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Quinic acid derivatives and coumarin glycoside from the roots and stems of *Erycibe obtusifolia*

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ABSTRACT

A new quinic acid derivative (**1**) and a new coumarin glycoside (**8**), together with six known compounds (**2–7**) were isolated from the roots and stems of *Erycibe obtusifolia*. The structures of the new compounds were elucidated by spectroscopic and chemical analyses. The *in vitro* antiviral activity against the respiratory syncytial virus (RSV) of seven quinic acid derivatives was evaluated by cytopathic effect (CPE) reduction assay. Among them, the dicaffeoylquinic acids (**6** and **7**) displayed potent *in vitro* anti-RSV activity.

1. Introduction

The genus *Erycibe* (Convolvulaceae) comprises more than 60 species, most of which are distributed in tropical Asia (Fang and Huang, 1979), and some species are used as traditional medicines in China and Thailand (Hsu et al., 1998; Matsuda et al., 2004; Tanawan et al., 2007). Previous phytochemical investigations have revealed that the main constituents of this genus plants are coumarins, quinic acid derivatives, alkaloids, flavonoids, and diglycosides (Liu et al., 2007, 2014; Lu et al., 1986; Morikawa et al., 2006; Matsuda et al., 2004, 2007; Song et al., 2010; Yao and Chen, 1979; Pan et al., 2009; Fan et al., 2013). The roots and stems of *Erycibe obtusifolia* are commonly used as a traditional Chinese medicine to treat various rheumatoid diseases (Hsu et al., 1998). In a previous study, we reported the isolation of several quinic acid derivatives and coumarin glycosides from the roots and stems of *E. obtusifolia*, together with their *in vitro* antiviral effect against the respiratory syncytial virus (RSV) (Fan et al., 2013). In order to search for more anti-RSV constituents from this plant, our continuing phytochemical studies have led to identification of a new quinic acid derivative (**1**) and a new coumarin glycoside (**8**), together with six known compounds (**2**–**7**) (Fig. 1). In addition, seven quinic acid derivatives were tested for their *in vitro* anti-RSV activity. Here, we describe the isolation and structural elucidation of two new compounds, as well as the *in vitro* anti-RSV activity of the quinic acid derivatives.

2. Results and discussion

Compound **1** was obtained as yellow oil. The molecular formula of **1** was determined to be $C_{27}H_{30}O_{13}$ based on HR–ESI–MS at *m/z* 563.1759 $[M+H]^+$ (calculated for $C_{27}H_{31}O_{13}$, 563.1759). The UV spectrum of **1** showed absorption maxima at 216, 288 and 327 nm. The IR spectrum implied the presence of hydroxyl (3414 cm^{-1}) and carbonyl (1716 cm^{-1}) groups, as well as aromatic ring (1603 and 1517 cm^{-1}). The ^1H NMR spectrum of **1** showed signals for three oxygenated methine protons at δ_H 5.54 (1H, m), 5.01 (1H, dd, J = 7.2, 3.2 Hz) and 4.13 (1H, m), as well as four methylene protons at δ_H 2.30 (1H, dd, J = 13.6, 3.9 Hz), 2.04 (2H, m) and 2.01 (1H, m), suggesting the presence of a quinic acid moiety. In addition, the ^1H NMR spectrum displayed signals for two aromatic protons at δ_H

