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Three new prenylflavonol glycosides from heat-processed *Epimedium koreanum*

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Abstract: Phytochemical investigation of the *n*-BuOH extract of the leaves of processed *Epimedium koreanum* led to the isolation of three new prenylflavonol glycosides, epimedkoresides A–C (**1**–**3**), along with the 15 known ones (**4**–**18**). The structures of the three new compounds were established on the basis of chemical and spectroscopic methods as 8-γ-hydroxy-γ,γ-dimethylpropyl-5,7,4′-trihydroxy-flavonol-3-*O*-β-D-glucopyranosyl(1 → 2)-α-L-rhamnopyranoside (**1**), 8-γ-hydroxy-γ,γ-dimethylpropyl-5,7,4′-trihydroxy-flavonol-3-*O*-α-L-rhamnopyranosyl(1 → 2)-α-L-rhamnopyranoside (**2**), and anhydroicaritin 3-*O*-α-L-rhamnopyranosyl(1 → 2)-α-L-rhamnopyranosyl-7-*O*-α-L-rhamnopyranosyl(1 → 2)-β-D-glucopyranoside (**3**), respectively. Additionally, partial structures were evaluated for their anti-inflammatory activity.

Keywords: anti-inflammatory; *Epimedium koreanum;* epimedkoresides A–C; prenylflavonol glycosides.

1 Introduction

Herba Epimedii (Yinyanghuo) is one of the most well-known and frequently used Chinese herbal medicine with tonic, anti-rheumatic, and aphrodisiac effects. The genus *Epimedium* (Berberidaceae) is widely distributed in China, with about 30 species, 15 of which are circulated in the crude drug markets used as Yinyanghuo [1]. The aerial parts of *Epimedium koreanum*, *E. brevicornu*, *E. sagittatum*, and *E. pubescens* are designated in *Chinese Pharmacopoeia* (*2010 Edition*) as the official source of Herba Epimedii [2]. In addition to the aerial parts, the underground parts of *Epimedium* plants are widely used as anti-rheumatic medicine in Chinese folk medicines [1]. Previous investigations on *Epimedium* plants have reported prenylflavonoids with various pharmacological effects, such as anti-inflammatory [3], anti-osteoporosis [4–6], antioxidant [7], estrogenic and anti-estrogenic [8], anti-tumor [9], anti-aging activities [10], and so forth. However, the pharmacological activities are different between crude and processed *Epimedium*. Crude *Epimedium* is mainly used against rheumatism and in strengthening the bones, whereas processed *Epimedium* possesses kidney-nourishing, aphrodisiac, and anti-inflammatory effects [11].

As a continuation of our research on *Epimedium* species [3, 12] and to search for anti-inflammatory prenylflavonoids from processed *Epimedium*, three new prenylflavonol glycosides, epimedkoresides A–C (**1**–**3**), along with 15 known ones, were isolated from the leaves of *E. koreanum* by heating with sheep fat (Fig. 1). The known compounds were identified as acuminatoside (**4**) [13], ikarisoside B (**5**) [14], icariin (**6**) [15], epimedins A–C (**7**–**9**) [16], epimedoside A (**10**) [15], desmethylicaritin 3-*O*-β-D-fucopyranosyl (1 → 2)-α-L-rhamnopyranoside-7-*O*-β-D-glucopyranoside (**11**) [17], baohuoside V (**12**) [18], diphylloside B (**13**) [19], diphylloside A (**14**) [19], epimedoside E (**15**) [19], anhydroicaritin 3-*O*-β-D-fucopyranosyl(1 → 2)-α-L-rhamnopyranoside-7-*O*-β-D-glucopyranoside (**16**) [17], hexandraside F (**17**) [20], and 4′,5-dihydroxyl-8-(3,3-dimethylallyl)-flavonol-3-*O*-[β-D-xylopyranosyl(1 → 3)-α-L-rhamnopyranozside]-7-*O*-β-D-glucopyranoside (**18**) [21] through comparison of their spectral data with those in the literature. Compounds **5**, **7**, **9**, **12**–**15** were evaluated for their anti-inflammatory activity. Unfortunately, the results failed to meet expectations. Herein, this article deals with the isolation, structural determination, and biological evaluation of these compounds.

