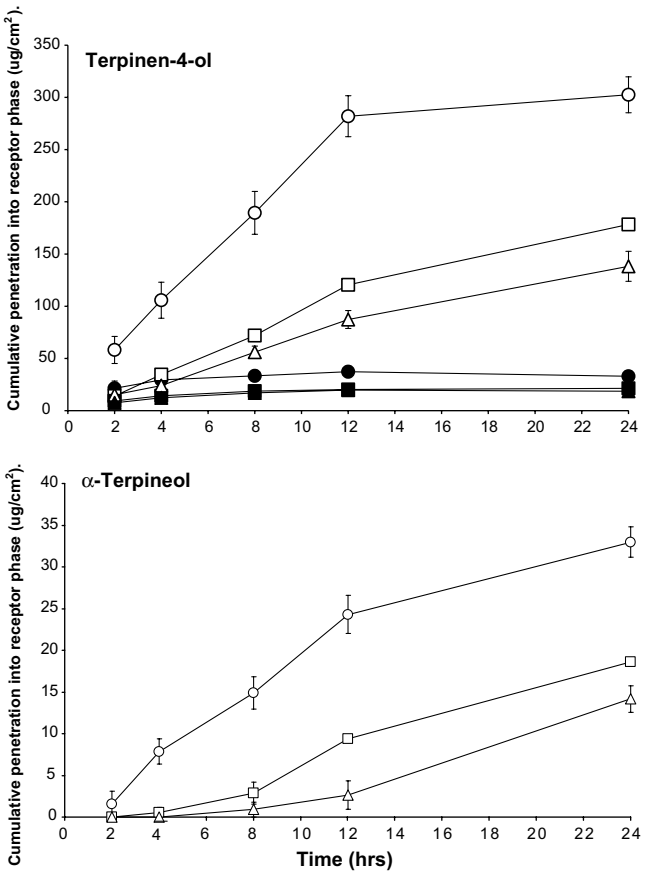

Fig. 1. Cumulative penetration of terpinen-4-ol and α-terpineol through human epidermal membranes in vitro following application of finite doses of pure TTO (open symbols) and a 20% TTO in ethanol formulation (closed symbols) in three different skin donors (Skin #1, ○; Skin #2, □; Skin #3, Δ), means ± SD, $n = 6$.

5. Epidermal retention of tea tree oil components

5.1. Non-occluded application using three different skin donors

The amount of terpinen-4-ol estimated to be remaining in the epidermal membranes from each of the skin samples at 24 h is shown in Table 2. Retention of terpinen-4-ol following application of pure TTO to the three skin donors varied between 4.1 and 6.6 μg/cm^2 (0.1–0.2% of the applied amount) and following application of the 20% formulation varied between 0.25 and 0.38 μg/cm^2 (<0.02% of the applied amount). There was a slight variability in the retention of TTO between the three different skin donors. There

Table 2
Penetration and epidermal retention (μg/cm^2, Mean ± SD) of terpinen-4-ol, α-terpineol and total TTO components following application of finite doses of pure TTO and a 20% TTO in ethanol formulation to human epidermis for 24 h under 'in use' and partially occluded conditions

Component	Open 'in use' application			Combined data	Partially occluded
	Skin #1	Skin #2	Skin #3		
Pure TTO					
Receptor phase (μg/cm^2)					
Terpinen-4-ol	302.5 ± 42.2	178.4 ± 11.9	138.23 ± 35.2	206.4 ± 78.2	531.4 ± 190.5
α-Terpineol	33.0 ± 4.4	18.6 ± 1.2	14.15 ± 3.9	21.9 ± 8.9	44.7 ± 16.4
1,8-Cineole	–	–	–	–	19.8 ± 8.5
Total	335.3 (3.82%)	197.0 (2.24%)	152.4 (1.73%)	228.2 (2.59%)	595.9 (6.77%)
Epidermis (μg/cm^2)					
Terpinen-4-ol	4.3	6.6	4.1	6.54 ± 2.04	4.3
α-Terpineol + others	23.3	25.7	16.3	28.29 ± 13.69	23.3
Total	27.6 (0.31%)	32.3 (0.37%)	20.4 (0.23%)	26.7 (0.30%)	27.6 (0.31%)
Combined receptor phase and epidermis (μg/cm^2)					
Potential total absorption	363.1 (4.13%)	229.3 (2.61%)	156.5 (1.96%)	249.6 (2.83%)	623.5 (7.08%)
20% TTO					
Receptor phase (μg/cm^2)					
Terpinen-4-ol	32.9 ± 12.5	21.4 ± 8.7	18.6 ± 7.9	24.3 ± 11.3	n.d.
α-Terpineol	0	0	0	0	n.d.
Epidermis					
Terpinen-4-ol	0.25 ± 0.18	0.38 ± 0.20	0.32 ± 0.23	0.32 ± 0.20	n.d.
α-Terpineol + others	1.15 ± 0.78	1.18 ± 0.69	0.50 ± 0.30	0.95 ± 0.67	n.d.
Total	1.4 (0.08%)	1.56 (0.09%)	0.82 (0.05%)	1.26 (0.07%)	n.d.
Combined receptor phase and epidermis (μg/cm^2)					
Potential total absorption	34.3 (1.94%)	23.0 (1.30%)	19.4 (1.10%)	25.6 (1.45%)	n.d.

