Topical formulation and dermal delivery of active phenolic compounds in the Thai medicinal plant - *Clerodendrum petasites* S. Moore

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Abstract

Purpose: To develop topical formulations of Clerodendrum petasites S. Moore (CP), and to optimise the skin permeability and topical bioavailability of the active phenolic compounds therein.

Methods: The skin uptake and delivery of active compounds from two formulations, an oil-in-water cream and a lotion (both containing 10% w/w CP extracts), were examined (a) in vitro using pig skin, and (b) in vivo in human volunteers. Stratum corneum (SC) was collected by tape stripping and the compounds were detected and quantified by high performance liquid chromatography coupled with either mass spectrometry (HPLC-MS) or ultraviolet and photodiode array (HPLC-UV-PDA) detection.

Results: From the in vitro results, vanillic acid, verbascoside, nepetin and hispidulin, were chosen as potential phenolic actives for topical delivery optimisation from the formulations. In vivo, vanillic acid, nepetin and hispidulin were appreciably taken up into the SC within 6 hours, while verbascoside did not penetrate beyond the most superficial layers. No significant differences in delivery were observed between the two formulations, both of which were well tolerated.

Conclusions: The detected topical absorption of hispidulin, nepetin, and vanillic acid, from the cream and lotion vehicles investigated, suggest that these compounds are potentially active compounds in Thai traditional medicine for the treatment of a wide range of skin diseases.

Keywords: Clerodendrum petasites S. Moore, hispidulin, skin permeation, topical bioavailability, mass spectrometry.


Abbreviations: CP, Clerodendrum petasites S. Moore; HPLC, high performance liquid chromatography; MS, mass spectrometry; PDA, photodiode array; SC, stratum corneum; TEWL, transepidermal water loss; $K_{SC,V}$, SC-vehicle partition coefficients; LOQ, limit of quantification.
Clerodendrum petasites S. Moore (Lamiaceae, English name: One Root Plant, Thai name: Thao-Yaai-Mom) is commonly found in the middle, north-eastern and southern parts of Thailand. It is extensively prepared as a tea, alcoholic extract, cigarette, or powder for oral administration to treat asthma (Hazekamp et al., 2001; Panthong et al., 2003; Panthong et al., 1986), inflammation (Panthong et al., 1986), fever, cough and vomiting (Panthong et al., 2003; Thai traditional medical textbook: Paet-Ta-Ya-Saat-Song-Kror 2007) (S. Tungjitaruen, pers. comm., 2011). For topical use, the traditional dosage form is a poultice to treat skin diseases, such as rash, abscess, urticaria, snakebites and insect bites (Panthong et al., 2003; Pongboonrot, 1965; Thai traditional medical textbook: Paet-Ta-Ya-Saat-Song-Kror 2007) (T. Tipcharoentham, pers. comm., 2011; S. Tungjitaruen, pers. comm., 2011). Alcohol, especially Thai rice whisky, is often used as a dispersing vehicle in many formulations before application. At present, only the plant itself and powders therefrom are found on the market with poor reliability and reproducibility with respect to the active ingredients. These forms are also inconvenient to use and transport. The nature of the active species remain poorly characterized (Hazekamp et al., 2001; Klaiklay, 2009; Singharachai et al., 2011; Thongchai et al., 2007) and there is no information concerning the topical bioavailability of active species from this plant.