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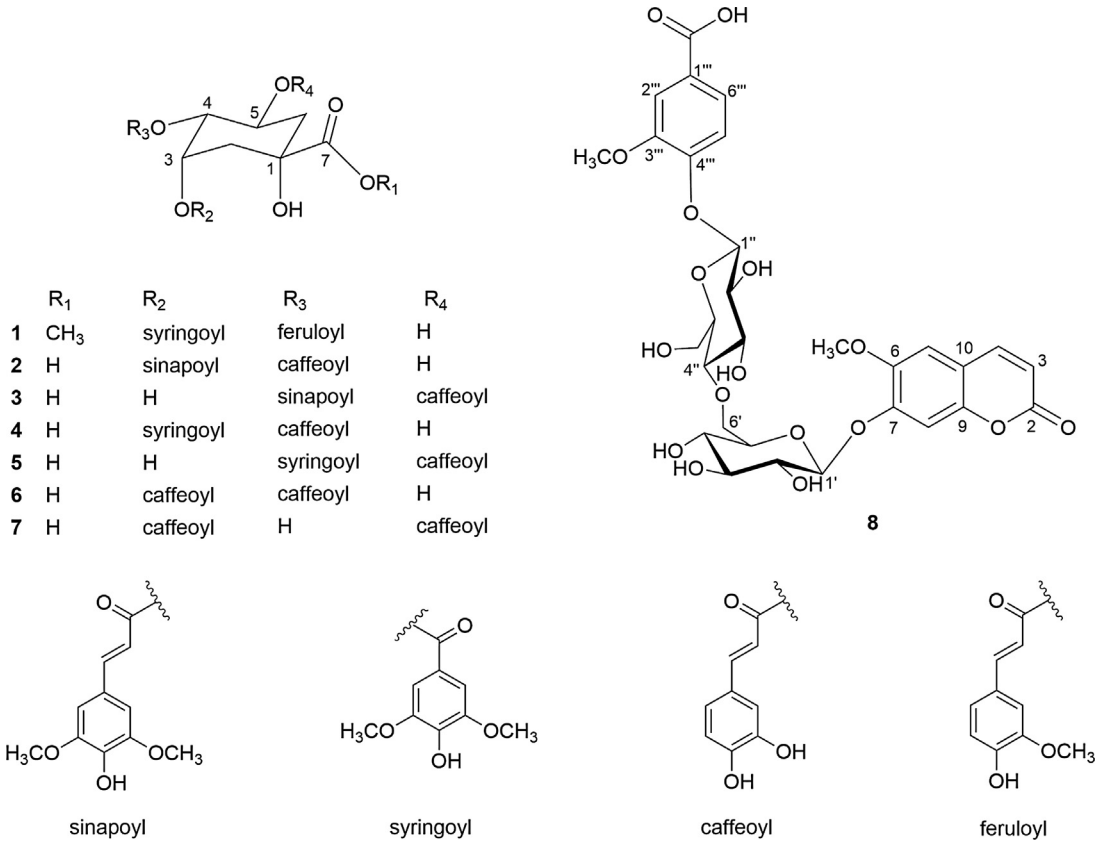

Fig. 1. Chemical structures of compounds **1–8**.

7.21 (2H, s) and two methoxy protons at δ_H 3.72 (6H, s), which indicated the presence of a syringoyl moiety in **1**. Furthermore, the ^{1}H NMR spectrum showed proton signals due to a 1,3,4-trisubstituted benzene ring at δ_H 7.24 (1H, d, J = 1.7 Hz), 7.03 (1H, dd, J = 8.2, 1.7 Hz) and 6.78 (1H, d, J = 8.2 Hz), a *trans*-disubstituted double bond at δ_H 7.49 (1H, d, J = 15.9 Hz) and 6.45 (1H, d, J = 15.9 Hz), and one methoxy protons at δ_H 3.79 (3H, s), suggesting the presence of a feruloyl moiety. The ^{13}C NMR and DEPT spectra of **1** revealed the presence of 27 carbon signals, including characteristic signals due to feruloyl, syringoyl and quinic acid moieties, respectively. With the aid of 1D and 2D NMR experiments, all of the ^{1}H and ^{13}C NMR signals of **1** were assigned (Table 1).

