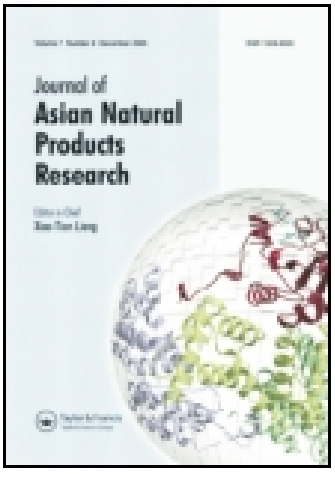

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Two new glycosides from the fruits of *Forsythia suspense*

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Two new glycosides suspensaside C (**1**) and 2,3,5,6-tetrahydro-jacaranone-4-*O*-β-D-glucopyranoside (**2**), together with four known compounds suspensaside A (**3**), rengynic acid-1′-*O*-β-D-glucopyranoside (**4**), forsythoside A (**5**), and rengynic acid (**6**), were isolated from the fruits of *Forsythia suspense* (Thunb.) Vahl. The structures of **1** and **2** were elucidated on the basis of chemical and spectral analysis, including 1D, 2D NMR analyses and HR-ESI-MS. All isolates were tested for their cytotoxicities against five human cancer cell lines (A549, Colo-205, Hep-3B, HL60, and KB). Compound **3** exhibited cytotoxicity against HL-60, Hep-3B, and A549 cancer cell lines.

Keywords: *Forsythia suspense*; glycosides; cytotoxicitiy

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 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 [2], phenylethanoid glycosides [3], lignans [4], terpenoids [5], alkaloids [6], and cyclohexylethanes [7]. In this paper, we describe the isolation and the structural elucidation of two new glycosides, along with four known compounds obtained from the 50% EtOH extract of *F. suspensa*. Their structures (Figure 1) were established by extensive spectroscopic data analysis and literature values. Meanwhile, all compounds were evaluated for their cytotoxicities against HL60, Hep-3B, Colo-205, KB, and A549 cancer cell lines.

2. Results and discussion

Compound **1** was isolated as an amorphous powder, and its molecular formula was established as $C_{20}H_{28}O_{12}$ (seven degrees of unsaturation) on the basis of HR-ESI-MS at *m/z* 483.1479 $[M + Na]^+$ and NMR spectral data (Table 1). The ^1H NMR spectrum showed proton signals of one ABX system aromatic ring [δ_H 6.68 (1H, d, $J = 8.1$ Hz), 6.65 (1H, dd, $J = 8.1$, 1.8 Hz), and 6.80 (1H, d, $J = 1.8$ Hz)], indicating the presence of one trisubstituted benzene ring. In addition, an oxygen-substituted methine [δ_H 4.46 (1H, dd, $J = 10.3$, 2.6 Hz)], one oxygen-substituted methylene [δ_H 3.57, 3.87 (each 1H, dd,

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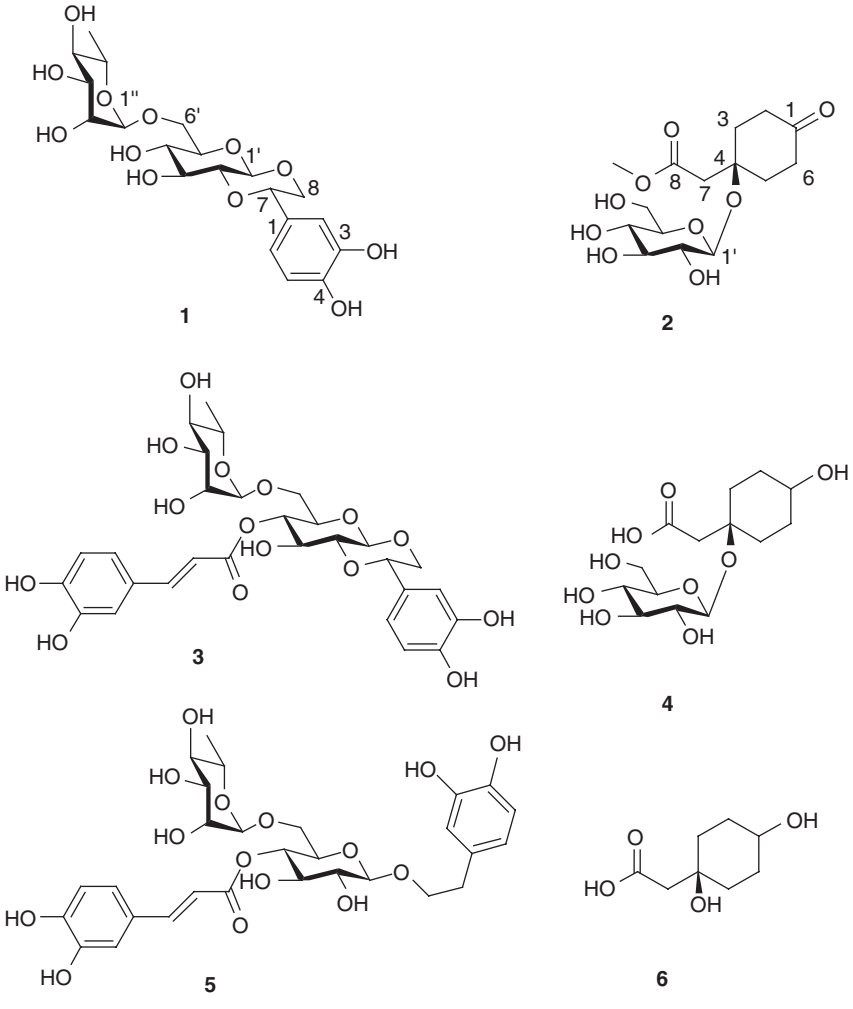