A preliminary study, reported elsewhere (Thitilertdecha et al., 2014), identified eleven phenolic compounds, vanillic acid, 4-coumaric acid, ferulic acid, verbascoside, nepetin, luteolin, chrysin, naringenin, hesperetin, apigenin, and hispidulin, in an ethanolic (80%) extract of the plant, predicted their maximum skin fluxes ($J_{\text{max}}$) and measured skin absorption in vitro from a 50% w/w CP paste and a 50 mg·mL$^{-1}$ CP solution. In the present study, suitable dosage forms for topical delivery to the skin were developed and optimised, and the topical bioavailability of the active substances was determined in vivo in human volunteers.
Materials and methods

1 Plant materials

Dried samples of CP were obtained from the Ayurved Siriraj Manufacturing Unit of Herbal Medicines and Products, Center of Applied Thai Traditional Medicine (CATTM), Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. An extract of powdered plant was produced by maceration using 80% ethanol and subsequently lyophilized to dryness (Alpha 2-4 Isc., Martin Christ Company, Germany). Prior to use, the extracts were kept separately in light protective and airtight containers and stored in a desiccator at room temperature. Batch-to-batch consistency of the ethanolic extracts was confirmed by quantification of phenolic constituents as described previously (Thitilertdecha et al., 2014).

2 Chemicals and reagents

Vanillic acid (Fluka Analytical, China), verbascoside, nepetin (Extrasynthese, France), and hispidulin (Tocris Bioscience, UK), were of analytical grade.

Mobile phases for HPLC-MS and HPLC-PDA consisted of chromatography grade acetonitrile (Fisher Scientific, UK), deionized water (Millipore, MA, USA) and MS grade acetic acid (Fluka Analytical, Germany). Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl, Acros Organics, USA), tris (hydroxymethyl) aminomethane (Trizma® base, Sigma-Aldrich, USA), methanol, and ethanol (Sigma-Aldrich, USA) were of analytical grade. Acetone was laboratory grade.

Excipients of the topical formulations were propyl paraben (propyl 4-hydroxybenzoate), stearyl alcohol (1-octadecanol), Tween 60 (polyethylene glycol sorbitan monostearate), Span 60 (sorbitan monooytadecanoate), glycerol, mineral oil, and triethanolamine (TEA) (Sigma-Aldrich, USA), methyl paraben (methyl 4-hydroxybenzoate) and cetyl alcohol (Fluka Analytical, Germany), butylated hydroxytoluene (BHT) (SAFC, Germany), propylene glycol (Acros Organics, UK), glycerol
monostearate-self emulsifier (GMS-SE) and Carbopol ultrez 21 (acrylates/C10-30 alkyl acrylate crosspolymer (The Lubrizol Corporation, USA).

3 Skin for the in vitro permeation study

Fresh porcine abdominal skin was obtained from B&J Pigs Ltd, Somerset, UK. Excess hair was carefully trimmed using scissors. After cleaning with running cold water, the skin was dermatomed (Zimmer electric dermatome, Oklahoma, USA) to a nominal thickness of 750 µm. The dermatomed skin was sealed in a plastic bag and stored at -20°C until use.

4 Human subjects

Six healthy volunteers aged between 25 and 31 years (4 females and 2 males), with no history of skin disease, no visible skin abnormalities and no prior skin treatment in the preceding 4 weeks, participated in the study. Written informed consent was obtained from each subject before the study, which was approved by the University of Bath Ethics Committee.

5 Cream and lotion formulations

Excipients of the cream and lotion are in Table I. They were formulated and then stored in a light-protective and airtight container at 4°C.

Table I: Excipients of cream and lotion formulations.

<table>
<thead>
<tr>
<th>Excipient</th>
<th>10% CP cream</th>
<th>Control cream</th>
<th>10% CP lotion</th>
<th>Control lotion</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. petasites (dried ethanolic extracts)</td>
<td>10</td>
<td>-</td>
<td>10</td>
<td>-</td>
</tr>
<tr>
<td>GMS-SE</td>
<td>5</td>
<td>5</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Cetyl alcohol</td>
<td>5</td>
<td>5</td>
<td>1.5</td>
<td>1.5</td>
</tr>
<tr>
<td>Stearyl alcohol</td>
<td>2.5</td>
<td>2.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mineral oil</td>
<td>15</td>
<td>15</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Tween 60</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Span 60</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Carbopol ultrez (2% w/w stock gel)*</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Propylene glycol</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Glycerol</td>
<td>3</td>
<td>3</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Concentrated paraben**</td>
<td>1% v/w</td>
<td>1% v/w</td>
<td>1% v/w</td>
<td>1% v/w</td>
</tr>
<tr>
<td>BHT</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
</tr>
<tr>
<td>Purified water qs. to</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

* Pre-preparation required before formulating cream and lotion.