–, below limit of assay detection; n.d.: not determined.

was a higher retention of terpinen-4-ol in skin donor #2 when TTO was applied as the pure oil, though there were no significant differences when applied as the 20% TTO formulation.

Peaks relating to other oil components, α-terpineol and a mixture of other sesqui-terpinenes, were also present in the extracted skin samples, but none of these components were found penetrating through the epidermal membrane into the receptor phase. The retention of a mixture of TTO components consisting of α-terpineol and other sesqui-terpinenes accounted for approximately 20 μg/cm^2 and 1 μg/cm^2 of material following application of the pure TTO and 20% formulation, respectively. The total retention of identifiable TTO components within the epidermis was between 0.23% and 0.37% of the applied dose following normal 'in use' application (Table 2).

5.2. Partially occluded application

Following partial occlusion of the donor chamber compartment of the Franz cell, there was a significant increase in the amount of terpinen-4-ol recovered from the epidermis following application of the pure oil (Table 3). In addition, all of the other 15 TTO components could now be identified in the membrane extraction samples. The concentration of each of the 15 identifiable tea tree oil components estimated to be remaining in the epidermal membranes, μg/cm^2, following partial occlusion is shown in detail in Table 3, however the levels of the majority of the TTO components were so low that they were below the official limit of quantification of the assay and have been estimated. Total epidermal retention of all 15 measured TTO components was estimated to be equivalent to 23.6 ± 7.0 μg/cm^2 or just 0.27% of the total dose applied to the membranes at time zero.

The increase in recovery of terpinen-4-ol from within the epidermis in the donor chamber partially occluded experimental system was approximately three times that seen following non-occluded application.

5.3. TTO total transdermal uptake

The total amount of identifiable TTO components taken up into the epidermis, estimated from that present in the receptor phase combined with that retained within the epidermis and potentially available for further absorption, is shown in Table 2. As known TTO components account for 88% of the liquid applied, total dose in a 10-μl/cm^2 total oil application can be considered to be loading dose of 8800 μg/cm^2. Our data show that, under normal 'in use' conditions, approximately 2–4% of identifiable applied TTO components can be found either permeating the skin into receptor fluid (1.7–3.8%) or retained within the epidermis (0.23–0.37%) after 24 h application under normal 'in use' conditions. Under partially occluded conditions, the transdermal uptake of identifiable TTO components

Table 3
Human epidermal retention of TTO components 24 h after application of pure TTO and a 20% TTO in ethanol formulation to membranes in vitro (means ± SE, $n = 6$), open application values for three skin donors combined

Component	Retention in epidermis at 24 h (μg/cm^2)
Pure TTO	
Open application	
Terpinen-4-ol	5.0 ± 0.4
Partially occluded application	
Terpinen-4-ol	14.6 ± 2.4
γ-Terpinene	1.9 ± 0.3
α-Terpinene	0.6 ± 0.1
1,8-Cineole	0.4 ± 0.0
Terpinolene	0.4 ± 0.1
α-Terpineol	1.5 ± 0.3
α-Pinene	0.1 ± 0.0
p-Cymene	0.3 ± 0.1
Aromadendrene	0.9 ± 0.1
δ-Cadinene	0.5 ± 0.3
Limonene	0.9 ± 0.0
Ledene	0.8 ± 0.1
Sabinene	0.1 ± 0.0
Globulol	0.3 ± 0.0
Viridiflorol	0.3 ± 0.0
20% TTO formulation	
Open application	
Terpinen-4-ol	0.3 ± 0.1

increased to 7% of the applied dose, 6.8% in the receptor phase and 0.3% remaining within the epidermis.

