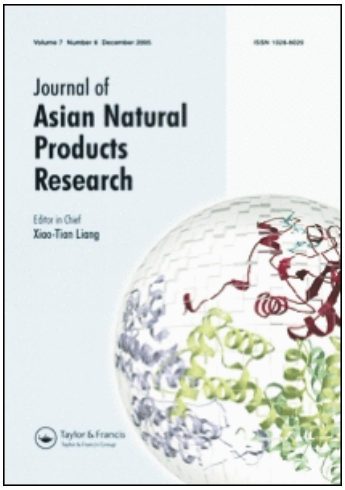

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Two new flavonol glycosides from *Epimedium koreanum* Nakai

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Two new flavonol glycosides, named icarisid B (**1**) and icarisid C (**2**), along with seven known flavonol glycosides were isolated from the bioassay-directed fractions of the aqueous extract of *Epimedium koreanum* Nakai. The structures of the two new compounds were established on the basis of chemical and spectroscopic methods (ESI-MS, 1D, and 2D NMR) as 5-hydroxyl-4′-methoxy-8-(γ-hydroxyl-γ,γ-dimethyl)-propyl-3-*O*-α-L-rhamnopyranosyl-flavonol-7-*O*-β-D-glucopyranoside (**1**) and 5-hydroxyl-4′-methoxy-8-(γ-methoxy-γ,γ-dimethyl)-propyl-3-*O*-β-D-glucopyranosyl(1 → 3)-α-L-rhamnopyranosyl-flavonol-7-*O*-β-D-glucopyranoside (**2**), respectively. All the nine compounds were tested for their effects on proliferation and alkaline phosphatase (ALP) activity using UMR106 cells. As a result, five compounds showed stimulating effects on both the proliferation and ALP activity, which suggested that they might be used as potential leading compounds to cure osteoporosis.

Keywords: *Epimedium koreanum* Nakai; icarisid B; icarisid C; UMR106; proliferation; ALP

1. Introduction

Epimedium koreanum Nakai (*E. koreanum*), one of the commonly used Chinese medicines, is believed to have various therapeutic properties including those against osteoporosis [1]. Previous investigation indicated that flavonoids were considered to be the main active components [2], such as icariin [3]. The present study was conducted to extend the research for active flavonol glycosides from *E. koreanum*. Stimulating effects on proliferation and differentiation of rat osteogenic sarcoma (UMR106) cells, with many osteoblast-like phenotypes, may indicate positive action on osteoblasts. In our bioactive screening assay, the aqueous extract of *E. koreanum* was found to show stimulating effects on the proliferation of UMR106 cells and indicated that it has the potential activity against osteoporosis. As the isolation was carried out, the ovariectomized (OVX) rat model and UMR106 cells were used to test the activities of the fractions *in vivo* and *in vitro*, respectively. In our present study, two new flavonol glycosides (**1** and **2**) and seven known compounds (**3**–**9**) were obtained (Figure 1). The effects of these nine compounds on proliferation and alkaline phosphatase (ALP) activity were tested.

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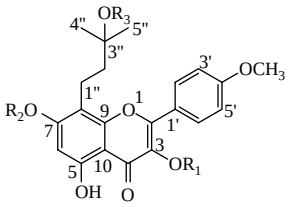
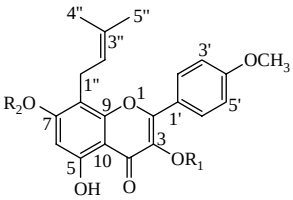


1 R_1 = α-Rha R_2 = β-Glc R_3 = H
2 R_1 = α-Rha^3-β-Glc R_2 = β-Glc R_3 = OCH_3
3 R_1 = H R_2 = β-Glc R_3 = H
4 R_1 = (4-acetyl-α-Rha)^3-β-Glc R_2 = β-Glc R_3 = H



5 R_1 = (4-acetyl-α-Rha)^3-(2-acetyl-β-Glc) R_2 = β-Glc
6 R_1 = α-Rha R_2 = β-Glc
7 R_1 = (4-acetyl-α-Rha)^3-β-Glc R_2 = β-Glc
8 R_1 = α-Rha^3-β-Glc R_2 = β-Glc
9 R_1 = (4-acetyl-α-Rha)^3-(6-acetyl-β-Glc) R_2 = β-Glc

Figure 1. Structures of compounds **1**–**9**.

