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Terpenoids and hexenes from the leaves of *Crataegus pinnatifida*

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ABSTRACT

Crataegus pinnatifida have long been used in traditional Chinese medicine and European herbal medicine, and are widely consumed as food, in the form of juice, drink, jam and canned fruit. Four new compounds, a sesquiterpene and its glycoside (**1–2**), two monoterpene glycosides (**3–4**), together with eight known compounds (**5–12**), were isolated from the leaves of *C. pinnatifida*. Their structures were elucidated as (5Z)-6-[5-(2-hydroxypropan-2-yl)-2-methyltetrahydrofuran-2-yl]-3-methylhexa-1,5-dien-3-ol (**1**), (5Z)-6-[5-(2-O-β-D-glucopyranosyl-propan-2-yl)-2-methyl tetrahydrofuran-2-yl]-3-methylhexa-1,5-dien-3-ol (**2**), 5-ethenyl-2-[2-O-β-D-glucopyranosyl-(1″ → 6′)-β-D-glucopyranosyl-propan-2-yl]-5-methyltetrahydrofuran-2-ol (**3**), 4-[4β-O-β-D-xylopyranosyl-(1″ → 6′)-β-D-glucopyranosyl-2,6,6-trimethyl-1-cyclohexen-1-yl]-butan-2-one (**4**), (Z)-3-hexenyl O-β-D-glucopyranosyl-(1″ → 6′)-β-D-glucopyranoside (**5**), (Z)-3-hexenyl O-β-D-xylopyranosyl-(1″ → 6′)-β-D-glucopyranoside (**6**), (Z)-3-hexenyl O-β-D-rhamnopyranosyl-(1″ → 6′)-β-D-glucopyranoside (**7**), (3R,5S,6S,7E,9S)-megastiman-7-ene-3,5,6,9-tetrol (**8**), (3R,5S,6S,7E,9S)-megastigman-7-ene-3,5,6,9-tetrol9-O-β-D-glucopyranoside (**9**), (6S,7E,9R)-6,9-dihydroxy-4,7-megastigmadien-3-one 9-O-[β-D-xylopyranosyl-(1″ → 6′)-β-D-glucopyranoside] (**10**), Linarionoside C (**11**), and (3S,9R)-3,9-dihydroxy-megastigman-5-ene 3-O-primeveroside (**12**), using a combination of mass spectroscopy, 1D and 2D NMR spectroscopy and chemical analysis. Cytotoxicity of the new compounds was assayed against selected human glioma (U87) cell lines.

1. Introduction

Crataegus pinnatifida Bge var *major* N.E.Br. or *Crataegus pinnatifida* Bge., locally called Hawthorn, is widely distributed throughout the northern temperate regions of the world with approximately 280 species, primarily in East Asia, Europe and North America (Zhang, Ho, Huang, & Chen, 2002). Hawthorn fruits have long been used in traditional Chinese medicine and European herbal medicine, and are widely consumed as food, in the form of juice, drink, jam and canned fruit (Chang, Zuo, Chow, & Ho, 2006). The extract of hawthorn has been shown to have many health benefits including being cardiovascular protective, hypotensive, hypocholesterolaemic and lowers serum cholesterol (Yao, Ritchie, & Brown-Woodman, 2008; Zhang et al., 2001, 2002). Pharmacological and toxicological studies have demonstrated that consumption of hawthorn fruits is associated with long-term medicinal benefits to cardiovascular function with little side effect (Ammon & Handel, 1981a, 1981b, 1981c). Hawthorn fruits and leaves have a curative effect on blood vessels of the heart which have been extensively reported (Frishman, Beravol, & Carosella, 2009; Frishman, Sinatra, & Moizuddin, 2004; Long, Carey, Crofoot, Proteau, & Filtz, 2006; Pittler, Schmidt, & Ernst, 2003). Previous studies on the chemical constituents of hawthorn led to the isolation of a series of diverse compounds, including flavonoids, terpenoids and organic acids. So far, more than 170 compounds have been isolated from *Crataegus* (Chen & Song, 2005; Dauguet, Bert, Dolley, Bekaert, & Lewin, 1993; Gao et al., 2010; Nikolov, Seligmann, Wagner, Horowitz, & Gentili, 1982; Zhang & Xu, 2001). In the present study, according to the pharmacokinetic data available, it appears that one of the potentially most active components of hawthorn, quercetin, is poorly bioavailable both *in vivo* and *in vitro*. However, quercetin is only one of many components of the hawthorn extract. The extent to which any of these other components might be available to the embryo is unknown (Yao et al., 2008). In order to evaluate the health protection value of hawthorn, the leaves of hawthorn was subjected to phytochemical investigation resulting in the isolation of a novel sesquiterpene and its glycoside (**1–2**), two novel monoterpene glycosides (**3–4**), together with eight known compounds (**5–12**). Their chemical structures (Fig. 1) were elucidated by their physicochemical properties and spectral data, such as 1D, 2D NMR and HRESI-MS.