In the HMBC spectrum of **1**, the correlations between H-3 (δ_H 5.54) of quinic acid moiety and C-7′ (δ_C 164.7) of syringoyl moiety, as well as between H-4 (δ_H 5.01) of quinic acid and C-9″ (δ_C 166.0) of feruloyl moiety were displayed, which suggested that the syringoyl and feruloyl moieties were attached to C-3 and C-4 positions of quinic acid moiety, respectively. The additional methoxy group (δ_H 3.65; δ_C 51.8) was located at C-7 of the quinic acid based on the HMBC correlation between methoxy protons (δ_H 3.65) and C-7 (δ_C 174.2) of quinic acid (Fig. 2). In the ROESY spectrum, the correlations between H-2′, 6′ (δ_H 7.21) and H-5 (δ_H 4.13)/ CH_3O-3″ (δ_H 3.79), as well as between H-4 (δ_H 5.01) and CH_3O-7 (δ_H 3.65) were observed, which indicated that H-3, H-4 and the carbonyl group at C-7 were on the β configuration, and H-5 was on the α configuration (Fig. 2). Hence, the structure of **1** was determined to be 4-*O*-feruloyl-3-*O*-syringoyl quinic acid methyl ester.

Although only one quinic acid derivative with methyl ester was reported in this paper, actually there were some references which have reported many other similar constituents with methyl ester, even ethyl ester or butyl ester (Liu et al., 2007, 2014; Song et al., 2010; Fan et al., 2013), indicating that the plants of the genus *Erycibe* are rich in this kind of quinic acid derivative. In addition, during the isolation procedure, silica gel which was acidic property and might lead to forming methyl ester if MeOH was used as the eluent avoided being used, and the liquid chromatography peak of compound **1** could be observed in the EtOH extract, fractions and

Table 1
^{1}H and ^{13}C NMR spectral data of compound **1** (DMSO-d_6, δ in ppm, J in Hz).

Position	δ_H	δ_C
1	–	73.0
2	a 2.30 dd (13.6, 3.9) b 2.01 m	35.6
3	5.54 m	68.5
4	5.01 dd (7.2, 3.2)	72.8
5	4.13 m	64.4
6	2.04 m	39.8
7	–	174.2
1′	–	119.6
2′, 6′	7.21 s	107.1
3′, 5′	–	147.4
4′	–	140.7
7′	–	164.7
1″	–	125.4
2″	7.24 d (1.7)	111.0
3″	–	148.0
4″	–	149.5
5″	6.78 d (8.2)	115.5
6″	7.03 dd (8.2, 1.7)	123.2
7″	7.49 d (15.9)	145.3
8″	6.45 d (15.9)	114.3
9″	–	166.0
7-OCH_3	3.65 s	51.8
3′, 5′-OCH_3	3.72 s	55.9
3″-OCH_3	3.79 s	55.7

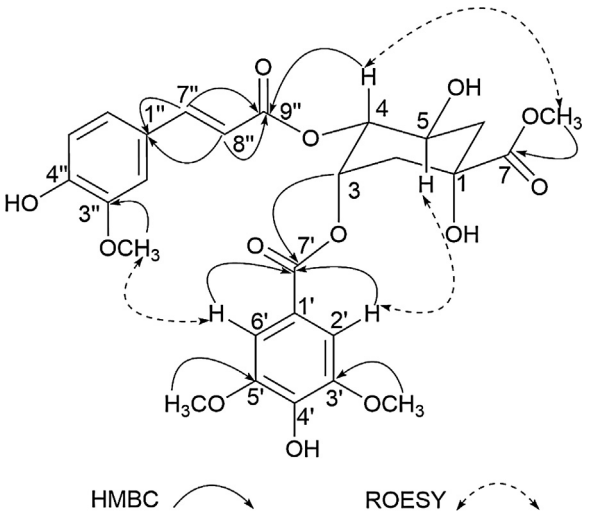

Fig. 2. Selected HMBC and ROESY correlations of **1**.

subfractions in the HPLC experiments. Therefore, compound **1** might not be an artifact product.

