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Three new glycosides from the whole plant of *Clematis lasiandra* Maxim and their cytotoxicity

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ABSTRACT

Phytochemical investigation on the whole plant of *Clematis lasiandra* Maxim led to the isolation of two new phenolic glycosides (**1** and **2**), one new lignanoid glycoside (**3**), together with three known lignanoid glycosides (**4**–**6**). The structures of the new compounds were elucidated as 4-*O*-β-D-galactopyranosyl-ethyl-*E*-caffeate (**1**), 4-*O*-β-D-galactopyranosyl-3-hydroxyl-phenylethene (**2**) and (8*R*)-3,3′-dimethoxy-4,4′,9,9′-tetrahydroxy-5′,8-lignan 3′-*O*-β-D-glucopyranoside (**3**), on the basis of extensive spectral analysis and chemical evidence. The characteristic of the polymerized C-5′–C-8 type lignanoid aglycone in glycoside **3** was found from genus *Clematis* for the first time. Compounds **1**–**6** were evaluated for their cytotoxicity against human tumor cell lines HL-60, Hep-G2 and SGC-7901, the new glycosides **1** and **2** showed significant cytotoxicity against those three tumor cell lines with IC_{50} in the range from 9.73 to 22.31 μM, while lignanoid glycosides **3**–**6** showed weak cytotoxicity to those test cell lines with IC_{50} value more than 52.71 μM.

1. Introduction

The genus *Clematis* has been taxonomically placed in the Ranunculaceae, and comprises about 150 species in China (Wang and Li, 2005). Some species in this genus have long been used as folk medicine for their analgesic, diuretic, anticancer, anti-inflammatory and antimicrobial activities (Hao et al., 2013). The chemical constituents recorded in the literature from genus *Clematis* are saponins, flavonoids, coumarins, lignans, *etc.*, and most of them exist in the form of their glycosides (Sun and Yang, 2009). *Clematis lasiandra* Maxim is a perennial herbaceous plant distributed widely in the north and south slopes of Tsinling Mountains, Shaanxi province of China (Zhang et al., 2010). The entire plants and rhizome of this species have been used as folk medicines for a long history to treatment of dehygrosis, antitoxic, diuretic, analgesic and antipyretic, *etc.* (Cai, 2000). Our previous chemical investigations on this genus from *C. lasiandra* (Tian et al., 2013), *C. argentilucida* (Hai et al., 2012; Zhao et al., 2012) and *C. tangutica* (Zhang et al., 2013) have led to the isolation of several new and known cytotoxic or anti-myocardial ischemia triterpenoid saponins. In the course of our ongoing search for new bioactive natural products from *C. lasiandra*, two new phenolic glycosides (**1** and **2**), one new lignanoid glycoside (**3**), together with three known lignanoid glycosides (**4**–**6**) were isolated (Fig. 1). This paper deals with the experimental details of isolation and structural elucidation of these compounds and their cytotoxic assay results.

2. Results and discussion

Compound **1** was obtained as a pale yellow solid. The positive ion mode HR-ESI-MS showed a pseudomolecular ion peak at *m/z* 393.1164 $[M+Na]^+$ (calcd. for $C_{17}H_{22}O_9Na$, 393.1162), enabled the determination of the molecular formula as $C_{17}H_{22}O_9$, with the help of NMR spectral data. The ^1H NMR spectrum showed three aromatic protons at δ_H 7.07 (1H, dd, J = 8.4, 2.0 Hz, H-6), 7.12 (1H, d, J = 2.0 Hz, H-2) and 7.22 (1H, d, J = 8.4 Hz, H-5), indicating an ABX system for *orth*- and *inter*-trisubstituted benzene ring. Two olefinic protons at δ_H 7.59 (1H, d, J = 16.0 Hz, H-7) and 6.37 (1H, d, J = 16.0 Hz, H-8) can be determined to *trans* configuration due to the large coupling constant ($J_{7,8}$ = 16.0 Hz) between H-7 and H-8. The ^{13}C NMR spectrum gave seventeen carbon signals, except for six aromatic carbons and two olefin carbons, the remainder carbon signals can be described to one ester carbonyl δ_C 169.1 (C-9), one