** A preservative mixture of methyl paraben (10 g) and propyl paraben (2 g) in propylene glycol (100 mL).
6 Analytical methods

6.1 High performance liquid chromatography coupled with mass spectrometry (HPLC-MS)

HPLC-MS was performed on a Shimadzu HPLC-2010A HT system (Shimadzu Corp., Kyoto, Japan) consisting of an autosampler, vacuum degasser, and UV detector operating at 260 and 330 nm.

The HPLC was interfaced to a Shimadzu MS-2010EV system (Shimadzu Corp., Kyoto, Japan) with a dual source of electrospray ionization and atmospheric pressure chemical ionization (ESI/APCI, DUIS-2010, Japan). Ionization was achieved in both negative- and positive-ion-modes with the detector voltage set at 1.5 kV. Nitrogen was used as a nebulising gas, heated to 480°C and delivered at a flow rate of 1.5 L·min⁻¹. MS signals collected in the single ion-monitoring (SIM) mode were used for quantification of individual compounds.

The column used was a Dionex Acclaim® 120 (C18, 5 µm, 150 x 4.6 mm i.d.). The mobile phase was a combination of acetonitrile (A) and 0.1% aqueous acetic acid (v/v, B) with an optimized gradient system of 20% A for 9 min, 20-60% A for 6 min, 60% A for 5 min, 60-95% A for 10 min, 95% A for 5 min and 20% A for 25 min. The injection volume was 20 µL and the flow rate was 0.5 mL·min⁻¹. The column temperature was maintained at 35°C throughout the analysis. All data acquired were processed by the LabSolutions LCMS Software (Shimadzu Corp., Kyoto, Japan).

6.2 HPLC coupled to ultraviolet and photodiode array detection (HPLC-UV-PDA)

HPLC-UV-PDA was carried out using an ASI-100 automated sample injector, thermostatted column compartment TCC-100 and PDA-100 photodiode array detector (Dionex® Ltd., UK). The UV detection wavelengths were 260 and 330 nm for quantification and the maximum wavelengths (λ_max) of each peak were confirmed by a wavelength scan from 240 to 360 nm.

A HiQ Sil C18 HS column (C18, 5 µm, 150 x 4.6 mm i.d., Kyatech, Japan) was used at a temperature of 35°C. The HPLC-PDA conditions differed only slightly from those optimized for HPLC-MS. Acetonitrile (A) and a mixture of 0.1% aqueous acetic acid and acetonitrile (v/v, 80:20, B) were combined as the
mobile phase in a gradient system of 0% A for 9 min, 0-50% A for 6 min, 50% A for 5 min, 50-94% A for 10 min, 94% A for 5 min and 0% A for 25 min with a flow rate of 0.5 mL·min⁻¹. 20 µL of each sample was injected. Chromatograms were interpreted with Chromeleon software (Dionex® Ltd., UK). Retention times (tᵣ) and UV peak detection using HPLC-PDA were compared with those using HPLC-MS.