Compound **8** was obtained as yellow oil. The HR–ESI–MS spectrum of **8** exhibited an $[M+Na]^+$ ion at *m/z* 689.1687 (calculated for $C_{30}H_{34}NaO_{17}$, 689.1688), corresponding to the molecular formula $C_{30}H_{34}O_{17}$. The UV spectrum of **8** showed absorption maxima at 204, 218, 255, 291 and 342 nm. The IR spectrum suggested the presence of hydroxyl (3395 cm^{-1}) and carbonyl (1715 cm^{-1}) groups, as well as aromatic ring (1614, 1571 and 1509 cm^{-1}). The ^1H NMR spectrum of **8** revealed the presence of signals due to a *cis*-disubstituted double bond at δ_H 7.98 (1H, d, J = 9.5 Hz) and 6.32 (1H, d, J = 9.5 Hz), and two aromatic proton signals at δ_H 7.31 (1H, s) and 7.26 (1H, s), suggesting the existence of a 6,7-disubstituted coumarin skeleton in **8** (Song et al., 2010). In addition, the ^1H NMR spectrum showed proton signals due to a 1,3,4-trisubstituted benzene ring at δ_H 7.57 (1H, dd, J = 8.8, 1.9 Hz), 7.44 (1H, d, J = 1.9 Hz) and 7.19 (1H, d, J = 8.8 Hz), two methoxy protons at δ_H 3.82 (3H, s) and 3.77 (3H, s), as well as two anomeric protons due to sugar moieties at δ_H 5.23 (1H, d, J = 7.1 Hz) and 5.05 (1H, d, J = 7.4 Hz).

Acid hydrolysis of **8** afforded D-glucose, which was identified by high-performance liquid chromatography (HPLC) analysis using an authentic sample as a reference. The relative anomeric configurations of the two sugar units were determined to be β based on their $^3J_{H1,H2}$ coupling constants (J = 7.1 Hz and J = 7.4 Hz). The ^{13}C NMR and DEPT spectra of **8** revealed the presence of 30 carbon signals, including signals due to a 6,7-disubstituted coumarin (δ_C 160.6, 149.7, 148.9, 145.9, 144.3, 113.3, 112.4, 109.9 and 102.8), a 1,3,4-trisubstituted benzene ring (δ_C 150.5, 148.5, 122.6, 113.9 and 112.5), and two β-D-glucopyranosyl units (δ_C 99.2, 99.0, 76.8, 76.7, 76.4, 73.7, 73.0, 72.8, 70.0, 69.2, 64.5 and 60.3). In addition to the above assigned signals, there were two methoxy (δ_C 56.0 and 55.5) and one carbonyl (δ_C 165.3) groups in **8**. With the aid of 1D and 2D NMR experiments, all of the ^1H and ^{13}C NMR signals of **8** were assigned (Table 2). In the HMBC spectrum of **8**, the correlations between H-2‴ (δ_H 7.44)/H-6‴ (δ_H 7.57) of 1,3,4-trisubstituted benzene ring and the carbonyl carbon (δ_C 165.3), as well as between the methoxy protons (δ_H 3.77) and C-3‴ (δ_C 148.5) of 1,3,4-trisubstituted benzene ring were observed, indicating the presence of a vanillic acid moiety. The additional methoxy group (δ_H 3.82; δ_C 56.0) was located at position C-6 of coumarin based on the HMBC correlation between methoxy protons (δ_H 3.82) and C-6 (δ_C 145.9) of coumarin. In addition, the HMBC correlations between H-1′ (δ_H 5.23) of D-glucose and C-7 (δ_C 149.7) of coumarin, between H-4″ (δ_H 3.94) of another D-glucose and C-6′ (δ_C 64.5) of D-glucose, as well as between H-1″ (δ_H 5.05) of D-glucose and C-4‴ (δ_C 150.5) of the vanillic acid moiety were observed, which led to the elucidation of linkage sequence and positions of these moieties as shown in Fig. 1. Hence, the structure of **8** was identified as 7-*O*-[6-*O*-(1-*O*-vanillic acid 4-*O*-β-D-glucopyranosyl)-β-D-glucopyranosyl]-6-methoxycoumarin.

