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New acylated triterpene saponins from the roots of *Securidaca inappendiculata* Hassk

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ABSTRACT

Two new acylated triterpene saponins, named as securioside **C** (**1**), securioside **D** (**2**), and one pair of isomers **3/4**, the (*Z*)–isomer securioside **E** (**3**) being new, together with a known triterpene saponin polygalasaponin XLIV (**4**) were isolated from the roots of *Securidaca inappendiculata* Hassk. Their structures were established by HRESIMS, 1D and 2D NMR experiments and comparison of their NMR data with previous reported data. In addition, Compounds **1–2**,**3/4**, **4** were evaluated for cytotoxicities against LLC (Lewis lung carcinoma) and MCF-7 (human breast cancer) cell lines. Compounds **1** and **2** exhibited moderate cytotoxic activities against LLC cells with IC_{50} values of 45.56 μM and 85.98 μM.

1. Introduction

Securidaca inappendiculata Hassk is a traditional Chinese herbal medicine, belonging to the Polygalaceae family, mainly distributed in Yunnan, Guangxi, Guangdong, and Hainan provinces of southern China and the tropical regions of Asia (Delecti Florae Reipublicae Popularis Sinicae Agendae Academiae Sinicae, 1997). The roots of *S. inappendiculata* are used as an anti-inflammatory, antibacterial, and antirheumatism agent in China (Jiangsu Institute of Botany, 1988). Our previous investigations on this species resulted in the isolation of benzophenones (Yang et al., 2003b) from the chloroform fraction, xanthones (Yang et al., 2001a) from the chloroform/ethyl acetate fractions, xanthone glycosides (Yang et al., 2002a) and organic acids (Yang et al., 2001b) from the ethyl acetate/acetone fractions, and oligosaccharide esters (Yang et al., 2003a) and terpenoic glycosides (Yang et al., 2002b) from the acetone fraction of ethanol extract. Two saponins (Kuroda et al., 2001) obtained from the water extract of the roots of the plant have been reported. Modern pharmacological evaluations revealed that the saponins from the roots of *S. inappendiculata* exhibited macrophage-oriented cytotoxic activity. In our preliminary experiment, the aqueous portion of ethanol extract from the roots of *S. inappendiculata* showed cytotoxic activities in vitro against LLC (Lewis lung carcinoma) and MCF-7 (human breast cancer) cell lines. Therefore, as part of our ongoing investigation of anti-tumor constituents, we reported the isolation and the structural elucidation of two new triterpene saponins, one pair of (*E*)/(*Z*) – isomers (the (*Z*) – isomer being new), and a known triterpene saponin with presenegenin as aglycon (see Fig. 1) isolated from the roots of *S. inappendiculata*. Cytotoxicities of compounds **1–2**, **3/4**, **4** were evaluated against LLC and MCF-7 cell lines, respectively.

2. Results and discussion

Compound **1**, obtained as a white amorphous powder, was assigned a molecular formula of $C_{65}H_{96}O_{28}$ which was deduced from quasi-molecular ion peak ($[M+Na]^+$) at *m*/*z* 1347.6020 in the positive ion mode HRESIMS. The ^1H NMR spectrum of **1** showed signals for five tertiary methyl groups at δ_H 0.76, 0.88, 1.11, 1.48, 1.90, which correlated with five sp^3 C-atoms at δ_C 34.0, 24.3, 19.0, 17.8, 14.6 respectively based on the HSQC spectrum, also an isolated oxymethylene at δ_H 3.78, 4.04 (H_2-27), an oxymethine proton at δ_H 4.68 (H-2), an olefinic proton at δ_H 5.78 (H-12) combined with two sp^2 olefinic carbons at δ_C 128.2 (C-12), 139.2 (C-13), and two carboxylic carbon signals at δ_C 181.0 (C-23), 177.0 (C-28) in the ^{13}C NMR spectrum. These data proved that the aglycone possessed an olean-12-ene-23,28-dioic acid skeleton (Tables 1 and 2), and was in full agreement with those of tenuifolin (3-*O*-β-D-glucopyranosyl presenegenin) in which presenegenin ((2β,3β,4α)-2,3,27-trihydroxyolean-12-ene-23,28-dioic acid)