7 In vitro skin penetration experiments
The skin permeation of compounds in the plant extracts was determined in vertical, glass Franz diffusion cells (PermeGear, Inc., Bethlehem, PA, USA). The exposed membrane surface area was 1.77 cm² and the receptor solution was 7.5 mL of a mixture of ethanol and 5 mM Tris buffer in ratio of 1:4 v/v, at pH 7.3 (i.e., slightly less than 7.4 due to the presence of ethanol). Frozen dermatomed pig abdominal skin was thawed for 30 minutes before use and examined visually for punctures or defects. The skin was stripped with one adhesive tape (3.5 cm x 3.5 cm, Scotch book tape, 3M, MN, USA) to remove SC disjunctum before being mounted into the Franz cell. After temperature equilibration at 37°C, approximately 0.2 g of the formulations was applied to the skin surface and occluded with Parafilm™ (Bemis Company, Inc., Neenah, WI). After 6 hours, the whole receptor solution volume was removed and stored at 4°C under light protection before quantitative analysis. The skin was subsequently taken out of the Franz cell and the remaining formulation was removed with an isopropyl alcohol swab (70% isopropyl alcohol; Sterets®, Medlock Medical Ltd., UK). The skin was then pinned to a polystyrene board and left to air dry for 3 hours. A template with a circular aperture (1.4 cm diameter, Scotch book tape, 3M, MN, USA) was positioned over the treated area before stripping the SC using tapes. Six replicates were performed with each formulation.

8 In vivo skin penetration experiments
The passive diffusion of constituents of the two plant extract formulations into the SC of healthy volunteers was studied over 2 days. On day 1, the first formulation was applied to one arm; on day 2, the second formulation was applied to the other. The ventral forearms were cleaned with an
alcohol wipe and the subject was acclimatized to the treatment venue for 30 minutes. Four skin sites
(3 treated + 1 blank, control) were delineated on each arm at least 4 cm above the wrist and a
minimum of 4 cm below the elbow. Each treatment site (6 cm² in area, Figure 1) was demarcated
with a rectangular frame cut from a self-adhesive foam (1.57 mm in thickness; 3M, USA) and applied
to the ventral forearm with the long dimension oriented across the forearm. One tape strip was
discarded before drug application to remove SC disjunctum. Good contact between tape and skin
was ensured by running a weighted roller (6 cm wide, 140 g cm⁻²) over the tape several times before
its removal. A fingertip cut from a laboratory glove (nitrile and powder-free glove; Kimtech, UK) was
used to distribute the cream/lotion over the demarcated area. The sites were treated at 45-minute
intervals. Approximately 0.2 g of the product (containing 10% w/w CP extracts) was applied to sites
1-3, while the blank (cream/lotion base without CP) was applied to site 4. The actual amount applied
was determined by weighing the fingertip before and after drug application. All sites were occluded
by covering the foam frame with Parafilm™ secured to the skin with a self-adhesive fabric dressing
(Mefix®, SCA Molnlycke Ltd., Sweden). The drug application time was 6 hours.

At the end of the experiment, the dressing, Parafilm™ and foam frame were removed. Excess
cream/lotion was gently wiped away with tissue (Fort James Ltd., UK), and then swabbed three
times with alcohol wipes. The skin was finally left to air dry for 1 minute before a new thin foam
frame (dimension of the cut out inside are 1.5 cm x 2 cm, 0.75 mm in thickness; 3M, USA) was placed
over each cleaned site in the same position as the original frame (Figure 1). Subsequently, the tape
stripping procedures were commenced.
Figure 1: Illustration of drug application and tape strip area (adapted from reference (N’Dri-Stempfer et al., 2009)).

9 Tape stripping and tape extraction

Tape stripping was performed at the end of the in vitro and in vivo percutaneous experiments. The stripped area was delineated by the templates. First, transepidermal water loss (TEWL) was measured (AquaFlux® evaporimeter, Biox System Ltd., UK) to obtain an initial value. An adhesive tape strip (2.5 cm x 2.5 cm; Permacel J-LAR®) was applied to the skin and pressed firmly down using the weighted roller. The tape was removed quickly from the skin and TEWL was measured again. The procedure was repeated until TEWL reached 4 times the initial value or when 30 strips had been taken.

The mass of skin removed on each tape was determined by weighing the tapes on a microbalance (Satorius model SE2-F, Sartorius AG, Germany), before and after application to the skin. Before weighing, the tapes were stored at room temperature for at least 12 hours and static electricity was discharged (R50 discharging bar and ES50 power supply, Eltex Elektrostatik GmbH, Weil am Rhein, Germany).