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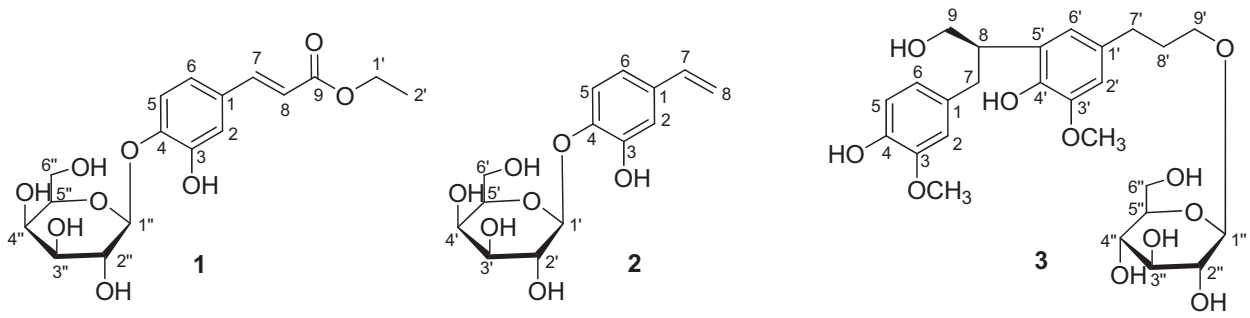

Fig. 1. Structures of new compounds **1**–**3** from *Clematis lasiandra*.

oxyethyl group δ_C 61.7 (C-1′) and 14.7 (C-2′), and one sugar unit (δ_C 101.7, 72.3, 73.0, 68.7, 76.2, 62.9). These ^{1}H and ^{13}C NMR signals were assigned with the aid of ^{1}H–^{1}H COSY, HSQC and HMBC spectra as shown in Table 1, and they suggested that compound **1** was a glycoside with caffeic acid as its aglycone. The position of the oxyethyl group was determined at C-9 since the observation of HMBC correlations from δ_H 1.34 (3H, t, J = 7.1 Hz, H_3-2′) to C-1′ (δ_C 61.7), and from δ_H 4.25 (2H, q, J = 7.1 Hz, H_2-1′) to C-9 (δ_C 169.1). The linkage of the sugar unit was deduced to be attached at C-4 (δ_C 148.8) due to HMBC correlation from anomeric proton δ_H 5.23 (1H, d, J = 8.0 Hz, Gal-1″) to C-4, and NOESY interaction between Gal H-1″ and H-5 (Fig. 2). The presence of the D-galactose in compound **1** was established by methanolysis followed by TLC analysis of the corresponding 1-*O*-methyl carbohydrate derivates (Tian et al., 2009). The D-configuration for Gal was determined by acidic hydrolysis after comparison of the GC retention times of the corresponding trimethylsilylated hydrolysate with those of the authentic samples prepared in the same manner (Ding et al., 2012; Gerwig et al., 1978), and the β-configuration for the sugar unit was deduced by the coupling constant (J = 8.1 Hz) of the anomeric proton signal and careful analysis of NOESY spectrum. Based on the above analysis, compound **1** was undoubtedly deduced as 4-*O*-β-D-galactopyranosyl-ethyl-*E*-caffeate.

Compound **2** was obtained as a pale yellow solid. The molecular formula of **2** was deduced as $C_{14}H_{18}O_7$ according to the positive HR-ESI-MS from the pseudomolecular ion peak at m/z 321.0946 $[M+Na]^+$ (calcd. for $C_{14}H_{18}O_7Na$, 321.0950). Detailed comparison of the ^{1}H and ^{13}C NMR data of compound **2** with **1** showed that the signals for the sugar moiety of the two compounds were in good agreement, except for the absence of a ester carbonyl and oxyethyl group in the aglycon in compound **2**. The ^{1}H NMR spectrum of **2** showed one vinyl methenyl proton at δ_H 6.63 (1H, d, J = 17.6 Hz, H-7), and two vinylic methine protons at δ_H 5.13 (1H, dd, J = 10.9, 17.6 Hz, H_a-8) and 5.63 (1H, dd, J = 10.9, 17.6 Hz, H_b-8), corresponding to two olefinic carbons at δ_C 137.9 (C-7) and 112.8 (C-8), with the help of HSQC spectrum. The position of the terminal vinyl group was assigned at C-1 (δ_C 134.9) due to HMBC correlations from H-7 (δ_H 6.63) to C-1 (δ_C 134.9), C-2 (δ_C 114.4) and C-6 (δ_C 119.6), and from H_2-8 (δ_H 5.13 and 5.63) to C-1 (δ_C 134.9). Methanolysis of **2** revealed the presence of D-galactose by comparison with an authentic sample. The D-configuration of the sugar unit was identified by the same method of **1**. The β-configuration of D-galactose in **2** was deduced on the coupling constant (J = 8.1 Hz) of the anomeric proton, and the linkage of the D-galactose at C-4 was also confirmed by the observation of the correlation between anomeric proton signal of Gal H-1′ δ_H 5.15 (1H, d, J = 8.1 Hz) to C-4 (δ_C 146.9) (Fig. 2). Hence, the structure of compound **2** was confirmed as 4-*O*-β-D-galactopyranosyl-3-hydroxyl-phenylethene.

