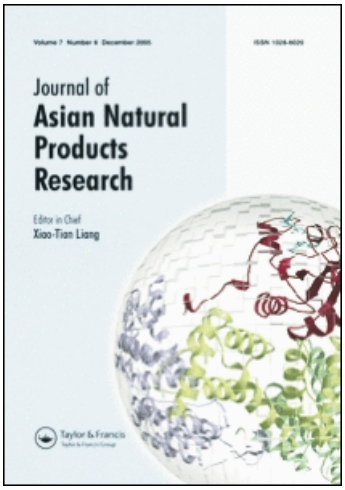

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Pu Liu[a]; Rui-Xue Deng[a]; Hong-Quan Duan[b]; Wei-Ping Yin[a]; Tian-Zeng Zhao[c]
[a] Chemical Engineering & Pharmaceutical College, Henan University of Science and Technology, Luoyang, China [b] School of Pharmaceutical Sciences, Basic Medical Research Center of Tianjin, Tianjin Medical University, Tianjin, China [c] Key Laboratory of Natural Products, Henan Academy of Sciences, Zhengzhou, China

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Pu Liu[a]*, Rui-Xue Deng[a], Hong-Quan Duan[b]*, Wei-Ping Yin[a] and Tian-Zeng Zhao[c]

[a]*Chemical Engineering & Pharmaceutical College, Henan University of Science and Technology, Luoyang, China;* [b]*School of Pharmaceutical Sciences, Basic Medical Research Center of Tianjin, Tianjin Medical University, Tianjin, China;* [c]*Key Laboratory of Natural Products, Henan Academy of Sciences, Zhengzhou, China*

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The phytochemical study of the roots of *Phlomis umbrosa* Turcz. afforded three new phenylethanoid glycosides, 3‴-acetyl-*O*-betonyoside D (**1**), 2‴, 3‴-di-acetyl-*O*- betonyoside D (**2**), and 3‴, 4‴-di-acetyl-*O*-betonyoside D (**3**), along with five known phenylethanoid glycosides. Their structures were elucidated on the basis of spectroscopic data. The antitumor activity of the isolated compounds was investigated.

Keywords: *Phlomis umbrosa* Turcz; 3‴-acetyl-*O*-betonyoside D; 2‴,3‴-di-acetyl-*O*-betonyoside D; 3‴,4‴-di-acetyl-*O*-betonyoside D; phenylethanoid glycosides

1. Introduction

Phlomis umbrosa Turcz. is a kind of perennial herbaceous plant growing in the North of China. Its roots have been used to treat cold, reduce swelling, and staunch bleeding in traditional Chinese medicine [1,2], Phytochemical studies carried out by different research groups have resulted in the isolation of various triterpenoids, iridoid glycosides, and phenylethanoid glycosides [3–11] from this plant. In the course of our investigations on the roots of *P. umbrosa*, we have studied on the chemical constituents and reported three new phenylethanoid glycosides **1**, **2**, and **3** from this plant. Their structures were elucidated on the basis of various 2D-NMR techniques, including HSQC, HMBC, $^{1}H-^{1}H$ COSY, and NOESY spectroscopy. The cytotoxic activity of the new compounds and the known compounds against tumor cells were carried out by MTT methods.

2. Results and discussion

The ethyl acetate (EtOAc)-soluble fraction from *P. umbrosa* was separated by silica gel, gel permeation chromatography and pre-HPLC (ODS-A) to give three new phenylethanoid glycosides, 3‴-acetyl-*O*-betonyoside D (**1**), 2‴, 3‴-di-acetyl-*O*-betonyoside D (**2**), and 3‴,4‴-di-acetyl-*O*-betonyoside D (**3**) (Figure 1), as well as five known phenylethanoid glycosides (**4**–**8**).