2 Result and discussion

Compound **1** was obtained as a yellow amorphous powder. The positive HR-ESI-MS showed a quasi-molecular ion peak at m/z = 703.2210 ([M + Na]$^+$), indicating the molecular formula $C_{32}H_{40}O_{16}$ and possessing 13 degrees of unsaturation. Its ^{1}H NMR spectrum (Tables 1 and 2) displayed a

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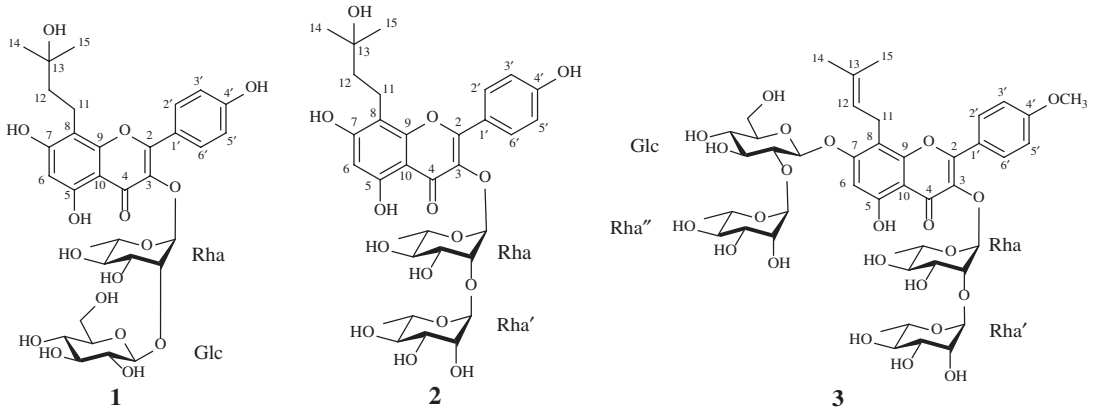

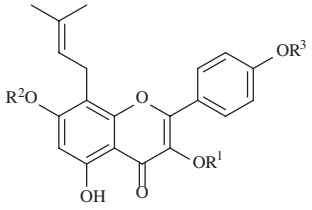

	R^1	R^2	R^3
4	Rha(2-1)Rha	Glc(2-1)Glc	CH_3
5	Rha(2-1)Glc	H	H
6	Rha	Glc	CH_3
7	Rha(2-1)Glc	Glc	CH_3
8	Rha(2-1)Xyl	Glc	CH_3
9	Rha(2-1)Rha	Glc	CH_3
10	Rha	Glc	H
11	Rha(2-1)Fuc	Glc	H
12	Rha(4-1)Rha	Glc	H
13	Rha(2-1)Rha	Glc	H
14	Rha(2-1)Glc	Glc	H
15	Rha(2-1)Xyl	Glc	H
16	Rha(2-1)Fuc	Glc	CH_3
17	Rha(3-1)Glc	Glc	CH_3
18	Rha(3-1)Xyl	Glc	H

Fig. 1: Structures of compounds **1–18**.

pair of broad doublets (δ_H = 7.85 [J = 8.6 Hz] and 6.94 ppm [J = 8.6 Hz]) attributed to the protons of an AA′BB′-type benzene ring, an aromatic singlet (δ_H = 6.24 ppm) attributed to the proton of a *penta*-substituted aromatic ring, as well as two anomeric protons (δ_H = 4.43 [d, J = 7.6 Hz] and 5.75 ppm [s]) of two sugar residues. Additionally, two sets of two-proton multiplets (δ_H = 2.82 [m, H-11] and 1.68 ppm [m, H-12]) as well as two tertiary methyls (δ_H = 1.26 [s, Me-14] and 1.27 ppm [s, Me-15]) showed a γ-hydroxy-γ,γ-dimethylpropyl group [22, 23], which was further supported by a series of signals at δ_C = 18.8 (t, C-11), 43.9 (t, C-12), 71.5 (s, C-13), 28.9 (q, C-14), and 29.0 ppm (q, C-15) in the ^{13}C NMR spectrum (Table 1). Except for 17 sp^3 carbons belonging to the γ-hydroxy-γ,γ-dimethylpropyl group and two sugar residues, the remaining 15 sp^2 carbons including 1 carbonyl group, 9 quaternary carbons (containing 5 oxygenated ones), and 5 methines, suggested that **1** possessed a flavonol skeleton.

