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Quinoid glycosides from *Forsythia suspensa*

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ABSTRACT

Phytochemical investigation on *Forsythia suspensa* (Thunb.) Vahl afforded 10 compounds, including quinoid glycosides, lignan glycosides, phenylethanoid glycoside and allylbenzene glycoside together with 13 known ones. Their structures were established based on extensive spectroscopic data analyses, including IR, UV, HRESIMS, 1D NMR and 2D NMR. Absolute configurations were determined by ECD calculation method and chemical degradation. In addition, all compounds were evaluated for their antiviral activity against influenza A (H1N1) virus and several were further evaluated against respiratory syncytial virus (RSV) *in vitro*. Among them, two previously known compounds showed significant activities against RSV with EC_{50} values of 3.43 and 6.72 μM.

1. Introduction

Forsythia suspensa (Thunb.) Vahl is widely distributed in China, Japan, Korea and many European countries. The dried fruit of this plant, locally named 'Lianqiao', is a well known herbal medicine in China for the treatment of pyrexia and infections (Cui et al., 2010). A number of phenylethanoid glycosides (Wang et al., 2009), lignan glycosides (Chang et al., 2008; Piao et al., 2008), quinoid glycosides (Ming et al., 1998), triterpenes (Rouf et al., 2001) and alkaloids (Zhang et al., 2002) have been isolated from the fruit of this plant. Some of them possess various bioactivities, including antiviral (Li et al., 2011), antibacterial (Qu et al., 2012) and anti-inflammatory activities (Dai et al., 2009). Our investigations on the fruit of *F. suspensa* in searching for antiviral components led to the isolation of 10 new compounds, along with 13 known ones. Herein, we mainly described the isolation and structural elucidation of the new compounds, as well as the antiviral activities against influenza A (H1N1) virus and respiratory syncytial virus (RSV).

2. Results and discussion

The dried fruit of *F. suspensa* was extracted with EtOH–H_2O (60:40), and then the extract was subjected to various column chromatographies to yield 10 new compounds, forsythensides G–L (**1**–**6**) and forsythialansides A–D (**7**–**10**) (Fig. 1). Moreover, 13 known compounds were also isolated, and their structures were identified as cornoside (**11**) (Lu et al., 2009), forsythenside A (**12**) (Ming et al., 1998), icariside E4 (**13**) (Miyase et al., 1989), dihydrodehydrodiconiferyl alcohol-4-*O*-*β*-D-glucoside (**14**) (Ou yang et al., 2011), calceolarioside B (**15**) (Nicoletti et al., 1986), forsythoside A (**16**) (Wang et al., 2009), sasanquin (**17**) (Zhao et al., 1999), epipinoresinol-4-*O*-*β*-D-glucoside (**18**) (Nishibe et al., 1984), phillyrin (**19**) (Nishibe et al., 1984), epipinoresinol-4-*O*-*β*-D-glucoside (**20**) (Rahman et al., 1990), pinoresinol-4-*O*-*β*-D-glucoside (**21**) (Kim et al., 2005), pinoresinol (**22**) (Li et al., 2012) and syringaresinol-4-*O*-*β*-D-glucoside (**23**) (Li et al., 2001), respectively.

Compound **1** was obtained as light yellow gum. Its molecular formula was deduced to be $C_{24}H_{34}O_{10}$ by HRESIMS. The ^{13}C NMR spectrum showed 24 carbon signals. The characteristic chemical shift of one carbonyl group (δ 187.8), along with four olefinic carbons (δ 154.4 × 2, 128.0, 127.9), two methylene carbons (δ 65.8, 41.0) and a quaternary carbon (δ 69.2) indicated that compound **1** had a 1-hydroxy-1-hydroxyethyl-2,5-cyclohexadienone moiety (Wang et al., 2008). This was further confirmed by HMBC correlations (Fig. 2).

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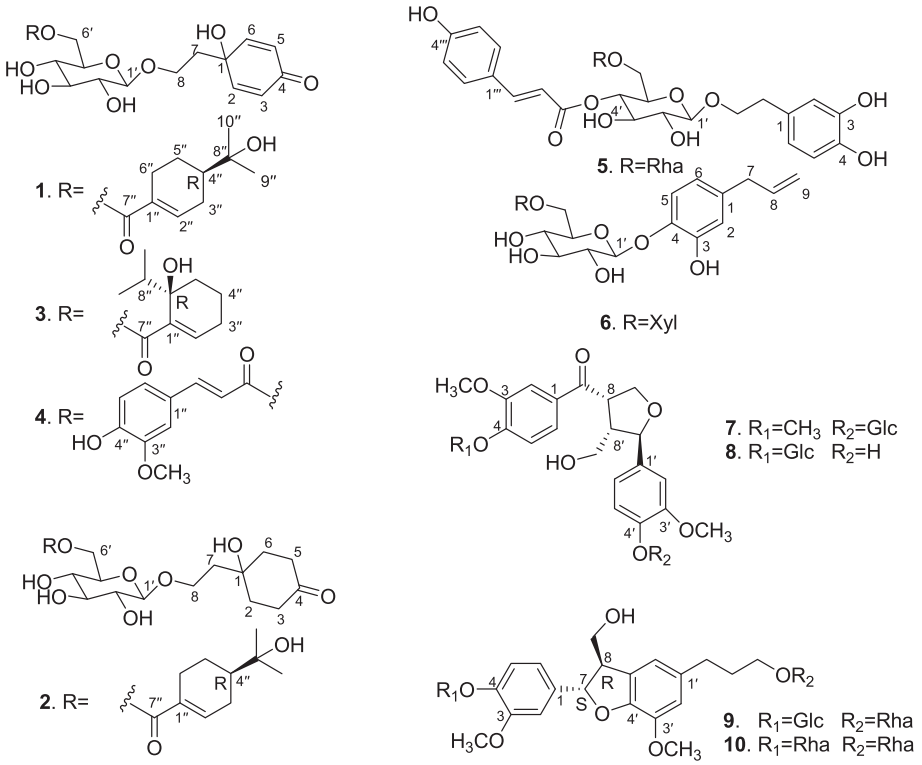
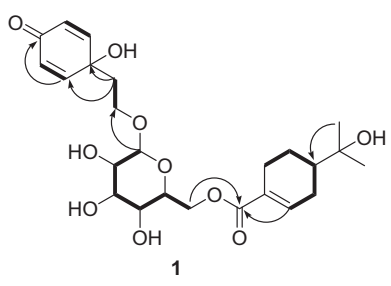


Fig. 1. Structural formulae of compounds **1**–**10**.



1

Fig. 2. Key HMBC (→) and ^{1}H, ^{1}H-COSY (▬) correlations of **1**.

The anomeric carbon resonance at δ 104.4 showed the existence of a monosaccharide moiety. Acid hydrolysis and HPLC analysis were performed according to the method of Tanaka et al. (2007), which suggested that the monosaccharide was D-glucose. Moreover, the β configuration was prompted by the large coupling constant of the anomeric proton (δ 4.24, d, J = 7.8 Hz).