Compound **1** was obtained as an amorphous yellowish powder. Its HRESIMS exhibited a pseudomolecular ion at *m/z* 825.2759 $[M - H]^{-}$, corresponding to the molecular formula $C_{38}H_{49}O_{20}$. The IR spectrum showed absorption bands of hydroxyl (3420 cm^{-1}), α,β-unsaturated ester (1714 and 1630 cm^{-1}), and aromatic rings (1630, 1596, and 1516 cm^{-1}). The ^{1}H and ^{13}C NMR spectra of **1** (Table 1), extensively analyzed with the aid of $^{1}H-^{1}H$ COSY and HSQC, exhibited proton signals characteristic

*Corresponding authors. Email: liudeng20022002@yahoo.com.cn; duanhq@tijmu.edu.cn

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Table 1. ^{1}H- and ^{13}C NMR spectral data of compounds **1**–**3** (^{1}H, 300 MHz; ^{13}C 75 MHz; in CD_3OD, δ ppm, J Hz).

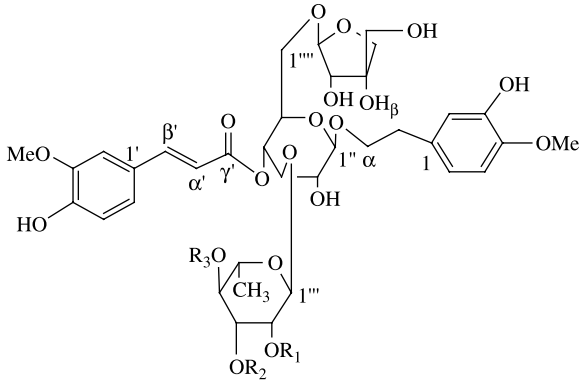
	1		**2**		**3**	
No.	δ_H J (Hz)	δ_C	δ_H J (Hz)	δ_C	δ_H J (Hz)	δ_C
1		133.0		133.0		132.9
2	6.73 (br s)	117.2	6.74 (br s)	117.2	6.74 (br s)	117.2
3		147.5		147.4		147.4
4		147.7		147.6		147.6
5	6.82 (d, 8.5)	113.0	6.82 (d, 8.5)	113.0	6.82 (d, 8.4)	113.0
6	6.69 (br d, 8.5)	121.3	6.69 (br d, 8.5)	121.3	6.69 (br d, 8.4)	121.3
α	3.75, 4.04 (m)	72.3	3.75, 4.03 (m)	72.3	3.76, 4.03 (m)	72.3
β	2.83 (t, 7.4)	36.7	2.82 (t, 7.4)	36.7	2.82 (t, 7.4)	36.6
OCH_3	3.81 (s)	56.6	3.81 (s)	56.6	3.81 (s)	56.6
1′		127.8		127.7		127.6
2′	7.21 (br s)	112.0	7.21 (br s)	111.9	7.22 (br s)	112.0
3′		149.5		149.5		149.5
4′		150.9		151.0		151.0
5′	6.83 (d, 8.5)	116.6	6.83 (d, 8.5)	116.6	6.82 (d, 7.0)	116.7
6′	7.09 (br d, 8.5)	124.5	7.10 (br d, 8.5)	124.5	7.11 (br d, 7.0)	124.4
α′	6.39 (d, 16.0)	115.3	6.39 (d, 15.8)	115.1	6.41 (d, 15.8)	115.0
β′	7.67 (d, 16.0)	148.1	7.68 (d, 15.8)	148.2	7.70 (d, 15.8)	148.1
γ′		168.2		169.1		168.0
OCH_3	3.89 (s)	56.6	3.89 (s)	56.6	3.88 (s)	56.6
1″	4.36 (d, 7.8)	104.3	4.35 (d, 7.8)	104.3	4.37 (d, 7.8)	104.2
2″	3.42–3.46 (m)	76.1	3.42–3.46 (m)	76.0	3.42–3.46 (m)	76.3
3″	3.80–3.85 (m)	82.1	3.80–3.85 (m)	82.4	3.85–3.90 (m)	80.0
4″	5.02 (m)	70.7	5.03 (m)	70.8	5.03 (m)	70.7
5″	Overlap	74.7	Overlap	74.6	Overlap	74.5
6″	3.75–3.80 (m)	68.6	3.75–3.80 (m)	68.5	3.75–3.80 (m)	68.4
1‴	5.18 (br s)	103.1	5.19 (br s)	100.4	5.37 (br s)	101.8
2‴	3.75 (br s)	75.7	5.35 (br s)	71.4	4.07 (m)	69.9
3‴	5.16 (m)	71.0	4.95 (m)	73.3	4.97 (m)	73.2
4‴	3.60 (m)	74.2	3.41 (m)	71.2	5.04 (m)	72.4
5‴	3.71 (m)	70.6	3.73 (m)	70.5	3.78 (m)	67.9
6‴	1.12 (d, 6.1)	18.6	1.13 (d, 6.1)	18.5	1.04 (d, 6.1)	18.2
$COCH_3$	2.08	172.8/21.2	2.08	171.8/20.8	1.65	171.9/20.5
			1.98	172.4/20.9	2.00	172.0/20.9
1⁗	4.92	111.2	4.92	111.1	4.92	111.1
2⁗	3.89 (m)	78.2	3.88 (m)	78.2	3.89 (m)	78.2
3⁗		80.7		80.7		80.7
4⁗	3.75–3.95 (m)	75.2	3.75–3.95 (m)	75.2	3.75–3.95 (m)	75.2
5⁗	3.50–3.60 (m)	65.8	3.50–3.60 (m)	65.8	3.50–3.60 (m)	65.8