Table 2
^1H and ^{13}C NMR spectral data of compound **8** (DMSO-d_6, δ in ppm, J in Hz)

Position	δ_H	δ_C
2	–	160.6
3	6.32 d (9.5)	113.3
4	7.98 d (9.5)	144.3
5	7.31 s	109.9
6	–	145.9
7	–	149.7
8	7.26 s	102.8
9	–	148.9
10	–	112.4
1′	5.23 d (7.1)	99.0
2′	3.36 m	72.8
3′	3.29 m	70.0
4′	3.30 m	76.8
5′	3.36 m	76.4
6′	a 4.65 br d (12.0)	64.5
	b 4.09 dd (12.0, 7.7)	
1″	5.05 d (7.4)	99.2
2″	3.26 m	73.0
3″	3.24 m	69.2
4″	3.94 t (8.2)	73.7
5″	3.30 m	76.7
6″	3.54 m	60.3
1‴	–	122.6
2‴	7.44 d (1.9)	112.5
3‴	–	148.5
4‴	–	150.5
5‴	7.19 d (8.8)	113.9
6‴	7.57 dd (8.8, 1.9)	122.6
7‴	–	165.3
6-OCH_3	3.82 s	56.0
3‴-OCH_3	3.77 s	55.5

The six other compounds were identified as 4-*O*-caffeoyl-3-*O*-sinapoylquinic acid (**2**) (Jaiswal et al., 2010), 5-*O*-caffeoyl-4-*O*-sinapoylquinic acid (**3**) (Nishizawa et al., 1988), 4-*O*-caffeoyl-3-*O*-syringoylquinic acid (**4**), 5-*O*-caffeoyl-4-*O*-syringoylquinic acid (**5**) (Song et al., 2010), 3,4-dicaffeoylquinic acid (**6**) (Ono et al., 2000), and 3,5-dicaffeoylquinic acid (**7**) (Wang and Liu, 2007) by comparison of their spectroscopic data with those reported in the literature.

Seven quinic acid derivatives were tested for their *in vitro* antiviral activities against RSV using a CPE reduction assay. The MTT method was applied to measure the cytotoxicity of the compounds. The selectivity index (SI) value calculated from the ratio of CC_{50}/IC_{50} was used as an important parameter to evaluate the antiviral activity of the active compounds. As shown in Table 3, among these quinic acid derivatives, the two dicaffeoylquinic acids (**6** and **7**) possessed strong anti-RSV activity with low IC_{50} values, and showed lower cytotoxicity to HEp-2 cells, resulting in much higher SI values than that of ribavirin. Based on our previous and present studies (Fan et al., 2013; Geng et al., 2011), the caffeoyl moiety may be important for antiviral activity. Moreover, dicaffeoylquinic acids show weaker activity than that of dicaffeoylquinic acid methyl esters, suggesting that the methoxy group at position C-7 provides an advantage in increasing antiviral activity. These results will be useful to further structural modifications of quinic acid derivatives.

Table 3
In vitro anti-RSV activity of compounds **1–7**.

Compounds[a]	RSV long strain		RSV A2 strain		CC_{50} (μg/mL)[c]
	IC_{50} (μg/mL)[b]	SI[d]	IC_{50} (μg/mL)	SI	
1	NA[f]	–	NA	–	>200.0
2	NA	–	NA	–	>200.0
3	NA	–	NA	–	>200.0
4	32.0	>6.3	32.0	>6.3	>200.0
5	NA	–	NA	–	>200.0
6	2.5	>80.0	3.0	>66.7	>200.0
7	2.5	>80.0	2.2	>90.9	>200.0
Ribavirin[e]	2.5	20.0	2.5	20.0	50.0 ± 2.3

"-" means no detection.
[a] The purity of all tested compounds is greater than 98%.
[b] IC_{50} is the concentration that reduced 50% of CPE in respect to virus control.
[c] CC_{50} is the concentration of sample with half maximal inhibition on the growth and survival of HEp-2 cells.
[d] SI is selective value = CC_{50}/IC_{50}.
[e] Ribavirin (purity ≥98%) is the positive control in the test.
[f] NA means no active.

