

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

## Authors

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## Key words

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

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## 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- $\beta$ -D-glucopyranoside (**1**), (*E*)-creoside I (**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 IC<sub>50</sub> value of 16.3 ± 1.6 μM, while its analogue 1,2,3,6-tetra-*O*-galloyl- $\beta$ -D-gluco-

pyranonoside (**17**) inhibited matrix metalloproteinase-2 with an IC<sub>50</sub> 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.

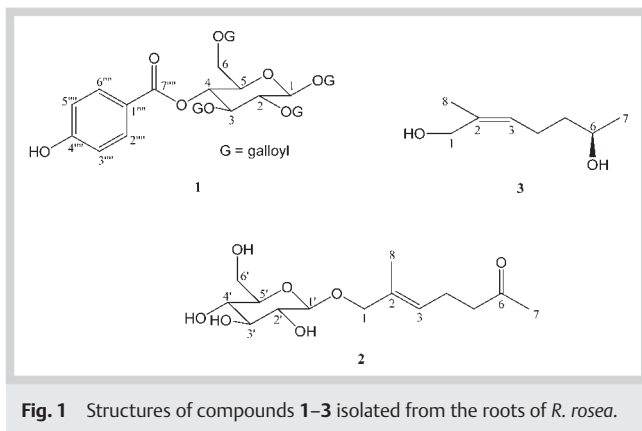
**Supporting information** available online at <http://www.thieme-connect.de/products>

## 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, 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], phenolethanoids [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



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 *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],  $\beta$ -sitosterol (5) [19], (*Z*)-2-methyl-6-oxo-2-hepten-1-ol (6) [20], gallic acid (7) [21], 4-hydroxyphenyl-2-ethyl  $\beta$ -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*R*,3*R*-dihydrokaempferol (14) [27], (*E*)-2-methyl-6-oxo-2-hepten-1-ol (15) [17], 1,2,6-tri-*O*-galloyl- $\beta$ -D-glucopyranoside (16) [28], 1,2,3,6-tetra-*O*-galloyl- $\beta$ -D-glucopyranoside (17) [28], *p*-hydroxyphenethyl alcohol 1-*O*-D-(6''-*O*-galloyl)-glucopyranoside (18) [29], herbacetin 7-*O*- $\alpha$ -L-rhamnopyranoside (19) [30], kaempferol 3-*O*- $\alpha$ -L-rhamnopyranoside (20) [30], epicatechin-3-*O*-gallate (21) [31], 1,1-dimethylprop-2-en-1-yl- $\beta$ -D-glucopyranoside (22) [20], 3-methyl-but-2-en-1-yl- $\beta$ -D-glucopyranoside (23) [32], (*Z*)-creoside 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  $C_{41}H_{32}O_{24}$ , as determined using  $^{13}C$  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 ( $1702\text{ cm}^{-1}$ ) along with aromatic functionalities ( $1609$  and  $1536\text{ cm}^{-1}$ ). In the  $^1H$  NMR spectrum of 1 (**Table 1**), signals at  $\delta_H$  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  $\beta$ -D-glucopyranoside bearing four galloyl groups at C-1, -2, -3, and C-