After weighing, the tapes were grouped for methanol extraction. The first and second tapes were individually analysed, while the remaining tape strips were combined into groups of 2-4 tapes depending on the total number collected in each experiment. Groups containing 1-2 tapes were extracted with 1 mL of methanol, those with 3-4 tapes were extracted into 1.5 mL. Extraction involved shaking overnight (IKA HS 260 Basic shaker, IKA® Werke GmbH & Co., KG, Germany). The
extracted solutions were then filtered through a 0.45 µm nylon membrane, and either directly injected, or concentrated by freeze-drying before injection into an HPLC-MS or HPLC-PDA. If not processed at once, the samples were freeze-dried and stored at 4°C before analysis.

10 Statistical analysis

All statistical analyses were performed using GraphPad Prism® version 5 (GraphPad Software Inc., CA, USA). Calibration curves were assessed by linear regression. Datasets were expressed as mean ± SD (standard deviation) and compared for statistical significance at P ≤ 0.05 with two-way ANOVA and Bonferroni post-tests.
Results

1  *In vitro* percutaneous absorption of CP constituents from a cream and a lotion

On the basis of preliminary results (Thitilertdecha et al., 2014), four compounds, vanillic acid, verbascoside, nepetin and hispidulin, all known to be present in the CP extract, were selected to monitor and compare the performance of the two formulations.

Equivalent sets of sample and control experiments were conducted with a 6-hour application to allow comparison with the results of *in vivo* studies carried out in human volunteers.

Table II: Quantities per unit area (and % penetration\(^a\)) of vanillic acid, verbascoside, nepetin and hispidulin detected in SC and in the receptor solution after 6 hours (average ± SD, 6 replicates).

<table>
<thead>
<tr>
<th>Compound</th>
<th>10% w/w CP cream</th>
<th>10% w/w CP lotion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Quantity (nmol·cm(^{-2})) % penetration</td>
<td>Quantity (nmol·cm(^{-2})) % penetration</td>
</tr>
<tr>
<td></td>
<td>In SC</td>
<td>In receptor</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>0.6 ± 0.3</td>
<td>0.6 ± 0.5</td>
</tr>
<tr>
<td>Verbascoside</td>
<td>0.3 ± 0.02 (^b)</td>
<td>-</td>
</tr>
<tr>
<td>Nepetin</td>
<td>0.8 ± 0.6</td>
<td>0.1 ± 0.02</td>
</tr>
<tr>
<td>Hispidulin</td>
<td>3.0 ± 0.4</td>
<td>1.1 ± 0.8</td>
</tr>
</tbody>
</table>

\(^a\) = Values were determined from the ratio of the cumulative amount of compound in the receptor solution to its original content in the formulation applied (calculated from the quantities of the compounds in a dried ethanolic extract, the amount of the dried extract used in the formulation, and the amount of the formulation applied).

\(^b\) = only 3 replicates were measurable.

All compounds except verbascoside were delivered from both cream and lotion vehicles and were detected in the receptor phase after 6 hours (Table II). Results from tape-stripping (Table II) were consistent with these findings. Vanillic acid and hispidulin were taken up into the SC and crossed the skin well. Verbascoside was only found in the SC and was not percutaneously absorbed. Little difference was observed between the two formulations.

2  *In vivo* SC uptake of CP constituents from a cream and a lotion

Cumulative amounts of vanillic acid, nepetin and hispidulin taken up into the SC of the individual volunteers from the cream and lotion are presented in Table III. Transport of hispidulin from the cream (1 nmol·cm\(^{-2}\)) was approximately double that of nepetin and vanillic acid (0.4, and 0.3
nmol·cm\(^{-2}\), respectively). The penetration of these three compounds from the lotion was similar to those from the cream. Verbascoside was not detectable in the SC following application of either the cream or the lotion. Reproducibility was good and the robustness of the in vivo methodology was demonstrated by low inter-subject variability: CVs were less than 29% for hispidulin, 44% for vanillic acid, and 55% for nepetin from both formulations.