Compound **3** was obtained as a white amorphous powder. The molecular formula was established as $C_{26}H_{36}O_{11}$ on the basis of its positive HR-ESI-MS m/z 547.2159 $[M+Na]^+$ (calcd. for $C_{26}H_{36}O_{11}Na$, 547.2155). The ^{1}H NMR spectrum of **3** showed one 1,3,4-trisubstituted benzene ring signals at δ_H 6.58 (1H, brs, H-2), 6.61 (1H, d, J = 8.0 Hz, H-5) and 6.53 (1H, d, J = 8.0 Hz, H-6), one 1′,3′,4′,5′-tetrasubstituted benzene ring signals at 6.68 (1H, brs, H-2′) and 6.50 (1H, brs, H-6′), two methoxyl proton signals at δ_H 3.70 (3H, s, 3-OCH_3) and 3.84 (3H, s, 3′-OCH_3), and a glucose (Glc)

Table 1
^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data of compounds **1** and **2** in CD_3OD.[a]

1			2		
Position	δ_C mult.	δ_H mult. (J in Hz)	Position	δ_C mult.	δ_H mult. (J in Hz)
1	131.2 s		1	134.9 s	
2	116.1 d	7.12 d (2.0)	2	114.4 d	6.96 d (2.0)
3	149.2 s		3	148.6 s	
4	148.8 s		4	146.9 s	
5	118.3 d	7.22 d (8.4)	5	118.6 d	7.15 d (8.3)
6	122.3 d	7.07 dd (8.4, 2.0)	6	119.6 d	6.87 dd (8.3, 2.0)
7	146.1 d	7.59 d (16.0)	7	137.9 d	6.63 d (17.6)
8	117.6 d	6.37 d (16.0)	8	112.8 t	a 5.13 dd (10.9, 17.6) b 5.63 dd (10.9, 17.6)
9	169.1 s				
1′	61.7 t	4.25 q (7.1)			
2′	14.7 q	1.34 t (7.1)			
Gal-1″	101.7 d	5.23 d (8.0)	Gal-1′	102.2 d	5.15 d (8.1)
2″	72.3 d	3.67 dd (3.0, 8.0)	2′	72.3 d	3.66 m
3″	73.0 d	4.19 m	3′	73.0 d	4.19 brt (2.8)
4″	68.7 d	3.63 dd (2.9, 9.6)	4′	68.7 d	3.63 dd (2.8, 9.2)
5″	76.2 d	3.87 m	5′	76.1 d	3.88 m
6″	62.9 t	a 3.72 dd (5.2, 11.7) b 3.89 m	6′	62.9 t	a 3.73 dd (5.3, 11.8) b 3.91 m

[a] Assignments aided by HSQC, ^{1}H–^{1}H COSY, HMBC and NOESY experiments.

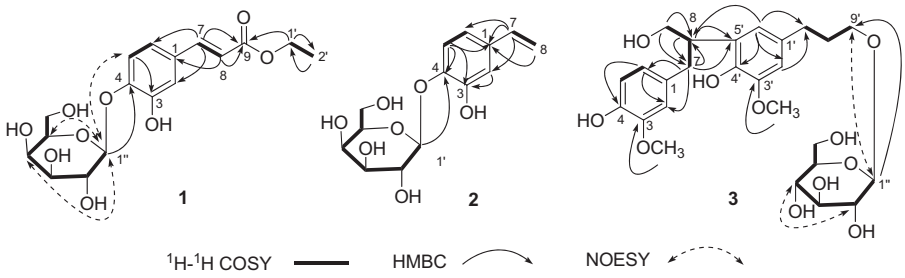

Fig. 2. Key ^{1}H–^{1}H COSY, HMBC and NOESY correlations of compounds **1**–**3**.

