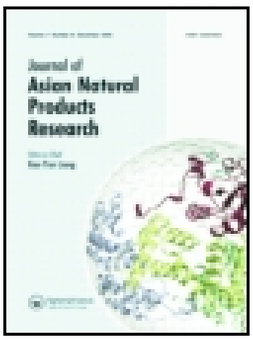

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Two new phenolic acids from the fruits of *Forsythia suspense*

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ABSTRACT

Two new phenolic acids forsythiayanoside C (**1**) and forsythiayanoside D (**2**), were isolated from the fruits of *Forsythia suspense* (Thunb.) Vahl. Their structures were elucidated on the basis of chemical and spectral analysis, including 1D, 2D NMR analyses, HRESIMS and CD spectrometry. The cytotoxic and antioxidant activities testing showed that compound **2** exhibited free radical scavenging activity.

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Oleaceae; *Forsythia suspense*; phenolic acid; cytotoxic activity; antioxidant activity

1. Introduction

Forsythia suspense (Thunb.) Vahl, a member of the family Oleaceae, is widely distributed in China including Henan, Shanxi, and Shandong Provinces. Its fruits are from one of the most important original plants of Traditional Chinese Medicine which have been used for antibacterial, antiviral, anti-inflammation, diuretic and antidotal purposes in oriental medicine [1]. The literature survey revealed that many kinds of compounds have been isolated from this plant, such as caffeoyl glycosides, phenylethanoid glycosides, lignans, terpenoids, alkaloids and cyclohexylethanes [2–4]. In this paper, we describe the isolation and the structural elucidation of two new phenolic acids obtained from 50% EtOH extract of *F. suspensa*. Their structures (Figure 1) were established by extensive spectroscopic data analysis and comparison with literature values. Furthermore, the cytotoxic and antioxidant activities of the isolated compounds were evaluated.

2. Results and discussion

Compound **1** was obtained as a sticky oil (MeOH), with the molecular formula $C_{15}H_{22}O_8$ as determined by the HRESIMS at *m/z* 353.1226 $[M + Na]^+$, indicating the presence of five degrees of unsaturation. The ^{1}H-NMR spectrum of compound **1** (Table 1) displayed three aromatic proton signals at δ_H 6.74 (^{1}H, d, J = 3.0 Hz, H-6), 6.60 (1H, dd, J = 8.7, 3.0 Hz, H-4), and 6.68 (1H, d, J = 8.7 Hz, H-3), which were assigned to one 1,3,4-trisubstituted

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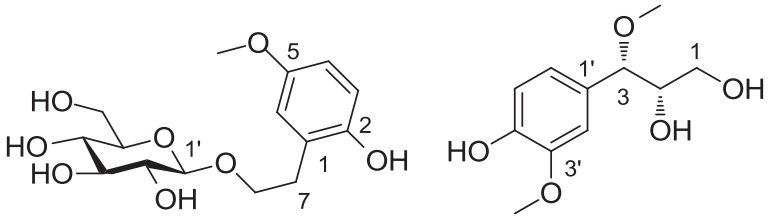

Figure 1. Structures of compounds **1** and **2**.

Table 1. ^{1}H-NMR (600 MHz) and ^{13}C-NMR (150 MHz) spectral data of compounds **1** and **2**.

	1[a]		**2**[b]	
No.	δ_H	δ_C	δ_H	δ_C
1		127.5	3.98 (d, 8.0)	62.9
			3.47–3.49 (m)	
2		150.7	4.35–4.37 (m)	75.6
3	6.68 (d, 8.7)	116.3	4.57 (d, 5.1)	84.3
4	6.60 (dd, 8.7, 3.0)	113.6		
5		154.4		
6	6.74 (d, 3.0)	117.4		
7	2.89–2.91 (m)	32.1		
8	3.75–3.79 (m)	70.7		
	4.03–4.07 (m)			
1′	4.32 (d, 7.7)	104.4		130.8
2′	3.50–3.52 (m)	75.2	6.81 (brs)	111.8
3′	3.41–3.42 (m)	78.1		147.8
4′	3.30–3.32 (m)	71.6		146.4
5′	3.36–3.37 (m)	78.0	6.65 (d, 8.1)	115.5
6′	3.70–3.72 (m)	62.7	6.73 (d, 8.1)	120.4
	3.85–3.87 (m)			
$-OCH_3$	3.70 (s)	56.5	3.11 (s)	56.6
$-OCH_3$			3.74 (s)	56.0

