Anti-MMP-2 Activity and Skin-Penetrating Capability of the Chemical Constituents from *Rhodiola rosea*

**Authors**

Tzong-Huei Lee¹, Chieh-Chih Hsu², George Hsiao³, Jia-You Fang⁴,⁵, Wei-Min Liu⁶, Ching-Kuo Lee²,⁷

**Affiliations**

The affiliations are listed at the end of the article

**Key words**

- *Rhodiola rosea*
- Crassulaceae
- matrix metalloproteinase (MMPs)
- collagenase
- 1,2,3,6-tetra-O-galloyl-4-O-hydroxybenzoyl-β-D-glucopyranoside (E)-creoside I
- (Z)-2-methyl-2-hepten-1,6-diol

**Abstract**

Based on the significant inhibitory activity toward matrix metalloproteinase-2 and collagenase noticed in preliminary studies, crude extracts of *Rhodiola rosea* were partitioned and chromatographed sequentially to afford three new compounds, 1,2,3,6-tetra-O-galloyl-4-O-hydroxybenzoyl-β-D-glucopyranoside (1), (E)-creoside I (2), and (RZ)-2-methylhept-2-ene-1,6-diol (3), along with twenty-four known compounds (4–27). Their structures were determined by spectroscopic data analyses. All isolated compounds were subjected to bioactivity assays. In these, 1 specifically inhibited matrix metalloproteinase-2 activity with an IC₅₀ value of 16.3 ± 1.6 µM, while its analogue 1,2,3,6-tetra-O-galloyl-β-D-glucopyranoside (17) inhibited matrix metalloproteinase-2 with an IC₅₀ value of 23.0 ± 4.8 µM. In the collagenase activity assay, the inhibitory effects of 1 and 17 at concentrations of both 20 and 40 µM were more potent than those of the positive control, 1,10-phenanthroline. In order to realize whether 17 could penetrate from the epidermal layer into the basal and dermal layers of the human skin to inhibit the activity of matrix metalloproteinase-2 and collagenase or not, a transdermal penetration test in nude and white mice skins was performed. Penetration percentages of 17 quantified by LC–MS were 27.8% and 74.8% in 24 hours, respectively.

**Supporting information** available online at http://www.thieme-connect.de/products

**Introduction**

Matrix metalloproteinases (MMPs) are a family of zinc- and calcium-dependent endopeptidases, which are grouped into the metzincin clan of metallopeptidases (MPs) together with other families such as ADAMs/adamalysins, astacins, fragilysins, and serralysins. MMPs are found throughout the animal and plant kingdoms, where their distribution is consistent with a Darwinian tree-based pathway [1]. Furthermore, polyplication has led to the presence of several paralogous MMP genes in the same organism: 24 in humans, 26 in sea urchins, 26 in zebrafish, 7 in sea squirts, and 2 in fruit flies. Overexpression of MMPs may cause various inflammatory, malignant, and degenerative symptoms [2]. Among the MMPs studied, gelatinase-A (MMP-2), gelatinase-B (MMP-9), and collagenase-1 (MMP-1) were reported to be responsible for the signal transduction of dermal photoaging [3], and inhibition of the activities of these two enzymes could potentially slow down skin aging. Thus, the search for bioactive compounds that can regulate the activities of MMP-1, -2, and -9 from natural resources is one of the key steps in delaying skin aging.