2. Results and discussion

The aqueous extract of *E. koreanum* was applied to a polyamide column eluating with H_2O, 20, 50, and 95% EtOH, successively. It was to afford four fractions: EP-A–D. As shown in Figure 2, EP-1 (EP-A and EP-B) and EP-2 (EP-C and EP-D) strikingly increased both the trabecular bone mineral density (BMD) and the total BMD of the mouse femoral head. And the effects of these four fractions on proliferation and ALP activity of UMR106 were evaluated (Figures 3 and 4). As a result, EP-B fraction promoted the cell proliferation and ALP activity most significantly at the concentration of 0.5 μg/ml after 48 h of co-culture with UMR106 cells, indicating that the EP-B fraction might contain active constituents stimulating proliferation and ALP activity of UMR106. Extensive column chromatography on silica gel, octadecylsilanized silica gel (ODS), and repeated pre-HPLC yielded the nine compounds. Compounds **1** and **2** were characterized as new flavonol glycosides by NMR spectral data analysis. Compounds **3**–**9** were determined as known flavonol glycosides by comparison with those reported data in literature.

Icarisid B (**1**) is a yellow amorphous powder and the positive-ion HR-ESI-MS displayed a pseudo-molecular ion at *m/z* 717.2405 $[M+Na]^+$, indicating the molecular formula to be $C_{33}H_{42}O_{16}$. The presence of a flavonol glycoside skeleton was suggested from the UV [λ_{max}^{MeOH} at 315 (log ε 3.71), 270 (log ε 3.52) nm] and IR [ν_{max}^{KBr} at 3361 (OH), 1651 (conjugated C═O), 1593, 1510 (C═C, aromatic) cm^{-1}] absorptions as well as the ^{13}C NMR signals for C-2 (δ 152.8) and C-3 (δ 134.5). In the aromatic region of ^1H NMR spectrum, a typical AA′BB′ spin coupling system of four aromatic protons at δ 7.95 (2H, $J = 8.9$ Hz, H-2′ and H-6′) and 7.11 (2H, $J = 8.9$ Hz, H-3′ and H-5′)

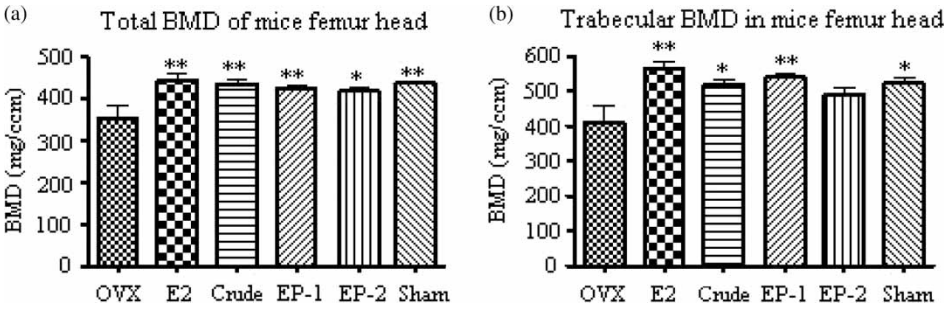


Figure 2. (a) Trabecular BMD in mice femur head. (b) Total BMD of mice femur head. $*p < 0.05$, $**p < 0.01$ when compared with OVX.