5.4. Mass balance

Recovery of TTO components within the experimental system (donor chamber, epidermis and receptor solution) was extremely low following non-occluded application onto human epidermis. The estimated total recoveries of terpinen-4-ol were 4901 and 1081.6 μg applied in the pure TTO and 20% formulations, respectively. The percentage of applied amount of terpinen-4-ol recovered in the system varied between 5.5% and 10% when applied in the neat oil, 2.4% and 4.4% following application as the 20% oil formulation. Recovery of non-terpinen-4-ol components was lower and ranged from 4.6% to 6.2% when applied in the neat oil, 0.4% to 2.3% following application as the 20% oil formulation. Under partially occluded conditions this recovery increased for each of the 15 identifiable TTO components, although levels were still low and ranged from around 25–30% for terpinen-4-ol, α-terpineol, ledene, δ-cadinine, globulol and viridiforol to less than 5% for α-terpinene, γ-terpinene, sabinene, limonene and α-pinene.

5.5. Oil evaporation rate

Two applications of tea tree oil of known chemical composition (Table 1) at different concentrations gave some quantitative measure of how rapidly tea tree oil evaporates from surface material. Using small filter papers at 30 °C, oil

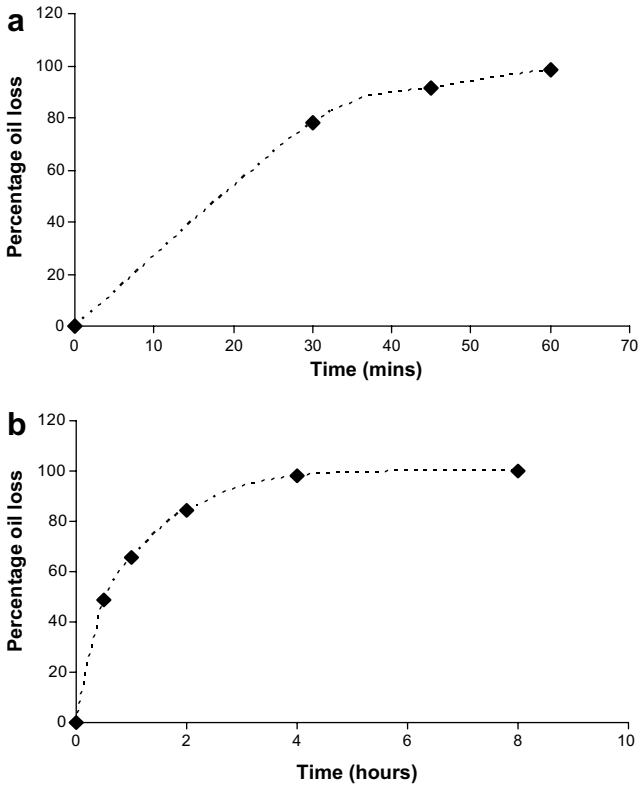

Fig. 2. Evaporation (%) of tea tree oil at (a) 1.4 mg/cm^2 and (b) 7.4 mg/cm^2 over 1 and 8 h, respectively.

applied at 1.43 mg/cm^2 evaporated within 1 h (Fig. 2a) whereas increasing to 7.37 mg/cm^2, approximating the dermal penetration application (8.9 mg/cm^2 = 10.0 μl/cm^2), retained oil for only 4 h (Fig. 2b).

6. Discussion

6.1. Epidermal penetration of TTO components

Our studies demonstrated that consistent with previous findings terpinen-4-ol was able to penetrate human epidermis and be recovered in the receptor phase solution. The plateauing effect seen in the penetration profiles was consistent with finite dosing and depletion of TTO components from the application site resulting in lowering of the concentration gradient across the membrane and slowing of the penetration rate (Fig. 1). One of the prerequisites in quantifying in vitro percutaneous penetration of topically applied solutes is that the receptor phase used acts as a perfect sink and does not provide any rate limitation to the diffusion of solutes through the skin [10]. The choice of phosphate-buffered saline containing 4% bovine serum albumin was selected in the current study as this receptor solution has been shown to be a suitable sink for a wide range of polar and lipophilic solutes [11]. Our study also confirmed that the solubility of the major components of TTO found penetrating the human epidermal membranes was far less than their solubility in the receptor solution,

confirming that receptor solubility was not a rate limiting step in these studies.