Note: Coupling constants (*J*) in Hz are given in parentheses; Chemical shift values are expressed in ppm.
[a]Measured in methanol-d_4.
[b]Measured in DMSO-d_6.

benzene ring. In addition, the 1H-NMR spectrum also revealed two methylene protons at δ_H 3.75–3.79, 4.03–4.07 (each 1H, m) and δ_H 2.89–2.91 (2H, m), one methoxyl group at δ_H 3.70 (3H, s), and a glucopyranosyl anomeric proton at δ_H 4.32 (1H, d, J = 7.7 Hz). The ^{13}C-NMR spectrum of **1** (Table 1) showed 15 carbon signals, including six aromatic (δ_C 127.5, 150.7, 116.3, 113.6, 154.4, and 117.4), a glucose unit (δ_C 104.4, 75.2, 78.1, 71.6, 78.0, and 62.7), one methoxy carbon at δ_C 56.5 and two methylenes (δ_C 32.1 and 70.7).

The HMBC correlation (Figure 2) between CH_3O group protons at δ_H 3.70 ($-OCH_3$) and C-5 (δ_C 154.4) indicated that the methoxyl group was located at C-5. The correlations from H-7 (δ_H 2.89–2.91) to C-8 (δ_C 70.7), C-1 (δ_C 127.5), C-2 (δ_C 150.7), and C-6 (δ_C 117.4) suggested that C-7 was connected with C-1 of the benzene ring. The anomeric proton of glucose at δ_H 4.32 (1H, d, J = 7.7 Hz) showed HMBC correlation with the aromatic C-8 (δ_C 70.7) indicated that C-1 should be connected with C-8. Accordingly, compound **1** was established as 2-(2-hydroxy-5-methoxyphenyl)-ethyl-β-D-glucopyranoside, named forsythiayanoside C.

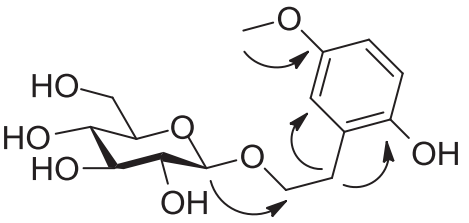

Figure 2. Key HMBC correlations of compound **1**.

Compound **2** was isolated as colorless needles (MeOH). HRESIMS gave a quasimolecular ion peak at *m/z* 251.0897 $[M + Na]^+$, corresponding to the molecular formula $C_{11}H_{16}O_5$. The ^{1}H-NMR spectrum (Table 1) showed one ABX system of aromatic protons [δ_H 6.65 (1H, d, J = 8.1 Hz, H-5′), 6.73 (1H, d, J = 8.1 Hz, H-6′), and 6.81 (1H, brs, H-2′)], indicating the presence of one trisubstituted benzene ring. In addition, the ^{1}H-NMR spectrum also revealed one oxymethylene at δ_H 3.98 (1H, d, J = 8.0 Hz), 3.47–3.49 (1H, m), two methine protons at δ_H 4.35–4.37 (1H, m), 4.57 (1H, d, J = 5.1 Hz), two methoxyl groups at δ_H 3.11 (3H, s, 3-OCH_3), 3.74 (3H, s, 3′-OCH_3). In the ^{13}C-NMR spectrum (Table 1), apart from six aromatic signals [δ_C 111.8, 115.5, 120.4, 130.8, 146.4 and 147.8], two methoxyl signals [δ_C 56.0 and 56.6], two oxymethines [δ_C 84.3 and 75.6] and one oxymethylene [δ_C 62.9] were observed. Comparison showed that the above spectroscopic data were similar to those of 3-(4-hydroxy-3-methoxyphenyl)-3-methoxypropane-1,2-diol [5]. Therefore, the structure of **2** was determined as shown in Figure 1. According to the literature, a smaller coupling constant ($J_{2,3}$ = 5.1 Hz) between H-2 and H-3 confirmed the *erythro* configuration of **2**, combining the negative *Cotton* effect at 280 nm [6,7], the absolute configurations C-2 and C-3 of **2** were determined as 2*S*,3*S*. Thus, the structure of **2** was characterized as (2*S*,3*S*)-3-(4-hydroxy-3-methoxyphenyl)-3-methoxypropane-1,2-diol, named forsythiayanoside D.

Compounds **1** and **2** were evaluated for their cytotoxic activities against breast cancer (Bcap-37), hepatoma (Hep-3B) and breast cancer (MCF-7) cell lines by MTT Method [8], using 5-fluorouracil as positive control. However, none of the isolated compounds exhibited cytotoxicity.