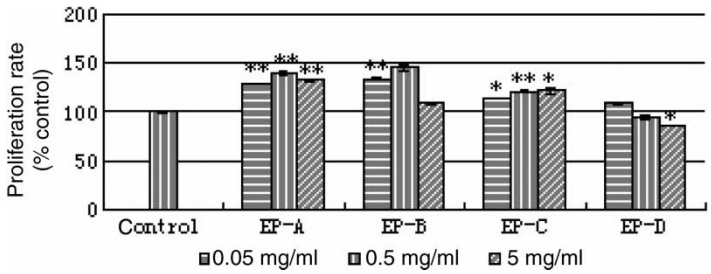


Figure 3. Effects of the four fractions of the extract chromatographed on a polyamide column on proliferation of UMR106 cells ($n = 3$) at dosages of 0.05, 0.5, and 5 μg/ml. $*p < 0.05$, $**p < 0.01$ when compared with control.

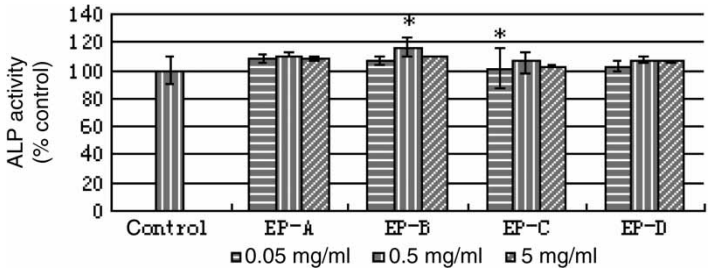


Figure 4. Effects of the four fractions of the extract chromatographed on a polyamide column on ALP activity of UMR106 cells ($n = 3$) at dosages of 0.05, 0.5, and 5 μg/ml. $*p < 0.05$, $**p < 0.01$ when compared with control.

suggested the presence of a *para*-substituted ring B. The HMBC correlation between the methoxy protons (δ 3.85) and C-4′ (δ 158.9) indicated that the *O*-methyl was attached to C-4′. In the ESI-IT-MS^n experiments, the MS^2 spectrum of the ion at *m/z* 717 $[M+Na]^+$ showed a positive fragment at *m/z* 571 $[M+Na-146]^+$ and the MS^3 spectrum of the ion at *m/z* 571 $[M+Na-146]^+$ showed a positive fragment at *m/z* 409 $[M+Na-146-162]^+$ suggesting that there were two hexose units in **1**. Acid hydrolysis of **1** with 2 M HCl was followed by GC–MS analysis of its aldononitrile peracetate derivatives using authentic samples as reference; the monosaccharides were assumed to be D-glucose and L-rhamnose in the relative proportions of 1:1. The HMBC correlation signals found for the carbon C-7 at δ 161.4 and the anomeric proton of glucose at δ 4.98 and for the carbon C-3 at δ 134.5 and the proton of rhamnose at δ 5.30 indicated that the glucose was attached at C-7 and the rhamnose was attached at C-3 of the aglycone by ester bond. The ^{1}H NMR spectrum displayed one singlet at δ 1.17 (6H, 4″, 5″-CH_3), two multiplets at δ 2.81 (2H, H-1″) and 1.55 (2H, H-2″) and the ^{13}C NMR spectrum showed peaks for C-1″–C-5″ at δ 17.4, 42.8, 69.0, 28.6, and 29.2, respectively, indicating the presence of a γ-hydroxyl-γ,γ-dimethyl-propyl moiety. It was attached to C-8 of the aglycone as indicated by the HMBC correlations between the H-1″ (δ 2.81) protons and C-7 (δ 161.4), C-8 (δ 109.7), and C-9 (δ 157.0). Analysis of the ^{13}C NMR spectral data of **1** and comparison with those of maohuoside A and icariin showed that the chemical shifts were similar to those of icariin except for the signals arising from C-1″ to C-5″ which were in good agreement with those of maohuoside A. On the basis of

the above data, the structure of **1** was established as 5-hydroxyl-4′-methoxy-8-(γ-hydroxyl-γ,γ-dimethyl)-propyl-3-*O*-α-L-rhamnopyranosyl-flavonol-7-*O*-β-D-glucopyranoside.