*Rhodiola rosea* L. (Crassulaceae), an herbaceous plant, is used in Asian and Eastern European traditional medicines for its pressure-reducing [4], neurosystem-stimulating [5], fatigue-removing [6], hypobaropathy-preventing [7], antidepressive [7], anticancer [8], antiaging [9], cardiovascular-protective [10], and hepatoprotective effects [11]. Chemical investigations on this plant have revealed many chemical entities, including phenolic acids [12], phenoletiolanoids [13], phenylpropenoids [14], flavonoids [15], monoterprenes [4], and glycosides [16]. In a preliminary biological evaluation, crude extracts of *R. rosea* roots exhibited inhibitory activities toward MMP-2 and collagenase at a concentration of 100 µg/mL [17]. An investigation of the active principles of this plant was thus undertaken by using a bioassay-guided method. Of the tested water, n-butanol, and ethyl acetate layers, the ethyl acetate layer...
was the most potent. The subsequent isolation and identification of bioactive components was focused on this layer and led to the isolation and characterization of three new compounds (1–3; [Fig. 1]) and twenty-four known compounds (4–27). The isolation and structural elucidation of the previously unreported compounds are described in this paper along with their bioactivities.

Results and Discussion

An ethanolic extract of the roots of <i>R. rosea</i> was partitioned in a ternary preparative manner to give an ethyl acetate soluble layer. Flash column separation of this layer over silica gel afforded three previously unreported chemical entities (1–3) along with twenty-four known compounds. The known compounds were identified to be methyl 4-hydroxybenzoate (4) [18], β-sitosterol (5) [19], (Z)-2-methyl-6-oxo-2-hepten-1-ol (6) [20], gallic acid (7) [21], 4-hydroxyphenyl-2-ethyl β-D-glucopyranoside (8) [22], tyrosol (9) [23], methyl gallate (10) [24], p-coumaric acid (11) [25], caffeic acid (12) [25], aspergillol B (13) [26], 2,3R-dihydrokaempferol (14) [27], (E)-2-methyl-6-oxo-2-hepten-1-ol (15) [17], 1,2,6-tri-O-galloyl-β-D-glucopyranoside (16) [28], 1,2,3,6-tetra-O-galloyl-β-D-glucopyranoside (17) [28], p-hydroxyphenethyl alcohol 1-O-D-β-D-galloyl-glucopyranoside (18) [29], herbacetin 7-O-α-L-rhamnopyranoside (19) [30], kaempferol 3-O-α-L-rhamnopyranoside (20) [30], epicatechin-3-O-gallate (21) [31], 1,1-dimethylprop-2-en-1-yl-β-D-glucopyranoside (22) [20], 3-methylbut-2-en-1-yl-β-D-glucopyranoside (23) [32], (Z)-croside I (24) [33], creoside III (25) [33], salidroside (26) [34], and icariside D2 (27) [35] based on spectroscopic data analysis and comparison to the literature.

Compound 1, obtained as amorphous white powder, had a formula of C₄₁H₃₂O₂₄ as determined using ¹³C NMR (Table 1), as well as a pseudo-molecular ion [M – H]⁺ at m/z 907.1241 in negative ion high-resolution electrospray ionization mass spectrometry (HR-ESIMS). IR absorption bands of 1 showed a conjugated ester carbonyl (1702 cm⁻¹) along with aromatic functionalities (1609 and 1536 cm⁻¹). In the ¹³C NMR spectrum of 1 (Table 1), signals at δ₁³C 62.44 (1 H, d, d, = 8.3 Hz, H-1), 5.58 (1 H, dd, d, = 8.3, 9.6 Hz, H-2), 5.91 (1 H, t, J = 9.6 Hz, H-3), 5.63 (1 H, t, J = 9.6 Hz, H-4), 4.40 (1 H, H-5), 4.41 (1 H, H-6a), 4.51 (1 H, d, d, = 10.3 Hz, H-6b), 7.05 (2 H, s, H-2′, -6′), 6.95 (2 H, s, H-2″, -6″), 6.80 (2 H, s, H-2‴, -6‴), and 7.10 (2 H, s, H-2‴‴, -6‴‴) were attributable to a β-D-glucopyranoside bearing four galloyl groups at C-1, -2, -3, and C-6 via ester linkages; this conclusion was corroborated by the large mutual coupled J values of H-1–H-5 and heteronuclear long-range correlations in the heteronuclear multiple-bond correlation (HMBC) experiment (Fig. 2A). Two additional mutual coupled resonances at δ₁³C 67.5 (H-2, H-4) and 7.78 (2 H, d, J = 8.7 Hz) were characteristic signals for p-hydroxybenzoyl located at C-4 as evidenced by a cross-peak of H-4/7‴‴‴ in the HMBC spectrum. Accordingly, 1 was determined to be 5,7-bis[(α-L-rhamnopyranosyl)-(2-O-D-glucopyranosyl)]-1,2,3,6-tetragalloyl-D-glucopyranose (5,7-dibromo-1,2,3,6-tetragalloyl-D-glucopyranose).