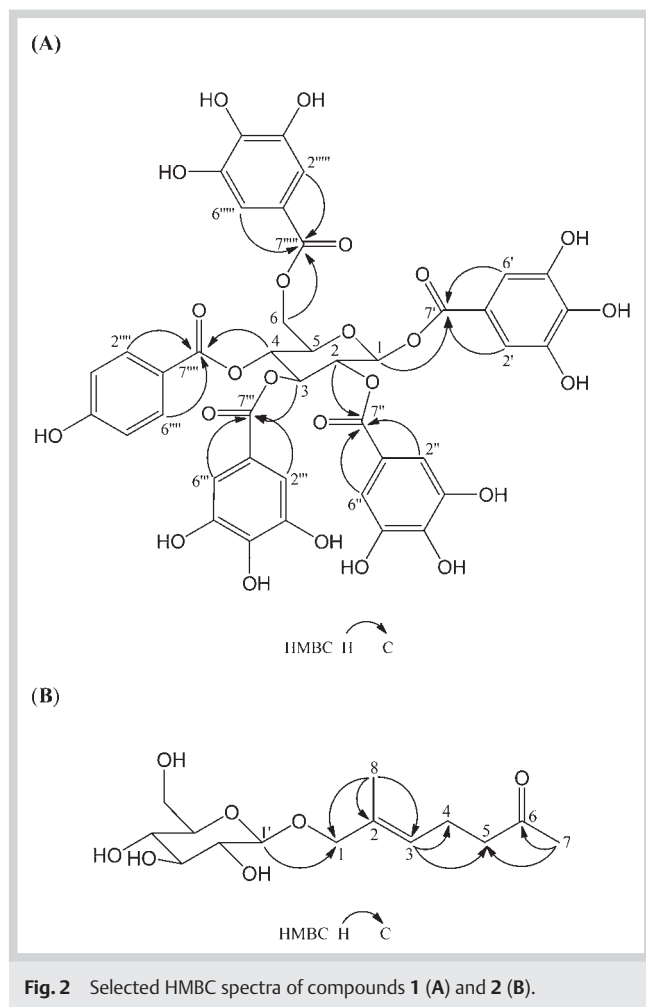
**Table 1**  $^{13}C$  (125 MHz) and  $^1H$  NMR (500 MHz) data for compound 1 (in  $CD_3OD$ ).

Position	$^{13}C$	$^1H$
1	94.3	6.24 (1 H, d, $J = 8.3$ Hz)
2	72.6	5.58 (1 H, dd, $J = 8.3, 9.6$ Hz)
3	74.5	5.91 (1 H, t, $J = 9.6$ Hz)
4	70.5	5.63 (1 H, t, $J = 9.6$ Hz)
5	74.7	4.40 (1 H) <sup>a</sup>
6	63.7	4.41 (1 H) <sup>a</sup> 4.51 (1 H, d, $J = 10.3$ Hz)
1'	120.2	
2', 6'	111.1	7.05 (2 H, s)
3', 5'	147.0	
4'	141.2	
7'	166.7	
1''	120.7	
2'', 6''	110.9	6.95 (2 H, s)
3'', 5''	146.8	
4''	140.8	
7''	167.5	
1'''	120.7	
2''', 6'''	110.8	6.80 (2 H, s)
3''', 5'''	146.7	
4'''	140.6	
7'''	167.8	
1''''	121.5	
2'''', 6''''	133.6	7.78 (2 H, d, $J = 8.7$ Hz)
3'''', 5''''	116.7	6.75 (2 H, d, $J = 8.7$ Hz)
4''''	163.3	
7''''	167.1	
1'''''	121.4	
2''''', 6'''''	110.8	7.10 (2 H, s)
3''''', 5'''''	146.9	
4'''''	140.4	
7'''''	168.4	

<sup>a</sup> Signals without multiplicity were overlapped and picked up from the HSQC spectrum

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  $\delta_H$  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- $\beta$ -D-glucopyranoside.

Compound 2 was isolated as amorphous white powder with the molecular formula  $C_{14}H_{24}O_7$ , as determined by positive ion HR-ESIMS, and showed an  $[M + Na]^+$  ion at  $m/z$  327.1428 (calcd. for  $C_{14}H_{24}O_7Na$ , 327.1420). Conspicuous absorptions at  $3389$  and  $1689\text{ cm}^{-1}$  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  $\delta_H$  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  $\beta$ -D-glucopyranoside moiety. Further, the  $^1H$ ,  $^{13}C$  NMR, COSY, and heteronuclear multiple-quantum correlation (HMQC) spectra of 2 indicated the presence of