In addition, typical carbon signals at δ 131.2, 141.5 and 168.7 showed the existence of an α,β-unsaturated carbonyl group. In the ^{1}H, ^{1}H-COSY spectrum, the ^{1}H, ^{1}H spin system (H2″/H3″, H3″/H4″, H4″/H5″, and H5″/H6″) enabled deduction of the fragment –CH–CH_2–CH–CH_2–CH_2–. In the HMBC spectrum, correlations of H-2″/C-6″, C-7″ and C-4″, and those of H-9″/C-8″ and C-4″ suggested the presence of an oleuropeic acid unit (Tian et al., 2009). Moreover, the linkages of three moieties were established on the basis of HMBC correlations of H-1′/C-8 and H-6′/C-7″ (Fig. 2).

The absolute configuration of **1** was determined by chemical degradation and optical rotation analysis. Methanolysis of **1** was performed with NaOMe in MeOH. After neutralization with HCOOH, the EtOAc extract was obtained and evaporated to dryness, then the residue was dissolved in MeOH and further purified by preparative HPLC to yield (+) oleuropeic acid (**1a**) {$[\alpha]_D^{20}$ +45.3 ($CHCl_3$)}, which indicated that the absolute configuration of C-4″ was *R* (Nakanishi et al., 2005). Thus, the structure of **1** was deduced as 6-*O*-[(*R*)-oleuropeoyl]-1-*O*-[2-(1-hydroxy-4-oxo-2,5-cyclohexadien-1-yl)ethyl]-β-D-glucopyranose, trivially named forsythenside G.

Compounds **2**–**5** were also isolated as light yellow gums. The molecular formula of **2**, $C_{24}H_{38}O_{10}$, was established by analysis of HRESIMS data. The ^{13}C NMR and DEPT-135 spectroscopic data of **2** were similar to those of **1** except that the two olefinic groups (δ 154.4 × 2, 128.0, 127.9) in **1**, were replaced by four methylenes (δ 37.9 × 2, 37.8 × 2) in **2**, which suggested that the 2,5-cyclohexadienone moiety in **1** was replaced by a cyclohexanone moiety in **2**. Methanolysis of **2** with NaOMe in MeOH also gave (+)-oleuropeic acid (**1a**) {$[\alpha]_D^{20}$ +38.0 ($CHCl_3$)}; therefore, the absolute configuration of C-4″ in **2** was assigned to be *R*. Thus, compound **2** was assigned as 6-*O*-[(*R*)-oleuropeoyl]-1-*O*-[2-(1-hydroxy-4-oxocyclohexyl)ethyl]-β-D-glucopyranose, it was given the trivial name forsythenside H.

Compound **3** had the same molecular formula with **1**, which was determined by HRESIMS data. The ^{13}C NMR and DEPT-135 data of **3** also showed the presence of a monosaccharide moiety and a 1-hydroxy-1-hydroxyethyl-2,5-cyclohexadienone unit, as same as compound **1**. The remaining 10 carbon signals consisted of one α,β-unsaturated carbonyl group (δ 168.6, 139.3, 130.6), two methyls (δ 17.3, 17.2), three methylenes (δ 35.6, 31.4, 22.4), one methine (δ 38.6) and one quaternary carbon signal (δ 72.4). Those resonances demonstrated the presence of a 6-hydroxy-6-isopropylcyclohex-1-enecarboxylic acid moiety (Dai et al., 2005). Furthermore, the linkages of the three moieties were based on the HMBC correlations of H-1′/C-8, and H-6′/C-7″.

Methanolysis of **3** was also performed with NaOMe in MeOH to yield 6-hydroxy-6-isopropylcyclohex-1-enecarboxylic acid (**3a**). The electronic circular dichroism (ECD) spectrum of **3a** was calculated by time dependent density functional theory (TDDFT). The calculated ECD data matched well with the experimental ECD data of **3a** (Fig. 3), allowing the assignment of the absolute configuration

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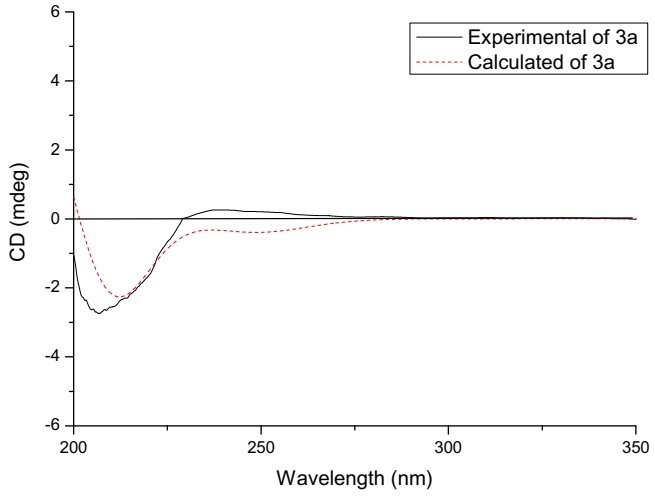

Fig. 3. Experimental and calculated ECD spectra for **3a**.

of 6″ in **3** as *R*. Therefore, compound **3** was elucidated as 6-*O*-[(*R*)-6-hydroxy-6-isopropylcyclohex-1-enecarbonyl]-1-*O*-[2-(1-hydroxy-4-oxo-2,5-cyclohexadien-1-yl)ethyl]-*β*-D-glucopyranose, named forsythenside I.

Compound **4** had a molecular formula of $C_{24}H_{28}O_{11}$ by HRESIMS. Compared to the NMR spectroscopic data of compound **4** with those of **1**, the same glucose moiety and the same 1-hydroxy-1-hydroxyethyl-2,5-cyclohexadienone unit were deduced. In addition, the ^{1}H NMR spectrum had a methyl proton signal at 3.90 (3H, s) and typical proton resonances at δ 7.23 (1H, d, J = 1.8 Hz), δ 6.82 (1H, d, J = 8.2 Hz), δ 7.09 (1H, dd, J = 1.8, 8.2 Hz) and δ 7.64 (1H, d, J = 15.9 Hz), δ 6.40 (1H, d, J = 15.9 Hz), which suggested the presence of one feruloyl moiety. This was further confirmed by the characteristic signals of a feruloyl moiety observed in the ^{13}C NMR spectrum (Table 1). Thus, compound **4** was elucidated as 6-*O*-feruloyl-1-*O*-[2-(1-hydroxy-4-oxo-2,5-cyclohexadien-1-yl) ethyl]-*β*-D-glucopyranose, named forsythenside J.

