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1 **Characterisation of polyphenolic compounds in *Clerodendrum petasites* S. Moore and their**
2 **potential for topical delivery through the skin**

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9 **Abstract**

10 Ethnopharmacological relevance: *Clerodendrum petasites* S. Moore (CP) has been widely prescribed
11 in Thailand and neighbouring countries for both oral and topical administration to treat asthma,
12 fever, cough, vomiting and skin diseases, for at least 30 years.

13 Aim of the study: To characterise polyphenolic compounds in the plant, to predict the feasibility of
14 their topical absorption and to test their ability to penetrate the skin.

15 Materials and Methods: Identification and quantification of flavonoids and phenolic acid derivatives
16 in an ethanolic extract of the aerial parts of the plant was carried out using high performance liquid
17 chromatography (HPLC) with photodiode array (PDA) and mass spectrometry (MS) detection.
18 Ambiguous isomeric compounds were distinguished by nuclear magnetic resonance (NMR)
19 spectroscopy. The feasibility of the compounds' topical permeability was evaluated by predicting
20 their maximum fluxes from their physicochemical properties. The skin penetration of compounds in
21 the plant extract was measured *in vitro* over 24 hours.

22 Results: Vanillic acid, verbascoside, 4-coumaric acid, ferulic acid, nepetin, luteolin, apigenin,
23 naringenin, hispidulin, hesperetin and chrysin, were identified in CP. All compounds except apigenin
24 and hispidulin are reported in this species for the first time. Hispidulin is the predominant compound
25 (1.2% w/w in a dried ethanolic extract) followed by nepetin, verbascoside, vanillic acid, and apigenin.
26 Across mammalian skin, hispidulin was percutaneously absorbed within 3 hours and vanillic acid and
27 nepetin permeated the skin after 6 hours. These experimental observations were consistent with the
28 predicted maximum fluxes of these compounds calculated from their physicochemical properties.

29 Conclusions: Many of the phenolic compounds reported in this study are well known to possess
30 antimicrobial, anti-inflammatory and anti-oxidant activities. The skin permeation studies reported
31 here support traditional topical uses of the plant in skin treatments and are useful for further topical
32 formulation optimisation.

33 **Abbreviations**

34 CP, *Clerodendrum petasites* S. Moore; HPLC, high performance liquid chromatography; PDA,
35 photodiode array; UV, ultraviolet; MS, mass spectrometry; NMR, nuclear magnetic resonance
36 spectroscopy.

37 **Chemical compounds studied in this article**

38 Vanillic acid (PubChem CID: 8468); verbascoside (PubChem CID: 5281800); nepetin (PubChem CID:
39 5317284); hispidulin (PubChem CID: 5281628).

40 **Keywords**

1 *Clerodendrum petasites*; Thai traditional medicine; HPLC-MS; topical delivery; phenolic compounds

2 1. Introduction

3 *Clerodendrum petasites* (English name: One Root Plant) is one of ~700 species of this genus in the
4 family Lamiaceae (*Clerodendrum petasites* S. Moore, 2005; The plant list, 2010). The plant is
5 widespread in the middle, north-eastern, and southern parts of Thailand. There are numerous Thai
6 names from each region, for instance, Ping-Khom and Ping-Luang in the north, Phaya-Rak-Deaw in
7 the south, Nang-Shon and Phom-Phee in the northeast. However, Thao-Yyai-Mom from the
8 midlands is the best known.

9 Thai traditional practitioners usually prepare aerial parts, leaves, or roots of *C. petasites* as a tea,
10 alcoholic extract or cigarette to treat asthma (Hazekamp *et al.*, 2001; Panthong *et al.*, 2003;
11 Panthong *et al.*, 1986). Leaves and roots are also ground into powders for treatment of inflammation
12 (Panthong *et al.*, 1986) as well as to treat fever, cough, and vomiting (Panthong *et al.*, 2003; Thai
13 traditional medical textbook: Paet-Ta-Ya-Saat-Song-Kror (แพทยศาสตร์สงคราะห์), 2007) (S. Tungjitaruen,
14 pers. comm., 2011). The plant is widely prescribed for oral administration and generally formulated
15 into multi-herb recipes. The most famous recipe is “Ha-Rak” (synonyms: Ben-Cha-Lo-Ka-Wi-Chian,
16 Kaew-Ha-Dueng, Phed-Sa-Wang), containing equal amounts by weight of five roots from *C. petasites*,
17 *Ficus racemosa* Linn, *Capparis micracantha* DC, *Harrisonia perforate* Merr, and *Tiliacora triandra*
18 Diels (Pichaensoonthon *et al.*, 2005). The recipe is currently registered by the Thai Food and Drug
19 Administration (FDA) for antipyretic activity (List of herbal medicinal products, 2006; National list of
20 essential medicines: Ha-Rak). Dosage forms of Ha-Rak are powders, tablets and capsules, but
21 decoction is conventionally served. There are fewer records for topical remedies. Poultices are most
22 often formulated for skin diseases, such as rash, abscess, urticaria, snakebites and insect bites
23 (Panthong *et al.*, 2003; Pongboonrot, 1965; Thai traditional medical textbook: Paet-Ta-Ya-Saat-Song-
24 Kror (แพทยศาสตร์สงคราะห์), 2007) (T. Tipcharoentham, pers. comm., 2011; S. Tungjitaruen, pers. comm.,
25 2011). Many recipes are dispersed in alcohol, especially Thai rice whisky, before application.

26 *C. petasites* is also widely distributed in many other countries, e.g., Malaysia, India, Southern China,
27 Sri Lanka, and Vietnam. Ethnomedical uses of the plant are found in their medical systems. For
28 example, root and leaf extracts of *C. petasites* have been documented for the treatment of
29 rheumatism, asthma and other inflammatory diseases (Shrivastava and Patel, 2007). In India, fruits
30 are reportedly used to reduce fertility in males and the plant is used to cure malaria in China
31 (Hazekamp *et al.*, 2001; Panthong *et al.*, 2003; Shrivastava and Patel, 2007).