anomeric H-atom signal at δ_H 4.23 (1H, d, J = 7.8 Hz, Glc H-1″). In the ^{13}C NMR spectrum, twenty-six carbon signals can be observed. Apart from six signals due to a glucopyranosyl unit (δ_C 104.6, 75.3, 78.3, 71.8, 78.0 and 62.9) and two methoxyl carbon signals (δ_C 56.4 and 56.7), the other eighteen carbons could be ascribed to a lignanoid skeleton for compound **3**. Except twelve carbon signals belonging to two benzenes, the remaining six carbon resonances in the up-field can be classified as five methylenes δ_C 37.6 (C-7), 66.0 (C-9), 33.0 (C-7′), 32.9 (C-8′) and 70.0 (C-9′), and one methine δ_C 45.8 (C-8) by DEPT 135 experiment (Table 2). The ^{13}C NMR data of **3** were similar to those of known compound **5**, (7*S*,8*R*)-3,3′,5-trimethoxy-4,9,9′-trihydroxy-4′,7-epoxy-5′,8-lignan 4-*O*-β-D-glucopyranoside, except for significant downfield shifts of C-4′ (δ_C 143.8, −3.8 ppm), C-5′ (δ_C 133.8, −3.3 ppm), C-7 (δ_C 37.6, −51.4 ppm) and C-8 (δ_C 45.8, −9.6 ppm). These findings suggested that the furan ring in **5** was cleavaged between oxygen atom and C-7. The two phenylpropanoid systems were confirmed to be polymerized between C-5′ and C-8 on the basis of key HMBC correlations from H-8 (δ_H 3.41) to C-7 (δ_C 37.6) and C-5′ (δ_C 133.8), and from H_2-7 (δ_H 2.91 and 3.00) to C-2 (δ_C 114.1), C-6 (δ_C 122.8) and C-5′ (δ_C 133.8). Methanolysis of **3** yielded methyl-β-D-glucose, which was identified on the basis of R_f value by TLC analysis, and the D-configuration for Glc was confirmed by the retention time by GC analysis with the same method of **1**. The β-configuration of D-glucose in **3** was determined on the coupling constant (J = 7.8 Hz) of the anomeric proton, and the linkage of the glucose unit attached at C-9′ was confirmed by the observation of the correlation between anomeric proton signal of Glc H-1″ (δ_H 4.23, d, J = 7.8 Hz) to C-9′ (δ_C 70.0) in the HMBC experiment, and NOESY correlation from Glc H-1″ (δ_H 4.23, d, J = 7.8 Hz) to H_2-9′ (δ_H 3.44 and 3.89) (Fig. 2). Thus, the planar structure of **3** was elucidated as 3,3′-dimethoxy-4,4′,9,9′-tetrahydroxy-5′,8-lignan 3′-*O*-β-D-glucopyranoside.

The stereochemistry of C-8 in the aglycone of **3** was determined as *R* configuration based on the ^{13}C NMR chemical shift of C-8 (δ_C 45.8), which was consistent with those reported for natural product, (8*R*)-isodehydrodiconiferyl alcohol-4′-*O*-(6″-vanilloyl)-β-D-glucopyranoside (δ_C 43.4) (Lee et al., 2009), while was different from its analog, 3-methoxy-4-hydroxy-5-[(8′*S*)-3′-methoxy-4′-hydroxyphenylpropyl alcohol]-*E*-cinnamic alcohol-4-*O*-β-D-glucopyranoside (δ_C 40.5) (Tan et al., 2004). Furthermore, the specific rotation ($[\alpha]^{25}_D$ = −21.7°) of its aglycon obtained by methanolysis of **3**, was in good accordance with (8*R*)-isodehydrodiconiferyl alcohol ($[\alpha]^{25}_D$ = −18.0°) (Iorizzi et al., 2001; Lee et al., 2009), while was different from 3-methoxy-4-hydroxy-5-[(8′*S*)-3′-methoxy-4′-hydroxyphenylpropyl alcohol]-*E*-cinnamic alcohol ($[\alpha]^{25}_D$ = +20.0°) (Tan et al., 2004). This further confirmed 8*R* configuration for compound **3**. Consequently, the structure of **3** was determined to be (8*R*)-3,3′-dimethoxy-4,4′,9,9′-tetrahydroxy-5′,8-lignan 3′-*O*-β-D-glucopyranoside. The new glycoside **3** structurally characterized with a polymerized C-5′–C-8 form lignanoid aglycone, which was isolated for the first time from genus *Clematis*.