Compound **5** had a molecular formula of $C_{29}H_{36}O_{14}$ by HRESIMS data analysis. Its ^{1}H NMR spectrum exhibited characteristic proton signals of a *p*-hydroxycinnamoyl moiety at δ 6.81 (2H, d, J = 8.6 Hz), 7.47 (2H, d, J = 8.6 Hz), 7.67 (1H, d, J = 15.9 Hz), and 6.37 (1H, d, J = 15.9 Hz). Additionally, one aromatic ABX coupling system was observed at δ 6.69 (1H, d, J = 1.9 Hz), 6.68 (1H, d, J = 8.1 Hz) and 6.57 (1H, d, J = 1.9, 8.1 Hz). These signals, together with two coupled methylene protons at δ 3.98, 3.71 (each 1H, m) and 2.78 (2H, m), indicated the existence of a phenylethanol moiety. Moreover, the ^{1}H NMR spectrum showed two anomeric proton signals at δ 4.37 (1H, d, J = 7.8 Hz) and 4.64 (1H, d, J = 1.2 Hz). Acid hydrolysis of **5** gave D-glucose and L-rhamnose as sugar residues. The ^{13}C NMR spectrum also displayed carbon signals belonged to one *p*-hydroxycinnamoyl and one phenylethanol moiety, besides those of glucose and rhamnose moieties. The linkages were established on the basis of the HMBC correlations of H-1′/C-8, H-4′/C-9‴ and H-1″/C-6′. Therefore, compound **5** was assigned as 3,4-dihydroxy-*β*-phenethyl-*O*-*α*-L-rhamnopyranosyl-(1→6)-4′-*O*-*p*-hydroxycinnamoyl-*β*-D-glucopyranoside, named forsythenside K.

Compound **6** was obtained as yellow amorphous powder. Its molecular formula was determined to be $C_{20}H_{28}O_{11}$ on the basis of HRESIMS data. The ^{1}H NMR spectrum indicated the presence of a 1,3,4-trisubstituted benzene ring [δ 6.68 (1H, d, J = 2.1 Hz), 7.13(1H, d, J = 8.2 Hz), 6.63 (1H, dd, J = 2.1, 8.2 Hz)] and a terminal olefinic bond [δ 5.03 (2H, m), 5.93 (1H, ddt, J = 6.7, 10.1, 16.9 Hz)]. In addition, two anomeric protons at δ 4.70 (1H, d, J = 7.5 Hz), 4.33 (1H, d, J = 7.3 Hz) suggested that there were two monosaccharide moieties. Acid hydrolysis of **6** gave D-glucose and D-xylose as sugar residues. Moreover, The ^{13}C NMR spectroscopic data of the aglycone moiety in Table 2 were in agreement with those of 3, 4-dihydroxy-1-allylbenzene moiety (Ly et al., 2002). The linkages

Table 1
^{1}H and ^{13}C NMR spectroscopic data for compounds **1**–**4** (Measured in CD_3OD).

Position	Compound **1**[a]		Compound **2**[b]		Compound **3**[b]		Compound **4**[a]	
	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	69.2, C		70.3, C		69.2, C		69.2, C	
2	154.4, CH	7.01, m	37.8, CH_2	2.00, m; 1.84, m	154.5, CH	7.01, m	154.4, CH	6.98, dd (10.2)
3	128.0, CH	6.10,d (10.1)	37.9, CH_2	2.66, m; 2.17, m	128.0, CH	6.10,d (10.2)	128.0, CH	6.08, dd (10.2)
4	187.8, C		214.8, C		187.8, C		187.8, C	
5	127.9, CH	6.10, d (10.1)	37.9, CH_2	2.66, m; 2.17, m	127.9, CH	6.10, d (10.2)	127.9, CH	6.08, dd (10.2)
6	154.4, CH	7.01, m	37.8, CH_2	2.00, m; 1.84, m	154.4, CH	7.01, m	154.3, CH	6.98, dd (10.2)
7	41.0, CH_2	2.04, t (6.5)	42.0, CH_2	1.91, m	41.0, CH_2	2.04, t (6.5)	41.0, CH_2	2.05, t (6.5)
8	65.8, CH_2	3.91,dt (6.6, 10.1) 3.64, dt (6.6, 10.1)	67.1, CH_2	4.03, m 3.77, m	65.8, CH_2	3.91, m 3.63, m	65.9, CH_2	3.91, m 3.65, m
1′	104.4, CH	4.24, d (7.8)	104.5, CH	4.30,d (7.8)	104.3, CH	4.23, d (7.8)	104.4, CH	4.25, d (7.8)
2′	75.0, CH	3.15, m	75.1, CH	3.16, m	75.0, CH	3.14, m	75.0, CH	3.18, m
3′	77.9, CH	3.35, m	78.0, CH	3.36, m	77.9, CH	3.33, m	77.9, CH	3.36, m
4′	71.7, CH	3.31, m	71.7, CH	3.35, m	71.7, CH	3.27, m	71.7, CH	3.35, m
5′	75.4, CH	3.47, m	75.5, CH	3.49, m	75.4, CH	3.46, m	75.4, CH	3.53, m
6′	64.6, CH_2	4.45, dd (2.0, 11.9) 4.21, dd (6.1, 11.9)	64.6, CH_2	4.45, dd (2.2, 11.9) 4.25, m	64.6, CH_2	4.46, dd (2.1, 11.9) 4.21, m	64.6, CH_2	4.50, dd (2.2, 11.9) 4.33, m
1″	131.2, C		131.2, C		130.6, C		127.7, C	
2″	141.5, CH	7.03, m	141.5,CH	7.04, m	139.3, CH	6.93, m	111.7, CH	7.23, d (1.8)
3″	28.6, CH_2	2.36, m; 2.04, m	28.6, CH_2	2.34, m; 2.02, m	35.6, CH_2	2.33, m; 2.16, m	149.4, C	
4″	45.5, CH	1.55, m	45.5, CH	1.53, m	22.4, CH_2	2.37, m	150.7, C	
5″	24.5, CH_2	2.03, m; 1.24, m	24.6, CH_2	2.00, m; 1.21, m	31.4, CH_2	1.74, m; 1.55, m	116.5, CH	6.82, d (8.2)
6″	26.3, CH_2	2.52, m; 2.18, m	26.4, CH_2	2.51, m; 2.14, m	72.4, C		124.3, CH	7.09, dd (1.8, 8.2)
7″	168.7, C		168.7, C		168.6, C		147.1, CH	7.64, d (15.9)
8″	72.8, C		72.8, C		38.6, CH	1.65, m	115.3, CH	6.40, d (15.9)
9″	27.1, CH_3	1.19, s	27.0, CH_3	1.18, s	17.3, CH_3	0.98, d (6.9)	169.1, C	
10″	26.4, CH_3	1.19, s	26.5, CH_3	1.18, s	17.2, CH_3	0.95, d (6.9)		
3″-OCH_3							56.5, CH_3	3.90, s

[a] 300 MHz for ^{1}H NMR, 75 MHz for ^{13}C NMR.
[b] 400 MHz for ^{1}H NMR, 100 MHz for ^{13}C NMR.

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Table 2
^{1}H and ^{13}C NMR spectroscopic data for compounds **5**–**6** (Measured in CD_3OD).