Phenolic acids have been claimed to possess antioxidant activity and could serve as lead compounds for the development of antioxidant agents [9]. The antioxidant effects of the two isolates were evaluated using 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay. Compound **1** (IC_{50} = 98.61 μg/ml) and compound **2** (IC_{50} = 45.12 μg/ml) were found to be less active than the positive control trolox (IC_{50} 8.29 μg/ml) in the DPPH assay.

3. Experimental

3.1. General experimental procedures

Optical rotations were measured on a JASCOP-1020 Polarimeter (Jasco Co., Tokyo, Japan). UV spectra were carried out on a Shimadzu UV-1700 Spectrophotometer (Shimadzu Corporation, Kyoto, Japan). IR spectra were obtained on a Shimadzu FTIR-8400s spectrophotometer (Shimadzu Corporation, Kyoto, Japan). NMR spectra were recorded on Bruker ARX-600 instruments (Bruker Co., Billerica, MA, USA). HR-ESI-TOF-MS experiments were performed on a Micro TOF spectrometer (Bruker Co., Karlsruhe, Germany). CD spectra

were measured by MOS 450 detector (Bio-Logic Co., Claix, France). High performance liquid chromatography (HPLC) preparation was performed on a Hitachi preparative HPLC system (Hitachi Ltd, Tokyo, Japan) equipped with Refractive Index Detector (L-2490) and prep-ODS column (10 × 250 mm). GC was done on an Agilent 7890A Gas Chromatograph (Agilent technologies, Inc., Santa Clara, CA, USA) equipped with HP-5 capillary column (30 m × 320 mm × 0.25 μm). Sephadex LH-20 (20–100 μm, Pharmacia Fine Chemical Co., Ltd, NJ, USA), silica gel (200–300 mesh, Qingdao Marine Chemistry Ltd, Qingdao, China), macroporous resin (D101, Cangzhou Bon Adsorber Technology Co., Ltd, Cangzhou, China) and Cosmosil octadecyl silane (ODS) (40–80 μm, Nacalai Tosoh, Inc., Uetikon, Switzerland) were used for column chromatography (CC). TLC was conducted on silica gel GF254 (Qingdao Marine Chemistry Ltd).

3.2. Plant material

The fruits (8.3 kg) of *F. Suspense* (Thunb.) Vahl were collected from Lushi County, Henan province of China in June 2009, and authenticated by Prof Jin-Cai Lu, Department of Pharmacognosy, Shenyang Pharmaceutical University. A voucher specimen (No. 20091011) is kept in the Nature Products Laboratory of Shenyang Pharmaceutical University, Shenyang, China.

3.3. Extraction and isolation

The fruits of *F. suspense* (8.3 kg) were crushed to pieces and refluxed with 50% EtOH for three times. The extract was evaporated under reduced pressure to afford a residue (350 g). The residue was suspended in H_2O and then successively extracted with $CHCl_3$, EtOAc and *n*-butanol. The *n*-butanol extract was evaporated *in vacuo* to give a residue (100 g), which was chromatographed over D101 macroporous resin with a gradient of aqueous EtOH (water, 30, 60 and 95%, v/v) to yield four major fractions (A-D). Fraction A (25.0 g) was subjected to silica gel column chromatography (12 × 60 cm) with a gradient mixture of CH_2Cl_2-MeOH (100:0–40:60) to afford five fractions (1–5). Fraction 1 (12.4 g) was further purified over an ODS column chromatography (4 × 45 cm) using MeOH and H_2O as the mobile phase with a gradient from 10 to 40% to afford fractions $F_{2\text{-}1}$–$F_{2\text{-}8}$ based on HPLC analysis. $F_{2\text{-}1}$ (9.5 g) was subjected to another silica gel column chromatography (2 × 30 cm) and eluted with CH_2Cl_2:MeOH:water (8:2:0.25) to afford fractions $F_{2\text{-}1\text{-}1}$–$F_{2\text{-}1\text{-}5}$ based on TLC analysis. $F_{2\text{-}1\text{-}2}$ (200 mg) was subjected to preparative HPLC eluted with CH_3OH-H_2O (10:90) at 3 ml/min (t_R 15 and 17 min), to yield compounds **1** (5 mg) and **2** (36 mg).