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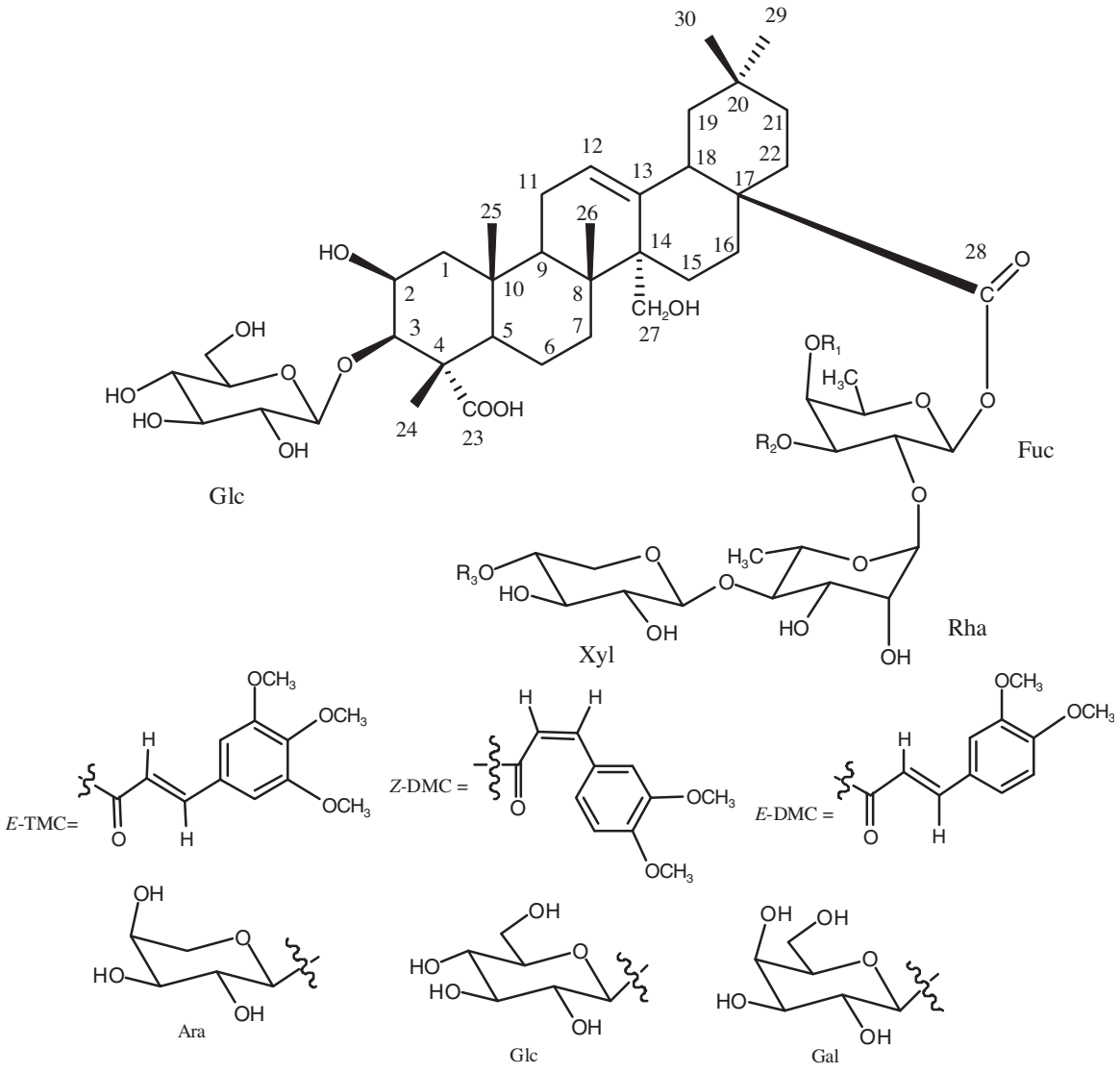

	R_1	R_2	R_3
1	*E*-TMC	H	H
2	*E*-DMC	Ara	H
3	*Z*-DMC	Glc	Gal

Fig. 1. Structures of compounds **1–3**.