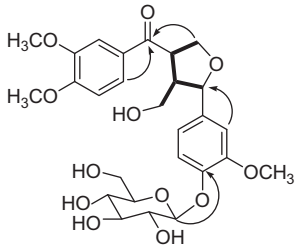
Position	Compound **5**[a]		Compound **6**[a]	
	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	131.4, C		137.3, C	
2	116.3, CH	6.69, d (1.9)	117.3, CH	6.68, d (2.1)
3	146.1, C		148.2, C	
4	144.7, C		145.0, C	
5	117.1, CH	6.68, d (8.1)	119.2, CH	7.13, d (8.2)
6	121.3, CH	6.57, d (1.9, 8.1)	121.2, CH	6.63, dd (2.1, 8.2)
7	36.7, CH_2	2.78, m	40.6, CH_2	3.24, m
8	72.4, CH_2	3.98, m	139.0, CH	5.93, ddt (6.7, 10.1, 16.9)
		3.71, m		
9			115.7, CH_2	5.03, m
1′	104.5, CH	4.37, d (7.8)	104.5, CH	4.70, d (7.5)
2′	75.2, CH	3.31, m	74.8, CH	3.45, m
3′	75.8, CH	3.64, m	77.6, CH	3.27, m
4′	72.2, CH	4.94, m	71.4, CH	3.40, m
5′	74.9, CH	3.60, m	77.3, CH	3.57, m
6′	67.7, CH_2	3.75, m	69.8, CH_2	4.12, dd (2.0, 11.5) 3.76, m
		3.49, m		
1″	102.1, CH	4.64, d (1.2)	105.4, CH	4.33, d (7.3)
2″	72.1, CH	3.85, m	75.0, CH	3.20, m
3″	72.3, CH	3.68, m	77.4, CH	3.43, m
4″	73.9, CH	3.35, m	71.2, CH	3.47, m
5″	69.8, CH	3.62, m	66.9, CH_2	3.84, m
				3.16, m
6″	18.0, CH_3	1.20, d (6.2)		
1‴	127.1, C			
2‴	116.9, CH	6.81, d (8.6)		
3‴	131.3, CH	7.47, d (8.6)		
4‴	161.4, C			
5‴	131.3, CH	7.47, d (8.6)		
6‴	116.9, CH	6.81, d (8.6)		
7‴	147.2, CH	7.67, d (15.9)		
8‴	114.8, CH	6.37, d (15.9)		
9‴	168.3, C			

[a] 300 MHz for ^{1}H NMR, 75 MHz for ^{13}C NMR.

of each moiety were established based on HMBC correlations of H-1′/C-4 and H-1″/C-6′. Thus, the structure of **6** was elucidated as 3,4-dihydroxy-1-allylbenzene-4-*O*-*β*-D-xylopyranosyl-(1→6)-*β*-D-glucopyranoside, trivially named forsythenside L.

Compound **7**, yellow gum, had a molecular formula of $C_{27}H_{34}O_{12}$ by HRESIMS. Its ^{1}H NMR spectrum gave three methoxy groups at δ 3.88, 3.89 and 3.91 (each 3H, s), and one anomeric proton signal at δ 4.89 (1H, d, J = 7.5 Hz). Signals belonging to two pairs of 1,3,4-trisubstituted benzene rings were also observed at δ 7.61 (1H, d, J = 2.0 Hz), 7.07 (1H, d, J = 8.5 Hz), 7.72 (1H, dd, J = 2.0, 8.5 Hz), and at δ 7.14 (1H, d, J = 2.0 Hz), 7.14 (1H, d, J = 8.3 Hz), 6.95 (1H, dd, J = 2.0, 8.3 Hz). The ^{13}C NMR and DEPT-135 spectra of **7** showed 27 carbon signals [three primary, three secondary, fourteen tertiary, and seven quaternary (including one ketone) carbon signals]. Acid hydrolysis of **7** gave D-glucose as sugar residue. From the above evidence, compound **7** was proposed as a lignan glycoside with three methoxy groups. The ^{13}C NMR spectroscopic data of the aglycone were similar to those of forsythialan B (Piao et al., 2008), except that the 3-methoxy-5-hydroxyphenyl moiety in forsythialan B was replaced by the 3-methoxy-4-hydroxyphenyl moiety in **7**. In addition, the glucose moiety was located at C-4′ of the aglycone due to the HMBC correlation of H-1″/C-4′ (Fig. 4).

The relative configurations of H-7′/H-8′ were determined on the basis of the characteristic chemical shift of H-7′. The *trans* configuration often gave the signal of H-7′ at δ 4.7, whereas the *cis* configuration gave a relative downfield shift at δ 5.5 (Guan et al., 2008). The chemical shift of H-7′ of **7** was observed at δ 4.69, indicating the *trans* configuration of H-7′/H-8′. The NOESY spectrum displayed a correlation between H-8 and H-8′. However, no NOESY correlation between H-7′/H-8 was observed. Thus, the relative configuration of H-7′/H-8 was determined as being a *trans* form. The calculated ECD spectrum for the aglycone of **7** was not in sufficiently good agreement with the experimental ECD spectrum of **7**. In addition, hydrolysis of compound **7** to obtain the aglycone of **7** was not successful. Thus, the absolute configuration of **7** was not determined. Compound **7** was deduced as 3,3′,4-trimethoxy-7′,9-epoxylignan-9′-ol-7-one-4′-*O*-*β*-D-glucopyranoside, trivially named forsythialanside A.



7

Fig. 4. Key HMBC (→) and ^{1}H, ^{1}H-COSY (▬) correlations of **7**.

Compound **8** was obtained as yellow gum. Its molecular formula was determined to be $C_{26}H_{32}O_{12}$ on the basis of HRESIMS data. The 1D NMR spectroscopic data of **8** resembled those of compound **7**, except for the absence of one methoxy group of the aglycone. In the HMBC spectrum, the correlation between the anomeric proton (δ 5.06) and *O*-linked carbon (δ 152.6) suggested the glucose moiety was linked to the C-4 of the aglycone.

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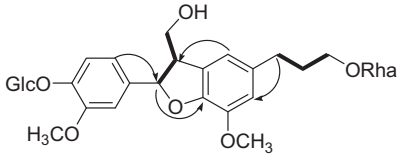
The chemical shift of H-7′ at δ 4.64 suggested a *trans* configuration for H-7′/H-8′. As no correlation between H-7′ and H-8 in the NOESY spectrum was observed, the relative configuration of H-7′/H-8 was determined to be *trans*. Consequently, compound **8** was elucidated as 4′-hydroxy-3,3′dimethoxy-7′,9-epoxylignan-9′-ol-7-one-4-*O*-β-D-glucopyranoside, namely forsythialanside B.

Compound **9** was isolated as yellow gum. Its molecular formula was deduced to be $C_{32}H_{44}O_{15}$ based on HRESIMS data. ^{1}H NMR spectrum showed two anomeric protons at δ 4.88 (1H, d, J = 7.6 Hz) and 4.64 (1H, d, J = 1.5 Hz). Acid hydrolysis of **9** yielded D-glucose and L-rhamnose as sugar residues. ^{13}C NMR spectrum showed 32 carbon signals. The resonances of the aglycone were similar to those of dihydrodehydrodiconiferyl alcohol (Takeda et al., 1998), which was further confirmed by analysis of the 2D NMR spectra (Fig. 5). Furthermore, the glucose and rhamnose moiety were located at C-4 and C-9′ of the aglycone, respectively, due to the obvious HMBC correlations of H-1″/C-4 and H-1‴/C-9′.