Icarisid C (**2**) was obtained as a yellow amorphous powder, and the positive-ion HR-ESI-MS showed the ion $[M+Na]^+$ at *m/z* 893.3074, corresponding to the molecular formula $C_{40}H_{54}O_{21}$. The ^{13}C NMR signals at δ 157.1, 134.3, and UV absorption maxima at λ_{max}^{MeOH} 352 (log ε 3.87) and 270 (log ε 3.66) nm were the characteristics of a flavonol. The ^1H NMR signals at δ 2.81 (1H, H-1″) and 2.70 (1H, H-1″), 1.56 (1H, H-2″) and 1.70 (1H, H-2″), and 1.15 (6H, 4″, 5″-CH_3) correlated with the carbon signals at δ 16.9 (C-1″), 38.1 (C-2″) and 24.9 (C-4″), and 24.9 (C-5″) in the HMQC spectrum, respectively. The HMBC correlations between H-4″, H-5″ and C-3″, C-2″, OCH_3-3″, and C-3″. $^1H-^1H$ COSY correlation between H-2″ and H-1″ suggested the presence of a γ-methoxy-γ,γ-dimethyl-propyl moiety. The C-1″ of this moiety is attached to C-8 of the aglycone, as indicated by the correlation between H-1″ (δ 2.81) and C-7 (δ 161.6), C-8 (δ 109.4), and C-9 (δ 153.0) of the aglycone in the HMBC spectrum. Acid hydrolysis of **2** with 2 M HCl was followed by GC–MS analysis of its aldononitrile peracetate derivatives using authentic samples as references. The result indicated the presence of D-glucose and L-rhamnose in a ratio of 2:1. The HMBC correlation signals found for the carbon C-7 at δ 161.6 and the anomeric proton of glucose at δ 5.01 and for the carbon C-3 at δ 134.3 and the anomeric proton of rhamnose at δ 5.33 indicated that one of the two glucoses was attached at C-7 and the rhamnose was attached at C-3 of the aglycone by ester bond. The HMBC correlation signals found for the C-3 at δ 81.1 of the rhamnose and the anomeric proton of another glucose at δ 4.30 indicated that the C-3 sugar chain was glucopyranosyl(1 → 3)-rhamnopyranosyl. Analysis of the ^{13}C NMR spectral data of **2** and comparison with those of hexandraside F and **1** revealed that the chemical shifts of **2** were similar to those of hexandraside F, except for the signals arising from C-1″ to C-5″ which were in good agreement with those of **1**. Therefore, the structure of **2** was concluded as 5-hydroxyl-4′-methoxy-8-(γ-methoxy-γ,γ-dimethyl)-propyl-3-*O*-β-D-glucopyranosyl(1 → 3)-α-L-rhamnopyranosyl-flavonol-7-*O*-β-D-glucopyranoside.

The structures of compounds **3**–**9** were determined as maohuoside A, caohuoside F, caohuoside E, icariin, korepimedoside C, hexandraside F, and epimedokoreanoside by comparison of their spectroscopic data with the literature values [4–10]. The *in vitro* activity of compounds **1**–**9** was evaluated by the proliferation and ALP assay. Compounds **1**–**4** showed no or weak stimulating effects on the proliferation and ALP activity of UMR106, while compounds **5**–**9** showed stimulating effects on the proliferation and ALP activity. The above result suggested that the presence of unsaturated linear prenyl group at the C-8 location contributes to the proliferation and ALP activity.

3. Experimental

3.1 General experimental procedures

Melting points were determined on a YANACO apparatus and are uncorrected. Optical rotations were taken on a P-1020 digital polarimeter (Jasco Corporation, Tokyo, Japan). UV spectra were recorded on a UV2401PC spectrophotometer (Shimadzu, Kyoto, Japan). IR spectra were measured on a FT/IR-8900 spectrophotometer (Shimadzu) in a KBr disk. The ^1H NMR spectra were obtained on a Bruker AVANCE 400 MHz spectrometer, while ^{13}C NMR spectra were recorded at 100 MHz on the same instrument, with TMS as an internal standard. ESI-MS spectra were acquired using a Bruker Esquire 2000 mass spectrometer.