3. Materials and methods

3.1. General experimental procedures

Optical rotations were recorded by using a JASCO P-1020 polarimeter. UV spectra were measured on a JASCO V-550 UV/vis spectrophotometer with a 1 cm length cell. IR spectra were recorded on a JASCO FT/IR-480 plus FT-IR spectrometer. NMR spectra were recorded on Bruker AV-400 and AV-300 spectrometers. HR–ESI–MS data were obtained on an Agilent 6210 ESI/TOF mass spectrometer. Macroporous resin Diaion HP-20 (Mitsubishi Chemical Corporation), MCI gel (75–150 μm; Mitsubishi), ODS (50 μm; YMC) and Sephadex LH-20 (Pharmacia) were used for column chromatography. TLC analyses were carried out using precoated silica gel GF_{254} plates (Yantai Chemical Industry Research Institute). Analytic high-performance liquid chromatography (HPLC) was performed on an Agilent chromatography equipped with a G1311C pump and a G1315D diode-array detector (DAD) with a Cosmosil $5C_{18}$-MS-II column (4.6 × 250 mm, 5 μm). Preparative HPLC was carried out on an Agilent instrument equipped with a G1310B pump and a G1365D detector with a Cosmosil $5C_{18}$-MS-II Waters column (20 × 250 mm, 5 μm).

3.1.1. Plant material

The roots and stems of *E. obtusifolia* were collected in Haikou city, Hainan province of PR China, in June of 2010 and authenticated by Prof. Guang-Xiong Zhou (Institute of Traditional Chinese Medicine & Natural Products, Jinan University). A voucher specimen (No. 100605) was deposited at the Institute of Traditional Chinese Medicine & Natural Products, Jinan University, Guangzhou, PR China.

3.1.2. Extraction and isolation

The air-dried roots and stems of *E. obtusifolia* (20.0 kg) were powdered and extracted with 95% (V/V) EtOH under reflux twice (2 × 200 L, 2 h each). The EtOH extract was concentrated under vacuum to afford a residue (963 g), which was suspended in H_2O and then partitioned successively with EtOAc and *n*-BuOH. After removing the solvent, the *n*-BuOH soluble fraction (273 g) was subjected to macroporous resin HP-20 column (15 × 60 cm) eluted with EtOH–H_2O mixtures (10:90; 30:70; 50:50; 70:30; 95:5, V/V, each 30 L). The 30% EtOH eluate (77 g) was subjected to an MCI gel column (7 × 60 cm) using gradient mixtures of MeOH–H_2O (0:100; 10:90; 15:85; 20:80; 25:75; 30:70; 40:60; 50:50; 100:0, each 15 L) as the eluent to afford twelve fractions (Fr. 1-Fr. 12). Fr. 2 (7 g) was separated by an ODS column (4 × 30 cm) with MeOH–H_2O (15:85; 20:80; 25:75; 30:70, each 4.5 L) as the eluent to give six subfractions (Fr. 2–1-Fr. 2–6). Fr. 2–3 (230 mg) was subsequently purified by preparative HPLC [CH_3CN–H_2O (0.1% TFA), 25:75, 6 mL/min] to yield compounds **1** (7 mg), **2** (17 mg), and **3** (11 mg). Fr. 2–2 (325 mg) was purified by preparative HPLC [CH_3CN–H_2O (0.1% TFA), 20:80, 6 mL/min] to obtain compounds **4** (15 mg) and **5** (12 mg). Fr. 1 (6 g) was then subjected to an ODS column (4 × 30 cm) eluted with gradient mixtures of MeOH–H_2O (5:95; 10:90; 15:85; 20:80; 25:75; 30:70, each 4.5 L) to afford six subfractions (Fr. 1–1-Fr. 1–6). Fr. 1–4 (563 mg) was purified by preparative HPLC [MeOH–H_2O (0.1% TFA), 45:55, 6 mL/min] to give compounds **6** (21 mg), **7** (30 mg), and **8** (7 mg).