Figure 1. The structures of compounds **1**–**6**.

$J = 12.0$, 2.6 Hz)], one secondary methyl signal [δ_H 1.21 (3H, d, $J = 6.2$ Hz)] and two anomeric protons [δ_H 4.34 (1H, d, $J = 7.8$ Hz), 4.68 (1H, s)] were also observed. The ^{13}C NMR spectrum displayed 20 carbon signals, including 6 aromatic carbons (δ_C 115.1, 116.2, 119.5, 129.9, 146.3, and 146.5), 1 oxygen-substituted methine (δ_C 78.8), 1 oxygen-substituted methylene (δ_C 72.8), and 12 carbon signals of two sugar units. These were confirmed by 2D NMR (HSQC and HMBC) data analysis of **1**. In the HMBC spectrum (Figure 2), correlations from H-1′ (δ_H 4.34) to C-8 (δ_C 72.8) of the aglycone, from H-8 (δ_H 3.87) to C-l′ (δ_C 99.5), and from H-1″ (δ_H 4.68) to C-6′ (δ_C 68.1) were observed. All of the above spectroscopic data were similar to those of suspensaside A (**3**) except for the absence of a caffeoyl moiety at C-4′, which suggested **1** was regarded as the decaffeoyl derivative of suspensaside A [2]. Acid hydrolysis of **1** afforded D-glucose and L-rhamnose in a ratio of 1:1 by thin layer chromatography (TLC) and gas chromatography (GC)

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		129.9		211.3
2	6.80 (d, 1.8)	115.1	1.96 (d, 11.5) 2.80 (td, 13.9, 6.2)	36.4
3		146.3	1.86 (dd, 13.2, 4.3) 2.20 (m)	32.5
4		146.5		74.9
5	6.68 (d, 8.1)	116.2	1.88 (dd, 13.2, 4.3) 2.18 (td, 13.9, 6.2)	34.9
6	6.65 (dd, 8.1, 1.8)	119.5	1.98 (d, 11.5) 2.84 (m)	36.9
7	4.46 (dd, 10.3, 2.6)	78.8	2.73 (d, 9.8)	43.6
8	3.57 (m) 3.87 (dd, 12.0, 2.6)	72.8		171.3
1′	4.34 (d, 7.8)	99.5	4.48 (d, 7.6)	97.5
2′	3.10 (m)	80.9	3.12 (m)	74.0
3′	3.53 (m)	75.0	3.06 (m)	77.2
4′	3.80 (m)	72.1	3.14 (m)	70.5
5′	3.54 (m)	78.6	3.13 (m)	76.9
6′	3.57 (m) 3.95 (d, 10.5)	68.1	3.40 (m) 3.64 (d, 11.5)	61.6
1″	4.68 (s)	102.3		
2″	3.32 (m)	72.0		
3″	3.61 (m)	72.3		
4″	3.31 (m)	74.0		
5″	3.59 (m)	69.9		
6″	1.21 (d, 6.2)	18.0		
—OCH_3			3.55 (s)	51.8

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.

analyses [8]. The α-anomeric configuration for the rhamnosyl group was determined by its C-5″ data (δ_C 69.9) [9]. The configuration of the anomeric proton of glucose was proposed as β on the basis of its coupling constant ($J = 7.8$ Hz). Thus, compound **1** was established as 1′,2′-(β-3,4-dihydroxylphenyl-α,β-dioxoethanol)-*O*-α-L-rhamnopyranosyl-(1 → 6)-β-D-glucopyranoside, and named suspensaside C.