32 Although the chemical constituents in the genus *Clerodendrum* have been widely investigated, there
33 have been only a few studies on *C. petasites*. The compounds previously reported in the aerial parts
34 and roots of *C. petasites* include apigenin, hispidulin, 6,4'-dimethoxyscutellarin, hispidulin 7-
35 methylglucuronide, nevadensin 7-glucoside, arbutin and bungene A (Hazekamp *et al.*, 2001; Klaiklay,
36 2009; Singharachai *et al.*, 2011; Thongchai *et al.*, 2007). There have been no clinical trials that
37 identify and verify the compounds that elicit useful pharmacological effects following topical
38 delivery. Thus, in this study, flavonoids and other phenolic compounds, which are well-known as
39 strong antioxidants with free radical scavenging and metal chelating activities (Perron and
40 Brumaghim, 2009; Robak and Gryglewski, 1996; Wuguo *et al.*, 1997), and are extensively used in
41 dermatological and cosmetological applications (Arct *et al.*, 2002; Arct and Pytkowska, 2008; Bonina
42 *et al.*, 1996; Cimino and Saija, 2005; Lin *et al.*, 2008), were characterised and their topical absorption
43 determined using a pig skin model. Experimental values were compared with theoretical
44 transdermal fluxes calculated from the physicochemical properties of the compounds (Potts and
45 Guy, 1992).

1 2. Materials and methods

2 2.1 Plant materials

3 Dried samples of the aerial parts of *C. petasites* were authenticated by macroscopic identification
4 and obtained from the Ayurved Siriraj Manufacturing Unit of Herbal Medicines and Products, Center
5 of Applied Thai Traditional Medicine (CATTM), Faculty of Medicine Siriraj Hospital, Mahidol
6 University, Thailand. Extracts were produced by maceration using 80% ethanol and subsequently
7 evaporated to dryness. Five batches of ethanolic extracts were kept separately in light protective
8 and airtight containers and stored in a desiccator at room temperature.

9 The ethanolic extracts were separated into water, butan-1-ol, ethyl acetate and petroleum ether
10 soluble fractions by liquid-liquid partition. Only the butanol and ethyl acetate fractions were further
11 separated by column chromatography using a step gradient of 100% ethyl acetate followed by 1%,
12 2%, 5%, 10%, 20%, 50% methanol in ethyl acetate and 100% methanol. All the fractions were kept in
13 light protective and airtight containers and stored at 4°C. The fractions were subsequently examined
14 by NMR to elucidate the structure of ambiguous isomers.

15 2.2 Chemicals and reagents

16 Caffeic acid, 4-coumaric acid, naringin, chrysin, 5,7-dimethoxycoumarin, gallic acid, rosmarinic acid,
17 kaempferol, cinnamic acid (Sigma-Aldrich, USA), vanillic acid, ferulic acid, apigenin (Fluka Analytical,
18 China), rutin, quercetin (Koch-Light Laboratories Ltd., UK), verbascoside, naringenin, chrysoeriol,
19 hesperetin, luteolin, diosmetin, nepetin, scutellarein (Extrasynthese, France), hispidulin (Tocris
20 Bioscience, UK), cirsimaritin (BioBioPha. Co; Ltd.), were of analytical grade.

21 Mobile phases for HPLC-MS and HPLC-PDA consisted of HPLC grade acetonitrile (Fisher Scientific,
22 UK), HPLC grade water obtained from a deionized water treatment system (Milli-pore, MA, USA) and
23 MS grade acetic acid (Fluka Analytical, Germany). Deuterated-methanol (methanol-D₄, CD₃OD),
24 deuterated-chloroform (chloroform-D, CDCl₃) and deuterium oxide (D₂O) were used for NMR
25 analysis and purchased from Cambridge Isotope Laboratories, Inc., UK. Other chemicals and
26 reagents, methanol, ethanol (Sigma-Aldrich, USA), butan-1-ol (Fisher Scientific, UK), ethyl acetate,
27 and petroleum ether, tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl, Acros Organics,
28 USA), tris aminomethane (Trizma® base, Sigma-Aldrich, USA), were of analytical grade.

29 Excipients of the preliminary topical formulations comprised propylene glycol (Acros Organics, UK)
30 and Vaseline white (Riedel-de Haën, Germany).

31 2.3 Skin

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

36 2.4 Preparation of standard solutions

37 Stock solutions (0.1 mg·mL⁻¹) of the phenolic standards were prepared by dissolution in methanol
38 followed by sonication for 30 minutes where necessary (Fisherbrand® FB11002, Thermo Fisher
39 Scientific Inc., UK). Each analyte stock solution was diluted with methanol to appropriate
40 concentrations for the establishment of calibration curves and validation tests. All standard solutions
41 were filtered through a 0.45 µm nylon membrane (Chronus® filter, LabHut Ltd., UK) before HPLC-MS
42 or HPLC-PDA analysis. Both stock and diluted solutions were stored at 4°C.

1 2.5 Preparation of plant sample solutions

2 The dried extract of *C. petasites* was accurately weighed and dissolved in methanol at a
3 concentration of 50 mg·mL⁻¹ and sonicated for 30 minutes. After centrifugation at 4000 rpm for 20
4 minutes (U-32, Boeco, Germany), the supernatant was filtered through a 0.45 µm nylon membrane
5 and diluted with methanol to appropriate concentrations prior to HPLC-MS or HPLC-PDA analysis.
6 The filtered plant sample solution was stored at 4°C.