According to the literature (Sergio et al., 2006), the chemical shift of H-7 was a competent tool to determine the relative configuration of H-7 and H-8 of benzofuran-type neolignans. The chemical shift at δ 5.84 suggested a *cis* configuration, while relative upfield chemical shift at δ 5.57 indicated a *trans* form. The chemical shift of H-7 in **9** was observed at δ 5.55 (1H, d, J = 5.9 Hz), suggesting a *trans* configuration of H-7/H-8. Acid hydrolysis of **9** with 2 M HCl at 90 °C produced dihydrodehydrodiconiferyl alcohol (**9a**). Then, the calculation of ECD of **9a** was applied in combination with the experimental ECD data. The calculated ECD spectrum matched very well with the experimental ECD spectrum of **9a** (Fig. 6), indicating the 7*S*, 8*R* configuration of **9**. Therefore, compound **9** was assigned as (7*S*, 8*R*)-dihydrodehydrodiconiferyl alcohol-4-*O*-β-D-glucopyranoside-9′-*O*-α-L-rhamnopyranoside, named forsythialanside C.

Compound **10** was also afforded as yellow gum. Its molecular formula was determined to be $C_{32}H_{44}O_{14}$ by HRESIMS. The 1D NMR spectroscopic data of **10** were similar to those of compound **9**, except that the glucose moiety was replaced by a rhamnose moiety. Acid hydrolysis of **10** gave L-rhamnose as sugar residue. The chemical shift of H-7 was at δ 5.55 (1H, d, J = 6.0 Hz), which demonstrated the *trans* configuration of H-7 and H-8. Acid hydrolysis of **10** with 2 M HCl at 90 °C also yielded **9a**. The calculated ECD spectrum of **9a** matched the experimental result very well, allowing the assignment of the absolute configuration of **10** as depicted. The structure of compound **10** was deduced to be (7*S*, 8*R*)-dihydrodehydrodiconiferyl alcohol-4,9′-di-*O*-α-L-rhamnopyranoside, trivially named forsythialanside D.

All compounds were evaluated for their antiviral activities against influenza A (H1N1) virus *in vitro*. Among them, only compounds **8**, **10** and **15** showed mild activities, of which the EC_{50} values were at 41.1, 60.5 and 91.8 μM with no cytotoxicities within 500 μM, respectively. In addition, the antiviral activities against RSV of compounds **1**–**10**, **15**–**16** were also tested. As a result, compounds **15** and **16** showed significant activities with EC_{50} values of 3.43 μM (CC_{50} = 193.2 μM) and 6.72 μM (CC_{50} = 230.0 μM), while compound **5** showed moderate activity with EC_{50} values of 36.2 μM (CC_{50} = 259.7 μM), compounds **4** and **7** showed weak activities with EC_{50} values of 153.9 and 166.4 μM (no cytotoxicity within 500 μM).



9

Fig. 5. Key HMBC (→) and ^{1}H, ^{1}H-COSY (▬) correlations of **9**.

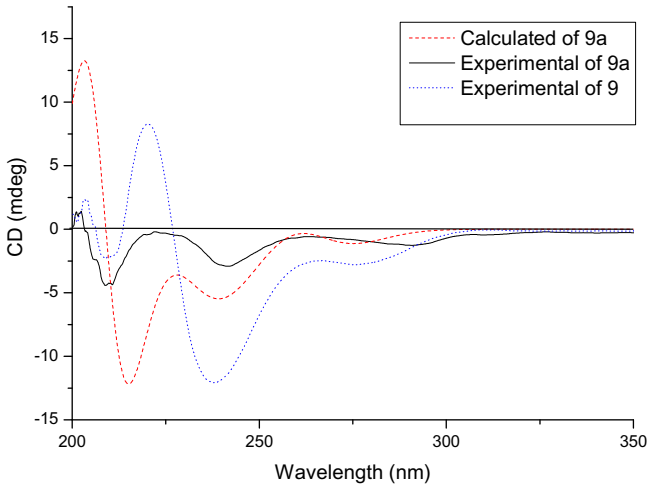


Fig. 6. Experimental and calculated ECD spectra for **9a**.

3. Conclusions

Phenylethanoid glycosides, lignan glycosides, quinoid glycosides were the typical constituents of *F. suspensa*. Our current study reported the isolation and elucidation of four new quinoid glycosides, one new phenylethanoid glycoside, one new allylbenzene glycoside and four new lignan glycosides, which added the diversity of these ingredients. Moreover, a number of oleuropeic acid glucose esters were reported from the family Myrtaceae (Goodger and Woodrow, 2011), but quinoid glycosides with oleuropeic acid (**1**–**2**) were found for the first time from the Oleaceae family.

Antiviral activity evaluation of the compounds suggested that five compounds (**4**, **5**, **7**, **15**, **16**) were active in inhibiting RSV. Three phenylethanoid glycosides showed good activity especially. Among them, the antiviral activity of forsythoside A (**16**) has previously been reported (Ma et al., 2012), but the result here first reported its anti-RSV activity with an EC_{50} value of 6.72 μM. Furthermore, the possible relationships between the chemical structures of phenylethanoid glycosides and their anti-RSV activities were not reported before, comparison of compound **5** with **15** and **16** found that the more phenolic hydroxyl groups the compound had, the better activity it showed. In conclusion, compounds isolated from *F. suspensa* showed structural diversity. Antiviral activity suggested that phenylethanoid glycosides were significantly active against RSV *in vitro*, thereby providing a basis for the activity of this plant.

4. Experimental section

4.1. General experimental procedures

Optical rotations were measured on JASCO P-1020 spectrometer, UV spectra were recorded on a JASCO V-550 UV/Vis spectrometer, CD spectra were acquired on a JASCO J-810 spectropolarimeter, whereas IR spectra were obtained using a JASCO FT/IR-480 plus spectrometer. 1D and 2D NMR spectra were measured on a Bruker AV-300/400 spectrometer. HRESIMS data were determined by a Waters Synapt G2 MS mass spectrometer. Open column chromatography (CC) was performed using silica gel (200–300 mesh, Qingdao Haiyang Chemical Goup Corp. Qingdao,

People's Republic of China), ODS (50 μm, YMC, Japan), and HW-40 (Tosoh, Japan). Thin-layer chromatography (TLC) was performed using precoated silica gel plates (silica gel GF_{254}, 1 mm, Yantai).

4.2. Plant material

Plant material provided by Heilongjiang Provincial Songhuajiang Pharmaceutical Co., Ltd., was identified as the fruit of *F. suspensa* by Professor Guang-Xiong Zhou (College of Pharmacy, Jinan University). A voucher specimen (20090919FS) is deposited at the Institute of Traditional Chinese Medicine and Natural Products, Jinan University, Guangzhou, China.