The three known lignanoid glycosides **4**–**6** were identified as (7*S*,8*R*)-3,3′,5-trimethoxy-4,9,9′-trihydroxy-4′,7-epoxy-5′,8-lignan 4-*O*-β-D-glucopyranoside (**4**) (Kuang et al., 2009), (7*S*,8*R*)-3,3′-dimethoxy-4,9,9′-trihydroxy-4′,7-epoxy-5′,8-lignan 9′-*O*-β-D-glucopyranoside (**5**) (Kuang et al., 2009) and (7*S*,8*R*)-3,3′-dimethoxy-4, 9,9′-trihydroxy-4′,7-epoxy-5′,8-lignan 9-*O*-β-D-glucopyranoside (**6**) (Abe and Yamauchi, 1986; Kuang et al., 2009), respectively, by comparing their spectroscopic data and chemical evidences with those published in the literature. All of them were isolated for the first time from the whole plants of *C. lasiandra*.

The cytotoxicities of the isolated glycosides **1**–**6** against human hepatocellular carcinoma Hep-G2, human leukemia HL-60 and human gastric carcinoma SGC-7901 cells were evaluated by MTT assay (Mosmann, 1983). The results of the cytotoxicities were shown in Table 3. Two new phenolic glycosides (**1** and **2**) characterized with a β-D-galactose at C-4 in the aglycone exhibited significant cytotoxicity against those three tumor cell lines with IC_{50} in the range from 9.73 to 22.31 μM, while lignanoid glycosides

Table 2
^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) data of compound **3** in CD_3OD.[a]

Position	δ_C mult.	δ_H mult. (J in Hz)	Position	δ_C mult.	δ_H mult. (J in Hz)
1	133.9 s		5′	133.8 s	
2	114.1 d	6.58 brs	6′	122.4 d	6.50 brs
3	148.5 s		7′	33.0 t	1.83 m
4	148.8 s		8′	32.9 t	2.60 m
5	115.8 d	6.61 d (8.0)	9′	70.0 t	3.44 m, 3.89 m
6	122.8 d	6.53 d (8.0)	3-OCH_3	56.4 q	3.70 s
7	37.6 t	2.91 dd (5.7, 13.7)	3′-OCH_3	56.7 q	3.84 s
		3.00 dd (5.7, 13.7)	Glc-1″	104.6 d	4.23 d (7.8)
8	45.8 d	3.41 m	2″	75.3 d	3.23 t (8.6)
9	66.0 d	3.77 m	3″	78.3 d	3.38 m
1′	129.5 s		4″	71.8 d	3.31 m
2′	110.9 d	6.68 brs	5″	78.0 d	3.29 m
3′	145.4 s		6″	62.9 t	3.69 m, 3.87 m
4′	143.8 s				

[a] Assignments aided by DEPT, HSQC, ^{1}H–^{1}H COSY, HMBC and NOESY experiments.

Table 3
Cytotoxicities of compounds **1**–**6** against three cancer cell lines *in vitro* (IC_{50}, μM).[a]

Cell line	1	2	3	4	5	6	Adriamycin[b]
HL-60	20.56 ± 0.32	22.31 ± 0.54	>100	87.32 ± 0.74	90.58 ± 0.82	87.46 ± 0.65	0.31 ± 0.17
Hep-G2	9.73 ± 0.47	12.24 ± 1.21	93.52 ± 0.13	52.71 ± 0.45	62.31 ± 1.45	56.12 ± 0.73	0.45 ± 0.22
SGC-7901	15.12 ± 0.39	18.72 ± 0.33	>100	68.27 ± 0.55	78.92 ± 0.95	81.47 ± 0.43	0.35 ± 0.13

[a] IC_{50} values are means from three independent experiments in which each compound concentration was tested in three replicate wells.
[b] Adriamycin as positive control.

3–**6** possessed a β-D-glucose in the aglycone showed relative weak cytotoxicity to those test cell lines with IC_{50} value more than 52.71 μM. Compared to structural features and cytotoxicities of the new lignanoid glycoside **3** with the known ones **4**–**6**, the normal 4′,7-epoxy furan ring in the lignanoid aglycone was collapsed between oxygen atom and C-7 in the new glycoside **3**, and its cytotoxicities against the test tumor cell lines were rather lower than the known glycosides **4**–**6**. It indicated that the cleavage of the 4′,7-epoxy furan ring between oxygen atom and C-7 can decrease their cytotoxicities. In fact, lignanoid glycosides often showed marginal cytotoxicities (Calis et al., 2005; Kuang et al., 2009). The results for the weak cytotoxicities of lignanoid glycosides (**4**–**6**) confirmed that these secondary metabolites may be recognized as non-toxic agent.

