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

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Key words

- *Rhodiola rosea*
- Crassulaceae
- matrix metalloproteinase
- collagenase
- 1,2,3,6-tetra-O-galloyl-4-O-p-hydroxybenzoyl-β-D-glucopyranoside
- (E)-croside 1
- (2Z)-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-p-hydroxybenzoyl-β-D-glucopyranoside (1), (E)-croside 1 (2), and (R,Z)-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 metalloproteinases (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, polyphyly 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], phenoethanoids [13], phenylpropenoids [14], flavonoids [15], monoterpenes [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...
Results and Discussion

An ethanolic extract of the roots of *R. rosea* was partitioned in a preliminary 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], 2R,3R-dihydrokämpferol (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-(6″-O-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-methyl-but-2-en-1-yl-β-D-glucopyranoside (23) [32], (Z)-croeside 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 C14H18O2Na 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 1H NMR spectrum of 1 (Table 1), signals at δH 6.24 (1 H, d, J = 8.3 Hz, H-1), 5.58 (1 H, dd, J = 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, J = 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 δH 6.75 (2 H, d, J = 8.7 Hz) and 7.78 (2 H, d, J = 8.7 Hz) were characteristic signals for p-hydroxybenzoyl located at CO-4 as evidenced by a cross-peak of H-4/7‴‴ in the HMBC spectrum. Accordingly, 1 was determined as shown and named 1,2,3,6-tetra-O-galloyl-4-O-p-hydroxybenzoyl-β-D-glucopyranoside.

Compound 2 was isolated as amorphous white powder with the molecular formula C16H24O7, as determined by positive ion HR-ESIMS, and showed an [M + Na]+ ion at m/z 327.1428 (calcld. for C16H24O7Na, 327.1420). Conspicuous absorptions at 3389 and 1689 cm−1 in the IR spectrum of 2 indicated the presence of hydroxyl and ketone functionalities, respectively. The 1H NMR (Table 2) spectrum coupled with the correlation spectroscopy (COSY) spectrum of 2 showed signals at δH 4.22 (1 H, d, J = 8.0 Hz, H-1′), 3.18 (1 H, dd, J = 8.0, 8.5 Hz, H-2′), 3.33 (1 H, t, J = 8.5 Hz, H-3′), 3.27 (1 H, t, J = 8.5 Hz, H-4′), 3.22 (1 H, m, H-5′), 3.65 (1 H, dd, J = 5.5, 11.5 Hz, H-6′a), and 3.85 (1 H, dd, J = 2.0, 11.5 Hz, H-6′b), indicating a β-D-glucopyranoside moiety. Further, the 1H, 13C NMR, COSY, and heteronuclear multiple-quantum correlation (HMQC) spectra of 2 indicated the presence of