HR-ESI-MS spectra were recorded using a Micromass Q-TOF mass spectrometer. GC–MS spectra were determined on a Shimadzu GCMS-QP2010 plus spectrometer and column chromatography was performed using silica gel (200–300 mesh; Qingdao Factory of Marine Chemical Industry, Qingdao, China) and ODS (40–75 μm; Fuji Silysia Chemical Ltd, Kasugai, Japan). Preparative HPLC was performed using an ODS column (250 × 20 mm, 10 μm, Shimadzu Pak; Detector: UV). D-Glucose, L-rhamnose, and 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetrazolium) (MTS) were purchased from Sigma Chemical Company (St Louis, MO, USA). The UMR106 cell line was purchased from American Type Culture Collection, No. CRL-1661. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Hyclone (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 0.25% trypsin-5.3 mM EDTA (1 ×) was purchased from Gibco BRL (Grand Island, NY, USA).

3.2 Plant material

The herb *E. koreanum* was collected in June 2004 at Xinbin (Liaoning Province, China) and identified by Prof. Qi-Shi Sun (Division of Pharmacognosy, Shengyang Pharmaceutical University, Shengyang, China). A voucher specimen (No. 20040608) is deposited in the Shenzhen Research Center of Traditional Chinese Medicines and Natural Products, Shenzhen (518057), China.

3.3 Extraction and isolation

The dried and powdered aerial parts of *E. koreanum* (10 kg) were extracted with H_2O twice for 2 h each. The aqueous extract was concentrated under reduced pressure and then applied to a polyamide column eluating with H_2O, 20, 50, and 95% EtOH, successively. Four fractions, EP-A (153.4 g), EP-B (144.0 g), EP-C (108.4 g), and EP-D (11.0 g) were obtained. Guided by the bio-assay results, the EP-B fraction was chromatographed on silica gel column (200–300 mesh) with gradient $CHCl_3$–MeOH (100:0; 97:3; 93:7; 95:5; 90:10; 85:15; 75:25; 70:30; 60:40) elution to yield 15 fractions (B1–B15). Fraction B13 (30.1 g) was separated by MPLC on ODS using MeOH–H_2O (4:6; 5:5; 6:4; 7:3) to yield 12 subfractions (B13-1–B13-12); subfraction B13-10 was subjected to RP-HPLC eluted with 60% MeOH–H_2O to obtain compound **3** (125.8 mg). Fraction B11 (9.8 g) was applied to an ODS column chromatography by MPLC eluted with MeOH–H_2O (4:6; 5:5; 6:4; 7:3) to yield subfractions B11-1–B11-14. The subfraction B11-7 was subjected to silica gel column chromatography with $CHCl_3$/MeOH (9:1; 8:2; 7:3) to yield 13 subfractions (B11-7-1–B11-7-13); the subfractions B11-7-9 and B11-7-10 were applied to RP-HPLC eluted with 55% MeOH–H_2O to afford compounds **1**, **2**, and **4** (7.6, 33.2, and 13.4 mg). The subfraction B11-10 was subjected to RP-HPLC eluted with 55% MeOH–H_2O to afford compounds **5**–**8** (70.8, 215.3, 322.2, and 324.1 mg). Fraction B10 (4.4 g) was separated by MPLC on ODS using MeOH–H_2O (4:6; 5:5; 6:4; 7:3) to yield eight subfractions (B10-1–B10-8). Subfraction B10-6 (412.8 mg) was subjected to RP-HPLC eluted with 60% MeOH–H_2O to afford eight subfractions (B10-6-1–B10-6-8) and subfraction B10-6-2 was purified by RP-HPLC (60% MeOH–H_2O) to obtain compound **9** (23.7 mg).