Table III: Amounts of vanillic acid, verbascoside, nepetin and hispidulin taken up into the SC after a 6-hour application of a cream and a lotion to human volunteers.

<table>
<thead>
<tr>
<th>Volunteer number</th>
<th>Amount in SC 6-hour post application of 10% w/w CP cream (average ± SD, nmol·cm(^{-2}), n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vanillic acid</td>
</tr>
<tr>
<td>1</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>4</td>
<td>0.2 ± 0.02</td>
</tr>
<tr>
<td>5</td>
<td>0.2 ± 0.04</td>
</tr>
<tr>
<td>6</td>
<td>0.6 ± 0.1(^a)</td>
</tr>
<tr>
<td>Average</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>%RSD</td>
<td>43.1</td>
</tr>
</tbody>
</table>

\(^a\)Measured with MS detection.

<table>
<thead>
<tr>
<th>Volunteer number</th>
<th>Amount in SC 6-hour post application of 10% w/w CP lotion (average ± SD, nmol·cm(^{-2}), n=3)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vanillic acid</td>
</tr>
<tr>
<td>1</td>
<td>0.5 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>0.4 ± 0.04</td>
</tr>
<tr>
<td>4</td>
<td>0.2 ± 0.03</td>
</tr>
<tr>
<td>5</td>
<td>0.3 ± 0.1(^a)</td>
</tr>
<tr>
<td>6</td>
<td>0.5 ± 0.1(^b)</td>
</tr>
<tr>
<td>Average</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>%RSD</td>
<td>29.0</td>
</tr>
</tbody>
</table>

\(^a\)Two sites were measured with UV detection, a third with MS; b = 3 sites were measured with MS detection.

When nepetin was detected by MS in volunteer 6, the level observed was small. While there should be little or no difference between the PDA and MS assays, it is possible that the PDA peak for nepetin was subject to interference by other compounds in the plant extract with a similar retention time (e.g., luteolin). It is possible, therefore, that the nepetin quantities found in volunteers 1-5 using PDA may over-estimate the actual amounts present in the SC. In contrast, no interfering peaks with those of vanillic acid and hispidulin were apparent.

Figure 2 compares in 1 subject the SC concentration versus depth profiles of vanillic acid, nepetin, and hispidulin, respectively, from the two formulations after a 6-hour application. Vanillic acid
showed a more or less constant low concentration throughout the SC. Concentration-depth profiles of nepetin and hispidulin were similar to those reported previously for other, unrelated compounds (Alberti et al., 2001; Herkenne et al., 2007; Wagner et al., 2000). There were consistent profiles observed for the 6 volunteers (see the Supplementary Data) except for nepetin in volunteer 6 when the MS assay was used.

**Figure 2:** SC concentration versus depth profiles of vanillic acid, nepetin, and hispidulin in 1 subject after a 6-hour application of 10% w/w CP cream (panel A) and 10% w/w CP lotion (panel B).
Discussion

Preliminary *in vitro* percutaneous penetration studies determined the topical absorption of the principal phenolic actives in CP (Thitilertdecha *et al.*, 2014). Vanillic acid, nepetin and hispidulin were the three compounds showing the most substantial absorption and thus were the likeliest candidates for eliciting CP's antimicrobial (Delaquis *et al.*, 2005; Sultana and Afolayan, 2007), anti-inflammatory (Clavin *et al.*, 2007; Gil *et al.*, 1994; Kim *et al.*, 2011), and antioxidant activities (Kang *et al.*, 2009). Verbascoside was included in the present study because of its apparent affinity to the SC and because its structure and physicochemical properties predict that it is a poor permeant (i.e., it serves as a “negative” control for the other three compounds).