7 2.6 Preparation of topical formulations

8 A solution containing the CP extract at a concentration of 50 mg·mL⁻¹ in 50% aqueous ethanol was
9 prepared. The solution was sonicated for 60 minutes and then centrifuged at 4000 rpm for 20
10 minutes. The supernatant was filtered through a 0.45 µm nylon membrane before use in the *in vitro*
11 permeation tests. The solution was used within 24 hours of preparation.

12 A paste consisting of 50% CP, 17% propylene glycol and 33% Vaseline (w/w) was prepared and well
13 mixed. The paste was used within 24 hours of preparation.

14 2.7 HPLC-MS

15 Experiments were performed on a Shimadzu HPLC-2010A HT system (Shimadzu Corp., Kyoto, Japan)
16 consisting of an autosampler, vacuum degasser, and UV detector which was set at the detection
17 wavelengths of 260 and 330 nm (chosen on the basis of HPLC-PDA results of individual standards).

18 The HPLC was connected to a Shimadzu MS-2010EV system (Shimadzu Corp., Kyoto, Japan) with a
19 dual source of electrospray ionization and atmospheric pressure chemical ionization (ESI/APCI, DUIS-
20 2010, Japan). Ionization was achieved in both negative- and positive-ion-modes with detector
21 voltage set at 1.5 kV. Nitrogen was used as the nebulising gas, heated to 480°C and delivered at a
22 flow rate of 1.5 L·min⁻¹. MS signals were collected in the scan mode between 50-1000 m/z for
23 identification of chemical components and the single ion-monitoring (SIM) mode was used for
24 quantification of individual compounds.

25 The column used was a Dionex Acclaim® 120 (C18, 5 µm, 150 x 4.6 mm i.d.). A combination of
26 acetonitrile (A) and 0.1% aqueous acetic acid (v/v, B) was used as mobile phase with an optimized
27 gradient system of 20% A, 80% B for 9 min, 20-60% A, 80-40% B for 6 min, 60% A, 40% B for 5 min,
28 60-95% A, 40-5% B for 10 min, 95% A, 5% B for 5 min and 20% A, 80% B for 25 min. The injection
29 volume was 20 µL and the flow rate was 0.5 mL·min⁻¹. The column temperature was maintained at
30 35°C throughout the analysis. All data acquired were processed by the LabSolutions LCMS Software
31 (Shimadzu Corp., Kyoto, Japan).

32 2.8 HPLC-PDA

33 The HPLC-PDA system comprised an ASI-100 automated sample injector, thermostatted column
34 compartment TCC-100 and PDA-100 photodiode array detector (Dionex® Ltd., UK). The UV detection
35 wavelengths were set at 260 and 330 nm for quantification and the maximum wavelengths (λ_{\max}) of
36 each peak were detected by a wavelength scan from 240 to 360 nm for peak confirmation.

37 A HiQ Sil C18 HS column (C18, 5 µm, 150 x 4.6 mm i.d., Kyatech, Japan) was used and the
38 temperature was maintained at 35°C. The HPLC-PDA conditions were slightly changed from those
39 which had been optimized for HPLC-MS. Acetonitrile (A) and a mixture of 0.1% aqueous acetic acid
40 and acetonitrile (v/v, 80:20, B) were combined as the mobile phase in a gradient system of 0% A,
41 100% B for 9 min, 0-50% A, 100-50% B for 6 min, 50% A, 50% B for 5 min, 50-94% A, 50-6% B for 10
42 min, 94% A, 6% B for 5 min and 0% A, 100% B for 25 min with a flow rate of 0.5 mL·min⁻¹. 20 µL of
43 each sample was injected. Chromatograms were interpreted with Chromeleon software (Dionex®

1 Ltd., UK). Retention times (t_R) and UV peak detection using HPLC-PDA were compared with those
2 using HPLC-MS.

3 2.9 Nuclear magnetic resonance spectroscopy (NMR)

4 All ethanolic extracts, solvent partition fractions, and phenolic standards were dissolved in
5 appropriate solvents (e.g., deuterated-methanol (CD_3OD), deuterated-chloroform ($CDCl_3$) and
6 deuterium oxide (D_2O)). 1H NMR (500 MHz) spectra were obtained on a Varian Mercury
7 spectrometer. Chemical shifts (δ) were recorded in parts per million (ppm).

8 2.10 Prediction of maximum flux (J_{max})

9 A maximum possible flux (J_{max} , $\mu g \cdot cm^{-2} \cdot h^{-1}$) of transport of each compound was calculated from an
10 algorithm derived from Fick's first law of diffusion as follows:

$$11 \quad J_{max} = k_p \cdot C_{sat,W} \quad \text{Eq. 1}$$

12 where k_p is the compound's permeability coefficient ($cm \cdot h^{-1}$) and $C_{sat,W}$ is the saturation solubility of
13 the compound in water ($\mu g \cdot cm^{-3}$). The k_p value is estimated by the Potts and Guy equation (Eq. 2)
14 (Potts and Guy, 1992).

$$15 \quad \log k_p = -2.72 + 0.71 \cdot \log P - 0.0061 \cdot MW \quad \text{Eq. 2}$$

16 where P is the compound's octanol-water partition coefficient and MW is its molecular weight (Da).

17 However, because the viable epidermis can represent a significant barrier to the penetration of
18 lipophilic compounds, the Potts and Guy estimated k_p (which assumes the transport across the skin
19 is controlled uniquely by the SC) is corrected as proposed by Cleek and Bunge (1993) as follows:

$$k_p^{corr} = \frac{k_p}{1 + \frac{k_p \cdot \sqrt{MW}}{2.6}} \quad \text{Eq. 3}$$

20 It follows that J_{max} for the putative active species in the plant extracts can be predicted from Eqs. 1-3
21 using available or calculable values of MW , $\log P$ and $C_{sat,W}$ (ALOGPS 2.1 algorithm, 2001;
22 Chempider).