Careful comparison of the NMR data with those of ikarisoside B (**5**) [14] showed high structural similarity except that the isoprenyl group in ikarisoside B was replaced a by γ-hydroxy-γ,γ-dimethylpropyl moiety in **1**. The γ-hydroxy-γ,γ-dimethylpropyl moiety was deduced to be located at C-8 (δ_C = 107.9 ppm) according to heteronuclear multiple bond correlation (HMBC) correlations (Fig. 2) of H-11 with C-7 (δ_C = 163.0), C-8, and C-9 (δ_C = 155.7 ppm) and of H-12 with C-8. In addition, two sugar residues were identified as D-glucose (Glc) and L-rhamnose (Rha) by acid hydrolysis and gas chromatography (GC) analysis of their corresponding trimethylsilylated L-cysteine derivatives. The β-pyranosyl configuration of the glycosidic bond of Glc moiety was deduced from the coupling constant (J = 7.6 Hz) of its anomeric proton. Simultaneously, the anomeric configuration of Rha moiety was determined as the α-position according to ^{13}C NMR chemical shifts (Table 2) [24]. The Rha moiety was located at C-3 (δ_C = 136.0 ppm) of the aglycone according to the HMBC correlation from H-1 (δ_H = 5.75 ppm) of Rha to C-3, whereas the Glc unit was linked to C-2 (δ_C = 82.7 ppm) of the Rha moiety due to the HMBC correlations from H-1 (δ_H = 4.43 ppm) of Glc to C-2 of Rha, as well as from H-2 (δ_H = 4.30 ppm) of Rha to C-1 (δ_C = 107.1 ppm) of Glc. Thus, the structure of **1** was unambiguously deduced as

Table 1: ^{1}H and ^{13}C NMR Data of aglycones for compounds **1–3** in CD_3OD.

Position	1		2		3	
	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
2	158.9 (s)		158.9 (s)		159.3 (s)	
3	136.0 (s)		136.1 (s)		136.5 (s)	
4	179.6 (s)		179.8 (s)		180.0 (s)	
5	160.7 (s)		160.8 (s)		160.9 (s)	
6	99.3 (d)	6.24 (s)	99.6 (d)	6.23 (s)	99.1 (d)	6.62 (s)
7	163.0 (s)		163.7 (s)		161.5 (s)	
8	107.9 (s)		108.8 (s)		110.9 (s)	
9	155.7 (s)		155.8 (s)		155.2 (s)	
10	105.9 (s)		105.6 (s)		107.6 (s)	
11	18.8 (t)	2.82 (m)	19.0 (t)	2.84 (m)	23.1 (t)	3.53 (m) 3.62 (m)
12	43.9 (t)	1.68 (m)	44.1 (t)	1.69 (m)	123.5 (d)	5.17 (dd, 5.8, 8.0)
13	71.5 (s)		71.7 (s)		132.7 (s)	
14	28.9 (q)	1.26 (s)	29.1 (q)	1.27 (s)	25.8 (q)	1.63 (s)
15	29.0 (q)	1.27 (s)	29.2 (q)	1.28 (s)	18.4 (q)	1.71 (s)
1′	122.7 (s)		122.8 (s)		123.8 (s)	
2′, 6′	132.0 (d)	7.85 (d, 8.6)	132.1 (d)	7.85 (d, 8.7)	131.9 (d)	7.89 (d, 8.9)
3′, 5′	116.7 (d)	6.94 (d, 8.6)	116.8 (d)	6.94 (d, 8.7)	115.2 (d)	7.10 (d, 8.9)
4′	162.0 (s)		162.0 (s)		163.5 (s)	
-OCH_3					56.0 (q)	3.89 (s)

The ^{1}H NMR spectra of **1–3** were recorded at 400, 600, and 500 MHz, respectively, and the ^{13}C NMR spectra of **1–3** at 100, 150, and 125 MHz, respectively. δ (in ppm); multiplicities and J (in Hz), in parentheses.