3.1.3. 4-O-*feruloyl*-3-O-*syringoyl quinic acid methyl ester* (**1**)

Yellow oil; $[\alpha]_D^{25}$: - 68.3 (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ε): 216 (4.45), 288 (4.15), 327 (4.10) nm; IR (KBr): ν_{max} 3414, 1716, 1603, 1517, 1458, 1424, 1334, 1274, 1210, 1119 cm^{-1}; HR–ESI–MS *m/z*: 563.1759 $[M+H]^+$ (calcd for $C_{27}H_{31}O_{13}$, 563.1759); ^1H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 100 MHz) data, see Table 1.

3.1.4. 7-O-[6-O-(1-O-*vanillic acid* 4-O-β-D-*glucopyranosyl*)-β-D-*glucopyranosyl*]-6-*methoxy coumarin* (**2**)

Yellow oil; $[\alpha]_D^{25}$: - 51.0 (*c* 0.23, MeOH); UV (MeOH) λ_{max} (log ε): 204 (4.26), 218 (4.11), 255 (3.81), 291 (3.64), 342 (3.55) nm; IR (KBr): ν_{max} 3395, 1715, 1614, 1571, 1509, 1456, 1420, 1279, 1086, 1035 cm^{-1}; HR-ESI–MS *m/z*: 689.1687 $[M+Na]^+$ (calcd for $C_{30}H_{34}NaO_{17}$, 689.1688); ^1H NMR (DMSO-d_6, 400 MHz) and ^{13}C NMR (DMSO-d_6, 100 MHz) data, see Table 2.

3.2. Acid hydrolysis and HPLC analysis of compound **8**

Compound **8** (2 mg) was hydrolyzed with 2 mol/L HCl (5 mL) at 80 °C for 2 h. The solution was then evaporated under reduced pressure, after which 1 mL of pyridine and 2 mg of L-cysteine methyl ester hydrochloride were added to the residue, and the reaction mixture was heated at 60 °C for 1 h. *O*-tolyl isothiocyanate (5 μL) was added to the mixture and heated at 60 °C for an additional 1 h. The reaction mixture was then analyzed by HPLC and detected at 250 nm. Analytical HPLC was performed on a Cosmosil $5C_{18}$-MS-II column (4.6 × 250 mm, 5 μm) at 20 °C using CH_3CN-0.05% CH_3COOH in H_2O (25:75, 1.0 mL/min) as the mobile phase. D-Glucose (t_R 16.36 min) was identified as the sugar moiety of compound **8** based on comparison with authentic samples of D-glucose (t_R 16.36 min) and L-glucose (t_R 14.99 min) (Tanaka et al., 2007).

3.3. Antiviral assay

3.3.1. Cells and viruses

Human larynx epidermoid carcinoma (HEp-2, ATCC CCL-23) cells, as well as RSV A2 (ATCC-VR-1540) and Long (ATCC-VR-26) strains were purchased from the Medicinal Virology Institute, Wuhan University. HEp-2 cells were grown in Dulbecco Modified Eagle Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) and antimicrobials (growth medium, GM), then cultured at 37 °C in a humidified atmosphere supplied with 5% CO_2. For RSV propagation, RSV stock diluted using DMEM with 2% FBS and antimicrobials (maintenance medium, MM) was added to the confluent HEp-2 cells and continually cultured in the incubator until the maximal RSV syncytia formation. Virus titers were determined by the 50% tissue culture-infective dose ($TCID_{50}$) method. The virus stock was stored at −80 °C until use.

3.3.2. Cytotoxicity assay

The cytotoxicity of the compounds on HEp-2 cells was tested by MTT assay as previously described (Wang et al., 2012). Due to the quantitative limitation of the isolated compounds, the initial concentrations of tested samples were set as 200 μg/mL.

3.3.3. CPE reduction assay

A cytopathic effect (CPE) reduction assay was adopted to evaluate the antiviral activities of the compounds as previously described (Wang et al., 2012). Ribavirin (Sigma) was used as a positive control.

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.phytol.2015.10.002.

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