3.3.1 Icarisid B (*1*)

A yellow amorphous powder; mp 229–231°C; $[\alpha]_D^{25}$ −81.7 ($c = 0.05$, MeOH). UV (MeOH) λ_{max} nm (log ε): 355 (3.75), 315 (3.71), 270 (3.52), 219 (3.29). IR (KBr) (ν cm^{-1}): 3361, 2925, 1651, 1593,

1510, 1487, 1373, 975, 937, and 908. ^{1}H NMR (DMSO-d_6) spectral data, see Table 1. ^{13}C NMR spectral data, see Table 2. HR-ESI-MS (positive mode): *m/z* 717.2405 $[M+Na]^+$ (calcd for $C_{33}H_{42}O_{16}Na$, 717.2371). ESI-MS (positive mode): *m/z* 717 $[M+Na]^+$, 571 $[M+Na-Rha]^+$, 409 $[M+Na-Rha-Glc]^+$.

3.3.2 *Icarisid C (**2**)*

A yellow amorphous powder; mp 193–195°C; $[\alpha]_D^{25}$ −110.5 ($c = 0.13$, MeOH). UV (MeOH) λ_{max} nm (log ε): 352 (3.87), 315 (3.83), 270 (3.66), 220 (3.45). IR (KBr) (ν cm^{-1}): 3382, 2927, 1651, 1596, 1508, 1491, 1437, 1215, 916, 839, and 808. ^{1}H NMR (DMSO-d_6) spectral data, see Table 1. ^{13}C NMR spectral data, see Table 2. HR-ESI-MS (positive mode): *m/z* 893.3074 $[M+Na]^+$ (calcd for $C_{40}H_{54}O_{21}Na$, 893.3055). ESI-MS (positive mode): *m/z* 893 $[M+Na]^+$, 731 $[M+Na-Glc]^+$, 585 $[M+Na-Glc-Rha]^+$, 423 $[M+Na-Glc\times 2-Rha]^+$.

3.4 *Acid hydrolysis*

Compounds **1** and **2** (each 5 mg) were dissolved in 2 M HCl (5 ml) and then stirred at 90°C for 2 h. After cooling, the reaction product was extracted with ethyl acetate twice and the aqueous layer was concentrated to dryness under reduced pressure. To the residue were added 1 ml of dry pyridine and 2 mg of $NH_2OH{\cdot}HCl$, and the reaction mixture was stirred at 90°C for 0.5 h. Subsequently, 0.5 ml of Ac_2O was added, followed by stirring at 90°C for 1 h. The resulting aldononitrile peracetates were directly subjected to GC–MS analysis using standard aldononitrile peracetates as reference samples [11].

3.5 *Animal study design*

After 1 week of acclimatization, 50 mice were OVX ($n = 50$) and 10 other mice were sham-operated (sham). After 10 days of recovery, OVX mice were randomly and equally divided into six groups:

Table 1. ^{1}H NMR spectral data of compounds **1** and **2** in DMSO-d_6 at 400 MHz.

No.	**1**	**2**	No.	**1**	**2**
2	–	–	C-3 Rha-1	5.30 (d, 1.4)	5.33 (s)
3	–	–	2	3.16 (d, 4.0)	4.20 (s)
6	6.63 (s)	6.62 (s)	3	4.01 (m)	3.59 (d, 3.0)
OH-5	12.5 (s)	12.5 (s)	4	3.14 (o)[a]	3.25 (o)
1′	–	–	5	3.50 (o)	3.36 (o)
2′	7.95 (d, 8.9)[b]	7.95 (d, 8.9)	6	0.79 (d, 5.7)	0.85 (d, 6.0)
3′	7.11 (d, 8.9)	7.12 (d, 8.9)	C-7 Glc-1	4.98 (d, 7.1)	5.01 (d, 7.0)
5′	7.11 (d, 8.9)	7.12 (d, 8.9)	2	3.31 (o)	3.28 (o)
6′	7.95 (d, 8.9)	7.95 (d, 8.9)	3	3.28 (o)	3.18 (o)
OCH_3-4′	3.85 (s)	3.85 (s)	4	3.14 (o)	3.33 (o)
1″	2.81 (m)	2.81 (m), 2.70 (m)	5	3.42 (o)	3.18 (o)
2″	1.55 (m)	1.70 (m), 1.56 (m)	6	3.72 (dd, 5, 10), 3.47(o)	3.70 (dd, 7.7, 10.0)
3″	–	–	Rha-Glc-1		4.30 (d, 7.7)
4″	1.17 (s)	1.15 (s)	2		3.06 (o)
5″	1.17 (s)	1.15 (s)	3		3.38 (o)
OCH_3-3″	–	3.16 (s)	4		4.20 (s)
			5		3.32 (o)
			6		3.50 (o)