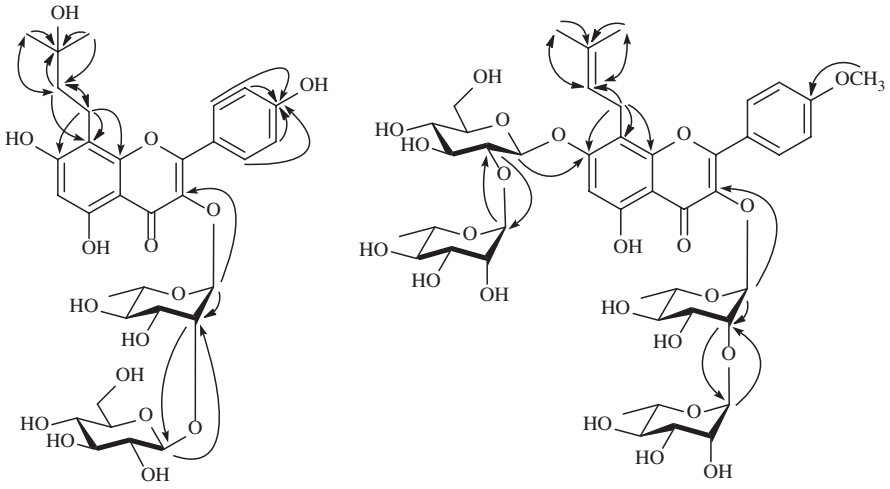

Fig. 2: Key HMBC correlations of compounds **1** and **3**.

8-γ-hydroxy-γ,γ-dimethylpropyl-5,7,4′-trihydroxy-flavonol-3-*O*-β-D-glucopyranosyl(1→2)-α-L-rhamnopyranoside and named as epimedkoreside A.

Compound **2** was isolated as a yellow amorphous powder, and the molecular formula $C_{32}H_{40}O_{15}$ was deduced from the pseudomolecular ion $[M + Na]^+$ at m/z = 687 in ESI-MS and further confirmed by the positive HR-ESI-MS (m/z = 687.2256 $[M + Na]^+$), requiring 13 degrees of unsaturation. Detailed analysis of the ^{1}H and ^{13}C NMR spectral data of **2** (Tables 1 and 2) and **1** made it clear that these two compounds were extremely similar except for the exchange of one monosaccharide. The protons at δ_H = 5.53 (s)/0.93 (d, J = 6.1 Hz) and 5.00 (s)/1.22 ppm (d, J = 6.2 Hz) in the ^{1}H NMR spectrum (Table 2) indicated the existence

Table 2: ^{1}H and ^{13}C NMR data of sugar moieties for compounds **1–3** in CD_3OD.

Position		1		2		3	
		δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
Rha							
	1	102.4 (d)	5.75 (s)	102.5 (d)	5.53 (s)	102.5 (d)	5.56 (d, 1.3)
	2	82.7 (d)	4.30 (br. s)	79.1 (d)	4.31 (br. s)	78.9 (d)	4.28 (br. s)
	3	71.8 (d)	3.82 (m)	72.2 (d)	3.87 (m)	72.2 (d)	3.85 (m)
	4	73.4 (d)	3.43 (m)	74.1 (d)	3.36 (m)	74.0 (d)	3.35 (m)
	5	72.0 (d)	3.30 (m)	72.1 (d)	3.36 (m)	72.1 (d)	3.35 (m)
	6	17.6 (q)	0.94 (d, 6.1)	17.9 (q)	0.93 (d, 6.1)	17.7 (d)	0.90 (d, 5.8)
Rha′							
	1			103.9 (d)	5.00 (s)	103.7 (d)	4.99 (d, 1.3)
	2			72.1 (d)	3.95 (m)	72.0 (d)	3.94 (br. s)
	3			72.3 (d)	3.61 (m)	72.3 (d)	3.60 (m)
	4			73.6 (d)	3.36 (m)	73.4 (d)	3.33 (m)
	5			70.5 (d)	3.61 (m)	70.3 (d)	3.66 (m)
	6			18.0 (q)	1.22 (d, 6.2)	17.8 (q)	1.22 (d, 6.2)
Glc							
	1	107.1 (d)	4.43 (d, 7.6)			100.0 (d)	5.30 (d, 7.5)
	2	75.3 (d)	3.25 (m)			80.2 (d)	3.73 (m)
	3	77.9 (d)	3.30 (m)			78.1 (d)	3.46 (m)
	4	70.9 (d)	3.35 (m)			71.3 (d)	3.85 (m)
	5	77.8 (d)	3.30 (m)			78.9 (d)	3.66 (m)
	6	62.3 (t)	3.84 (m)			62.4 (t)	3.85 (m)
			3.68 (m)				3.69 (m)
Rha″							
	1					102.3 (d)	5.34 (d, 1.3)
	2					71.9 (d)	3.98 (br. s)
	3					72.2 (d)	3.66 (m)
	4					73.9 (d)	3.36 (m)
	5					70.3 (d)	3.66 (m)
	6					18.2 (q)	1.15 (d, 6.2)