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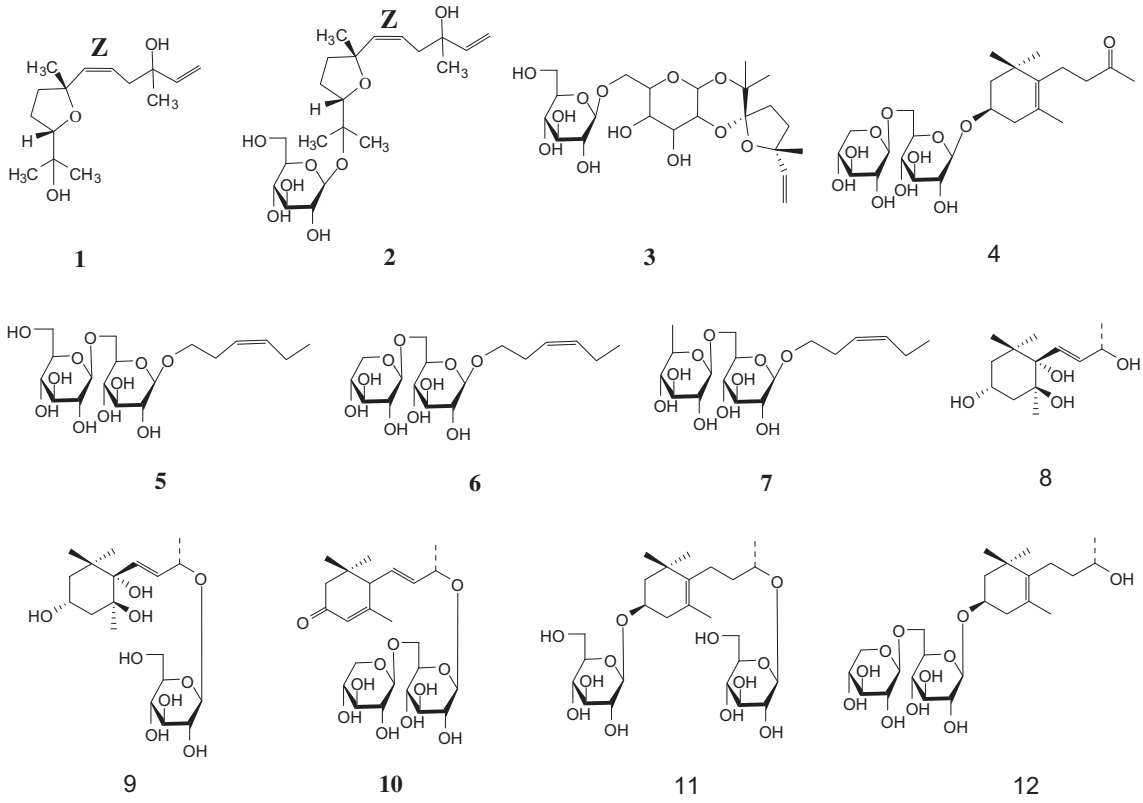

Fig. 1. The structures of compounds **1**–**12**.

2. Materials and methods

2.1. General methods

Optical rotations were measured with a Perkin–Elmer 241MC polarimeter. Silica gel (200–300 mesh, Qingdao Marine Chemical Co., China); Sephadex LH-20 (25–100 μm, Greenherbs Science and Technology Development Co., Ltd., China); MCI gel (CHP20P, 75–150 μm, Mitsubishi Chemical Corporation, Japan) and reversed-phase C_{18} silica gel (60–80 μm, Merck, Germany) were used for column chromatography and silica gel GF254 (Qingdao Marine Chemical Co., China) for TLC. Solvents were of industrial purity and distilled prior to use.