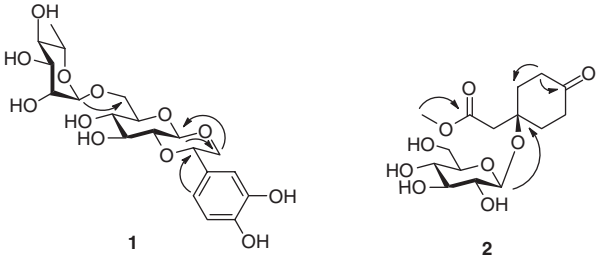

Figure 2. Key HMBC (→) correlations of **1** and **2**.

Compound **2** was isolated as colorless oil. HR-ESI-MS gave a quasimolecular ion peak at *m/z* 371.1311 $[M + Na]^+$, corresponding to the molecular formula $C_{15}H_{24}O_9$. The ^1H NMR spectrum (Table 1) of **2** showed signals for a methoxy group at δ_H 3.55 (3H, s) and an anomeric proton at δ_H 4.48 (1H, d, $J = 7.6$ Hz) of a sugar unit, along with other alkyl groups. The ^{13}C NMR spectrum of **2** exhibited signals due to a ketone group (δ_C 211.3), an ester carbonyl carbon (δ_C 171.3), an oxygenated quaternary carbon (δ_C 74.9), a methoxy carbon (δ_C 51.8), and one glucose unit (δ_C 97.5, 74.0, 77.2, 70.5, 76.9, and 61.5). The carbon signals assignable to the aglycone moiety were similar to those of 2,3,5,6-tetrahydrojacaranone [10], with glycosylation shifts at C-4 (+7.0), C-3 (−3.5), and C-5 (−1.1). The position of the sugar residue in **2** was defined by the HMBC correlations (Figure 2). A cross-peak between the ^1H NMR signal at δ_H 4.48 (H-1′, glucose group) and the carbon signal at δ_C 74.9 (C-4, aglycone) indicated glycosylation of the aglycone at C-4. The configuration of the anomeric proton of glucose was proposed as β on the basis of its coupling constant ($J = 7.6$ Hz). Acid hydrolysis of **2** afforded D-glucose by TLC and GC analyses [8]. On the basis of the ^1H, ^{13}C, and 2D NMR (HSQC, HMBC) data, the structure of **2** was established as 2,3,5,6-tetrahydro-jacaranone-4-*O*-β-D-glucopyranoside.

The known compounds were readily identified as suspensaside A (**3**) [2], rengynic acid-1′-*O*-β-D-glucopyranoside (**4**) [11], forsythoside A (**5**) [12], and rengynic acid (**6**) [13] by comparing NMR spectral data with those reported in the literature.

All compounds were evaluated for their cytotoxicities against five human cancer cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method [14], and 5-fluorouracil, a clinically prescribed antitumor drug, was co-assayed as a positive control. The bioassay results showed that compound **3** exhibited cytotoxic activities against HL60, Hep-3B, and A549 cancer cell lines and other isolates (**1**, **2**, **4**–**6**) did not show significant cytotoxicity.

3. Experimental

3.1 General experimental procedures

Optical rotations were measured on a JASCOP-1020 Polarimeter (Jasco Co., Tokyo, Japan). IR spectra were obtained on a Shimadzu ftir-8400s spectrophotometer (Shimadzu Corporation, Kyoto, Japan). NMR spectra were recorded on Bruker ARX-300 and ARX-600 instruments (Bruker Co., Billerica, MA, USA). HR-ESI-TOF-MS experiments were performed on a Micro TOF spectrometer (Bruker Co., Karlsruhe, Germany). 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 (10 mm × 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, Piscataway, 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