3.3.1. Forsythiayanoside C (1)

Sticky oil (MeOH), $[\alpha]_D^{25} - 10.1$ (*c* 0.05, MeOH). UV (MeOH) λ_{max}: 278.6, 227.4 and 207.0 nm; IR (KBr) ν_{max} (cm^{-1}): 3416, 1574, 1414, 1384, 1270, 1127 and 1074; ^1H and ^{13}C-NMR spectral data (MeOD-d_4), see Table 1. HRESIMS: *m/z* 353.1206 $[M + Na]^+$ (calcd for $C_{15}H_{22}O_8Na$, 353.1207).

3.3.2. Forsythiayanoside D (2)

Colorless needles (MeOH), $[\alpha]_D^{25} + 19.2$ (*c* 0.10, MeOH). UV (MeOH) λ_{max}: 280.2, 229.0 and 208.4 nm; IR (KBr) ν_{max} (cm^{-1}): 3439, 1631, 1466, 1384, 1271, 1123, and 1032; CD (MeOH) :

$\Delta\varepsilon_{212.4\,nm}$ −4.12, $\Delta\varepsilon_{236.0\,nm}$ −5.53, $\Delta\varepsilon_{278.6\,nm}$ −8.12; ^{1}H and ^{13}C-NMR spectral data (DMSO-d_6), see Table 1; HRESIMS: *m/z* 251.0897 $[M + Na]^+$ (calcd for $C_{11}H_{16}O_5Na$, 251.0890).

3.4. Acid hydrolysis of compound 1

Compound **1** (3.0 mg) was hydrolyzed with 2 M HCl (5.0 ml), heated for 4 h at 95 °C and extracted with $CHCl_3$ (3 × 5.0 ml). Then the aqueous layer was concentrated *in vacuo* to appropriate volume, and the solution was examined by TLC (EtOAc-BuOH-H_2O-HOAc, 4:4:1:1), and compared with the authentic sample, glucose was detected. Each remaining aqueous layer was concentrated to dryness to give a residue, which was dissolved in pyridine (1.0 ml), and then L-cysteine methyl ester hydrochloride (2.0 mg) was added to the solution. The mixture was heated at 60 °C for 2 h, and 0.5 ml TMSI (*N*-Trimethylsilyimidazole) was added, followed by heating at 60 °C for 2 h. The reaction product was subjected to GC analysis on Agilent 7890A (HP-5, 30 m × 320 mm, 0.25 μm) with flame ionization detector (FID) detection. Column temperature was kept at 120–280 °C with the rate of 8 °C/min, and the carrier gas was N_2 (1.4 ml/min), injection temperature: 250 °C; injection volume: 1 μl. The absolute configuration of the monosaccharide was confirmed to be D-Glu by comparison of the retention time of its Me_3Si ethers with standard sample [t_R (D-glucose) = 25.873 min].

3.5. Cytotoxicity assay

Breast cancer (Bcap-37), hepatoma (Hep-3B) and breast cancer (MCF-7) cells were obtained from the National Center for Medical Culture Collection (Shanghai, CHN). They were routinely cultured in Roswell Park Memorial Institute (RPMI) 1640 or DMEM (Dulbecco's Modified Eagle Medium) supplemented with 10% fetal bovine serum (FBS) and maintained at 37 °C in a humidified incubator with 5% CO_2. The *in vitro* cell viability effects of compounds were determined by MTT assay. The cells (1×10^5 cells/ml) were seeded into 96-well culture plates. After overnight incubation, the cells were treated with various concentrations of agents for 72 h. Then 10 μl MTT solution (2.5 mg/ml in PBS) was added to each well, and the plates were incubated for an additional 4 h at 37 °C. After centrifugation (200 g, 10 min), the medium with MTT was aspirated, followed by the addition of 100 μl DMSO. The optical density of each well was measured at 492 nm with a Biotek Synergy TM HT Reader.

3.6. Antioxidant assay

The DPPH radical scavenging activity of the samples was measured using a published method with some modifications. The samples in different concentrations of EtOH (100 μl) were added to 0.2 mM DPPH in EtOH (150 μl). Each mixture was shaken vigorously and then immediately incubated in darkness for 1 h. The absorbance of the reaction solution was determined in a varioskan flash instrument (Thermo scientific, USA) at 515 nm. Trolox, a stable antioxidant, was used as a positive reference. The DPPH radical scavenging activity in terms of the percentage of sample was calculated as follows: DPPH scavenging activity (%) = $[1 - (S - S_B)/(C - C_B)] \times 100\%$. Where S, S_B, C and C_B is the absorption of the sample, the blank sample, the control, and the blank control, respectively. Tests were performed in triplicate.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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