^{1}H NMR spectra of **1–3** were recorded at 400, 600, and 500 MHz, respectively, and the ^{13}C NMR spectra of **1–3** at 100, 150, and 125 MHz, respectively. δ (in ppm); multiplicities and J (in Hz), in parentheses.

of two rhamnose residues (Rha and Rha′), which was further confirmed by hydrolysis and GC analysis. Moreover, one rhamnose moiety (Rha) was located at C-3 (δ_C = 136.1 ppm) of the aglycone according to the HMBC correlation from H-1 (δ_H = 5.53 ppm) of Rha to C-3. The other rhamnose moiety (Rha′) was located at C-2 (δ_C = 79.1 ppm) of the inner one (Rha) due to the HMBC correlations from H-1 (δ_H = 5.00 ppm) of Rha′ to C-2 of Rha and from H-2 (δ_H = 4.31 ppm) of Rha to C-1 (δ_C = 103.9 ppm) of Rha′. Based on the above evidence, compound **2** was unequivocally identified as 8-γ-hydroxy-γ,γ-dimethylpropyl-5,7,4′-trihydroxy-flavonol-3-*O*-α-L-rhamnopyranosyl(1 → 2)-α-L-rhamnopyranoside and named epimedkoreside B.

Compound **3**, a yellow amorphous powder, was found to possess the molecular formula $C_{45}H_{60}O_{23}$ based on its HR-ESI-MS (m/z = 991.3409 $[M + Na]^+$), in combination with ^{1}H and ^{13}C NMR spectra. The ^{1}H NMR spectrum (Table 1) of **3** exhibited an *O*-methyl group at δ_H = 3.89 ppm (s), a singlet proton at δ_H = 6.62 ppm of a *penta*-substituted ring A, a pair of doublets at δ_H = 7.89 (J = 8.9 Hz), and 7.10 ppm (J = 8.9 Hz) of typical AA′BB′ system attributed to the protons of *para*-substituted ring B. In addition, a group of protons at δ_H = 3.53 (m, H_a-11), 3.62 (m, H_b-11), 5.17 (dd, J = 5.8 and 8.0 Hz, H-12), 1.63 (s, Me-14), and 1.71 ppm (s, Me-15), correlated with carbon signals at δ_C = 23.1 (t, C-11), 123.5 (d, C-12), 25.8 (q, C-14), and 18.4 ppm (q, C-15) in HSQC spectrum, respectively, suggesting the presence of a prenyl group [13, 14, 25]. Moreover, a series of signals at δ_H = 5.30 (d, J = 7.5 Hz), 5.56 (d, J = 1.3 Hz)/0.90 (d, J = 5.8 Hz), 4.99 (d, J = 1.3 Hz)/1.22 ppm (d, J = 6.2 Hz), as well as 5.34 (d, J = 1.3 Hz)/1.15 ppm (d, J = 6.2 Hz) (Table 2), revealed the presence of one glucose (Glc) and three rhamnose (Rha, Rha′, and Rha″) residues, in accordance with its acid hydrolysis and GC analysis results. As the NMR signals of four sugar units overlapped undesirably, the HSQC-TOCSY experiment was successfully used to distinguish and assign the

^{1}H and ^{13}C NMR signals of each sugar unit. Besides the signals belonging to one methoxyl, one prenyl group, and four monosaccharides, the ^{13}C NMR spectrum (Table 1) of **3** showed 15 sp^2 carbons (including 1 carbonyl, 9 quaternary ones, and 5 methines) for the flavonol skeleton.