Table 1 ¹³C (125 MHz) and ¹H NMR (500 MHz) data for compound 1 (in CD₃OD).

<table>
<thead>
<tr>
<th>Position</th>
<th>¹³C</th>
<th>¹H</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>94.3</td>
<td>6.24 (1 H, d, J = 8.3 Hz)</td>
</tr>
<tr>
<td>2</td>
<td>72.6</td>
<td>5.58 (1 H, dd, d, = 8.3, 9.6 Hz)</td>
</tr>
<tr>
<td>3</td>
<td>74.5</td>
<td>5.91 (1 H, t, J = 9.6 Hz)</td>
</tr>
<tr>
<td>4</td>
<td>70.5</td>
<td>5.63 (1 H, t, J = 9.6 Hz)</td>
</tr>
<tr>
<td>5</td>
<td>74.7</td>
<td>4.40 (1 H)</td>
</tr>
<tr>
<td>6</td>
<td>63.7</td>
<td>8.41 (1 H)</td>
</tr>
<tr>
<td>1‴</td>
<td>120.2</td>
<td></td>
</tr>
<tr>
<td>2‴, 6‴</td>
<td>111.1</td>
<td>7.05 (2 H, s)</td>
</tr>
<tr>
<td>3‴, 5‴</td>
<td>147.0</td>
<td></td>
</tr>
<tr>
<td>4‴</td>
<td>141.2</td>
<td></td>
</tr>
<tr>
<td>7‴</td>
<td>166.7</td>
<td></td>
</tr>
<tr>
<td>1‴‴</td>
<td>120.7</td>
<td></td>
</tr>
<tr>
<td>2‴‴, 6‴‴</td>
<td>110.8</td>
<td>6.80 (2 H, s)</td>
</tr>
<tr>
<td>3‴‴, 5‴‴</td>
<td>146.7</td>
<td></td>
</tr>
<tr>
<td>4‴‴</td>
<td>140.6</td>
<td></td>
</tr>
<tr>
<td>7‴‴</td>
<td>167.8</td>
<td></td>
</tr>
<tr>
<td>1‴‴‴</td>
<td>121.5</td>
<td></td>
</tr>
<tr>
<td>2‴‴‴, 6‴‴‴</td>
<td>133.6</td>
<td>7.78 (2 H, d, J = 8.7 Hz)</td>
</tr>
<tr>
<td>3‴‴‴, 5‴‴‴</td>
<td>116.7</td>
<td>6.75 (2 H, d, J = 8.7 Hz)</td>
</tr>
<tr>
<td>4‴‴‴</td>
<td>163.3</td>
<td></td>
</tr>
<tr>
<td>7‴‴‴</td>
<td>167.1</td>
<td></td>
</tr>
<tr>
<td>1‴‴‴‴</td>
<td>121.4</td>
<td></td>
</tr>
<tr>
<td>2‴‴‴‴, 6‴‴‴‴</td>
<td>110.8</td>
<td>7.10 (2 H, s)</td>
</tr>
<tr>
<td>3‴‴‴‴, 5‴‴‴‴</td>
<td>146.9</td>
<td></td>
</tr>
<tr>
<td>4‴‴‴‴</td>
<td>140.4</td>
<td></td>
</tr>
<tr>
<td>7‴‴‴‴</td>
<td>168.4</td>
<td></td>
</tr>
</tbody>
</table>

*Signals without multiplicity were overlapped and picked up from the HSQC spectrum.