Recorded on a Bruker-400 MHz NMR spectrometer.
[a] Overlapped signals indicated by (o).
[b] *J* values (in parentheses) are in Hz and chemical shifts (δ) are in ppm. All assignments made by the combination of 1D and 2D NMR measurements (DEPT135, COSY, HMQC, and HMBC).

Table 2. ^{13}C NMR spectral data of compounds **1** and **2** in DMSO-d_6 at 100 MHz.

No.	1	2	No.	1	2
2	152.8	157.1	C-3 Rha-1	102.0	101.4
3	134.5	134.3	2	69.7	69.2
4	178.3	178.3	3	70.0	81.1
5	160.6	160.7	4	70.7	70.5
6	98.1	98.0	5	70.3	69.9
7	161.4	161.6	6	17.4	17.5
8	109.7	109.4	C-7 Glc-1	100.5	100.4
9	157.0	153.0	2	73.4	73.5
10	105.5	104.8	3	76.4	76.3
1′	122.3	122.2	4	71.1	69.7
2′	130.5	130.7	5	77.1	76.3
3′	114.0	114.1	6	60.6	61.0
4′	158.9	159.0	Rha-Glc-1		104.8
5′	114.0	114.1	2		73.9
6′	130.5	130.7	3		77.2
OCH_3-4′	55.5	55.6	4		69.2
1″	17.4	16.9	5		76.8
2″	42.8	38.1	6		60.7
3″	69.0	74.0			
4″	28.6	24.9			
5″	29.2	24.9			
OCH_3-3″	–	48.6			

Recorded on a Bruker-400 MHz (100 MHz for ^{13}C) NMR spectrometer.

OVX+vehicle; OVX+17β estradiol (4 mg/kg); OVX+crude (1400 mg/kg); OVX+EP-1 (1053 mg/kg); and OVX+EP-2 (600 mg/kg). The oral administration to all rats continued for 6 weeks. All rats were fed the regular diet in the experimental period. After killing, the femurs were collected and stored at −20°C. When measuring BMD, the femurs were scanned and the trabecular BMD and total BMD in femoral heads were measured using pQCT [12].

3.6 Cell culture

The UMR106 cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2 in DMEM supplemented with 5% FBS. At around 80% confluence observed under the light microscope (after ~4 days), the cells were transferred to 96-well microtiter plates at a density of 7500 cells/well by the standard trypsinization method.

3.7 Proliferation assay

The effects of these fractions and compounds on the proliferation of UMR106 were evaluated. Proliferative effects of the samples against the UMR106 cell line were determined in the MTS colorimetric assay. The cells were seeded into 96-well plates at a concentration of 7500 cells/well. The cells were allowed to attach for 48 h. The medium was removed and replaced with the experimental medium (1% FBS in DMEM) containing various concentrations of each sample. After 48 h of incubation, the MTS/PMS master mix solution containing 1.1 ml MTS and 55 μl PMS was added to 5.5 ml of PBS solution and then 60 μl of the final mixture was added to each well. The cells were incubated at 37°C in darkness for 1 h, and then the optical density was measured on Spectra MAX 340PC at 490 nm. The negative control was 0.1% DMSO. The 17β estradiol was dissolved in DMEM with 0.1% DMSO to give concentrations