of an *E*-feruloyl group indicating three aromatic protons resonating at δ 7.21 (br s), 7.09 (br d, $J = 8.5$ Hz), 6.83 (d, $J = 8.5$ Hz) as an ABX system and two *trans* olefinic protons as an AB system at δ 7.67, 6.39 (d, $J = 16.0$ Hz), a 3-hydroxy-4-methoxyphenylethanol moiety indicating three aromatic protons at δ 6.82 (d, $J = 8.5$ Hz), 6.73 (br s), 6.69 (d, $J = 8.5$ Hz) as an ABX system, a broad triplet signal at δ 2.83 (t, $J = 7.4$ Hz) and two non-equivalent protons at δ 4.04 and 3.75 due to the side chain of the aglycone moiety, and an acetyl group. Additionally, three signals assignable to anomeric protons indicated the presence of three sugar moieties in **1**: a doublet proton at δ 4.36 (d, $J = 7.8$ Hz, H-1′ of β-glucosyl), a broad singlet proton at δ 5.18 (br s, 1″ of α-rhamnosyl), and a broad

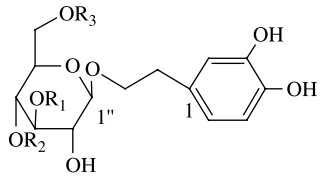
singlet proton at δ 4.92 (overlapped, 1‴ of β-apiosyl). The sugars were detected after the acid hydrolysis and compared with the authentic sugars on TLC. Thus, compound **1** was deduced as a phenylethanol glycoside, with three sugar moieties, one feruloyl group and an acetyl group. Its ^{13}C NMR spectral data were similar to those of betonyoside D [12], except for an acetyl group.

In the HMBC spectrum (Figure 2) of **1**, H-4″ (δ 5.02) correlated with the ketone of feruloyl group, and H-1″ (δ 4.36, d, $J = 7.8$ Hz) with C-α(δ 72.3), which indicated that the feruloyl group and phenylethanol moiety were linked to C-4″ and C-1″ of the glucosyl, respectively. Moreover, the clear correlations of H-1‴ (δ 5.18, br s) to C-3″, and H-1⁗ (δ 4.92) to C-6″ indicated that rhamnosyl should be attached to C-3″ of glucosyl and apiosyl to C-6″ of the glucosyl group. The correlations between H-3‴ (δ 5.16, br s) and C-1‴, C-2‴, C-5‴, and C═O (δ 172.8) indicated that an acetyl group was attached to C-3‴ of rhamnosyl unit. Therefore, the structure of **1** was elucidated as 3-hydroxy-4-methoxy-β-phenylethoxy-*O*-[3-acetyl-α-L-rhamnopyranosyl-(1 → 3)]-4-*O*-feruloyl-[β-D-apiofuranosyl-(1 → 6)]-β-D-glucopyranoside (3‴-acetyl-*O*-betonyoside D).