23 2.11 *In vitro* skin permeation

24 The skin permeation of compounds in the plant extracts was determined using vertical, glass Franz
25 diffusion cells (PermeGear, Inc., Bethlehem, PA, USA). The exposed membrane surface area was 1.77
26 cm^2 and the receptor volume was 7.5 mL. The receptor solution was a mixture of ethanol and 5 mM
27 Tris buffer in ratio of 1:4 v/v, at pH 7.3 (slightly less than 7.4 due to the presence of ethanol). Frozen
28 dermatomed pig abdominal skin was thawed for 30 minutes before use and examined visually for
29 punctures or defects. The skin was stripped with one adhesive tape (3.5 cm x 3.5 cm, Scotch book
30 tape, 3M, MN, USA) to remove SC disjunctum before being mounted into the Franz cell. After
31 temperature equilibration at 37°C, the formulations were applied to the skin surface and occluded
32 with Parafilm™ (Bemis®, USA). The amounts of drug applied were 1 mL for the CP solution and
33 approximately 0.2 g for the CP paste. Samples were withdrawn at 3, 6, and 24 hours. At each
34 sampling time, the whole receptor solution volume was removed and replaced with fresh buffer.
35 The samples were stored at 4°C under light protection before quantitative analysis. Six replicates
36 were performed with each formulation.

1 2.12 Validation and statistical analysis

2 2.12.1 Limits of detection and quantification (LOD and LOQ)

3 Each standard solution was diluted and measured in triplicate to assess a signal-to-noise ratio (S/N).
4 The S/N was the ratio of the height of the chromatographic signal above the baseline and the height
5 of the baseline noise measured more than 30 seconds before and after the peak to avoid any peak
6 tails. The concentration with $S/N \geq 3$ was defined as LOD and that with $S/N \geq 10$ was identified as
7 LOQ.

8 2.12.2 Calibration curves

9 Separate calibrations were carried out for HPLC-MS and HPLC-PDA assays. At least six concentrations
10 and three independent preparations of phenolic standards in the range of 0.25 – 20 ng in methanol
11 were injected into the HPLC-MS detector. 0.25 ng – 2 µg of standard mixtures in methanol were
12 subjected to HPLC-PDA detection. Calibration curves were obtained by plotting the areas under the
13 curves (AUCs) against concentration and the equation of the line determined by linear regression.
14 The curves were used only within the linear range.

15 2.12.3 Precision

16 Three different concentrations (low, middle, and high examples on the calibration curves) of
17 individual phenolic standards were measured five times a day to determine intra-day variability. The
18 standards were also analysed twice a day on three consecutive days in order to obtain inter-day
19 variability. The results were expressed in terms of relative standard deviation (RSD).

20 2.12.4 Statistical analysis

21 All statistical analyses were performed using GraphPad Prism® version 5 (GraphPad Software Inc.,
22 CA, USA). Calibration curves were analysed with linear regression. Datasets were expressed as mean
23 \pm SD (standard deviation) and compared for statistical significance at $P \leq 0.05$ with two-way ANOVA
24 and Bonferroni post-tests.

25 **3. Results and discussion**

26 3.1 Analytical method optimisation and validations of phenolic standards

27 Twenty four phenolic standards were selected for preliminary qualitative analysis and detected by
28 the optimised HPLC-MS and HPLC-PDA (Table 1).

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1 **Table 1.** Retention times (t_R), mass to charge ratios (m/z), ion modes of MS detection, and maximum
 2 wavelengths (λ_{max}) of phenolic standards from HPLC-MS and HPLC-PDA analyses.

Compound	HPLC-MS			HPLC-PDA	
	t_R (min)	m/z	Ion mode	t_R (min)	λ_{max} (nm)
Gallic acid	4.6	169	(-)ve	5.0	272
Caffeic acid	7.6	179	(-)ve	9.0	324
Vanillic acid	7.8	167	(-)ve	9.9	261
Rutin	9.1	609	(-)ve	11.8	257
Verbascoside	9.9	623	(-)ve	12.7	330
4-Coumaric acid	11.5	163	(-)ve	15.3	310
Ferulic acid	13.9	193	(-)ve	16.4	322
Rosemarinic acid	15.1	359	(-)ve	16.8	329
Naringin	16.2	579	(-)ve	16.3	284
Scutellarein	17.5	287	(+)ve	17.9	334
Luteolin	18.3	287	(+)ve	18.6	347
Nepetin	18.4	315	(-)ve	18.7	345
Quercetin	18.6	301	(-)ve	18.8	256
Cinnamic acid	19.3	147	(-)ve	19.6	282
Apigenin	19.3	271	(+)ve	19.8	334
Naringenin	19.4	271	(-)ve	19.7	291
Hispidulin	19.4	301	(+)ve	20.0	334
Kaempferol	19.5	287	(+)ve	20.0	out of scanning range
Chrysoeriol	19.6	299	(-)ve	20.1	345
Diosmetin	19.6	301	(+)ve	20.1	344
Hesperetin	19.8	301	(-)ve	20.1	289
Cirsimaritin	21.1	315	(+)ve	22.1	333
5,7-Dimethoxycoumarin	21.6	207	(+)ve	22.2	327
Chrysin	22.9	253	(-)ve	24.0	268

3
 4 From preliminary MS spectra of the plant samples, eleven phenolic compounds were tentatively
 5 identified and selected as characteristic markers. Vanillic acid, verbascoside, 4-coumaric acid, ferulic
 6 acid, nepetin, naringenin, hesperetin and chrysin were detected in the negative ion mode as $[M-H]^-$
 7 ions, whereas luteolin, apigenin, and hispidulin were detected in the positive ion mode as $[M+H]^+$
 8 ions.