4.3. Extraction and isolation

The dried fruit of *F. suspensa* (2 kg) was extracted with EtOH–H_2O (60:40, v/v) twice, under conditions of reflux, two hours each time, and then the combined extracts were concentrated under reduced pressure to yield a brownish extract (540 g). An aliquot (~500 g) of the latter was applied to a Diaion HP-20 resin column eluted with EtOH–H_2O in gradient. The 95% EtOH–H_2O eluate (D, 100 g) was then subjected to silica-gel CC, eluted with a $CHCl_3$–MeOH gradient to give 10 fractions (D1–D10). Fraction D1 ($CHCl_3$–MeOH 10:0 eluate, 2.8 g) was subjected to CC over Sephadex LH-20 and silica gel to give compounds **11** (8.0 mg), and **22** (33.2 mg). Fraction D5 ($CHCl_3$–MeOH 9:1 eluate, 3.3 g) was subjected to ODS CC, eluted with a MeOH–H_2O gradient, to yield 10 subfractions (D5A–D5J). Subfraction D5B (MeOH–H_2O (30:70, V/V) eluate, 183.7 mg) was subjected to HW-40 CC eluted with a MeOH–H_2O gradient (20:80→60:40, V/V–). The fine fraction D5B3 (MeOH–H_2O (20:80, V/V) eluate, 96.5 mg) was purified by preparative HPLC (Ultimate™ XB-C18 5 μm, 21.2 × 250 mm, Welch) with MeOH–H_2O (30:70, V/V) to afford compounds **4** (15.2 mg), **12**(20.2 mg) and **21** (47.9 mg). The fine fraction D5B2 (MeOH–H_2O (20:80, V/V) eluate, 21.6 mg) was also purified by preparative HPLC with MeOH–H_2O (30:70, V/V) to yield compounds **7** (11.6 mg) and **8** (6.5 mg). Subfraction D5C (MeOH–H_2O (50:50, V/V) eluate, 442.4 mg) was further separated by HW-40 CC eluted with a MeOH–H_2O gradient. The fine fraction D5C1 and D5C3 (MeOH–H_2O (20:80, V/V) eluate, 184.7 and 43.4 mg) were subjected to ODS CC eluted with MeOH–H_2O (30:70, V/V), and further purified by preparative HPLC with MeOH–H_2O (30:70, V/V) to afford compounds **13** (4.0 mg), **1** (8.0 mg), **2** (4.0 mg) and **3** (3.6 mg), respectively. Fraction D8 ($CHCl_3$–MeOH, 80:20 V/V, eluate, 3.3 g) was applied to ODS CC eluted with a MeOH–H_2O gradient. Subfraction D8C and D8D (MeOH–H_2O (50:50, V/V) eluate, 541.2 and 184.4 mg) were subjected to HW-40 CC, and then purifed by preparative HPLC with MeOH–H_2O (40:60, V/V) to yield compounds **17** (13.7 mg), **5** (20.0 mg), **15** (34.3 mg) and **10** (7.9 mg), respectively. Fraction D9 ($CHCl_3$–MeOH 70:30, V/V eluate, 9.1 g) was divided into 6 fractions by ODS CC with a MeOH–H_2O gradient. Subfraction D9D (MeOH–H_2O (40:60, V/V) eluate, 1.2 g) was separated using HW-40 CC, and purified by preparative HPLC with MeOH–H_2O (40:60, V/V) to afford compounds **16** (105.2 mg), **6** (14.3 mg) and **9** (6.4 mg). By similar procedures, compounds **14** (3.1 mg), **18** (11.6 mg), **19** (31.8 mg), **20** (11.8 mg) and **23** (21.9 mg) were isolated from fraction D2 ($CHCl_3$–MeOH, 90:10, V/V, eluate, 3.0 g) and D3 ($CHCl_3$–MeOH, 90:10, V/V eluate, 3.1 g), respectively.

4.3.1. Forsythenside G (**1**)

Light yellow gum; $[\alpha]_D^{20}$ −40.4 (c 0.7, MeOH); UV (MeOH) λ_{max} (log ε) 223 (4.19) nm; CD (MeOH) 250 (Δε −0.19) nm; IR (KBr) ν_{max} 3428, 2925, 1675 cm^{-1}. For ^1H and ^{13}C NMR spectroscopic data, see Table 1; HRESIMS *m/z* 505.2042 $[M+Na]^+$ (calcd for $C_{24}H_{34}O_{10}Na$, 505.2050).

4.3.2. Forsythenside H (**2**)

Light yellow gum; $[\alpha]_D^{20}$ −58.6 (c 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 218 (3.86) nm; CD (MeOH) 230 (Δε −0.31) nm; IR (KBr) ν_{max} 3411, 2926, 1705 cm^{-1}. For ^1H and ^{13}C NMR spectroscopic data, see Table 1; HRESIMS *m/z* 509.2375 $[M+Na]^+$ (calcd for $C_{24}H_{38}O_{10}Na$, 509.2363).

4.3.3. Forsythenside I (**3**)

Light yellow gum; $[\alpha]_D^{20}$ −8.5 (c 0.2, MeOH); UV (MeOH) λ_{max} (log ε) 212 (4.02) nm; CD (MeOH) 227 (Δε −0.35) nm; IR (KBr) ν_{max} 3426, 2925, 1645 cm^{-1}. For ^1H and ^{13}C NMR spectroscopic data, see Table 1; HRESIMS *m/z* 481.2054 $[M-H]^-$ (calcd for $C_{24}H_{33}O_{10}$, 481.2074).

4.3.4. Forsythenside J (**4**)

Light yellow gum; $[\alpha]_D^{20}$ −31.6 (c 0.25, MeOH); UV (MeOH) λ_{max} (log ε) 234 (4.06), 296 (3.90), 323 (4.05) nm; IR (KBr) ν_{max} 3284, 2928, 1675 cm^{-1}. For ^1H and ^{13}C NMR spectroscopic data, see Table 1; HRESIMS *m/z* 491.1523 $[M-H]^-$ (calcd for $C_{24}H_{27}O_{11}$, 491.1553).

4.3.5. Forsythenside K (**5**)

Light yellow gum; $[\alpha]_D^{20}$ −11.2 (c 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.34), 226 (4.11), 291 (4.09), 314 (4.16) nm; IR (KBr) ν_{max} 3410, 2925, 1697, 1601 cm^{-1}. For ^1H and ^{13}C NMR spectroscopic data, see Table 2; HRESIMS *m/z* 607.2038 $[M-H]^-$ (calcd for $C_{29}H_{35}O_{14}$, 607.2027).

4.3.6. Forsythenside L (**6**)

Yellow amorphous powder; $[\alpha]_D^{20}$ −54.6 (c 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 205 (4.52), 254 (4.56) nm; IR (KBr) ν_{max} 3400, 2884, 1074 cm^{-1}; For ^1H and ^{13}C NMR spectroscopic data, see Table 2; HRESIMS *m/z* 443.1555 $[M-H]^-$ (calcd for $C_{20}H_{27}O_{11}$, 443.1553).