Compound **2** was obtained as an amorphous yellowish powder. Its HRESIMS exhibited a pseudomolecular ion at *m*/*z* 891.2964 $[M + Na]^+$, which was compatible with the molecular formula $C_{40}H_{52}O_{21}$. The ^{1}H and ^{13}C NMR spectra of **2** (Table 1) showed that its structure is closely related to that of **1**. Comparing the NMR and MS spectral data of **2** with those of **1**, it is obvious that **2** bears one more acetyl group than **1**. In the HMBC spectrum, H-4″ (δ 5.03) correlated with the ketone of feruloyl group, and H-1″ (δ 4.35, d, $J = 7.8$ Hz) with C-α(δ 72.3), which indicated that the feruloyl group and phenylethanol moiety were linked to C-4″ and C-1″ of the glucosyl, respectively. Moreover, the clear correlations of H-1‴ (δ 5.19, br s) to C-3″, and H-1⁗ (δ 4.92) to C-6″ indicated that the rhamnosyl should be attached to C-3″ of the glucosyl group and



	R_1	R_2	R_3
1	H	Ac	H
2	Ac	Ac	H
3	H	Ac	Ac



	R_1	R_2	R_3
4	a-L-Rha	H	H
5	H	H	caffeoyl
6	a-L-Rha	H	caffeoyl
7	a-L-Rha	caffeoyl	H
8	a-L-Rha	Api	feruloyl

Figure 1. Structures of compounds **1**–**8**.

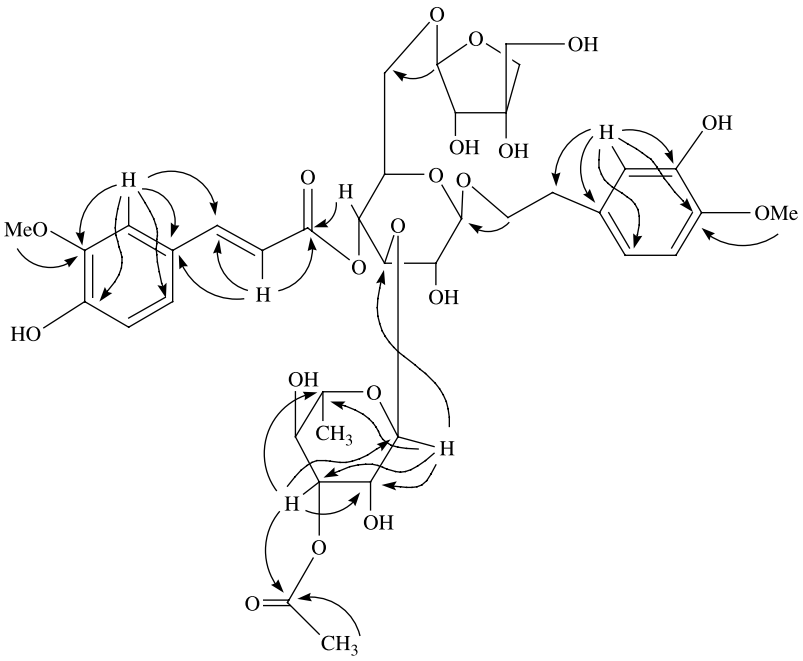


Figure 2. The key HMBC correlations of compound **1**.

the apiosyl group to C-6″ of the glucosyl group. The correlations between H-2‴ (δ 5.35, br s) and C-1‴, C-3‴, C-4‴, and C═O (δ 171.8) indicated that an acetyl group was attached to C-2‴ of the rhamnosyl unit. In the same manner, the other acetyl group was assigned to the position C-3‴ of rhamnosyl unit. Therefore, the structure of **2** was elucidated as 3-hydroxy-4-methoxy-β-phenylethoxy-*O*-[2,3-diacetyl-α-L-rhamnopyranosyl-(1 → 3)]-4-*O*-feruloyl-[β-D-apiofuranosyl-(1 → 6)]-β-D-glucopyranoside (2‴,3‴-di -acetyl-*O*-betonyoside D).