was commonly encountered in triterpene saponins isolated from the family Polygalaceae (Mitaine-Offer et al., 2002; Teng et al., 2002; Zhang et al., 1996). The chemical shift values at δ_C 86.3 (C-3) and δ_C 177 (C-28) suggested that the saponin was a bisdesmosidic glycoside with saccharide units attached to these positions. Beside these, the ^{1}H NMR spectrum was also displayed signals of four anomeric protons at δ_H 6.35 (1H, s), 6.17 (1H, d, J = 8.15 Hz), 5.03 (1H, d, J = 5.8 Hz), and 5.02 (1H, s), which gave correlations with four anomeric carbon signals at δ_C 102.0, 94.9, 107.7, and 105.7 in the HSQC spectrum respectively. The COSY, TOCSY, HSQC, and HMBC spectroscopic experiments identified the direct correlations of protons of sugar ring from the readily identifiable anomeric protons (Tables 1 and 2). The coupling constant confirmed the presence of the units of one β-glucopyranosyl, one β-fucopyranosyl, one β-xylopyranosyl, and one α-rhamnopyranosyl. After acid hydrolysis, the sugar units were confirmed to be D-glucose, D-fucose, L-rhamnose, and D-xylose by HPLC analysis of their thiocarbamoyl-thiazolidine derivatives compared with authentic samples.

The HMBC correlation between δ_H 5.02 (1H, s, Glc-1) and δ_C 86.3 (Agly-C-3) and NOE correlation between δ_H 5.02 (Glc-1) and δ_H 4.54 (Agly-H-3) in the NOESY spectrum revealed a linkage between the aglycon and a glucopyranosyl moiety. A correlation in the HMBC spectrum between δ_H 6.17 (1H, d, J = 8.15 Hz) (Fuc-1) and δ_C 177 (Agly-C-28) indicated that a fucose was attached to C-28 of the aglycon. Moreover, in the HMBC spectrum, long-range correlations were observed between the following protons and carbons: δ_H 4.70 (Fuc-2) and δ_C 102.0 (Rha-1), δ_H 4.35 (Rha-4) and δ_C 107.7 (Xyl-1),

Table 1
^{1}H NMR spectral data (500 Hz, pyridine-d_5) for compounds **1**–**3** (δ ppm).

	1	2	3		1	2	3
Aglycone-1	1.32, 2.24	1.31, 2.24	1.33, 2.24	Xyl-1	5.03(d, 5.8)	5.02(d, 6.5)	4.96(d, 7.0)
2	4.68	4.68	4.72	2	4.03	4.04	3.98
3	4.54	4.57	4.58	3	4.02	4.02	4.00
5	2.16	2.17	2.17	4	4.15	4.02	4.27
6	1.75,1.91	1.75,1.91	1.76,1.93	5	3.78, 4.18	3.46, 4.33	3.44, 4.28
7	1.15,1.20	1.11,1.22	1.14,1.25	Gal-1			4.94(d, 7.5)
9	2.30	2.32	2.33	2			4.44
11	1.92, 2.11	1.92, 2.07	1.92, nd	3			3.89
12	5.78	5.80	5.82	4			4.47
15	1.63, 2.10	1.63, 2.11	1.62, 2.07	5			4.13
16	1.62, 2.04	1.62, 2.03	1.65, 2.15	6			4.33
18	3.18 (d,13.5)	3.19 (d,13.5)	3.21 (d,13.5)	Glc-1			5.10(d, 7.2)
19	1.26, 1.70	1.27, 1.71	1.30, 1.75	2			3.95
21	1.17	1.21	1.17	3			4.14
22	nd, 2.08	nd, 2.08	nd, 2.01	4			4.02
24	1.90(s)	1.94(s)	1.93(s)	5			3.90
25	1.48(s)	1.53(s)	1.53(s)	6			4.39
26	1.11(s)	1.11(s)	1.10(s)	Ara-1		4.97(d, 6.9)	
27	3.78, 4.04	3.78, 4.04	3.78, 4.04	2		4.40	
29	0.76(s)	0.76(s)	0.76(s)	3		4.04	
30	0.88(s)	0.89(s)	0.88(s)	4		4.26	
3-Glc-1	5.02 (s)	5.02(d, 6.5)	5.04(d, 7.5)	5		3.46, 4.19	
2	3.93	3.90	3.92	MC			
3	4.14	4.14	4.15	H-C (2)	6.84(s)	7.04(s)	7.92(br s)
4	4.13	4.13	3.89	H-C (5)		6.94(d, 8.5)	6.93(d, 8.4)
5	3.91	3.90	4.26	H-C (6)	6.84(s)	7.05(d, 8.5)	7.50(d, 8.4)
6	4.29	4.24	4.29, 4.43	H-C (β)	6.61 (d,16.0)	6.49(d, 16.0)	5.91(d, 12.6)
28-Fuc-1	6.17(d, 8.15)	6.13(d, 8.3)	6.13(d, 7.7)	H-C (γ)	7.95 (d,16.0)	7.85(d, 16.0)	6.88(d, 12.6)
2	4.70	4.73	4.77	MeO-C(3)	3.81(s)	3.81(s)	3.68(s)
3	4.48	4.51	4.47	MeO-C(4)	3.90(s)	3.83(s)	3.86(s)
4	5.75	5.95	5.97	MeO-C(5)	3.81(s)		
5	4.13	4.11	4.13				
6	1.34(d, 6.0)	1.30(d, 6.0)	1.26(d, 6.0)				
Rha-1	6.35(s)	6.28(s)	6.36(br s)				
2	4.79	4.81	4.82(br s)				
3	4.68	4.64	4.63				
4	4.35	4.28	4.24				
5	4.55	4.44	4.45				
6	1.79(d, 5.5)	1.74(d, 5.5)	1.71(d, 5.5)				