3. Experimental

3.1. General

Melting points were determined on an XT5-XMT apparatus and uncorrected. Specific rotations were measured on a Perkin-Elmer 343 polarimeter. The ESI-MS and HR-ESI-MS spectra were obtained on a Micromass Quattro mass spectrometer. 1D and 2D NMR spectral experiments were measured in CD_3OD on Bruker AVANCE-500 NMR spectrometer with tetramethylsilane (TMS) as an internal standard. GC analysis was performed on a Finnigan Voyager apparatus using an l-Chirasil-Val column (0.32 mm × 25 m) with an initial temperature of 180 °C at the rate of 5 °C/min. Separations and purifications were performed by column chromatography (CC) on silica gel H (10–40 μm, Qingdao Marine Chemical Inc., Qingdao, China), reversed-phase silica gel (Lichroprep RP-18, 40–63 μm, Merck Inc., Darmstadt, Germany) and Sephadex LH-20 (GE Inc., USA). HPLC was carried out on a Shimadzu LC-10ATVP liquid chromatograph equipped with a SPD-10ADVP (UV–Vis) detector at 206 nm using a YMC-Pack R&D ODS-A column (250 mm × 10 mm i.d.) for semi-preparation. TLC detection was achieved by spraying the silica gel plates (Qingdao Marine Chemical Inc., Qingdao, China) with 20% H_2SO_4 in EtOH followed by heating.

3.2. Plant material

The whole plants of *Clematis lasiandra* Maxim were collected in the north of Tsinling Mountain, Shaanxi Province of China in September 2009, and were identified by Prof. Ji-Tao Wang from Shaanxi University of Chinese Medicine. A voucher specimen (No. 20090904) was deposited in the Department of Pharmacy, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi, PR China.

3.3. Extraction and isolation

The air-dried whole plants of *C. lasiandra* (7.2 kg) were crushed and then extracted with 70% EtOH (3 times × 56 L, 6 h/time) under reflux at room temperature. The concentrated EtOH extract (1.4 kg) was suspended in H_2O and then partitioned successively with petroleum ether (3 times × 6 L), EtOAc (3 times × 6 L) and *n*-BuOH (5 times × 6 L), respectively. The *n*-BuOH extract (180 g) was separated into 24 fractions (Fr. A–Y) on a silica gel column using a step gradient elution of $CHCl_3$–MeOH–H_2O (30:1:0, 20:1:0, 15:1:0, 10:1:0.1, 9:1:0.1, 8.5:1.5:0.15, 8:2:0.2, 7.5:2.5:0.25, 7:3:0.3 and 6.5:3.5:0.35) according to their TLC profiles. Fractions G and J were selected to our principal study objectives as their different TLC profiles compared with triterpenoid saponins, which we previously obtained from *C. lasiandra*, *C. argentilucida* and *C. tangutica*. Fr. G (1.2 g) was eluted with $CHCl_3$–MeOH (1:1) on Sephadex LH-20 to give three subfractions (Fr. G_1–Fr. G_3). Fr. G_2 (167.3 mg) was further purified by semi-preparative HPLC using MeOH–H_2O (34:66) as the mobile phase at a flow rate of 2.0 mL/min to afford compounds **1** (7.2 mg, t_R = 59.5 min) and **2** (6.8 mg, t_R = 28.9 min). Fr. J (4.7 g) was subjected to CC over reversed-phase silica gel eluting with MeOH–H_2O (10:90, 40:60, 70:30, 100:0) to give four subfractions (Fr. J_1–Fr. J_4). Fr. J_2 (0.56 g) was eluted with $CHCl_3$–MeOH (1:1) on Sephadex LH-20 to remove pigments, and then was further purified by semi-preparative HPLC using MeOH–H_2O (32:68) as the mobile phase at a flow rate of 2.0 mL/min to afford compounds **3** (23.8 mg, t_R = 56.7 min), **4** (7.6 mg, t_R = 49.3 min), **5** (110.5 mg, t_R = 59.6 min) and **6** (26.3 mg, t_R = 71.5 min).