Compound **3** was obtained as an amorphous yellowish powder. The positive ion HRESIMS of **3** exhibited a pseudomolecular ion at *m/z* 891.2865 $[M + Na]^+$ suggesting the molecular formula $C_{40}H_{52}O_{21}$. The ^1H and ^{13}C NMR spectra of **3** (Table 1) showed that its structure is closely related to those of **2**. Compound **3** has the same MS data and similar NMR spectral data of **2**, except for the positions of two acetyl groups. In the HMBC spectrum, the signal of H-3‴ (δ 4.97) correlated with the carbon signals of C═O (171.9), C-1‴, C-2‴, and C-4‴, the methyl signal of H_3-6‴ (δ 1.04, d, $J = 6.1$ Hz) correlated with the signals of C-4‴ and C-5‴, and the signal of H-4‴ (δ 5.04) correlated with the signals of C═O (172.0), C-2‴, C-3‴, and C-5‴. Thus, two acetyl groups were located at positions C-3‴ and C-4‴ of the rhamnosyl unit. Therefore, the structure of **3** was elucidated as 3-hydroxy-4-methoxy-β-phenylethoxy-*O*-[3,4-di-acetyl-α-L-rhamnopyranosyl-(1 → 3)]-4-*O*-feruloyl-[β-D-apiofuranosy-(1 → 6)]-β-D-glucopyranoside (3‴, 4‴-di- acetyl-*O*-betonyoside D).

Five known compounds, decaffeoylverbascoside (**4**) [13,14], calcelarioside B (**5**) [15], verbascoside (**6**) [16,17], isoverbascoside (**7**) [15,17], and alyssonoside (**8**) [18], were identified by comparison of their spectroscopic data with those of the literature values.

In search for antitumor compounds, we examined the cytotoxic activity against tumor cells for these compounds with the reported procedure [19,20]. The results of inhibitory percent of compounds **1**–**8** were shown in Table 2.

Table 2. Cytotoxic activities of compounds **1**–**8**.

	Inhibition (%, $n = 6$)			
	HeLa		L929	
No.	10 (μg/ml)	30 (μg/ml)	10 (μg/ml)	30 (μg/ml)
1	16.62	26.00	48.63	70.70
2	26.80	28.60	29.35	30.00
3	52.00	53.86	68.19	70.23
4	61.85	69.26	54.23	54.73
5	69.63	58.02	48.01	55.72
6	−0.74	10.37	68.00	61.76
7	−33.33	17.41	66.24	64.48
8	67.90	57.53	49.50	57.46

3. Experimental

3.1 General experimental procedures

Optical rotation was measured with a MC 241 digital polarimeter (Perkin-Elmer, Waltham, USA). UV spectra were recorded on a Uvikon$_{xs}$ recording spectrometer (Secomam, ALES Cedex, France). IR spectra were recorded on a FTS3000 Infrared Fourier Transform spectrometer (BIO-RAD, San Francisco, USA). NMR spectra were performed on a Bruker AVANCE 300 instrument with tetramethylsilane as an internal standard (Rheinstetten, Germany). HRFTMS and EIMS were obtained on a Bruker ApexIII 7.0 TESLA and VG ZAB-HS instrument (Rheinstetten), respectively. HPLC was performed using a JASCO Gulliver Series with PU-1580 pump, RI-1530 and UV-1575 detector (Tokyo, Japan). Preparative HPLC column was used as below: ODS (YMC-Pack ODS-A, SH-343-5, Tokyo), GPC (Shodex, Asahipak GS-310, 20G, Kawasaki, Japan). Column chromatography was performed on silica gel (Qingdao Marine Chemical Co., Ltd, Qingdao, China), Sephadex LH-20 (Amersham Pharmacia Biotech, Stockholm, Sweden) and Toyopearl HW-40 (TOSOH, Tokyo).

3.2 Plant material

The rhizomes of *P. umbrosa* Turcz. were collected in Jianshi County, Hubei Province, China, in January 2005. The plant was identified by Prof. Ding-rong Wan, School of Life Sciences, South Central University for Nationalities, China. A voucher specimen (No. D20050110) is deposited at the School of Pharmaceutical Sciences, Tianjin Medical University, Tianjin, China.