**Fig. 2** Selected HMBC spectra of compounds **1** (A) and **2** (B).

two methyls [ $\delta_{\text{H}}$  2.12 (3 H, s, H<sub>3</sub>-7) and 1.69 (3 H, s, H<sub>3</sub>-8)] in the residual aglycone part, three methylenes [ $\delta_{\text{H}}$  germinal coupled 4.02 and 4.18 (each 1 H, d,  $J = 11.5$  Hz, H<sub>2</sub>-1), vicinal coupling [2.28 (2 H, m, H<sub>2</sub>-4) and 2.53 (2 H, t,  $J = 7.0$  Hz, H<sub>2</sub>-5)], and an olefinic methine [ $\delta_{\text{H}}$  5.42 (1 H, t,  $J = 6.5$  Hz, H-3)]. In the HMBC spectrum of **2** (● Fig. 2B), cross-peaks of  $\delta_{\text{H}}$  1.69 (H<sub>3</sub>-8)/ $\delta_{\text{C}}$  75.6 (C-1), 133.9 (C-2) and 128.3 (C-3),  $\delta_{\text{H}}$  5.42 (H-3)/ $\delta_{\text{C}}$  23.0 (C-4) and 43.7

(C-5),  $\delta_{\text{H}}$  2.12 (H<sub>3</sub>-7)/ $\delta_{\text{C}}$  211.4 (C-6) and 43.7 (C-5) and  $\delta_{\text{H}}$  4.22 (H-1')/ $\delta_{\text{C}}$  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  $\delta_{\text{C}}$  14.1 of C-8 in **2** in contrast with  $\delta_{\text{C}}$  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  $[\alpha]_{\text{D}}^{25} = -42.2^\circ$  (c 0.23, MeOH). Comparison of the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra of **3** and **2** revealed the major distinctive differences to be primary alcohol ( $\delta_{\text{C}}$  60.0, C-1), a secondary hydroxyl group ( $\delta_{\text{C}}$  66.5, C-6), and Z form  $\Delta^2$  ( $\delta_{\text{C}}$  20.2, C-8) in **3** as opposed to the ether group ( $\delta_{\text{C}}$  75.6, C-1), ketone group ( $\delta_{\text{C}}$  211.4, C-6), and E form  $\Delta^2$  ( $\delta_{\text{C}}$  14.1, 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<sub>3</sub>-8 at  $\delta_{\text{H}}$  1.75 after radiation of H-3 at  $\delta_{\text{H}}$  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**,  $[\alpha]_{\text{D}}^{25} -42.2^\circ$ , with that of (R)-6-methylhept-5-en-2-ol,  $[\alpha]_{\text{D}} -14.5^\circ$  [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- $\beta$ -D-glucopyranoside (**1**) inhibited MMP-2 activity with an IC<sub>50</sub> of  $16.3 \pm 1.6 \mu\text{M}$ , while its analog 1,2,3,6-tetra-O-galloyl- $\beta$ -D-glucopyranoside (**17**) inhibited MMP-2 with an IC<sub>50</sub> value of  $23.0 \pm 4.8 \mu\text{M}$ . In the collagenase inhibitory activity assay, the effects of **1** and **17** at concentrations of both 20 and 40  $\mu\text{M}$  were significant and more potent than those of the positive control, 1,10-phenanthroline (● Table 3). When a concentration of 20  $\mu\text{M}$  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 **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 pene-