4.3.7. Forsythialanside A (**7**)

Yellow gum; $[\alpha]_D^{20}$ −36.8 (c 0.25, MeOH); UV (MeOH) λ_{max} (log ε) 229 (4.18), 276 (3.92), 304 (3.72) nm; CD (MeOH) 240 (Δε −0.22), 272 (Δε −0.30), 313 (Δε −0.25) nm; IR (KBr) ν_{max} 3301, 2869, 1685 cm^{-1}; For ^1H and ^{13}C NMR spectroscopic data, see Table 3; HRESIMS *m/z* 573.1947 $[M+Na]^+$ (calcd for $C_{27}H_{34}O_{12}Na$, 573.1948).

4.3.8. Forsythialanside B (**8**)

Yellow gum; $[\alpha]_D^{20}$ −23.2 (c 0.25, MeOH); UV (MeOH) λ_{max} (log ε) 227 (3.65), 273 (3.42), 305 (3.16) nm; CD (MeOH) 244 (Δε −0.42), 266 (Δε −0.30), 320 (Δε −0.40) nm; IR (KBr) ν_{max} 3328, 1671 cm^{-1}. For ^1H and ^{13}C NMR spectroscopic data, see Table 3; HRESIMS *m/z* 559.1785 $[M+Na]^+$ (calcd for $C_{26}H_{32}O_{12}Na$, 559.1757).

4.3.9. Forsythialanside C (**9**)

Yellow gum; $[\alpha]_D^{20}$ −53.5 (c 0.6, MeOH); UV (MeOH) λ_{max} (log ε) 206 (4.31), 230 (3.92), 279 (3.50) nm; CD (MeOH) 238 (Δε −5.42), 275 (Δε −1.26) nm; IR (KBr) ν_{max} 3397, 2926, 2346, 1619 cm^{-1}. For ^1H and ^{13}C NMR spectroscopic data, see Table 3; HRESIMS *m/z* 691.2565 $[M+Na]^+$ (calcd for $C_{32}H_{44}O_{15}Na$, 691.2578).

4.3.10. Forsythialanside D (**10**)

Yellow gum; $[\alpha]_D^{20}$ −32.8 (c 0.4, MeOH); UV (MeOH) λ_{max} (log ε) 206 (4.57), 233 (4.08), 279 (3.71) nm; CD (MeOH) 241 (Δε −0.79), 277 (Δε −0.54) nm; IR (KBr) ν_{max} 3425, 2925, 2359, 1617 cm^{-1}; For ^1H and ^{13}C NMR spectroscopic data, see Table 3); HRESIMS *m/z* 651.2630 $[M-H]^-$ (calcd for $C_{32}H_{43}O_{14}$, 651.2653).

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Table 3
^{1}H and ^{13}C NMR spectroscopic data for compounds 7–**10** (Measured in CD_3OD).

Position	Compound **7**[a]		Compound **8**[b]		Compound **9**[b]		Compound **10**[b]	
	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)	δ_C, type	δ_H (J in Hz)
1	130.9, C		132.5, C		138.3, C		138.8, C	
2	112.2, CH	7.61, d (2.0)	113.0, CH	7.66, d (1.9)	111.2, CH	7.03, d (1.9)	111.2, CH	7.03, d (1.9)
3	150.6, C		150.8, C		151.0, C		152.1, C	
4	155.3, C		152.6, C		147.6, C		146.6, C	
5	111.8, CH	7.07, d (8.5)	116.3, CH	7.26, d (8.5)	118.1, CH	7.14, d (8.3)	119.6, CH	7.08, d (8.2)
6	124.8, CH	7.72, dd (2.0, 8.5)	124.3, CH	7.69, dd (1.9, 8.5)	119.4, CH	6.93, dd (1.9, 8.3)	119.1, CH	6.92, dd (1.9, 8.2)
7	200.2, C		200.4, C		88.5, CH	5.55, d (5.9)	88.5, CH	5.55, d (6.0)
8	50.4, CH	4.26, m	50.5, CH	4.27, m	55.7, CH	3.45, m	55.6, CH	3.46, m
9	71.8, CH_2	4.22, m	71.6, CH_2	4.23, dd (6.3, 13.0) 4.17, m	65.1, CH_2	3.84, m	65.1, CH_2	3.84, m
						3.76, m		3.76, m
1′	137.3, C		133.5, C		136.7, C		136.7, C	
2′	112.1, CH	7.14, d (2.0)	111.4, CH	7.05, d (1.8)	114.1, CH	6.72, brs	114.0, CH	6.72, brs
3′	150.9, C		149.1, C		145.3, C		145.3,C	
4′	147.7, C		147.5, C		147.5, C		147.6, C	
5′	117.7, CH	7.14, d (8.3)	115.9, CH	6.76, d (8.1)	129.6, C		129.6, C	
6′	120.7, CH	6.95, dd (2.0, 8.3)	120.9, CH	6.84, dd (1.8, 8.1)	118.1, CH	6.72, brs	118.1, CH	6.72, brs
7′	85.0, CH	4.69, d (8.0)	85.4, CH	4.64, d (8.4)	33.1, CH_2	2.67, m	33.1, CH_2	2.64, m
8′	54.7, CH	2.69, m	54.6, CH	2.70, m	32.6, CH_2	1.88, m	32.6, CH_2	1.88, m
9′	61.4, CH_2	3.65, m	61.4, CH_2	3.63, m	67.6, CH_2	3.66, m	67.6, CH_2	3.66, m
						3.38, m		3.38, m
1″	102.8, CH	4.89, d (7.5)	101.9, CH	5.06, d (7.4)	102.8, CH	4.88, d (7.6)	101.4, CH	5.34, d (1.6)
2″	74.9, CH	3.50, m	74.7, CH	3.51, m	74.9, CH	3.47, m	72.2, CH	4.05, m
3″	78.2, CH	3.47, m	78.4, CH	3.48, m	78.2, CH	3.39, m	72.0, CH	3.86, m
4″	71.3, CH	3.41, m	71.2, CH	3.40, m	71.3, CH	3.38, m	73.8, CH	3.43, m
5″	77.8, CH	3.48, m	77.9, CH	3.49, m	77.9, CH	3.46, m	70.8, CH	3.46, m
6″	62.5, CH_2	3.87, m	62.5, CH_2	3.90, m	62.5, CH_2	3.85, m	18.0, CH_3	1.20, d (6.2)
		3.72, m		3.67, m		3.68, m		
1‴					101.8, CH	4.64, d (1.5)	101.7, CH	4.64, d (1.5)
2‴					72.5, CH	3.81, m	72.5, CH	3.81, m
3‴					72.4, CH	3.67, m	72.4, CH	3.65, m
4‴					74.0, CH	3.35, m	74.0, CH	3.36, m
5‴					69.8, CH	3.56, m	69.8, CH	3.56, m
6‴					18.0, CH_3	1.21, d (6.2)	18.0, CH_3	1.20, d (6.2)
3′-OCH_3	56.4, CH_3	3.88, s	56.4, CH_3	3.88, s	56.8, CH_3	3.87, s	56.8, CH_3	3.87, s
3-OCH_3	56.5, CH_3	3.89, s	56.7, CH_3	3.93, s	56.7, CH_3	3.83, s	56.4, CH_3	3.80, s
4-OCH_3	56.7, CH_3	3.91, s						

[a] 300 MHz for ^{1}H NMR, 75 MHz for ^{13}C NMR.
[b] 400 MHz for ^{1}H NMR, 100 MHz for ^{13}C NMR.