The one- (1D) and two-dimensional (2D) NMR spectral data of **3** resembled those of acuminatoside (**4**) [13], except for one sugar moiety linked to C-7. The glycosidic linkages of **3** were determined by detailed analysis of HMBC spectrum (Fig. 2). A correlation of the proton at δ_H = 5.56 (d, J = 1.3 Hz, H-1 of Rha) to C-3 (δ_C = 136.5 ppm) of the aglycone indicated that the Rha moiety was attached to C-3. The Rha′ moiety was located at C-2 (δ_C = 78.9 ppm) of Rha due to the HMBC correlations from H-1 (δ_H = 4.99 ppm) of Rha′ to C-2 of Rha and from H-2 (δ_H = 4.28 ppm) of Rha to C-1 (δ_C = 103.7 ppm) of Rha″. The Glc unit was linked to C-7 (δ_C = 161.5 ppm) of the aglycone according to the HMBC correlation from H-1 (δ_H = 5.30 ppm) of Glc to C-7. The Rha″ moiety was connected with C-2 (δ_C = 80.2 ppm) of Glc based on the HMBC correlations from H-1 (δ_H = 5.34 ppm) of Rha″ to C-2 of Glc and from H-2 (δ_H = 3.73) of Glc to C-1 (δ_C = 102.3 ppm) of Rha″. Epimedkoreside C (**3**) was therefore elucidated as anhydroicaritin 3-*O*-α-L-rhamnopyranosyl(1 → 2)-α-L-rhamnopyranosyl-7-*O*-α-L-rhamnopyranosyl(1 → 2)β-D-glucopyranoside.

Compounds **5**, **7**, **9**, and **12**–**15** were examined for their inhibition effect on superoxide anion generation and elastase release in formyl-L-methionyl-L-leucyl-L-phenylalanine/cytochalasin B (FMLP/CB)-induced human neutrophils to evaluate their anti-inflammatory potential. However, all compounds at 10-μM concentration exhibited inhibition lower than 50 %.

3 Experimental section

3.1 General

The optical rotations were measured with a Jasco DIP-370 digital polarimeter (JASCO Corporation, Tokyo, Japan). 1D and 2D NMR spectra were recorded using Bruker AM-400, DRX-500, or Avance III-600 instruments with tetramethylsilane (TMS) as an internal standard (Bruker BioSpin Group, Karlsruhe, Germany). Electrospray ionization mass spectrometry (ESIMS) and high resolution electrospray ionization mass spectroscopy (HRESIMS) were measured on API-Qstar-TOF instrument (Allen-Bradley, Milwaukee, WI, USA). GC analysis was run on Agilent Technologies HP5890 GC with flame ionization detector (Agilent, USA). Semi-preparative HPLC was performed on an Agilent 1200 liquid chromatography with a ZORBAX SB-C18 (5 μm, 9.4 × 250 mm) column (Agilent, Santa Clara, CA, USA). Column chromatography (CC) was carried out on silica gel (80–100, 100–200, or 200–300 mesh; Qingdao Haiyang Chemical, Qingdao, China), Lichroprep RP-18 (43–63 μm; Merck, Darmstadt, Germany), Toyopeal HW-40 (Tosoh), Sephadex LH-20 (Amersham Biosciences, Uppsala, Sweden), Diaion HP-20ss (Mitsubishi, Japan), and YMC*-GEL ODS-A (12 nm, S-50 μm, YMC, Japan). Fractions were monitored by TLC plates (Si gel G and GF_{254}; Qingdao Haiyang Chemical), and spots were visualized by heating Si gel plates with 5 % H_2SO_4-EtOH.