2.2. Plant material

The leaves (5 kg) of *C. pinnatifida* were collected from Liaoning province, China in June 2007, and authenticated by Prof. Qi-Shi Sun, Department of Pharmacognosy, Shenyang Pharmaceutical University. A voucher specimen is kept in the Nature Products Laboratory of Shenyang Pharmaceutical University, Shenyang, China.

2.3. Extraction and isolation

Air-dried leaves (5 kg) of *C. pinnatifida* were extracted with 70% ethanol by reflux (95 °C, 2 h) three times. The ethanol extract was concentrated *in vacuo* to yield a brownish-dark crude extract (492 g), which was subjected to the macroporous resin (ethanol/water 0:100 → 95:5) to yield 4 main fractions: *Frs. A–D* according to chemical monitoring by TLC. *Fr. C* (288 g) was further separated by CC (SiO_2; $CHCl_3$/MeOH 100:1 → 2:1) to provide 6 fractions (*Frs. D1–D6*), among which *Fr. D2* (18 g) was purified by CC (SiO_2; $CHCl_3$/MeOH 30:1), sequentially by CC (Sephadex LH-20; MeOH) to yield compounds **1** (12 mg) and **5** (36 mg). *Frs. D3* (20 g), *D4* (35 g) and *D5* (40 g) were applied to a column of MCI gel (methanol/water 0:100 → 100:0), respectively, to collect major fractions. After removal of a large amount of water-soluble components, the major fractions of *Frs. D3* were then combined on the basis of TLC analysis, and finally performed by reversed-phase C_{18} silica gel using MeOH/H_2O with a gradient solvent system of (30:70 → 90:10) and HPLC to obtain compounds **2** (16 mg), **6** (13 mg), **10** (22 mg), and **12** (17 mg). In the same way, compounds **3** (11 mg), **4** (38 mg), **7** (18 mg) were isolated from *Frs. D4*, and compounds **8** (27 mg), **9** (46 mg), **11** (15 mg) from *Frs. D5*.

2.4. Mass spectrometry

The HRESI-TOF-MS was obtained on a MicroTOF spectrometer (Bruker Daltonics, CA).

2.5. NMR spectroscopy

^1H, ^{13}C NMR and 2D NMR data, including HSQC, HMBC, and NOESY, were measured on a Bruker ARX-600 spectrometer (298 K) instrument (Bruker Corporation, Bremen, Germany) operating at 600 MHz for ^1H and 150 MHz for ^{13}C. The chemical shifts (δ) are reported in ppm down-field from tetramethylsilane (TMS) using TMS or the solvent signal as the standard in chloroform-d_1 or dimethyl sulfoxide-d_6 (DMSO-d_6).

2.6. Cytotoxic assay

Cytotoxic activities were performed according to the MTT method reported by Hussain, Nouri, and Oliver (1993). Tested-compounds were dissolved in DMSO and the final concentration of DMSO in the culture medium was controlled at less than 0.1%

(*v*/*v*). Cultivated human glioma (U87) cells were seeded in a 96-well plate with 1×10^4 cells/well. The plate was incubated with the tested compounds for 48 h, the MTT solution (2.5 mg/ml in PBS) was then added (10 μl/well) and the plates were incubated for an additional 4 h at 37 °C. The produced formazan crystals were dissolved in 100 μl of DMSO and the optical density of the solution was measured at 492 nm using a microplate reader (Synergy HT (Biotek)). The percentage of cytotoxic activity was determined by comparison with the control (DMSO).

The cytotoxicity of samples on tumour cells were expressed as IC_{50} values (the drug concentration reducing the absorbance by 50% in treated cells, with respect to untreated cells), which were calculated by the LOGIT method.