Lee T-H et al. Anti-MMP Activity and...
two methyls [δH 2.12 (3 H, s, H3-7) and 1.69 (3 H, s, H3-8)] in the residual aglycone part, three methylenes [δH germinal coupled 4.02 and 4.18 (each 1 H, d, J = 11.5 Hz, H2-1), vicinal coupling [2.28 (2 H, m, H2-4) and 2.53 (2 H, t, J = 7.0 Hz, H2-5)], and an olefinic methine [δH 5.42 (1 H, t, J = 6.5 Hz, H-3)]. In the HMBC spectrum of 2 (Fig. 2B), cross-peaks of δH 1.69 (H3-8)/δC 75.6 (C-1), 133.9 (C-2) and 128.3 (C-3), δH 5.42 (H-3)/δC 23.0 (C-4) and 43.7 (C-5), δH 2.12 (H2-7)/δC 211.4 (C-6) and 43.7 (C-5) and δH 4.22 (H-1′)/δC 75.6 (C-1) indicated that the aglycone of 2 was connected with the C-1′ of β-D-glucopyranoside via an ether linkage to form creoside I. The configuration of Δ2 was determined to be in E form owing to the δC 14.1 of C-8 in 2 in contrast with the δC 21.9 of C-8 in (Z)-creoside I (25). Consequently, the structure of 2 was elucidated as shown and the compound was named (E)-creoside I. Compound 3 was obtained as oil with an optical rotation [α]25D = -42.2° (c 0.23, MeOH). Comparison of the 1H and 13C NMR spectra of 3 and 2 revealed the major distinctive differences to be primary alcohol (δC 60.0, C-1), a secondary hydroxyl group (δC 66.5, C-6), and Z form Δ2 (δC 20.2, C-8) in 3 as opposed to the ether group (δC 75.6, C-1), ketone group (δC 211.4, C-6), and E form Δ2 (δC 14.1, C-8) in 2, respectively. The configuration of the Δ2 in 3 was also verified by a nuclear Overhauser effect (NOE) enhancement signal of H-3 at δH 1.75 after radiation of H-3 at δH 5.27, which indicated a Z form double bond. The chirality of C-6 was determined to be R by comparing the optical rotation of 3, [α]25D = -42.2°, with that of (R)-6-methylhept-5-en-2-ol, [α]25D = -14.5° [36]. Thus, compound 3 was assigned as shown and named (R,Z)-2-methylhept-2-ene-1,6-diol.

In the MMP inhibitory activity assay, 1,2,3,6-tetra-O-galloyl-4-O-p-hydroxybenzoyl-β-D-glucopyranoside (1) inhibited MMP-2 activity with an IC50 of 16.3 ± 1.6 µM, while its analog 1,2,3,6-tetra-O-galloyl-β-D-glucopyranoside (17) inhibited MMP-2 with an IC50 value of 23.0 ± 4.8 µM. In the collagenase inhibitory activity assay, the effects of 1 and 17 at concentrations of both 20 and 40 µM were significant and more potent than those of the positive control, 1,10-phenanthroline (Table 3). When a concentration of 20 µM of compound 17 was applied, an inhibitory activity of over 50% could be achieved. Owing to its promising activity in the enzymatic assay and in an attempt to realize whether 17 could penetrate from the epidermal layer into the basal and dermal layers of human skin or not, a transdermal penetration assay was performed using nude and white mouse skins. The nude mouse skin with sparse hair follicles mimicked human skin, and the white mouse skin with compact hair follicles was used for comparison. The transdermal penetration percentages of 17 in the nude mouse skin and the white mouse skin were 27.8% and 17% for comparison. The transdermal penetration percentages of 17 was performed using nude and white mouse skins. The nude mouse skin with sparse hair follicles mimicked human skin, and the white mouse skin with compact hair follicles was used for comparison. The transdermal penetration percentages of 17 in the nude mouse skin and the white mouse skin were 27.8% and 17% for comparison. The transdermal penetration percentages of 17 in the nude mouse skin and the white mouse skin were 27.8% and 17% for comparison.
trate from the epidermal layer into the basal and dermal layers of human skin to inhibit the activity of MMP-2 and collagenase, and to mitigate skin aging.