![Fig. 1 Structures of compounds 1-3 isolated from the roots of R. rosea.](image-url)
two methyls \( \delta_{11} 2.12 \) (3 H, s, H3-7) and 1.69 (3 H, s, H3-8) \( \) in the residual aglycone part, three methylenes \( \delta_{11} \) germainal 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 \( \delta_{11} 5.42 \) (1 H, t, \( J = 6.5 \) Hz, H-3)]. In the HMBC spectrum of 2 \( \) (Fig. 2B), cross-peaks of \( \delta_{11} 1.69 \) (H3-8)\( /\delta_C 75.6 \) (C-1), 133.9 (C-2) and 128.3 (C-3), \( \delta_{11} 5.42 \) (H-3)\( /\delta_C 23.0 \) (C-4) and 43.7 (C-5), \( \delta_{11} 2.12 \) (H3-7)\( /\delta_C 211.4 \) (C-6) and 43.7 (C-5) and \( \delta_{11} 4.22 \) (H-1)\( /\delta_C 75.6 \) (C-1) indicated that the aglycone of 2 was connected with the C-1 of \( \beta\)-D-glucopyranoside via an ether linkage to form creoside I. The configuration of \( \Delta^2 \) was determined to be in E form owing to the \( \delta_C 14.1 \) of C-8 in 2 in contrast with \( \delta_C 21.9 \) 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 \( [\alpha]_D^{25} = -42.2^\circ \) (c 0.23, MeOH). Comparison of the \( ^1H \) and \( ^{13}C \) NMR spectra of 3 and 2 revealed the major distinctive differences to be primary alcohol \( (\delta_C 60.0, \text{C-1}) \), a secondary hydroxyl group \( (\delta_C 66.5, \text{C-6}) \), and Z form \( \Delta^2 \) (\( \delta_C 20.2, \text{C-8} \) ) in 3 as opposed to the ether group \( (\delta_C 75.6, \text{C-1}) \), ketone group \( (\delta_C 211.4, \text{C-6}) \), and E form \( \Delta^2 \) \( (\delta_C 14.1, \text{C-8}) \) in 2, respectively. The configuration of the \( \Delta^2 \) in 3 was also verified by a nuclear Overhauser effect (NOE) enhancement signal of H-3-8 at \( \delta_H 1.75 \) after radiation of H-3 at \( \delta_H 5.27 \), which indicated a Z form double bond. The chirality of C-6 was determined to be \( R \) by comparing the optical rotation of 3, \( [\alpha]_D^{25} = -42.2^\circ \), with that of \( (R)-6\)-methylhept-5-en-2-ol, \( [\alpha]_D = -14.5^\circ \) \( [36] \).

In the MMP inhibitory activity assay, 1,2,3,6-tetra-O-galloyl-4-O-p-hydroxybenzoyl-\( \beta\)-D-glucopyranoside \( (1) \) inhibited MMP-2 activity with an \( IC_{50} \) of 16.3 ± 1.6 µM, while its analog 1,2,3,6-tetra-O-galloyl-\( \beta\)-D-glucopyranoside \( (17) \) inhibited MMP-2 with an \( IC_{50} \) 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 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 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 74.8% (Table 4), respectively, in 24 h as determined by using LC-MS. In the same conditions, the transdermal penetration percentage of genistein, a positive control used in this study, was 3.7%. Compound 17 was thus speculated to be able to penen-
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 acetonitrile 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 evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g). This residue was then evaporated to give a black residue (1758 g).

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

<table>
<thead>
<tr>
<th>Samples</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>1. 1.42</td>
<td>1.17 ± 0.21</td>
<td>27.8</td>
</tr>
<tr>
<td></td>
<td>2. 1.02</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>3. 1.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td>White mice</td>
<td>4. 8.82</td>
<td>6.07 ± 2.43</td>
<td>74.8</td>
</tr>
<tr>
<td></td>
<td>5. 4.23</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>6. 5.16</td>
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<td></td>
</tr>
</tbody>
</table>

**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>20 µM</th>
<th>40 µM</th>
<th>Positive control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>Nude mice</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td></td>
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<td></td>
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<tr>
<td>3</td>
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</tr>
<tr>
<td>White mice</td>
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<tr>
<td>4</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>5</td>
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<td></td>
<td></td>
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<tr>
<td>6</td>
<td></td>
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</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.
Identification of isolated compounds

All the isolated compounds were identified by interpreting their $^1$H, $^13$C, and 2D NMR spectra, including COSY, NOESY, HMBC, and HMQC spectra, which were further supported by IR, LR-, HR-MS, and optical rotation data.

$^1$H, $^13$C NMR data see Table 1.

Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>$^1$H NMR (δ in ppm)</th>
<th>$^13$C NMR (δ in ppm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Compound 1</td>
<td>3.65 (s), 3.95 (s)</td>
<td>32.5 (s), 70.8 (m)</td>
</tr>
<tr>
<td>Compound 2</td>
<td>2.45 (q), 3.05 (t)</td>
<td>26.5 (q), 31.5 (t)</td>
</tr>
</tbody>
</table>

Matrix metalloproteinase-2 inhibitory activity assay

To evaluate the MMP-2 inhibitory activity of the galloyl derivatives 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. Subsequently, the cells were treated with the vehicle (DMSO), compounds 1, 7, and 16-19 (10, 20, 50, and 100 µM), or epigallocatechin 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 compounds 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% bromophenol blue; pH 6.8) in a volume ratio of 1:2, followed by polycrylamide gel electrophoresis (PAGE). The PAGE gel contained 1% gelatin 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% methanol) was subsequently applied to the gel for 30 min, and the gel was stained with Brilliant Blue G-Colloidal, and then destained with a destain solution (10% acetic acid and 40% methanol). Finally, the gelatinolytic zone was analyzed using an image analysis system (Vilber Lourmat). The analysis software used was Bio-1 version 99. The formula used to determine the inhibitory effectiveness of the compounds was as follows: [(the values of blank – the values of experimental group)/the values of blank] × 100%.

Collagenase inhibitory activity assay

DQ™ (EnzChek Gelatinase/Collagenase Assay Kit, E-12055) is a type of fluorescent material that can bond with gelatin to form DQ-gelatin. Since the capability of collagenase type IV to induce gelatin hydrolysis has been demonstrated, DQ-gelatin can be a substrate for collagenase. Thus, the principle of this experiment was to measure the activity of collagenase by determining the emission intensity of DQ fluorescence because the chemical bond between DQ-gelatin could be enzymatically digested by collagenase. The fluorescence intensity can be measured using a fluorescence microplate reader equipped with standard fluorescence filters. The fluorescence absorption and emission wavelengths for the digestion product from DQ-gelatin and the DQ collagen substrate were 495 nm and 515 nm, respectively. Therefore, a fluorescence emission intensity higher than that of the blank at a wavelength of 515 nm is an indicator of collagenase activity. In other words, the resulting fluorescence emission intensity would decrease at 515 nm if the collagenase inhibitors, compounds 1 and 17, were applied. The results of the collagenase assay are presented as mean ± SD values.

Transdermal penetration test

To evaluate the effectiveness of a topical agent of compound 17 within a larger amount, a transdermal penetration test was performed. 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 surface of the donor chamber is provided for close integration. The lower receptor chamber is a double-layered hollow cylindrical diffusion container, of which the inner layer is made of glass, containing the fluid for sampling; the outer layer of the receptor chamber is filled with circulating water, maintained at 37 ± 1 °C to simulate the human body. The contact area between the donor chamber and the receptor chamber was 0.785 cm² (the actual penetration area), and nude or white mouse (n = 3 skin (epidermis facing upwards) was used to 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 Parafilm™, and the inner receptor chamber was filled with 5.5 mL of the buffer solution (30 wt% ethanol and phosphate buffer solution; 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 solution in the receptor chamber was analyzed by liquid chromatography-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 compound 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 performing LC-MS. Genistein was used as a positive control following the same method.

Transdermal penetration analysis 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 quadrupole 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-
rination was performed on a C18 Phenomenex column (100 × 4.6 mm, 5 μm). The injection volume was 10 μL. The flow rate was set to 0.5 mL/min and the gradient used was as follows (where A = water and B = acetonitrile): t = 0–1.2 min, A:B (80:10 v/v); t = 1.2–2.5 min, A:B (80:10 v/v); t = 2.5–3.5 min, A:B (80:10 v/v) to A:B (10:80 v/v); t = 3.5–6.5 min, A:B (80:10 v/v). Mass detection and quantification were performed in the negative mode using multiple reaction monitoring (MRM) of the precursor/product ion pair at m/z 787/617 and 269/133 for compound 17 and genistein, respectively. The optimized parameters for mass detection were as follows: curtain gas, 10 psi; nebulizer gas (A 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.

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Conflict of Interest
The authors declare no conflict of interest.

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