An o/w cream and an o/w lotion containing excipients expected to enhance skin penetration (e.g., propylene glycol, glycerol, and the surfactants Tween 60 and Span 60) were formulated to improve permeability of the four selected actives. The concentration of CP was controlled at 10% by weight which has been reported as the maximal concentration of plant ethanolic extracts in topical products without measurable toxicity (Diwan *et al.*, 2001). A secondary objective was to avoid the use of ethanol as this co-solvent may alter the barrier function and irritate the skin. Hispidulin penetrated in the greatest amount followed by vanillic acid and nepetin. Verbascoside was only taken up into the SC and was not percutaneously absorbed. No significant difference was observed between the two formulations, the excipients of which were similar.

The theoretically predicted uptake (determined from the calculated $J_{\text{max}}$ values determined in the preliminary study (Thitilertdecha *et al.*, 2014)) can be qualitatively compared with the experimental data in Table II. Verbascoside’s poor permeability was as anticipated (predicted uptake of only 0.003 nmol·cm$^{-2}$). Vanillic acid was indeed well-absorbed but its penetration was much lower than that expected from the predicted $J_{\text{max}}$ (440 nmol·cm$^{-2}$). This is most likely because the formulations contained vanillic acid at much less than its saturation concentration. The same conclusion fits with
the results for nepetin and hispidulin, the experimental absorption of which was lower than that predicted from their calculated $J_{\text{max}}$ values (1.2 and 2.4 nmol·cm$^{-2}$, respectively).

Although delivery of the compounds from the CP cream and lotion were less than those predicted from saturated solution, the percentage penetration observed (Table II) was greater than that from the hydroalcoholic solution used in the preliminary study (Thitilertdecha et al., 2014). This may be due to the presence of surfactants in the cream and lotion formulations facilitating the solubilisation of the compounds in the SC.

The in vivo and in vitro SC uptake data are compared in Figure 3 and show good overall agreement. SC uptake in vitro was generally higher than in vivo and statistically significant differences were found for nepetin and hispidulin ($P < 0.01$ and $< 0.001$, respectively). Verbascoside taken up into the SC never reached the analytical LOQ in vivo. Thus, while quantitative extrapolation from in vitro results to the in vivo situation may not be possible, it appears that in vitro experiments would be useful for formulation development.

**Figure 3**: Amounts (mean ± SD) of vanillic acid, verbascoside, nepetin and hispidulin taken up into the SC from cream and lotion formulations in vitro and in vivo (filled and open bars, respectively; n = 6).
The cream and lotion bases without plant extracts were stable for at least 1 month under accelerated conditions at 30°C and 75%RH (as recommended for Thailand by the World Health Organization). These stability tests were conducted at the Center of Applied Thai Traditional Medicine, Faculty of Medicine Siriraj Hospital in Thailand. However, the formulations containing the plant extracts were unstable under the same stress conditions. The plant extracts in the lotion separated more easily from the base than in the cream. Nevertheless, the separation was reversible upon shaking. Formulation reproducibility was shown by consistent viscosity profiles between different batches of the two formulations.
Conclusion

This is the first investigation of the topical delivery of products containing extracts of *C. petasites* in *vitro* and *in vivo* in humans. Four naturally-occurring active compounds in the plant, vanillic acid, verbascoside, nepetin and hispidulin, were identified as relevant actives for skin permeation studies. The four compounds, based on their uptake and penetration into the skin, together with their known biological activities, may be considered as feasible candidates for the development of novel and effective antimicrobial, anti-inflammatory, and antioxidant formulations and support the ethnomedical uses of *C. petasites* in Thailand. It was also found that the *in vitro* model and the tape-stripping method were robust and effective to establish guidelines for topical delivery studies of natural products. Both lotion and cream formulations containing *C. petasites* extracts are potential standardised medicines for the Thai market, but show some stability problems, that require further development. Overall, this study demonstrates a feasible approach to developing a series of topical medicines that retain the herbal ingredients that many patients value whilst achieving higher levels of standardisation and quality than currently exist on the market.

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