Materials and Methods

General experimental procedures

Optical rotations were measured on a JASCO P-1020 polarimeter. 1H and 13C NMR were performed with a Bruker Avance DRX-500 spectrometer. Low- and high-resolution mass spectra were obtained using an ABI API 4000 Q-TRAP ESI-MS and a Waters LCT Premier™XE HR-ESI-MS, respectively. IR spectra were recorded on a JASCO FT/IR 4100 spectrometer.

Chemicals and reagents

HPLC grade solvents, n-hexane, ethyl acetate, methanol, and acetone, triethyl were purchased from J.T. Baker. Epigallocatechin gallate, genistein, and 1,10-phenanthroline (all purities > 95%) were purchased from Sigma-Aldrich.

Plant materials

Dried roots of R. rosea were purchased from Scientific Pharmaceutical Elite Company (Batch No. 106), Taipei, Taiwan, on September 8, 2009. A voucher specimen (No. TMU090908) was identified by Hsiu-Wen Huang, Taiwan Endemic Species Research Institute, ChiChi, Taiwan, and deposited at the Institute of Pharmacognosy, Taipei Medical University, Taipei, Taiwan.

Extraction and isolation

Dried roots (10.0 kg) of R. rosea were smashed and extracted three times with 40 L of ethanol, which was filtered and rotary evaporated to give a black residue (1758 g). This residue was then suspended in H2O (3 L) and partitioned with an equal volume of ethyl acetate three times. The ethyl acetate layer was evaporated to dryness under vacuum (415 g). Subsequently, the dried ethyl acetate layer (250 g) was mixed with 375 g of silica gel (70–230 mesh, Merck), and was loaded onto a conditioned open column packed with 3550 g of silica gel and eluted via a stepwise gradient method by using mixtures of n-hexane, ethyl acetate, and methanol. Five hundred ml were collected for each fraction and analyzed by TLC. TLC was performed on silica gel 60 F254 plates (Merck) by using mixtures of n-hexane–ethyl acetate for development, and spots were detected by spraying with vanillin-sulfuric acid followed by heating. Then, all fractions were combined into six portions (I–VI) according to the results of the TLC analyses; they were then redissolved in a minimum volume of the n-hexane/ethyl acetate mixtures for subsequent HPLC analysis. Portion II eluted by n-hexane/ethyl acetate (95:5) was purified by performing semipreparative HPLC (Hibar® Fertigsäule, 10 × 250 mm) using n-hexane/ethyl acetate (96:4) as the eluent at a flow rate of 3 mL/min to afford 6 (42.5 mg, tR = 19.8 min), 3 (6.1 mg, tR = 21.2 min), 5 (32 mg, tR = 23.5 min), and 7 (79 mg, tR = 28.0 min). The same portion was purified by performing semipreparative HPLC (Phenomenex® Luna, 10 × 250 mm) using n-hexane/ethyl acetate (99:1) as the eluent at a flow rate of 3 mL/min to afford 4 (36 mg, tR = 32.5 min). Portion III, eluted by n-hexane/ethyl acetate (90:10), was purified by performing semipreparative HPLC (Phenomenex® Luna, 10 × 250 mm) using n-hexane/acetone (99:1) as the eluent at a flow rate of 3 mL/min to afford 8 (1.7 mg, tR = 19.3 min). Portion IV, eluted by n-hexane/ethyl acetate (80:20), was purified by performing semipreparative HPLC (Phenomenex® Luna, 10 × 250 mm) using n-hexane/ethyl acetate (85:15) as the eluent at a flow rate of 3 mL/min to afford 10 (19 mg, tR = 24.4 min), 11 (12 mg, tR = 26.9 min), 9 (25 mg, tR = 33.8 min), 10 (31 mg, tR = 37.0 min), and 11 (24 mg, tR = 42.5 min). The same portion was purified using the same column by using n-hexane/ethyl acetate (78:22) as the eluent at a flow rate of 3 mL/min to obtain 14 (10 mg, tR = 20.1 min) and 13 (22 mg, tR = 24.6 min). The same portion was purified by the same column using n-hexane/ethyl acetate/acetone (85:10:10) as the eluent at a flow rate of 3 mL/min to obtain 12 (25 mg, tR = 13.2 min), 17 (55 mg, tR = 16.3 min), 16 (132 mg, tR = 21.8 min), and 18 (39 mg, tR = 25.6 min). Portion V, eluted by n-hexane/ethyl acetate (60:40), was purified by performing semipreparative HPLC (Hibar® Fertigsäule, 10 × 250 mm) using n-hexane/ethyl acetate/acetone (68:27:5) as the eluent at a flow rate of 3 mL/min to afford 20 (5.3 mg, tR = 10.2 min), 22 (63 mg, tR = 20.0 min), 21 (84 mg, tR = 22.0 min), and 23 (33 mg, tR = 26.5 min). The same portion was purified by performing semipreparative HPLC (Hibar® Fertigsäule, 10 × 250 mm) using n-hexane/ethyl acetate (72:28) as the eluent at a flow rate of 3 mL/min to afford 2 (24 mg, tR = 24.3 min), 24 (4.6 mg, tR = 32.3 min), and 25 (33 mg, tR = 38.7 min). Portion VI, eluted by n-hexane/ethyl acetate (40:60), was purified by performing semipreparative HPLC (Hibar® Fertigsäule, 10 × 250 mm) using n-hexane/ethyl acetate