Table 2
^{13}C NMR spectral data (125 Hz, pyridine-d_5) for compounds **1**–**3** (δ ppm).

	1	2	3		1	2	3
Aglycone-1	44.5	44.3	44.0	Rha-1	102.0	101.7	101.5
2	70.6	70.1	70.3	2	72.1	71.9	71.8
3	86.3	86.0	85.8	3	72.8	72.3	72.9
4	53.2	52.9	52.6	4	85.3	84.4	84.5
5	52.8	52.5	52.2	5	68.8	68.7	68.4
6	21.7	21.4	21.4	6	18.8	18.7	18.7
7	33.9	33.5	33.7	Xyl-1	107.7	107.1	106.8
8	41.5	41.1	41.0	2	76.5	74.2	75.6
9	49.6	49.3	49.1	3	79.1	76.1	76.5
10	37.3	37.0	36.9	4	71.2	78.6	78.2
11	23.9	23.5	23.4	5	67.8	67.0	64.8
12	128.2	127.9	127.6	Gal-1			104.3
13	139.2	139.1	139.0	2			71.4
14	48.3	48.0	47.9	3			75.0
15	24.1	24.5	24.4	4			70.1
16	24.2	24.0	23.8	5			77.0
17	47.3	47.0	46.9	6			62.1
18	42.3	42.0	42.0	Glc-1			105.6
19	45.6	45.2	45.2	2			74.9
20	31.1	30.8	30.6	3			78.2
21	33.6	33.7	33.6	4			71.2
22	32.1	32.3	32.1	5			78.2
23	181.0	181.2	181.0	6			62.7
24	14.6	14.2	14.2	Ara-1		106.5	
25	17.8	17.5	17.3	2		72.4	
26	19.0	18.9	18.7	3		74.6	
27	64.8	64.2	64.4	4		69.1	
28	177	176.9	176.8	5		67.2	
29	34.0	32.8	33.0	MC			
30	24.3	24.0	23.8	C(1)	130.7	128.0	127.5
3-*O*-Glc-1	105.7	105.3	105.2	C(2)	105.8	111.3	115.0
2	75.6	75.1	74.8	C(3)	154.4	149.5	151.4
3	78.7	78.3	78.0	C(4)	141.5	152.4	152.0
4	71.9	71.5	71.1	C(5)	154.4	112.1	111.6
5	78.7	78.3	77.9	C(6)	106.7	123.4	125.8
6	63.0	62.4	62.6	C(α)	167.8	167.4	166.6
28-*O*-Fuc-1	94.9	94.1	94.7	C(β)	118.1	116.1	116.6
2	74.6	73.8	73.2	C(γ)	146.2	146.2	145.1
3	74.6	81.6	82.7	MeO-C(3)	56.7	56.1	55.5
4	75.3	74.4	74.2	MeO-C(4)	61.0	56.4	55.9
5	71.2	71.1	70.9	MeO-C(5)	56.7		
6	17.0	16.1	16.5				

which revealed the (1 → 2) linkage between rhamnose and fucose, and a xylopyranosyl unit was linked to rhamnose by a (1 → 4) linkage. Above conclusions also can be confirmed by the NOE correlations between δ_H 6.35 (1H, d, J = 1.0 Hz, Rha-1) and δ_H 4.70 (Fuc-2), δ_H 5.03 (1H, d, J = 5.8 Hz) (Xyl-1) and δ_H 4.35 (Rha-4) (see Fig. 2).