Position	<b>2</b>		<b>3</b>	
	$^{13}\text{C}$	$^1\text{H}$	$^{13}\text{C}$	$^1\text{H}$
1	75.6	4.02 (1 H, d, $J = 11.5$ Hz) 4.18 (1 H, d, $J = 11.5$ Hz)	60.0	4.04 (1 H, d, $J = 12.0$ Hz) 4.08 (1 H, d, $J = 12.0$ Hz)
2	133.9		134.6	
3	128.3	5.42 (1 H, t, $J = 6.5$ Hz)	127.2	5.27 (1 H, t, $J = 7.0$ Hz)
4	23.0	2.28 (2 H, m)	23.5	2.11 (2 H, m)
5	43.7	2.53 (2 H, t, $J = 7.0$ Hz)	38.9	1.45 (1 H, m)
6	211.4		66.5	3.70 (2 H, m)
7	29.8	2.12 (3 H, s)	22.1	1.14 (3 H, d, $J = 6.5$ Hz)
8	14.1	1.69 (3 H, s)	20.2	1.75 (3 H, s)
1'	102.6	4.22 (1 H, d, $J = 8.0$ Hz)		
2'	75.1	3.18 (1 H, dd, $J = 8.0, 8.5$ Hz)		
3'	78.2	3.33 (1 H, t, $J = 8.5$ Hz)		
4'	71.7	3.27 (1 H, t, $J = 8.5$ Hz)		
5'	77.9	3.22 (1 H, m)		
6'	62.8	3.65 (1 H, dd, $J = 5.5, 11.5$ Hz) 3.85 (1 H, dd, $J = 2.0, 11.5$ Hz)		

**Table 2**  $^{13}\text{C}$  (125 MHz) and  $^1\text{H}$  NMR (500 MHz) data for compounds **2** and **3** (in CD<sub>3</sub>OD).

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

Samples	<b>1</b>		<b>17</b>		Positive control	
	20 $\mu$ M	40 $\mu$ M	20 $\mu$ M	40 $\mu$ M	20 $\mu$ M	40 $\mu$ M
Inhibitory effectiveness (%)	23.51 $\pm$ 2.81*	47.50 $\pm$ 2.90	52.12 $\pm$ 4.30***	61.73 $\pm$ 1.98**	12.80 $\pm$ 5.40	38.41 $\pm$ 1.43

The percentual inhibition is shown as mean  $\pm$  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

Sample mice		The amount of <b>17</b> applied onto the skins ( $\mu$ g)	Average ( $\mu$ g)	Penetration percentage of <b>17</b> into the mouse skins (%)
Nude mice	1.	1.42	1.17 $\pm$ 0.21	27.8
	2.	1.02		
	3.	1.09		
White mice	4.	8.82	6.07 $\pm$ 2.43	74.8
	5.	4.23		
	6.	5.16		

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

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.  $^1\text{H}$  and  $^{13}\text{C}$  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 suspended in  $\text{H}_2\text{O}$  (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 F<sub>254</sub> plates (Merck) by using mixtures of *n*-hexane-ethyl acetate for develop-

ment, 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  $\times$  250 mm) using *n*-hexane/ethyl acetate (96:4) as the eluent at a flow rate of 3 mL/min to afford **6** (4.2 mg,  $t_R$  = 19.8 min), **3** (6.1 mg,  $t_R$  = 21.2 min), **5** (32 mg,  $t_R$  = 23.5 min), and **7** (79 mg,  $t_R$  = 28.0 min). The same portion was purified by performing semipreparative HPLC (Phenomenex® Luna, 10  $\times$  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,  $t_R$  = 32.5 min). Portion III, eluted by *n*-hexane/ethyl acetate (90:10), was purified by performing semipreparative HPLC (Phenomenex® Luna, 10  $\times$  250 mm) using *n*-hexane/acetone (99:1) as the eluent at a flow rate of 3 mL/min to afford **8** (1.7 g,  $t_R$  = 19.3 min). Portion IV, eluted by *n*-hexane/ethyl acetate (80:20), was purified by performing semipreparative HPLC (Phenomenex® Luna, 10  $\times$  250 mm) using *n*-hexane/ethyl acetate (85:15) as the eluent at a flow rate of 3 mL/min to afford **15** (19 mg,  $t_R$  = 24.4 min), **12** (11 mg,  $t_R$  = 26.9 min), **9** (25 mg,  $t_R$  = 33.8 min), **10** (31 mg,  $t_R$  = 37.0 min), and **11** (24 mg,  $t_R$  = 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,  $t_R$  = 20.1 min) and **13** (22 mg,  $t_R$  = 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 **19** (25 mg,  $t_R$  = 13.2 min), **17** (55 mg,  $t_R$  = 16.3 min), **16** (132 mg,  $t_R$  = 21.8 min), and **18** (39 mg,  $t_R$  = 25.6 min). Portion V, eluted by *n*-hexane/ethyl acetate (60:40), was purified by performing semipreparative HPLC (Hibar® Fertigsäule, 10  $\times$  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 g,  $t_R$  = 10.2 min), **22** (63 mg,  $t_R$  = 20.0 min), **21** (84 mg,  $t_R$  = 22.0 min), and **23** (33 mg,  $t_R$  = 26.5 min). The same portion was purified by performing semipreparative HPLC (Hibar® Fertigsäule, 10  $\times$  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,  $t_R$  = 24.3 min), **24** (4.6 mg,  $t_R$  = 32.3 min), and **25** (3.3 mg,  $t_R$  = 38.7 min). Portion VI, eluted by *n*-hexane/ethyl acetate (40:60), was purified by performing semipreparative HPLC (Hibar® Fertigsäule, 10  $\times$  250 mm) using *n*-hexane/ethyl acetate