Table 3  The inhibitory effects of compounds 1 and 17 on collagenase activity.

<table>
<thead>
<tr>
<th>Samples</th>
<th>1</th>
<th>17</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>20 µM</td>
<td>40 µM</td>
<td>20 µM</td>
</tr>
<tr>
<td>Inhibitory effectiveness (%)</td>
<td>23.51 ± 2.81*</td>
<td>47.50 ± 2.90</td>
<td>52.12 ± 4.30***</td>
</tr>
</tbody>
</table>

The percentual inhibition is shown as mean ± SD of three independent experiments. * P < 0.05, ** p < 0.01, and *** p < 0.0001 compared with the positive control group. Positive control: 1,10-phenanthroline

Table 4  Results of the transdermal penetration test of compound 17 through nude mice and white mice skins.

<table>
<thead>
<tr>
<th>Sample mice</th>
<th>The amount of 17 applied onto the skins (µg)</th>
<th>Average (µg)</th>
<th>Penetration percentage of 17 into the mouse skins (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nude mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.42</td>
<td>1.17 ± 0.21</td>
<td>27.8</td>
</tr>
<tr>
<td>2</td>
<td>1.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>1.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White mice</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>8.82</td>
<td>6.07 ± 2.43</td>
<td>74.8</td>
</tr>
<tr>
<td>5</td>
<td>4.23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>5.16</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Tris-base, 200 mM NaCl, 5 mM CaCl₂, and 0.02% Brij 35; pH 7.5) at
Afterwards, the gel was incubated with a reacting buffer (50 mM
amide gel electrophoresis (PAGE). The PAGE gel contained 1% gel-
pounds was higher than 95% as checked by HPLC. Supernatants
chin gallate (EGCG, 100 µM) as a positive control followed by an
ν
for 1 Ha n d13 C NMR data see
–
version 99. The formula used to determine the inhibitory effec-
tives
1 H, 13 C, and 2D NMR spectra, including COSY, NOESY, HMQC, and
HMBC spectra, which were further supported by IR, LR-, HR-MS,
and optical rotation data.
1,2,3,6-Tetra-O-galloyl-4-O-p-hydroxybezoyl-β-D-glucopyra-
side (1): Amorphous white powder; [α]_
D
25
+ 38.75 (c 0.08, CH₂OH); IR (neat) ν
max
3375, 1702, 1569, 1396; negative HRESIMS m/z
907.1241 [M − H]⁺ (calcld. for C₄₅H₃₁O₂₄, 907.1205); for 1 H and
13 C NMR data see Table 1. (E)-Creoside I (2): Amorphous white powder; [α]_
D
20
− 58.0 (c 0.05, CH₂OH); IR (neat) ν
max
3389, 1698; ESIMS m/z 327.3 [M + Na]⁺;
for 3 H and 13 C NMR data see Table 2. (R,Z)-2-Methylhept-2-ene-1,6-diol (3): Colorless oil; [α]_
D
17
− 42.2 (c 0.23, CH₂OH); ESIMS m/z 166.2 [M + Na]⁺; for 1 H and 13 C NMR data see Table 2.
Matrix metalloproteinase-2 inhibitory activity assay
To evaluate the MMP-2 inhibitory activity of the galloyl deriva-
tives 1, 7, and 16–19, gelatin zymography was conducted [37]. Briefly, HT1080 cell suspension (5 × 10⁵ cells/mL) was placed in 24-well cell culture plates for 24 h of incubation at 37 °C. Subse-
tively, the cells were treated with the vehicle (DMSO), com-
pounds 1, 7, and 16–19 (10, 20, 50, and 100 µM), or epigallocate-
chin gallate (EGCG, 100 µM) as a positive control followed by an
incubation at 37 °C for 24 h. The purity of all of the tested com-
pounds was higher than 95% as checked by HPLC. Supernatants
were mixed with a sample-loading dye (the composition was
500 mM Tris-HCl, 25% glycerol, 10% SDS, and 0.32% bromopho-
ramide gel electrophoresis (PAGE). The PAGE gel contained 1% gel-