The ^{1}H-^{1}H COSY experiment allowed us to identify the *E*-olefinic protons of 3,4,5-trimethoxycinnamoyl moiety, which appeared as two double peaks at δ_H 7.95 (1H, d, J = 16.0 Hz) and 6.61 (1H, d, J = 16.0 Hz). Single peak signal at δ_H 6.84 (2H, s) belongs to H-2 and H-6 of benzene and signals at δ_H 3.81 (6H, s) and δ_H 3.90 (3H, s) belong to the three methoxy groups of benzene. Further investigations based on the HMBC and HSQC spectra allowed the complete assignments of all protons and carbons of the trisubstituted benzene ring (Tables 1 and 2) which were in good agreement with those described in literatures (Elbandy et al., 2004; Mitaine-Offeret al., 2010). The downfield shifts observed in the HSQC spectrum for the H-4 and C-4 resonances of fucose at δ_H 5.75 and δ_C 75.3 established the position at C-4 of fucose to be acylated by the 3,4,5-trimethoxycinnamic acid. For the above evidences, the structure of **1** was elucidated as 3-*O*-β-D-glucopyranosyl presenegenin 28-*O*-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyransyl-(1 → 2)-4-*O*-[(*E*)-3,4,5-trimethoxycinnamoyl]-β-D-fucopyranosyl ester.

Compound **2**, obtained as a white amorphous powder, was assigned a molecular formula of $C_{69}H_{102}O_{31}$ which was deduced from the quasi-molecular ion peak ($[M+Na]^+$) at m/z 1449.6368 in the positive ion mode HRESIMS. After acid hydrolysis, the sugar units were confirmed to be D-glucose, D-fucose, L-rhamnose, D-xylose, and L-arabinose by HPLC analysis of their thiocarbamoyl-thiazolidine derivatives compared with authentic samples. Comparison of the spectroscopic data of **2** (Tables 1 and 2) with those of **1** showed they have the same structure of 3-*O*-β-D-glucopyranosyl presenegenin 28-*O*-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-β-D-fucopyranosyl ester, except for two differences

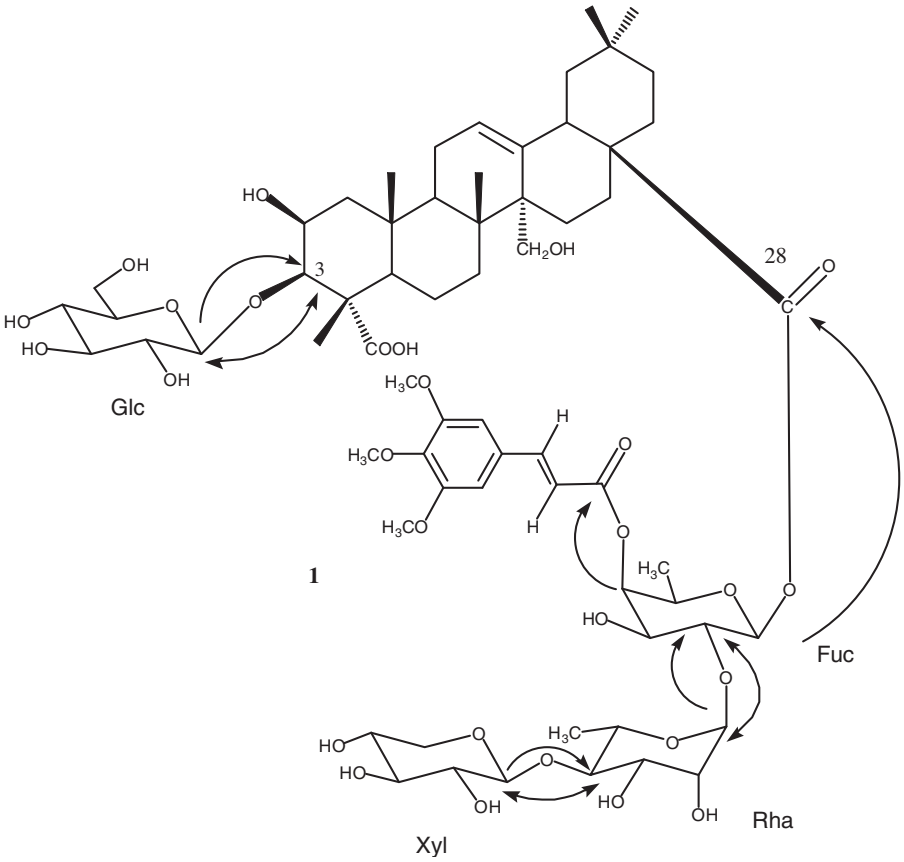

Fig. 2. Key HMBC and NOESY correlations for compound **1**.