The infinite TTO oil dosing study of Reichling et al. [9], with the use of aqueous ethanol as a receptor solution, reported concentrations of approximately 4 μl/cm^2 of terpinen-4-ol penetrating human epidermis in vitro over a 24-h period, equating to, assuming a specific gravity of 0.933, a concentration of 4.28 mg/cm^2 absorbed in 24 h. Our current study found penetration rates under 'in use' non-occluded conditions at least 10-fold lower than this in each of the three different skin donors we examined with concentrations of 140–300 μg/cm^2, with means ± SE of the three found to be 206.4 ± 73.9 μg/cm^2. The most likely explanation for the 10-fold difference is the use of infinite and finite doses in the two studies. We have found a similar difference for the human skin penetration of oxybenzone (Log *P* 2.63) which has a similar lipophilicity to terpinen-4-ol (Log *P* 2.99) [12]. This difference arises mainly from the much greater exposure associated with infinite dose application [9] relative to 'in use' conditions (this study). A further source for the greater exposure is a reduced evaporation in the infinite donor case relative to the 'in use' study here, where the recovery was 5.5% to 10%. The higher penetration of terpinen-4-ol is also likely to be due to the inclusion of 50% ethanol in the receptor solution [9] that may have diffused into the epidermal membrane and affected its barrier function [10].

The epidermal penetration of terpinen-4-ol dropped to around 24 μg/cm^2 following application of the 20% formulation, which is less than the approx. 40 μg/cm^2 predicted from a simple 5-fold drop in its concentration and its penetration after application of pure TTO. This finding is consistent with the ethanol causing a greater than expected reduction in the thermodynamic activity of terpinen-4-ol in the 20% solution relative to the pure TTO, as well as a greater potential change in penetration enhancement in the two vehicles. There may be components present in the pure TTO in sufficient concentrations to facilitate its penetration through the skin that are not capable of having the same effect in their diluted form. The ability of terpenes, present in TTO, to enhance the skin penetration of topically applied solutes has been known for many years. Williams and Barry [13] demonstrated that both α-terpineol and terpinen-4-ol enhanced the human skin penetration of 5-fluorouracil by a factor of 10-fold and that 1,8-cineole could enhance its penetration almost 100-fold following application of the terpene to the skin 12 h prior to application of the drug. The mechanism of action of the terpenes on penetration was found to be through their interaction with the lipids of the stratum corneum and subsequent enhancement of diffusivity of 5-fluorouracil within the membrane and not as a result of increased partitioning into the stratum corneum.

Consistent with the presence of higher concentrations of terpenes, we were able to detect the presence of α-terpineol in the receptor phase following application of the pure oil, although not following application of the 20% TTO formulation. This finding is also consistent with the faster penetration of components from the pure TTO compared to the 20% formulation, as concentrations of α-terpineol 5-fold lower than those seen following application of the pure oil would have been easily detectable in the receptor phase following application of the 20% diluted TTO formulation.

The 2-fold increase in the epidermal penetration of both terpinen-4-ol and α-terpineol following partial occlusion of the application site for the pure oil is consistent with the enhancement of terpene penetration into the epidermis following a reduction in TTO evaporation and subsequent loss from the donor chamber. This build up of vapours within the donor chamber would cause a longer maintenance of higher concentrations of TTO on the skin compared to non-occluded 'in use' application conditions. The detection of 1,8-cineole in the receptor phase following partial occlusion also supports the assumption that the maintenance of higher concentrations of terpenes on the skin results in higher diffusivity within the stratum corneum lipids and facilitated penetration of solutes. In this case 1,8-cineole was one of the most active terpenes identified by Williams and Barry [13] in disrupting the barrier properties of the stratum corneum, and facilitation of its own absorption is not an entirely unexpected finding. Despite the apparent decrease in stratum corneum barrier properties following application of pure TTO under partial occlusion, none of the remaining 12 standard TTO components could be detected penetrating the epidermis and entering the receptor phase. This finding suggests that the majority of TTO components have minimal ability to penetrate through human epidermis. It is to be noted that the major TTO components penetrating the skin were not the most lipophilic, but those with a Log *P* of about 2.5. Roberts and Walters [14] have previously suggested that a Log *P* in this region is associated with optimal skin permeability behaviour, before and after which skin flux tends to decline. It is therefore evident that these moderately polar components, and not the more lipophilic, could penetrate the skin slightly more readily.

7. Epidermal retention of TTO components

A greater array of TTO components could be found retained within the epidermis than were detected penetrating all the way through it. Under open, 'in use' application conditions terpinen-4-ol could be seen in the epidermis following application of both the pure TTO and 20% formulation, though again the retention following application of the pure TTO was higher than would have been expected from the 5-fold change in concentration of the 20% formulation, 5 and 0.3 μg/cm^2, respectively. The detection of a mixture of components consisting of α-terpineol and sesquiterpenes within the epidermis following application of TTO in both the pure form and as the 20% formulations shows that these components are also capable of partitioning into the stratum corneum, but not able to penetrate all the way through into the receptor solution, representative

of the systemic circulation. One drawback of the current study is that we were not able to determine with which region of the epidermis the retained TTO components were associated. It is most likely that most components coming into contact with the skin, particularly the more lipophilic species, would be able to partition into the upper layers of the stratum corneum but not diffuse any further into the membrane.