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 and 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 CC and eluted with water and aqueous EtOH (in gradient, 30%, 60%, and 95%, v/v) to yield four major fractions (A–D). Fraction B (53.0 g) was subjected to silica gel CC (12 × 60 cm) with a gradient mixture of CH_2Cl_2–MeOH (100:0–50:50) to afford five fractions (1–5). Fraction 4 (16.3 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 5% to 50% to afford fractions $F_{4\text{-}1}$–$F_{4\text{-}8}$ based on HPLC analysis. $F_{4\text{-}6}$ (2.1 g) was subjected to another silica gel CC (2 × 30 cm) and eluted with CH_2Cl_2: MeOH:water (8:2:0.25) to afford fractions $F_{4\text{-}6\text{-}1}$–$F_{4\text{-}6\text{-}5}$ based on TLC analysis. $F_{4\text{-}6\text{-}2}$ was subjected to preparative HPLC eluted with CH_3OH–H_2O (10:90) at 3 ml/min (t_R 18 and 24 min) to yield compounds **1** (10 mg) and **5** (36 mg). $F_{4\text{-}6\text{-}3}$ was subjected to semi-preparative HPLC eluted with CH_3CN–H_2O (10:90) at 2 ml/min (t_R 25 min) to yield **2** (20 mg) and at 2 ml/min (t_R 40 min) to yield **3** (16 mg). $F_{4\text{-}6\text{-}4}$ was subjected to semi-preparative HPLC eluted with CH_3CN–H_2O (20:80) at 2 ml/min (t_R 30 min) to yield **4** (6 mg) and at 2 ml/min (t_R 45 min) to yield **6** (30 mg).

3.3.1 Suspensaside C (*1*)

Amorphous powder (MeOH); $[\alpha]_D^{25}$ + 16.4 (c = 0.10, MeOH). IR (KBr) ν_{max} (cm^{-1}): 3374, 1613, 1516, 1384, 1243, 1077, 1023, and 830; for ^1H and ^{13}C NMR spectral data (methanol-d_4), see Table 1; HR-ESI-MS: *m/z* 483.1479 $[M + Na]^+$ (calcd for $C_{20}H_{28}O_{12}Na$, 483.1473).

3.3.2 2,3,5,6-Tetrahydro-jacaranone-4-*O*-β-D-glucopyranoside (*2*)

Colorless oil (MeOH); $[\alpha]_D^{25}$ −18.1 (c = 0.10, MeOH). IR (KBr) ν_{max} (cm^{-1}): 3385, 1712, 1162, and 1025; for ^1H and ^{13}C NMR spectral data (DMSO-d_6), see Table 1; HR-ESI-MS: *m/z* 371.1311 $[M + Na]^+$ (calcd for $C_{15}H_{24}O_9Na$, 371.1313).

3.4 Acid hydrolysis of compounds 1 and 2

Each compound (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 samples, rhamnose and glucose were 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 *N*-(tremethylsily)imidazole (TMSI) 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. Column temperature was set 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

Table 2. Cytotoxicities[a] of compounds **1**–**6** against cultured HL60, A549, Hep-3B, Colo-205, and KB cancer cell lines.

Compounds	Growth inhibition constant (IC_{50})[a] [μM]				
	HL60	A549	Hep-3B	Colo-205	KB
1	>100	>100	>100	>100	>100
2	>100	>100	>100	>100	>100
3	46.52 ± 6.4	71.6 ± 5.0	60.28 ± 8.2	>100	>100
4	>100	>100	>100	>100	>100
5	>100	>100	>100	>100	>100
6	>100	>100	>100	>100	>100
5-Fu[b]	11.52 ± 3.5	25.72 ± 5.9	4.8 ± 2.1	39.3 ± 4.1	44.35 ± 2.4

[a] IC_{50} represents means ± SD of three independent replicates. The IC_{50} greater than 100 μM was considered to indicate no cytotoxicity.
[b] Positive control substance.

configurations of the monosaccharides were confirmed to be D-Glu and L-Rha by comparison of the retention times of its Me_3Si ethers with those of standard samples [t_R (D-glucose) 25.873 min, t_R (L-rhamnose) 22.165 min)].

3.5 *Cytotoxicity assay*

Human malignant leucocythemia cell lines HL-60, hepatoma cell lines Hep-3B, colon cancer cell lines Colo-205, cervical cancer lines KB, and lung cancer cell lines A549 were obtained from the National Center for Medical Culture Collection (Shanghai, China). They were routinely cultured in Roswell Park Memorial Institute (RPMI) 1640 or Dulbecco's modified eagle medium (DMEM) supplemented with 10% fetal bovine serum 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 [14]. 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 phosphate buffered saline, 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. The results are given in Table 2.

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