located at the saccharide chain linked to C-28 of aglycone. The first difference was at the fucopyranosyl moiety. The ^{13}C NMR spectrum showed the deshielded signal of the Fuc-3 position at δ_C 81.6 in comparison with the signal of the free Fuc-3 position of **1**. The signals of an arabinopyranosyl moiety can be deduced from the NMR data at the same time. In the HMBC spectrum, a cross-peak between δ_H 4.51 (Fuc-3) and δ_C 106.5 (Ara-1), and a correlation between δ_H 4.97 (1H, d, J = 6.9 Hz, Ara-1) and δ_H 4.51 (Fuc-3) in the NOESY spectrum, proved that an arabinose was linked to the C-3 position of the fucose. The HMBC correlations and NOESY correlations of other sugars also can be observed (see Fig. 2). Another difference was at the acyl group linked to the C-4 position of the fucose. The acyl group was substituted by an *E*-3,4-dimethoxycinnamoyl group instead of an *E*-3,4,5-trimethoxycinnamoyl group. The ^{1}H NMR spectrum showed two double peaks at δ_H 6.49 and 7.85, coupling constant of them is 16.0 Hz, which belong to the *E*-olefinic protons, a single peak at δ_H 7.04 that belongs to H-2 of benzene, and two double peaks at δ_H 6.94 (d, J = 8.5 Hz) and 7.05 (d, J = 8.5 Hz) which belong to H-5 and H-6 of benzene. The HMBC and HSQC spectrum confirmed the complete assignments of all protons and carbons of the disubstituted benzene ring (Tables 1 and 2) which were in good agreement with those described in literature (Mitaine-Offer et al., 2003). For the above evidence, the structure of **2** was elucidated as 3-*O*-β-D-glucopyranosyl presenegenin 28-*O*-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnosyl-(1 → 2)-(α-L-arabinopyranosyl)-4-*O*-[(*E*)-3,4-dimethoxycinnamoyl]-β-D-fucopyranosyl ester.

Compounds **3/4** were isolated as a white amorphous powder. The molecular formula was determined to be $C_{76}H_{114}O_{37}$ on the basis of the positive ion mode HRESIMS (*m*/*z* 1641.7035 for quasi-molecular ion [M + Na]$^+$). The ^{1}H NMR and ^{13}C NMR signals of **3/4** (Tables 1 and 2) assigned from 2D NMR spectra were almost superimposable on those of known compound **4**, namely polygalasaponin XLIV (Zhang et al., 1998). Except for the signals of **4**, compounds **3/4** also contain signals of the *Z*-4,5-dimethoxycinnamoyl moiety at δ_H 5.91 (d, J = 12.6 Hz) and 6.88 (d, J = 12.6 Hz) which belong to *Z*-olefinic protons, and the substituted benzene ring protons at δ_H 7.92 (br. s H-2), δ_H 7.50 (d, J = 8.4 Hz, H-6), and 6.93 (d, J = 8.4 Hz, H-5). The relative NMR experiments and HPLC intensities indicated that **3/4** are a mixture of *E*- and *Z*-3,4-dimethoxycinnamoyl-substituted triterpene saponins. All attempts to separate **3** and **4** by semi-preparative HPLC were unsuccessful. This phenomenon of isomerization referred to the effect of light on the 3,4-methoxycinnamoyl group in aqueous methanolic solution has already been observed for the saponins isolated from Polygalaceae (Mitaine-Offer et al., 2003). On the basis of above results, the structure of **3** was elucidated as 3-*O*-β-D-glucopyranosyl presenegenin 28-*O*-β-D-galactopyranosyl-(1 → 4)-β-D-xylopyranosyl-(1 → 4)-α-L-rhamnopyranosyl-(1 → 2)-[β-D-glucopyranosyl-(1 → 3)]-4-*O*-[(*Z*)-3,4-dimethoxycinnamoyl]-β-D-fucopyranosyl ester.

Saponins, acylated bidesmosidic presenegenin glycosides by methoxy-substituted cinnamic acids, isolated from the roots of *S. inappendiculata* showed potent selective cytotoxic activity against M-CSF-stimulated macrophages (Kuroda et al., 2001; Yui et al., 2001). Compounds **1–2**, **3/4**, **4** were evaluated for their cytotoxic activity in vitro against LLC (Lewis lung carcinoma) and MCF-7 (human breast cancer) cell lines using the MTT assay method. Compounds **1** and **2** exhibited moderate cytotoxic activity against LLC cells with an IC_{50} value of 45.56, 85.98 μM respectively using cisplatin as a positive control (IC_{50} = 26.67 μM), and did not show cytotoxicity against MCF-7 cells (IC_{50} > 100 μM). Compounds **3/4** (as a mixture) and **4** were inactive against all cell lines tested (IC_{50} > 100 μM). It is reported that the cinnamoyl group at the