3.2 Plant material

The leaves of heat-processed *E. koreanum* were purchased from Jiling Province, China, on September 2010 and identified by Prof. Hai-Zhou Li, Kunming University of Science and Technology, Kunming, P. R. China. A voucher specimen (KUMST 20100901) was deposited at the Laboratory of Phytochemistry, Kunming University of Science and Technology.

3.3 Extraction and isolation

Air-dried and powdered leaves of heat-processed *E. koreanum* (10 kg) was extracted with 75 % aq. Me_2CO (3 × 20 L, 1 day, each) at room temperature and then concentrated under vacuum to yield an extract that was suspended in H_2O and then successively extracted with petroleum ether (3 × 3 L), EtOAc (3 × 3 L), and *n*-BuOH (3 × 4 L). The *n*-BuOH extract (164 g) was chromatographed over Toyopeal column eluted with MeOH-H_2O gradient system (0, 30, 60, and 90 %) to afford six fractions, A–F. Fraction B (60 g) was then subjected to MPLC (ODS), eluted with gradient MeOH-H_2O (20–70 %, 200 min, flow rate 10 mL min^{-1}), to give six subfractions, B1–B6. Fraction B4 (16 g) was subjected to silica gel CC and eluted with gradient $CHCl_3$-MeOH system (10:1, 7:1, 6:1, 4:1, and 1:1) to yield six fractions (B4-1 to B4-6). Fraction B4-2 (1.2 g) was recrystallized (in MeOH) to give compound **6** (150 mg). Fraction B4-4 (2.6 g) was separated over MPLC (ODS), eluting with 60 % MeOH-H_2O (100 min, flow rate 10 mL min^{-1}) to afford seven subfractions (B4-4-1 to B4-4-7). Fraction B4-4-2 (100 mg) was purified over Sephadex LH-20 ($CHCl_3$-MeOH 1:1) followed by semi-preparative HPLC with 23 % CH_3CN-H_2O to yield compounds **1** (7.9 mg, t_R = 13.1 min) and **2** (3.5 mg, t_R = 15.3 min). Fraction B4-4-5 (1.2 g) was chromatographed on silica gel CC ($CHCl_3$-MeOH, gradient 10:1, 7:1, 5:1, 1:1) and then applied to semi-preparative HPLC with 25 % CH_3CN-H_2O to

obtain compounds **17** (4.5 mg, t_R = 18.6 min), **7** (46.4 mg, t_R = 18.9 min), **8** (30.1 mg, t_R = 20.4 min), **9** (48.7 mg, t_R = 21.3 min), and **16** (31.6 mg, t_R = 22.4 min). Similarly, purification of fraction B4-4-6 (800 mg) applied to semi-preparative HPLC with 26 % CH_3CN-H_2O to yield compounds **3** (9.0 mg, t_R = 16.3 min) and **4** (8.0 mg, t_R = 16.8 min). Fraction C (40 g) was subjected to MPLC (ODS) (25–75 % MeOH-H_2O, 250 min, flow rate 10 mL min^{-1}) to give five fractions, C1–C5. Fraction C3 (8.0 g) was purified over Sephadex LH-20 (MeOH), then further separated on silica gel CC eluted with $CHCl_3$-MeOH (10:1, 7:1, 4:1, 3:1) to afford eight fractions (C3-1 to C3-8). Fraction C3-3 (720 mg) was further separated by MPLC (ODS) eluting with 55 % MeOH-H_2O (100 min, flow rate 10 mL min^{-1}) to give six fractions (C3-3-1 to C3-3-6). Compounds **10** (30 mg, t_R = 9.4 min) and **18** (4.2 mg, t_R = 12.6 min) were obtained from subfraction C3-3-3 (100 mg) by semi-preparative HPLC with 21 % CH_3CN-H_2O as mobile phase. Fraction C3-3-4 (50 mg) was chromatographed over semi-preparative HPLC with 22 % CH_3CN/H_2O to yield compounds **12** (3.5 mg, t_R = 13.6 min) and **11** (4.3 mg, t_R = 13.9 min). Fraction D (25 g) was applied to Diaion HP-20ss eluting with MeOH-H_2O gradient system (30, 60, and 90 %) to give six fractions, D1–D6. Fraction D3 (8.0 g) was chromatographed over Sephadex LH-20 ($CHCl_3$-MeOH 1:1) to get six fractions (D3-1 to D3-6), and fraction D3-4 (760 mg) was separated on silica gel CC eluted with gradient $CHCl_3$-MeOH (10:1 to 0:1) to afford five fractions (D3-4-1 to D3-4-5). Compounds **14** (35 mg, t_R = 11.5 min), **15** (30 mg, t_R = 12.0 min), and **13** (19.2 mg, t_R = 13.0 min) were obtained from subfraction D3-4-2 (630 mg) by semi-preparative HPLC using 22 % CH_3CN-H_2O as mobile phase. Fraction D5 (1.7 g) was chromatographed over silica gel CC employing $CHCl_3$-MeOH gradient system (7:1, 6:1, 4:1, 3:1, 1:1) as eluent and repeated Sephadex LH-20 ($CHCl_3$-MeOH 1:1) to yield compound **5** (19.2 mg).