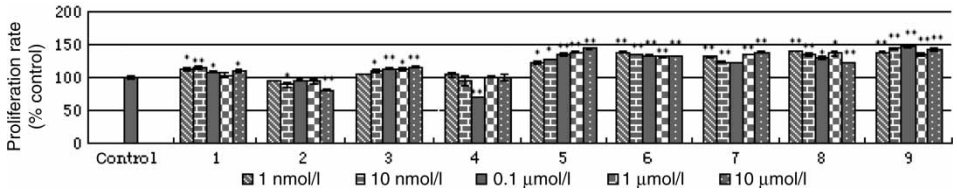


Figure 5. Effects of pure compounds **1**–**9** on the proliferation of UMR106 cells ($n = 3$) at dosage of 1, 10 nmol/l, 0.1, 1, and 10 μmol/l. $*p < 0.05$, $**p < 0.01$ when compared with control.

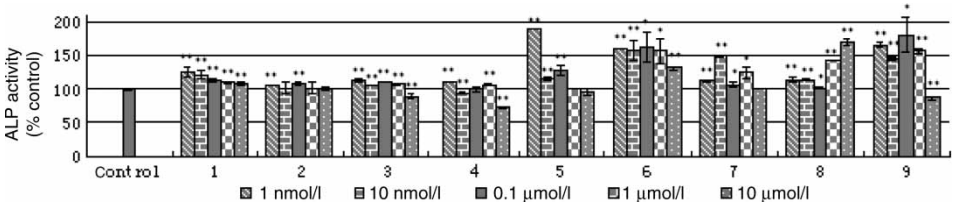


Figure 6. Effects of pure compounds **1**–**9** on the ALP activity of UMR106 cells ($n = 3$) at dosage of 1, 10 nmol/l, 0.1, 1, and 10 μmol/l. $*p < 0.05$, $**p < 0.01$ when compared with control.

of 0.1 μmol/l as positive control. The proliferation ratio was expressed as the percentage of the difference of the optical density at 490 nm of the cells treated with samples, i.e., proliferation ratio $= A_{\text{sample}}/A_{\text{control}}$. The results of pure compounds are shown in Figure 5.

3.8 ALP activity assay

The cells were seeded to 96-well plate at 7500 cells/well in 5% FBS/DMEM and cultured for 48 h. Then, followed by treatment with various concentrations of test samples in 1% FBS/DMEM for another 48 h, the medium was removed and the cells were washed with the ice-cold PBS. Six wells of the same column of the plate contained cells that underwent the same treatment. Then 20 μl/well lysis buffer was added to lyse the cells and release the cell content. Then the lysate was frozen at −80°C for at least 5 min and thawed back to room temperature. The freeze-and-thaw cycle helped in homogenizing the cellular material. To half the number of wells which were subjected to ALP assay, 100 μl alkaline buffer containing 15 mmol/l *p*-nitrophenyl phosphate was added. The reaction solution was then incubated for 30 min at 37°C in darkness. The absorbance was measured on Spectra MAX 340PC at 405 nm. The other half of the individual wells were used for the total protein determination by Bradford method with the addition of 200 μl/well, Bradford reagent, and the absorbance was measured at 595 nm after 5-min incubation at room temperature. The negative control was 0.1% DMSO. The 17β estradiol was dissolved in DMEM with 0.1% DMSO to give concentrations of 0.1 μmol/l as positive control. The inducing effects of ALP activity were quantified as $(A_{405,\ \text{test sample}}/\mu\text{g total cellular protein})/(A_{405,\ \text{control}}/\mu\text{g total cellular protein})$. The results of pure compounds are shown in Figure 6.

3.9 Statistical analysis

Results were expressed as mean ± SD ($n = 3$). The statistical significance was assessed by Student's *t*-test, $*p < 0.05$, $**p < 0.01$.

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