position 4 of the fucopyranosyl residue was important for the cytotoxicity (Yui et al., 2001). Compounds **1** and **2** all have a cinnamoyl group at the position 4 of the fucopyranosyl residue. The structural difference between two compounds mainly was at the position of Fuc-3, compound **2** has a more α-L-arabinopyranosyl than compound **1**. So, the α-L-arabinopyranosyl maybe led to the cytotoxicity decrease of compound **2**, which need to be supported by more research results.

3. Experimental

3.1. General

Optical rotations were recorded on an AUTOPOL II Polarimeter from Rudolph. UV spectra were determined on Agilent Cary 60 UV–vis spectrometer. IR spectra were recorded on Bruker TENSOR 27 spectrometer. The high resolution electrospray ionization mass spectroscopy (HRESIMS) was measured with a Bruker MicroTOF-QII spectrometer. The 1D and 2D NMR spectra (^{1}H-^{1}H COSY, TOCSY, NOESY, HSQC, and HMBC) were performed on Bruker spectrometer (500 MHz) using tetramethylsilane (TMS) as internal standard. Vacuum liquid chromatography (VLC) was carried out using Silica gel (100–200 mesh, Qingdao Marine, China). Analytical TLC was carried out with GF254 plates (Qingdao Marine, China). The spray reagent for saponins was anisaldehyde reagent (0.5 mL Anisaldehyde, 10 mL Acetic acid, 85 mL MeOH and 5 mL H_2SO_4). Medium pressure liquid chromatography (MPLC) was performed on Dr Flash II (Suzhou Lisui, China) equipped with a UV3000 UV–vis Detector and a High Techsil glass column (500 × 50 mm, RP-18, 50 μm). Preparative HPLC and semi-preparative HPLC were carried out on a LC3000 P instrument (Beijing Chuangxin Tongheng, China) equipped with a P3000 pump, UV3000 UV–vis Detector, a YMC-Pack ODS-A column (250 × 20 mm, 5 μm) or a Cosmosil πNAP column (250 × 10 mm, 5 μm) respectively. Analytical HPLC was performed on a LC3000 instrument (Beijing Chuangxin Tongheng, China) equipped with a P3000 gradient pump, UV3000 UV–vis detector, and CXTH-3000 chromatography workstation using a Sharpsil-T C18 column (250 × 4.6 mm, 5 μm).

3.2. Plant material

The roots of *S. inappendiculata* were collected from Yunnan Province, People's Republic of China, in 2010, collected and identified by Hong Wang, plant taxonomist of the Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences. A voucher specimen (Y201011) was deposited in School of Pharmaceutical Science and Technology, Tianjin University.

3.3. Extraction and isolation

Dried roots of *S. inappendiculata* (9.85 kg) were extracted three times with 95% EtOH for 2 h each time, to give residue (1.25 kg) on removal of the solvent. The residue was suspended in H_2O (5.0 L) and partitioned with dichloromethane sat. H_2O and ethyl acetate sat. H_2O successively (each 3 × 3.0 L). The aqueous portion was applied to D101 macroporous resin column chromatography (10.6 × 94.0 cm) and then washed with H_2O, 30% EtOH, 60% EtOH, and 95% EtOH, respectively. The 60% EtOH eluted part (180 g) was subjected to vacuum liquid chromatography (VLC, 40 × 14 cm) using silica-gel (100–200 mesh), and eluted with a gradient of $CHCl_3$:MeOH:H_2O (85:15:2, 75:20:5, 68:24:8, 60:32:8, 50:42:8, low phase) to afford 30 fractions, namely C1 to C30. Then, fraction C25 (14.0 g) was separated by MPLC (RP-18, 500 × 50 mm, 35 mL/min) eluted with MeCN: 0.02% CF_3COOH-H_2O (30:70, 32:6, 34:66) to yield 5 subfractions. Subfraction C25-2 (2.0 g) were further separated by preparative HPLC (RP-18, 250 × 20 mm, 5 μm, 12 mL/min) and purified by semi-preparative HPLC (πNAP, 250 × 10 mm, 5 μm, 4 mL/min) to afford two new compounds **1**–**2**, named as securioside **C** (11 mg), securioside **D** (18 mg), one pair of isomers **3**/**4** (17 mg) (the (*Z*)—isomer (**3**) being new, named as securioside **E**), and a known compound **4**, Polygalasaponin XLIV (20 mg). As securioside **A** and **B** were already isolated from this plant (Kuroda et al., 2001), we adopted this nomenclature to keep consistency.