atin and 10% polyacrylamide, where gelatin acted as a substrate
for MMP-2. After electrophoresis, the gel was washed twice with
2.5% Triton X-100 at 24 °C for 30 min to remove the dye and SDS.
Afterwards, the gel was incubated with a reacting buffer (50 mM
Tris-base, 200 mM NaCl, 5 mM CaCl₂, and 0.02% Brij 35; pH 7.5) at
37 °C for 24 h. A fixing solution (7% acetic acid and 40% metha-
nol) was subsequently applied to the gel for 30 min, and the
gel was stained with Brilliant Blue G-Colloidial, and then destained
within a larger amount, a transdermal penetration test was per-
formed. The apparatus used for this experiment was a Franz-type
diffusion cell, consisting of a set of vertical double-diffusion and
detachable glass containers. The upper donor chamber of the cell
is a hollow cylinder for sample injection, the bottom contact sur-
facing upwards) was fixed using metal clips to serve as the in
vitro transdermal penetration barrier. The experimental method
is presented below. First, compound 17 was dissolved in 30 wt%
propylene glycol aqueous solution, followed by a transfer of
0.5 mL of aqueous solution (containing 0.25 mg) of compound 17
into the donor chamber of the Franz-type diffusion cell. The top
of the open-ended donor chamber was covered tightly with Par-
afilm®, and the inner receptor chamber was filled with 5.5 mL of
the buffer solution (30 wt% ethanol and phosphate buffer solu-
tion; pH 7.4), under continuously stirring at 600 rpm. The buffer
solution was sampled (0.3 mL) at time points of 1, 2, 4, 6, 8, 10, 12,
24, 36, and 48 h, and was replaced with an equal volume (0.3 mL)
of the buffer solution to maintain the same volume of fluid in the
diffusion cell. Finally, the accumulated compound 17 in the solu-
tion in the receptor chamber was analyzed by liquid chromato-
graphy-tandem mass spectrometry (LC-MS/MS), and the results
represented the amount of compound 17 that could penetrate
through the skin and reach the blood vessels. After completing
this experimental step, deionized water was used to wash compo-
dound 17 off the nude and white mouse skin surfaces. Thereafter,
the skins were taken out of the apparatus and trimmed to obtain a
circle with a diameter equal to that of the donor chamber by
using surgical scissors, and the weight of the sample was recorded.
The skin samples were then homogenized with 1 mL of alcohol at
300 rpm for 5 min, followed by centrifugation at 10 000 rpm for
5 min. The supernatant was filtered using 0.45 µm PVDF and the
amount of compound 17 in the skin was quantified by perform-
ing LC-MS. Genistein was used as a positive control following the
same method.
Transdermal penetration test using
high-performance liquid chromatography
tandem mass spectrometry
The HPLC-MS/MS apparatus used consisted of an Agilent 1100
HPLC system coupled to an Applied Biosystems 4000 triple quad-
rupole mass spectrometer with an electrospray ionization source
for the simultaneous detection of 1,2,3,6-tetra-O-galloyl-β-D-
glucopyranoside (17) and genistein. The chromatographic sepa-
performed using Analyst 1.4 software (AB SCIEX). The spray temperature was 425 °C. Data acquisition and processing for mass detection were as follows: curtain gas, 10 psi; nebulizer gas (Gas 1), 60 psi; auxiliary gas (Gas 2), 65 psi; and turbo ion spray temperature, 425 °C. Data acquisition and processing were performed using Analyst 1.4 software (AB SCIEX).