Epidermal membranes were chosen over full-thickness human skin for these studies as in the in-vivo situation the dermis is constantly perfused with blood via the dermal vascular networks (upper and lower plexus and associated capillary beds). This perfusion process creates an effective sink for the clearance of solutes penetrating the epidermal membrane. In the in-vitro situation, the dermis is no longer perfused and it is possible that substances penetrating the epidermal membrane can artificially accumulate in the dermal tissue due to lack of effective clearance [15]. This accumulation would then change the effective concentration gradient across the epidermis and have the potential to significantly change observed absorption kinetics. As we cannot determine effectively which components of TTO may be susceptible to the dermal accumulation artifact, epidermal membranes that eliminate the possibility of this effect occurring were purposely selected for the study.

Following partial occlusion of the donor chamber during application of the pure TTO we observed a significant increase in the epidermal retention of TTO components. Again, we cannot specify into which regions of the epidermis these components have distributed, but their lack of detection in the receptor solution suggests that they are most likely associated with the upper regions of the stratum corneum directly below the site of the applied formulation. From a practical perspective, this finding suggests that partial occlusion of the skin produced by semi-solid formulations rich in components such as petrolatum would also have the ability to increase epidermal hydration and therefore potentially facilitate the penetration of TTO components.

8. TTO total transdermal uptake and potential systemic exposure

The total transdermal uptake of TTO components into the skin following topical application, taken as that in the receptor fluid combined with that retained within the epidermis, was almost doubled following partial occlusion (Table 2). It was interesting to note that although the epidermal retention of TTO components remained relatively constant under both conditions, 0.30% for non-occluded and 0.27% for partially occluded conditions, the percentage of terpinen-4-ol making up this amount was relatively higher under partially occluded conditions.

The total recovery of TTO components within the entire experimental system (mass balance) at the end of the 24-h application period was found to be low. In previous publications the total recovery of TTO components, or even terpinen-4-ol alone, was not mentioned [9] making our findings hard to compare to the work of other groups. However, we were not entirely surprised by this finding, due to the volatile nature of many of the TTO components and the length of application to the epidermal membrane in the diffusion apparatus. Under donor chamber partially occluded conditions we saw an increase in recovery of TTO components which is consistent with a reduction in their ability to escape from the system due to vapourisation. As we did not design the study with a fully enclosed vapour trap system, the reason for the disappearance of the TTO components remains unproven. In a recent report by Green and Brain [16], estimation of the extent of evaporative loss of volatile components during skin permeation highlighted that the use of such vapour traps in in vitro systems can lead to altered penetration kinetics due to their occlusive effect and that within a 24-h study period recoveries of only 10% are not unexpected for volatile substances. This finding is consistent with the low recovery of TTO components in the current study. However, the evaporation rate of TTO measured from filter paper in this investigation confirmed that approx. 98% of the topically applied oil evaporates within a matter of hours (Fig. 2).

Our data also show that the penetration of TTO components through the epidermal membrane occurred over a much longer time period than the open evaporation studies from paper discs imply that TTO components would be remaining on the surface of the skin. We anticipate that the difference between the rate of loss of TTO components from the open paper study and the diffusion cell apparatus would have been due to a combination of the presence of the walls of the diffusion chamber and the increased humidity within the diffusion chamber due to the natural transepidermal water loss through the epidermal membrane from the receptor chamber. In addition, the epidermal membrane would be at a much higher hydration state than the paper used in our open evaporation studies which would be expected to reduce the rate of vapourisation of the TTO components. These findings support the suggestion that TTO appears to be of little potential risk of transdermal absorption following topical application as most of the oil applied is lost into the atmosphere, however exposure due to inhalation and absorption through respiratory epithelia needs to be addressed as a separate issue.

In conclusion, the current study has shown that following application of pure TTO under normal ‘in use’ conditions, a small quantity of TTO components, 1.1–1.9% and 2–4% of the applied amount following application of a 20% TTO solution and pure TTO respectively, were found to penetrate into or through human epidermis. The largest TTO component penetrating the skin was terpinen-4-ol. Following partial occlusion of the application site, this penetration increased to approximately 7% of the applied TTO. These data should now be used to expand risk assessment profiles of the use of TTO and reduce spec-

ulation that all lipophilic components of TTO are assumed to freely penetrate the skin.

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