3.3.1. 4-O-β-D-galactopyranosyl-ethyl-E-caffeate (**1**)

Pale yellow solid (CH_3OH), mp 206–207 °C, $[\alpha]^{23}_D$ +49.6° (*c* 0.10, MeOH); ^1H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD) data, see Table 1; Positive ESI-MS *m/z* 763 $[2M+Na]^+$, 393 $[M+Na]^+$; positive HR-ESI-MS *m/z* 393.1164 $[M+Na]^+$ (calcd. for $C_{17}H_{22}O_9Na$, 393.1162).

3.3.2. 4-O-β-D-galactopyranosyl-3-hydroxyl-phenylethene (**2**)

Pale yellow solid (CH_3OH), mp 181–182 °C, $[\alpha]^{25}_D$ +47.8° (*c* 0.10, MeOH); ^1H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD) data, see Table 1; Positive HR-ESI-MS *m/z* 321.0946 $[M+Na]^+$ (calcd. for $C_{14}H_{18}O_7Na$, 321.0950).

3.3.3. (8R)-3,3′-dimethoxy-4,4′,9,9′-tetrahydroxy-5′,8-lignan 3′-O-β-D-glucopyranoside (**3**)

White amphous powder (CH_3COCH_3), mp 242–243 °C, $[\alpha]^{25}_D$ −89.1° (*c* 0.35, MeOH); ^1H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD) data, see Table 2; positive ESI-MS *m/z* 1071 $[2M+Na]^+$, 547 $[M+Na]^+$; positive HR-ESI-MS *m/z* 547.2159 $[M+Na]^+$ (calcd. for $C_{26}H_{36}O_{11}Na$, 547.2155).

3.4. Methanolysis of glycosides **1**–**3**

Each glycoside (3 mg) was dissolved in 5% HCl–MeOH (3 mL) and refluxed for 12 h (80 °C). The reaction mixture was evaporated under reduced pressure to remove residual HCl. The resulting residue was partitioned between H_2O and benzene. The benzene layer of glycoside **3** was concentrated *in vacuo* to yield major (8*R*)-3,3′-dimethoxy-4,4′,9,9′-tetrahydroxy-5′,8-lignan with the optical rotation of $[\alpha]^{25}_D$ −21.7°. The H_2O layer of glycosides **1** and **2** were concentrated to afford the major methyl-β-D-galactosylpyranoside with R_f value of 0.54, while glycoside **3** give methyl-β-D-glucopyranoside with R_f value of 0.63, which were subjected to

co-TLC analysis with authentic sugars by developed with *n*-BuOH–Pyridine–H_2O (6:4:3) and detected with aniline phthalate spray (Ding et al., 2012; Tian et al., 2009).

3.5. Acid hydrolysis of glycosides **1**–**3**

Each glycoside (1.5 mg) was heated with 2 mol/L CF_3COOH (1.5 mL) at 120 °C for 2 h. The reaction mixture was partitioned with $CHCl_3$–H_2O (1:1). The aqueous phase was concentrated and dissolved in 1-(trimethylsilyl)-imidazole and pyridine (0.1 mL). Then, the solution was stirred at 60 °C for 5 min and dried with a stream of N_2. The organic layer was analyzed by GC using an l-Chirasil-Val column. Retention times for authentic sugars after being derivatized were 14.54 min (D-Glc), 14.49 min (L-Glc), 16.05 min (D-Gal) and 16.11 min (L-Gal), respectively. The results of the GC analysis showed that D-Gal was identified for glycosides **1** and **2**, while D-Glc was identified for glycoside **3**.

3.6. Cytotoxic assay for glycosides **1**–**6**

The 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) colorimetric assay was used for *in vitro* evaluation of the cytotoxic potential of the isolated glycosides **1**–**6** against three cultured human tumor cell lines. The tumor cell lines of Hep-G2, HL-60 and SGC-7901 were obtained from American Type Culture Collection (ATCC), and were seeded to RPMI-1640 medium with 10% fetal bovine serum and 100 U/mL benzyl penicillin-streptomycin solutions at 37 °C in a humidified atmosphere containing in 5% CO_2/air for 24 h. Then the test glycosides **1**–**6** were added and incubated at 37 °C in another 72 h, respectively. The detailed experimental procedures have been described in our previous published literature (Tian et al., 2013). The inhibition was expressed as IC_{50} value using adriamycin as positive control.

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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.phytol.2014.09.004.

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