3.4. Acid hydrolysis of the saponins and HPLC analysis

Each saponin (2 mg) was hydrolyzed with 2 M aq. CF_3COOH (2 mL) for 2 h at 90 °C. After extraction with CH_2Cl_2 (3 × 2 mL), the aqueous layer was repeatedly evaporated to dryness with MeOH until neutral, and then analyzed by TLC over silica gel ($CHCl_3$:MeOH:H_2O, 8:5:1) by comparison with authentic samples. Then, the residue was dissolved in pyridine (0.1 mL) containing L-cysteine methyl ester hydrochloride (0.5 mg) and heated at 60 °C for 1 h. A 0.1 mL solution of *o*-tolyl isothiocyanate (0.5 mg) in pyridine was added to the mixture, which was heated at 60 °C for 1 h. The reaction mixture was directly analyzed by reversed-phase HPLC at 35 °C with isocratic elution of 25% MeCN in 50 mM H_3PO_4 for 50 min and subsequent washing of the column with 90% MeCN at a flow rate 0.8 mL/min and detected at 250 nm (Tanaka et al., 2007). Identification of D-glucose, D-xylose, D-fucose, and L-rhamnose for **1**; D-glucose, D-xylose, D-fucose, L-rhamnose, and L-arabinose for **2**; and D-glucose, D-xylose, D-fucose, L-rhamnose, and D-galactose for **3/4** were carried out by co-injection of the hydrolysate with standard thiazolidine samples prepared in a same way from standard sugars, giving peaks at t_R 22.6 min (D-galactose), 26.2 min (D-glucose), 29.4 min (L-arabinose), 30.5 min (D-xylose), 35.5 min (D-fucose), and 44.8 min (L-rhamnose).

3.5. MTT cytotoxicity bioassays

Cytotoxicities of compounds **1–2**, **3/4**, **4** against LLC and MCF-7 cells were measured using methylthiazole tetrazolium (MTT) assay (Mosmann, 1983). IC_{50} values were determined as concentration of a compound resulting in cell growth inhibition by 50% and cisplatin was used as a positive control. All samples were assayed in triplicate.

3.6. Securioside *C*

A white amorphous powder, $[\alpha]_D$—12 (*c* = 0.08, MeOH); UV (MeOH) λ_{max} (log ε): 206 (5.97), 226 (5.92), 302 (6.07) nm; IR ν_{max} 3393, 2937, 1740, 1710, 1679, 1637, 1456, 1073, 837. HRESIMS *m*/*z* 1347.6020 $[M+Na]^+$ (calcd. for $C_{65}H_{96}O_{28}Na$ 1347.6088); ^{1}H NMR (pyridine-d_5, 500 MHz) and ^{13}C NMR (pyridine-d_5, 125 MHz) data, see Tables 1 and 2.

3.7. Securioside *D*

A white amorphous powder, $[\alpha]_D$—9.8 (*c* = 0.05, MeOH); UV (MeOH) λ_{max} (log ε): 206 (5.86), 226 (5.91), 308 (6.04) nm; IR ν_{max} 3429, 2935, 1723, 1710, 1680, 1632, 1454, 1070, 841; HRESIMS *m*/*z* 1449.6368 $[M+Na]^+$ (calcd. for $C_{69}H_{102}O_{31}Na$ 1449.6405); ^{1}H NMR (pyridine-d_5, 500 MHz) and ^{13}C NMR (pyridine-d_5, 125 MHz) data, see Tables 1 and 2.

3.8. Compounds *3/4*

A white amorphous powder, $[\alpha]_D$—2.73 (*c* = 0.092, MeCN:H_2O = 3:7); UV (MeOH) λ_{max} (log ε): 206 (4.86), 226 (4.91), 325 (5.08) nm; IR ν_{max} 3500, 2937, 1723, 1710, 1678, 1632, 1424, 1055, 801; HRESIMS at *m*/*z* 1614.6838 $[M+Na]^+$ (calcd. for $C_{76}H_{114}O_{37}Na$

1614.6937); ^{1}H NMR (pyridine-d_5, 500 MHz) and ^{13}C NMR (pyridine-d_5, 125 MHz) data, see Tables 1 and 2.

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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.2015.05.022.

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