3.3 Extraction and isolation

The dried rhizomes (3.2 kg) of *P. umbrosa* were crushed and then extracted with 95% aq. EtOH (10 l) for 6 h at reflux (3 ×). The pooled EtOH solutions were concentrated *in vacuo*, and the resulting residue (500 g) was suspended in H_2O, and then successively extracted with petroleum ether (PE), EtOAc and *n*-BuOH. The EtOAc-soluble fraction afforded, upon evaporation, a residue (20 g), which was further separated by column chromatography (1 kg silica gel; PE/EtOAc 3:1, 2:1, 1:1, 1:2, 1:3, EtOAc only, then EtOAc/MeOH 9:1, 4:1, 7:3, MeOH only) to yield 20 fractions (1–20) according to TLC. Fraction 10 (1460 mg) was subjected to column chromatography (Toyopearl HW-40; $CHCl_3$/MeOH 2:1) to afford six subfractions (10.1–10.6). Subfraction 10.3 was purified by HPLC (ODS-A; MeOH/H_2O 9:1, 3.0 ml/min) to provide compound **2** (35.4 mg), **3** (32.3 mg), **4** (17.8 mg), and **5** (34.4 mg). Fraction 12 (520 mg) was subjected to column chromatography (Toyopearl HW-40; $CHCl_3$/MeOH 2:1) to afford five subfractions (12.1–12.5). Subfraction 12.3 (258 mg) was further purified by HPLC (ODS-A; MeOH/H_2O 9:1, 3.0 ml/min) to afford compounds **1** (17.8 mg) and **8** (56.8 mg). Fraction 14–15 (1580 mg) was subjected to column chromatography (Toyopearl HW-40; $CHCl_3$/MeOH 2:1) and HPLC (ODS-A; MeOH/H_2O 9:1, 3.0 ml/min) to afford compounds **6** (81.4 mg) and **7** (22.1 mg).

3.3.1 *3‴-Acetyl-O-betonyoside D* (**1**)

Amorphous yellowish powder. $[\alpha]_D^{25}$ − 80.2 (*c* 0.70, MeOH). UVλ_{max} (nm): 329 (ε 9870), 205 (ε 16340). IR (KBr) ν_{max} cm^{-1}: 3420, 2932,

1714, 1630, 1596, 1515, 1437, 1270, 1159, 1130, 1043, 811. HRESIMS *m/z*:825.2759 $[M - H]^-$ (calcd for $C_{38}H_{49}O_{20}$, 825.2817). ^{1}H- and ^{13}C-NMR spectral data (Table 1).

3.3.2 2‴, 3‴-Di-acetyl-O-*betonyoside D* (**2**)

Amorphous yellowish powder. $[\alpha]_D^{25} - 75.2$ (*c* 0.53, MeOH). UVλ_{ma} (nm): 329 (ε 11997), 202 (ε 25110). IR (KBr) ν_{max} cm^{-1}: 3435, 2938, 1729, 1630, 1594, 1515, 1373, 1249, 1130, 1036, 810. HRESIMS *m/z*: 891.2964 $[M + Na]^+$ (calcd for $C_{40}H_{52}NaO_{21}$, 891.2899). ^{1}H- and ^{13}C-NMR spectral data (Table 1).

3.3.3 3‴, 4‴-Di-acetyl-O-*betonyoside D* (**3**)

Amorphous yellowish powder. $[\alpha]_D^{25} - 88.8$ (*c* 1.7, MeOH). UVλ_{max} (nm): 329 (ε 10540), 201 (ε 27280). IR (KBr) ν_{max} cm^{-1}: 3432, 2937, 1729, 1629, 1595, 1515, 1374, 1248, 1132,1036, 812. HRESIMS *m/z*: 891.2964 $[M + Na]^+$ (calcd for $C_{40}H_{52}NaO_{21}$, 891.2899). ^{1}H- and ^{13}C-NMR spectral data (Table 1).

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