9 The linearity of the concentration versus peak area relationships was determined over the range of
 10 0.01-6 μ M. Linear correlations (r^2) were obtained with $r^2 > 0.95$ for all the eleven phenolic standards
 11 except hesperetin ($r^2 = 0.90$). Based on a 20- μ L injection, the limits of detection (LOD) and
 12 quantification (LOQ) for each standard were determined to be 20-25 nM and 41-50 nM, respectively
 13 for luteolin, apigenin, naringenin, hispidulin, hesperetin and chrysin. LODs of vanillic acid, 4-coumaric
 14 acid and ferulic acid were 32-38 nM and those of verbascoside and nepetin were 60 and 79 nM,
 15 respectively; the LOQs of these compounds were in the range of 63-80 nM except for nepetin (158
 16 nM).

17 Multiple injections were carried out to determine the precision of the assay for each standard. The
 18 intra-day RSD (relative standard deviation) values at medium and high concentrations of each of the
 19 standard calibration curves were less than 5% for the eleven phenolic standards except for 4-
 20 coumaric acid (6.3%), while the intra-day RSDs at low concentration were relatively higher (less than
 21 9% for the eight phenolic compounds, 13% for 4-coumaric acid and hispidulin, and 19% for ferulic
 22 acid). The inter-day RSD values were not significantly different among the low, medium and high

1 concentrations (below 10% for all compounds except 4-coumaric acid, luteolin, apigenin, and
2 hesperetin for which the RSD was less than 18.5%).

3 3.2 Identification of chemical compounds in CP

4 To characterise naturally-occurring chemical compounds in CP (Table 2), three independent criteria
5 corresponding with pure standards were routinely applied: (a) retention time (t_R), (b) mass to charge
6 ratio (m/z), (c) maximum wavelength (λ_{max}). Peaks with a retention time identical to one of the
7 standards and one other criterion in common with that standard were regarded as tentatively
8 identified in this study; peaks with two other criteria in common with the standard were regarded as
9 identified.

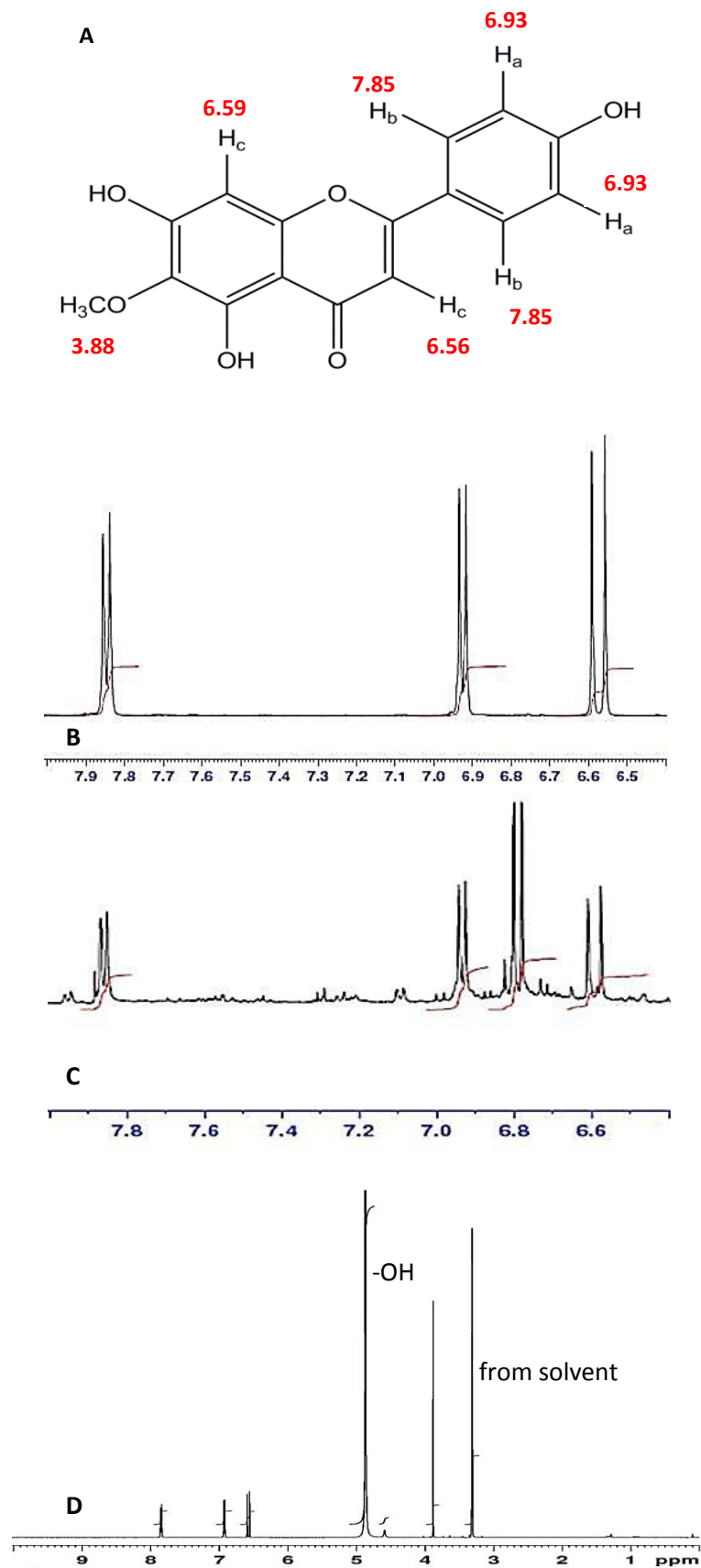
10 **Table 2.** The characteristic peaks of ethanolic extracts of *C. petasites* ($0.1 \text{ mg}\cdot\text{mL}^{-1}$ in methanol) with
11 MS detection in both negative and positive ion modes. Three independent criteria corresponding
12 with pure standards: (a) retention time (t_R), (b) mass to charge ratio (m/z), and (c) maximum
13 wavelength (λ_{max}), were applied.