### Statistical analysis

The experimental results are expressed as mean ± standard error. Data were assessed using the Student-Newman-Keuls test and p < 0.05, p < 0.01, and p < 0.001 was considered significant.

### Supporting information

Spectral data of compounds 1–3 are available as Supporting Information.

### Acknowledgments

We are grateful to Dr. Shwu-Huey Wang, Ms. Shou-Ling Huang, and Ms. Shyu-Yun Sun of the Instrumentation Center of Taipei Medical University and the Instrumentation Center of the College of Science, National Taiwan University, respectively, for NMR and MS data acquisition. We also want to thank Taipei Medical University Hospital (101 TMU-TMUH-18) and the Ministry of Science and Technology of the Republic of China for financial support (98–2320-B-038-015-MY3 and 101–2320-B-038-013-MY3).

### Conflict of Interest

The authors declare no conflict of interest.

### Affiliations

1 Institute of Fisheries Science, National Taiwan University, Taipei, Taiwan
2 Graduate Institute of Pharmacognosy, Taipei Medical University, Taipei, Taiwan
3 Graduate Institute of Medical Science and Department of Pharmacology, College of Medicine, Taipei Medical University, Taipei, Taiwan
4 Research Center for Industry of Human Ecology, Chang Gung University of Science and Technology, Kweishan, Taoyuan, Taiwan
5 Pharmaceuticals Laboratory, Graduate Institute of Natural Products, Chang Gung University, Kweishan, Taoyuan, Taiwan
6 Department of Obstetrics and Gynecology, Taipei Medical University Hospital, Taipei, Taiwan
7 School of Pharmacy, Taipei Medical University, Taipei, Taiwan

### References

17 Hsu CC. Anti-aggregating agents of Rhodiola rosea [master thesis]. Taipei, Taiwan: Taipei Medical University; 2004
18 Penner GH, Wasylisien RE. A carbon-13 CP/MAS nuclear magnetic resonance study of several 1,4-disubstituted benzenes in the solid state. Can J Chem 1989; 67: 525–534

Original Papers