3.3.1 Epimedkoreside A (1)

Yellow amorphous powder. $-[\alpha]_D^{23.3} = -120.2$ (c = 0.01, MeOH). –^{1}H and ^{13}C NMR data: see Tables 1 and 2. –MS ((+)-ESI): m/z = 703 $[M + Na]^+$. –HRMS ((+)-ESI): m/z = 703.2210 (calcd. 703.2214 for $C_{32}H_{40}O_{16}Na$ $[M + Na]^+$).

3.3.2 Epimedkoreside B (2)

Yellow amorphous powder. $-[\alpha]_D^{23.1} = -108.0$ (c = 0.01, MeOH). –^{1}H and ^{13}C NMR data: see Tables 1 and 2. –MS ((+)-ESI): m/z = 687 $[M + Na]^+$. –HRMS ((+)-ESI): m/z = 687.2256 (calcd. 687.2265 for $C_{32}H_{40}O_{15}Na$ $[M + Na]^+$).

3.3.3 Epimedkoreside C (3)

Yellow amorphous powder. $-[\alpha]_D^{23.0} = -115.1$ (c = 0.008, MeOH). –^{1}H and ^{13}C NMR data: see Tables 1 and 2. –MS ((+)-ESI): m/z = 991 $[M + Na]^+$. –HRMS ((+)-ESI): m/z = 991.3409 (calcd. 991.3423 for $C_{45}H_{60}O_{23}$ Na $[M + Na]^+$).

3.4 Acid hydrolysis of compounds 1–3

Compounds **1**–**3** (2 mg, each) were hydrolyzed with 2 M HCl-DMSO 1:1 (2 mL) on water bath for 6 h, respectively. The reaction mixture was extracted with $CHCl_3$ (4 × 2 mL). The remaining aq. layer was neutralized by addition of Amberlite IRA401 and filtered. The filtrate was dried in vacuo, then dissolved in pyridine (1 mL) containing L-cysteine methyl ester (10 mg mL^{-1}, 1 mL) and kept at 60 °C for 1 h. To this mixture, a solution of trimethylsilylimidazole (1 mL) was added, and it was heated at 60 °C for 30 min. The mixture was subjected to GC analysis under the following conditions: 30QC2/AC-5 quartz capillary column (30 m × 0.32 mm); column temperature, 180 °C/280 °C; programmed increase, 3 °C min^{-1}; carrier gas, N_2 (1 mL min^{-1}); injection and detector temperature, 250 °C; injection volume, 4 μL; split ratio, 1:50. The configurations of sugar residues were determined by comparison of the retention times of their corresponding trimethylsilylated L-cysteine derivatives with the derivatives of the authentic samples (retention times for D-glucose, L-glucose, and L-rhamnose were 18.29, 18.87, and 14.97, respectively).

3.5 Preparation of human neutrophils and measurement of superoxide anion generation and elastase release

The preparation of human neutrophils and measurement of superoxide anion generation and elastase release were carried out following the method of the literature [26].

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