R_t (min)	m/z (-) <i>ve</i>	m/z (+) <i>ve</i>	λ_{max} (nm)	Identification	Type of identification
6.2	-	476	-	Unknown	-
7.4	179	-	-	Unknown	-
7.8	-	564	-	Unknown	-
8.0	167	-	261	Vanillic acid	a, b, c
10.1	623	-	330	Verbascoside	a, b, c
11.9	163	-	-	4-Coumaric acid	a, b
14.1	193	-	322	Ferulic acid	a, b, c
18.4	-	287	-	Luteolin	a, b
18.5	315	317	345	Nepetin	a, b, c
19.3	-	271	333	Apigenin	a, b, c
19.4	271	-	-	Naringenin	a, b
19.6	299	301	334	Hispidulin	a, b, c
19.8	301	-	-	Hesperetin	a, b
20.5	-	297	-	Unknown	-
23.0	253	-	-	Chrysin	a, b
23.8	-	315	-	Unknown	-
25.8	-	415	-	Unknown	-
26.8	-	505	-	Unknown	-
34.5	-	458	-	Unknown	-

14

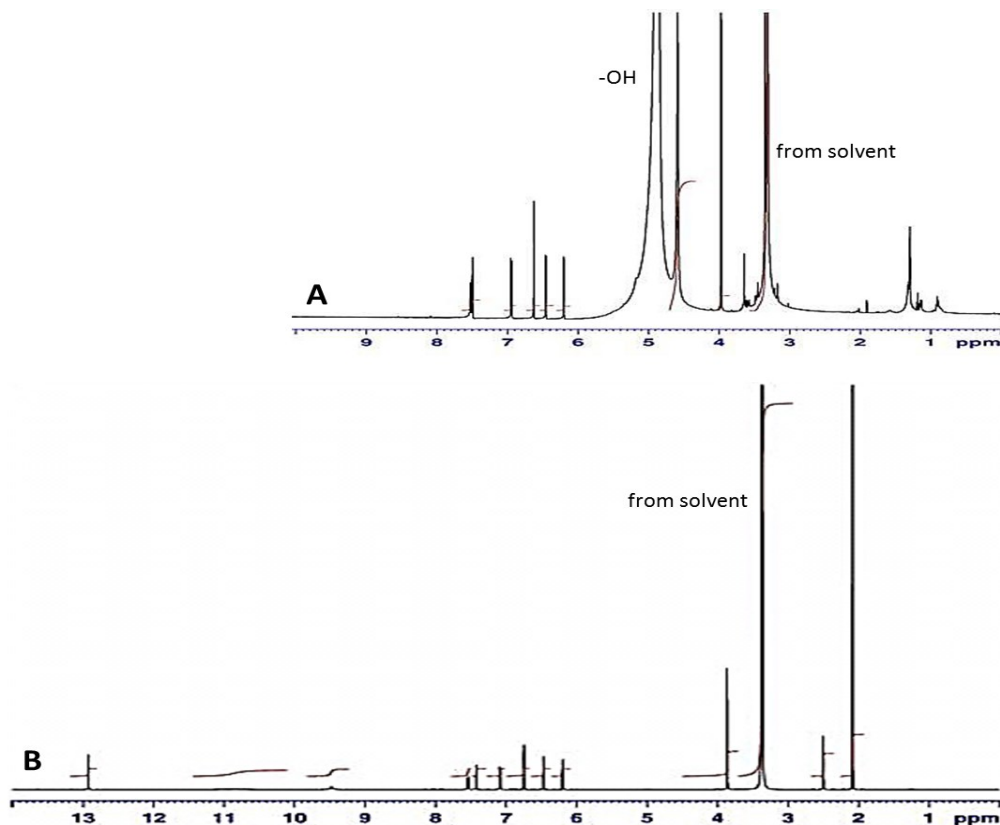
15 Eleven compounds of CP were characterised as vanillic acid, verbascoside, 4-coumaric acid, ferulic
16 acid, luteolin, nepetin, naringenin, hispidulin, hesperetin, and chrysin. However, using the above
17 criteria, hispidulin could not be reliably separated from the two isomeric forms: chrysoeriol and
18 diosmetin. They share the same MW, polarity and chromophores, and thus they cannot be
19 unambiguously identified by either MS or UV detection. ^1H NMR analysis of an enriched fraction of
20 the extract obtained by column chromatography was used to elucidate which of these isomers was
21 present. Fig. 1 shows that peaks observed in the enriched fraction matched those of hispidulin. No
22 matching NMR peaks for chrysoeriol and diosmetin were observed in the enriched extract (Fig. 2).
23 Thus, the molecule with MW 300 Da in CP is confidently confirmed as hispidulin by four independent
24 criteria of identification. Although hispidulin has been previously reported (Hazekamp *et al.*, 2001;
25 Klaiklay, 2009; Singharachai *et al.*, 2011), none of this earlier work has unambiguously excluded the
26 isomers by NMR spectroscopy.

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2 **Fig. 1.** NMR spectra (1H , 500 MHz in CD_3OD) of *C. petasites* extract and that of a hispidulin standard:
 3 (A) chemical structure of hispidulin, (B) NMR spectrum of hispidulin standard (aromatic region), (C)
 4 NMR spectrum (range 6-8 ppm) of an enriched fraction from the *C. petasites*, (D) full NMR spectrum
 5 (range 0-10 ppm) of hispidulin.



1 Fig. 2. NMR spectra (^1H , 500 MHz in CD_3OD) of (A) chrysoeriol and (B) diosmetin standards in the
 2 ranges of 0-10 ppm and 0-14 ppm, respectively.