(53 : 47) as the eluent at a flow rate of 3 mL/min to afford **1** (5 mg,  $t_R = 13.2$  min), **26** (11.4 mg,  $t_R = 21.5$  min), and **27** (3.6 mg,  $t_R = 30.2$  min).

### Identification of isolated compounds

All the isolated compounds were identified by interpreting their  $^1\text{H}$ ,  $^{13}\text{C}$ , 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-hydroxybenzoyl- $\beta$ -D-glucopyranoside* (**1**): Amorphous white powder;  $[\alpha]_D^{24} + 38.75$  (c 0.08,  $\text{CH}_3\text{OH}$ ); IR (neat)  $\nu_{\text{max}}$  3375, 1702, 1609, 1536; negative HRESIMS  $m/z$  907.1241  $[\text{M} - \text{H}]^-$  (calcd. for  $\text{C}_{41}\text{H}_{31}\text{O}_{24}$ , 907.1205); for  $^1\text{H}$  and  $^{13}\text{C}$  NMR data see **Table 1**.

(*E*)-*Creoside I* (**2**): Amorphous white powder;  $[\alpha]_D^{25} - 58.0$  (c 0.05,  $\text{CH}_3\text{OH}$ ); IR (neat)  $\nu_{\text{max}}$  3389, 1698; ESIMS  $m/z$  327.3  $[\text{M} + \text{Na}]^+$ ; for  $^1\text{H}$  and  $^{13}\text{C}$  NMR data see **Table 2**.

(*R,Z*)-*2-Methylhept-2-ene-1,6-diol* (**3**): Colorless oil;  $[\alpha]_D^{25} - 42.2$  (c 0.23,  $\text{CH}_3\text{OH}$ ); ESIMS  $m/z$  166.2  $[\text{M} + \text{Na}]^+$ ; for  $^1\text{H}$  and  $^{13}\text{C}$  NMR data see **Table 2**.

### 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 ( $5 \times 10^5$  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  $\mu\text{M}$ ), or epigallocatechin gallate (EGCG, 100  $\mu\text{M}$ ) 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 polyacrylamide 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  $\text{CaCl}_2$ , 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 Lounmat). 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]  $\times$  100%.

### Collagenase inhibitory activity assay

DQ<sup>TM</sup> (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<sup>TM</sup>-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 fluorescein 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  $\pm$  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  $37 \pm 1$  °C to simulate the human body. The contact area between the donor chamber and the receptor chamber was 0.785 cm<sup>2</sup> (the actual penetration area), and nude or white mouse ( $n = 3$ ) skin (epidermis 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 Parafilm<sup>®</sup>, 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 10000 rpm for 5 min. The supernatant was filtered using 0.45  $\mu\text{m}$  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- $\beta$ -D-glucopyranoside (**17**) and genistein. The chromatographic sepa-

ration was performed on a C<sub>18</sub> 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 (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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