4.4. Acid hydrolysis and HPLC analysis of compounds *1–10*

The absolute configuration of the sugar units were determined by the method of Tanaka et al. (Tu et al., 2011). Compounds **1–5** and **7–8** afforded D-glucose (t_R = 19.236 min); compound **6** gave D-glucose (t_R = 19.236 min) and D-xylose (t_R = 22.351 min); compound **9** liberated D-glucose (t_R = 19.236 min) and L-rhamnose (t_R = 32.407 min), compound **10** afforded L-rhamnose (t_R = 32.407 min).

4.5. Acid hydrolysis of *9* and *10*

A solution of **9** (1.5 mg) was hydrolyzed with 2 M HCl for 2 h at 90 °C. The mixture was then extracted with EtOAc, the EtOAc layer was evaporated to dryness and dissolved in MeOH, with the mixture subjected to p-HPLC (Cosmosil C_{18}-MS-II column, 250 × 4.6 mm i.d., 5 μm) at 35 °C with a 50:50 mixture of MeOH–H_2O containing 0.1% HCO_2H) to give **9a**. Its structure was confirmed by analysis of HRESIMS and ^{1}H NMR spectroscopic data (Jiang et al., 2005). Acid hydrolysis of **10** (2.0 mg) also gave **9a**.

4.6. Methanolysis of compounds *1–3*

A solution of **1** (2.0 mg) in 0.02 M NaOMe–MeOH (1 ml) was placed at room temperature for 12 h respectively (Tian et al., 2009). After neutralizing with HCOOH (5M) and extracting with EtOAc, the EtOAc layer was evaporated to dryness and dissolved in MeOH. Then the mixture was subjected to preparative HPLC (cosmosil C_{18}-MS-II column, 250 × 4.6 mm i.d., 5μm at 35 °C with MeOH–H_2O, 40:60, V/V) to give (+)-oleuropeic acid (**1a**). **1a** (0.6 mg): colorless oil, $[\alpha]_D^{20}$ +45.3 (c 0.3, $CHCl_3$). ^{1}H NMR (CD_3OD): 6.75 (1H, brs, H-2), 2.28 (1H, m, H-3a) and 1.96 (1H, m, H-3b), 1.53 (1H, m, H-4), 2.00 (1H, m, H-5a) and 1.21 (1H, m, H-5b), 2.49 (1H, m, H-6a) and 2.12 (1H, m, H-6b), 1.17 (6H, s, H-9 and H-10). Methanolysis of **2** also gave (**1a**) {$[\alpha]_D^{20}$ +38.0 (c 0.25, $CHCl_3$)}. Similar methanolysis of **3** (1.6 mg) gave 6-hydroxy-6-isopropylcyclohex-1-enecarboxylic acid (**3a**). **3a** (0.5 mg): colorless oil, CD (207 nm, $\Delta\varepsilon$ −0.06), ^{1}H NMR (CD_3OD): 6.68 (1H, brs, H-2), 2.30 (1H, m, H-3a) and 2.12 (1H, m, H-3b), 2.36 (2H, m, H-4), 1.72 (1H, m, H-5a) and 1.55 (1H, m, H-5b), 1.65 (1H, m, H-8), 0.87 (3H, d, J = 7.6 Hz, H-9), 0.89 (3H, d, J = 7.6 Hz, H-10).

4.7. Computation methods

The conformational search of **3a** and **9a** was performed using the Random search method, MMFF94 force field and charged with MMFF94 as implemented in the program package Sybyl 8.0. 27 and 14 minimum geometries within an energy of 10 kcal/mol above the presumably global minimum, each for **3a** and **9a** respectively. These were selected and further optimized by using DFT at the B3LYP/6-31G(d) level as implemented in the Gaussian 09 software. The stable conformers obtained were used to CD calculation by

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TDDFT [B3LYP/6-31G (d)] method with 50 excited states in gas phase. The overall predicted CD curves of **3a** and **9a** were simulated as sums of Gaussian functions in accordance to the Boltzmann weighting with the half width at 0.2 eV, which revealed good agreement to the measured CD curves.

4.8. Cell culture, virus strains and bioassay

4.8.1. Neuraminidase inhibition assay

Madin-Darby canine kidney (MDCK) cells were grown in Dulbeccos modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) with 5% CO_2 at 37 °C. The influenza virus strain A/PR/8/34/H1N1 was kept and cultured by Wuhan Institute of Virology, Chinese Academy of Science.

Influenza virus NA activity was assayed by quantifying the fluorescent product resulting from cleavage of the substrate 4-methylumbellifery-α-D-*N*-acetyl-neur-aminate (MUNANA) (Zu et al., 2012; Potier et al., 1979). The reaction mixture consisted of the tested compounds, virus and MUNANA in a 96-well plate. After incubation for 60 min at 37 °C, the reaction was terminated and the fluorescence of the mixture was recorded for the excitation wavelength 355 nm and the emission wavelength 485 nm. The inhibition ratio was obtained using the equation:

$$\text{Inhibition activity } (\%) = (F_{\text{sample}} - F_{\text{virus}})/(F_{\text{substrate}} - F_{\text{virus}}) * 100$$

F_{sample} is the fluorescence of the tested samples. F_{virus} is the fluorescence of the virus control. $F_{\text{substrate}}$ is the fluorescence of the substrate control.

The 50% effective concentration (EC_{50}) was determined by extrapolation of the results from various doses tested using a linear equation.

4.8.2. Cytopathic effect inhibition assay

Hep-2 cells were grown in RPMI-1640 medium containing 10% fetal bovine serum (FBS) with 5% CO_2 at 37 °C. The virus strain RSV was kept and cultured by Wuhan Institute of Virology, Chinese Academy of Science.

The anti-RSV assay of tested compounds was measured by the CPE inhibition assay (Ma et al., 2001), virus suspension and two fold serial dilutions of the compounds were added to each test well, and the plates were reincubated for 5 days to allow development of a CPE if any. A non-infection control was made and ribavirin was used for drug control. The concentration reducing CPE by 50% with respect to virus control was estimated from graphic plots and was defined EC_{50}.

4.8.3. Cytotoxicity assay

The method using alamar blue has been developed for detecting cell-mediated cytotoxicity *in vitro* (Nociari et al., 1998). Alamar blue detected cell viability by utilizing a nonfluorescent dye resazurin, which was converted to a fluorescent dye resorufin in response to chemical reduction of growth medium resulting from cell growth. The fluorescent signal generated from the assay was proportional to the number of living cells in the sample.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.04.010.

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