3 3.3 Quantification of natural constituents in CP

4 Vanillic acid, verbascoside, nepetin, apigenin and hispidulin were quantified in the ethanolic extracts
 5 (Table 3). The amounts of the other six compounds, 4-coumaric acid, ferulic acid, luteolin,
 6 naringenin, hesperetin and chrysin, fell below their LOQs. Hispidulin was predominant at $39 \mu\text{mol/g}$
 7 followed by nepetin ($15 \mu\text{mol/g}$), verbascoside ($4 \mu\text{mol/g}$), vanillic acid ($3 \mu\text{mol/g}$) and apigenin (1
 8 $\mu\text{mol/g}$), respectively. Reproducibility of the ethanolic extraction among five different batches was
 9 good.

10 Table 3. Amounts of phenolic constituents in *C. petasites* from five extracts with MS detection in
 11 both negative and positive ion modes.

Batch no.	Amount ($\mu\text{mol/g}$) in dried extract of <i>C. petasites</i>				
	Vanillic acid	Verbascoside	Nepetin	Apigenin	Hispidulin
1	3.4	4.8	17.9	0.7	35.7
2	2.5	3.9	12.8	-	29.8
3	3.7	5.3	17.4	1.2	49.3
4	2.0	2.9	11.5	0.6	35.0
5	2.0	2.5	12.8	1.0	42.5
Average \pm SD	2.7 ± 0.8	3.9 ± 1.2	14.5 ± 2.9	0.9 ± 0.3	38.5 ± 7.6

12

13 3.4 Prediction of maximum fluxes (J_{max}) of the phenolic reference compounds

14 The feasibility of skin absorption of the eleven naturally-occurring phenolic compounds was
 15 evaluated by predicting their maximum fluxes (J_{max}) (Table 4) from their physicochemical properties:
 16 molecular weight (MW), octanol-water partition coefficient ($\log P$), and water solubility ($C_{\text{sat,w}}$)
 17 (ALOGPS 2.1 algorithm, 2001; Chempidder).

1 **Table 4.** Physicochemical properties (molecular weight (MW), octanol-water partition coefficient (log
 2 P), and water solubility ($C_{\text{sat,w}}$)) of the eleven phenolic compounds and their predicted maximum
 3 permeation rate (J_{max}).

Compound	MW (Da)	log P	$C_{\text{sat,w}}^{\text{b}}$ (mM)	Predicted J_{max} ($\text{nmol}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$)
Vanillic acid	168.2	1.4 ^a	38.0	73.3
Verbascoside	624.6	-0.03 ± 1.0 ^b	1.5	0.0005
4-Coumaric acid	164.2	1.5 ^a	11.0	23.5
Ferulic acid	194.2	1.5 ^a	9.5	14.6
Nepetin	316.3	2.0 ± 0.8 ^b	0.4	0.2
Luteolin	286.2	2.5 ^a	0.6	1.3
Apigenin	270.2	2.3 ± 0.6 ^b	0.8	1.5
Naringenin	272.3	2.5 ^a	1.1	3.0
Hispidulin	300.3	2.2 ± 0.7 ^b	0.4	0.4
Hesperetin	302.3	2.1 ^a	0.8	0.7
Chrysin	254.2	3.5 ^a	0.5	8.0

4 a = experimental value; b = calculated value

5
 6 The eleven compounds may be broadly categorised into three groups: phenolic acids, flavonoid
 7 aglycones, and a phenolic glycoside. Phenolic acids including vanillic acid, 4-coumaric acid and ferulic
 8 acid, have the highest predicted J_{max} because of their smaller size and greater water solubility.
 9 Flavonoid aglycones, nepetin, luteolin, apigenin, naringenin, hispidulin, hesperetin, and chrysin, have
 10 slightly larger MW and are less soluble in water than the phenolic acids; hence, their predicted
 11 penetration rates are slower. Verbascoside is the only phenolic glycoside in this study and contains
 12 two sugars, rhamnose and β -glucose; it has the highest MW as a result and a lower log P which
 13 (despite its reasonable water solubility) means that this compound has the lowest predicted J_{max} .

14 Overall, ten of the eleven compounds have predicted fluxes of at least $\sim 0.1 \mu\text{g}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$; that of
 15 verbascoside is so small that it seems very unlikely to result in any measurable therapeutic effect.
 16 The phenolic acids have physicochemical properties consistent with a reasonable skin permeability,
 17 i.e., modest MW and log P in the range of 1-3 (Hadgraft and Guy, 2003).

18 3.5 *In vitro* percutaneous absorption

19 To identify which of the naturally-occurring compounds in CP are able to penetrate through the skin
 20 and to evaluate their permeability, *in vitro* experiments using a pig skin model with simple
 21 formulations (50% w/w CP paste and a 50 mg·mL⁻¹ CP solution in 50:50 v/v ethanol:water) were
 22 performed over a 24 hour period. Both formulations represented models for Thai traditional
 23 preparations based on natural oils and alcohol. From a pharmaceutical view point, a paste can
 24 contain the highest amount of a powdered plant (up to 50% of the total recipe) whereas a solution is
 25 the dosage form that might be manipulated easily to achieve the maximum fluxes of the ingredients.
 26 Propylene glycol in the paste was used to disperse the powdered plant, to facilitate dissolution of
 27 hydrophobic ingredients and to increase skin penetration. 50% aqueous ethanol in the solution also
 28 acted as a powerful cosolvent and potential skin penetration enhancer; in addition, it has been
 29 reported to be an acceptable donor vehicle for *in vitro* diffusion experiments. Samples were
 30 withdrawn from the receptor solution at 3, 6 and 24 hours. Control experiments, in which no
 31 formulations were applied, revealed no penetration of any of the compounds present in CP.

32 Hispidulin was the only compound that penetrated from both formulations through the skin in an
 33 amount that could be quantified after 3 hours (Table 5). While both vanillic acid and nepetin applied
 34 as a CP solution were detectable in the receptor phase at 3 hours, they could not be quantified

1 because of their relatively high LOQs (74 and 158 mM, respectively) compared to that of hispidulin
 2 (42 mM). Verbascoside delivered from the CP paste was not detected in the receptor solution after
 3 24 hours because it is not soluble in the fatty base of this formulation; however, it was detected
 4 when delivered from the hydroalcoholic solution reflecting a better solubility.

5 **Table 5.** Quantities per unit area of skin surface of the eleven phenolic compounds detected in the
 6 receptor solution after 3, 6 and 24 hours.

Compound	Quantity per unit area of skin detected in receptor solution (average \pm SD, nmol·cm ⁻² , n=6)					
	50% w/w CP paste			50 mg·mL ⁻¹ CP solution (ethanol/water; 50:50)		
	3 h	6 h	24 h	3 h	6 h	24 h
Vanillic acid	0.1 \pm 0.07	0.5 \pm 0.3	4.4 \pm 2.9	-	0.4 \pm 0.2	8.0 \pm 3.7 ^a
Verbascoside	-	-	-	-	-	0.2 \pm 0.1
4-Coumaric acid	-	-	0.2 \pm 0.2	-	-	0.5 \pm 0.2
Ferulic acid	-	-	0.3 \pm 0.3	-	-	1.1 \pm 0.6
Nepetin	0.1 \pm 0.02	0.1 \pm 0.03	0.4 \pm 0.2	-	0.03 \pm 0.01	3.2 \pm 1.6 ^b
Luteolin	-	-	0.05 \pm 0.02	-	-	0.3 \pm 0.1
Apigenin	-	-	0.1 \pm 0.1	-	-	0.9 \pm 0.2
Naringenin	-	-	-	-	-	0.01 \pm 0.002
Hispidulin	0.2 \pm 0.2	0.7 \pm 0.5	4.0 \pm 2.4	0.1 \pm 0.1	1.5 \pm 0.8	21.4 \pm 3.7 ^b
Hesperetin	-	-	0.2 \pm 0.1	-	-	0.3 \pm 0.1
Chrysin	-	-	-	-	-	-

7 a = significant difference at P < 0.05; b = significant difference at P < 0.001 when compare the two formulations at the same
 8 sampling time.

9 Direct comparison of the theoretically predicted J_{max} values in Table 4 with the experimental data in
 10 Table 5 is not possible because the degrees of saturation of the different compounds in the two
 11 formulations are unknown. However, from a qualitative standpoint, it is perhaps reassuring to
 12 observe that verbascoside was expected to penetrate the skin very poorly and this was indeed the
 13 case. Equally, vanillic acid was well-absorbed and this was consistent with the relatively high J_{max}
 14 predicted from the model; nonetheless, the measured penetration of this compound was well below
 15 that anticipated from J_{max} suggesting that vanillic acid was present in the formulations at levels much
 16 less than the saturation concentration. The same is almost certainly true for 4-coumaric acid, ferulic
 17 acid and chrysin, for which no detectable skin penetration was found. Interestingly, the predicted
 18 J_{max} values of nepetin and hispidulin would roughly suggest absorptions of 4.8 and 9.6 nmol·cm⁻²,
 19 respectively, in 24 hours, values not that different from those observed experimentally (and
 20 suggesting, therefore, that these constituents were close to saturation in the vehicles).

21 Various types of bioactivity have been reported for vanillic acid, nepetin and hispidulin, such as
 22 antimicrobial (Delaquis *et al.*, 2005; Sultana and Afolayan, 2007), anti-inflammatory (Clavin *et al.*,
 23 2007; Gil *et al.*, 1994; Kim *et al.*, 2011), and antioxidant (Kang *et al.*, 2009), that may be used to
 24 predict the potential pharmacological activities of topical CP products in Thai traditional medicine.
 25 Interestingly, even though verbascoside is unlikely to penetrate through the skin, it possesses
 26 antimicrobial activity against *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli*, and
 27 *Pseudomonas aeruginosa* (Shikanga *et al.*, 2010). Thus, it may at least be beneficial as a topical
 28 antimicrobial compound.

29 4. Conclusions

30 We have examined the profile of occurrence and skin penetration of polyphenolic compounds of *C.*
 31 *petasites* to evaluate the topical uses of this plant in Thai traditional medicine. Nine phenolic
 32 compounds, vanillic acid, 4-coumaric acid, ferulic acid, verbascoside, nepetin, luteolin, chrysin,
 33 naringenin, and hesperetin that have not previously been reported from the species, plus apigenin

1 and hispidulin, were identified in CP extracts. Hispidulin was quantified as a predominant compound,
2 being present at 39 $\mu\text{mol/g}$ in a dried ethanolic extract (equal to 0.04% by weight in dried crude
3 plant material). Using a pig skin model, vanillic acid, nepetin and hispidulin were found to be able to
4 penetrate the skin. Reasonable predicted maximum fluxes and together with biological activities
5 reported elsewhere, of these compounds potentially support the topical clinical uses of this plant in
6 Thai folklore.

7 The results in this study illustrate that topical bioavailability of individual constituents of herbal
8 preparations may limit which compounds actually act at a pharmacological level, and that this is
9 determined by both the levels in the plant and the ability of the compound to penetrate the skin. In
10 particular, various glycosides, which commonly occur in plant materials and which possess a wide
11 range of potentially useful biological properties, may not be absorbed transdermally. The results
12 reported here show that such compounds are readily identified from their physicochemical
13 properties. However, compounds with modest molecular weight and a balanced lipophilicity (as
14 measured by log P), are both predicted and demonstrated to have reasonable percutaneous
15 absorbability. Taken together, a quantitative analysis of the plant material, the application of the
16 Potts and Guy equation, and knowledge of the *in vitro* biological activities of individual chemical
17 components provide a useful approach to the evaluation of traditional topical herbal preparations
18 and for further formulation optimisation of those products.

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