3. Results and discussion

3.1. Phytochemical investigation

Compound **1** was obtained as a colourless oil. $[\alpha]_D^{20} = -12.0$ ($c = 0.14$, methanol). The molecular formula was established as $C_{15}H_{26}O_3$ by HRESI-MS, which showed a sodium adduct ion peak $[M+Na]^+$ at *m*/*z* 277.1776 (calculated 277.1774). ^{13}C NMR spectrum of **1** displayed 15 carbon signals assigned to four methyls, four methylenes, four methines and three quaternary carbons (Table 1). Two olefin signals (δ_C 144.7, 139.5, 122.1 and 111.9) and four carbons bearing oxygen (δ_C 85.4, 82.6, 72.6 and 71.1) were evident. Furthermore, ^1H NMR spectrum of **1** showed the signals of terminal olefin signals at δ_H 5.19 (1H, $J = 17.1$, 1.8 Hz), 5.04 (1H, $J = 10.8$, 1.8 Hz) and 5.91 (1H, $J = 17.1$, 10.8 Hz), a *cis*-olefin signals at δ_H 5.54 (1H, $J = 7.2$, 3.9 Hz) and 5.53 (1H, $J = 7.2$ Hz), a carbon signal bearing oxygen at δ_H 3.76 (1H, $J = 6.6$ Hz) and four methyl groups attached to the quaternary carbon at δ_H 1.26 (3H, s), 1.31 (3H, s), 1.21 (3H, s) and 1.12 (3H, s).

Analysis of the ^1H, ^{13}C NMR and HSQC data helped us to allot H- to their bonded C-atoms, and further demonstration of the planar structure of **1** (Fig. 2) was obtained from the HMBC experiment. In the HMBC spectrum, the key correlations of a methylene unit with a terminal olefin group and a *cis*-olefin group, δ_C 144.7 (C-2)/H-1a, H-1b, H-4a, H-4b, H-15; 72.6 (C-3)/H-1a, H-1b, H-4a, H-4b, H-15; δ_C 45.1 (C-4)/H-2, H-5, H-6, H-15; δ_C 122.1 (C-5)/H-4a, H-4b, H-6; and δ_C 139.5 (C-6)/H-4a, H-4b, H-5, suggested the presence of Part 1 (Fig. 2). Furthermore, the key correlations of two methylene units with three carbons bearing oxygen and a *cis*-olefin groups, δ_C 139.5 (C-6)/H-5, H-8a, H-8b, H-14; δ_C 82.6 (C-7)/H-5, H-6, H-8a, H-8b, H-9a, H-9b, H-14; δ_C 37.8 (C-8)/H-9a, H-9b, H-14; δ_C 26.3 (C-9)/H-8a, H-8b, H-10; δ_C 85.4 (C-10)/H-8a, H-8b, H-9a, H-9b, H-12, H-13; and 71.1 (C-11)/H-9a, H-9b, H-10, H-12, H-13, indicated that two iso-pentenyl units were connected as Part 2 (Fig. 2). Unambiguously, C-5 and C-6 were allocated to the linkage site of Part 1 and Part 2. The stereochemistry of H-5 and H-6 was completely established by the coupling constants δ_H 5.54 (1H, m, $J = 7.2$, 3.9 Hz, H-5) and 5.53 (1H, m, $J = 7.2$ Hz, H-6) (Meng, Wu, & Zhao, 2010). Hence, the structure of compound **1** is deduced as (5Z)-6-[5-(2-hydroxypropan-2-yl)-2-methyl tetrahydrofuran-2-yl]-3-methylhexa-1,5-dien-3-ol (**1**), a novel sesquiterpene named shanyegenin B.

Compound **2** was obtained as a colourless oil. $[\alpha]_D^{20} = -4.0$ ($c = 0.25$, methanol). The molecular formula was established as $C_{21}H_{36}O_8$ by HRESI-MS, which showed a sodium adduct ion peak $[M+Na]^+$ at *m*/*z* 439.2307 (calculated 439.2302). The ^1H and ^{13}C NMR spectral data of **2** were very similar to those of **1** (Tables 1 and 2), except for the presence of one more glucopyranosyl which was determined by ^1H, ^{13}C NMR and ESI-MS. Furthermore, the H-1′

Table 1
^1H NMR data for **1**–**4**.

No.	**1**[a]	**2**[a]	No.	**3**[a]	No.	**4**[a]
1a	5.19 dd (17.1, 1.8)	5.10 dd (17.1, 1.8)	1a	5.16 dd (17.1, 1.5)	1	
1b	5.04 dd (10.8, 1.8)	4.91 dd (10.8, 1.8)	1b	4.94 dd (10.5, 1.5)	2a	1.35 dd (12.3, 12.0)
2	5.91 dd (17.1, 10.8)	5.84 dd (17.1, 10.8)	2	5.91 dd (17.1, 10.5)	2b	1.73 brd (10.2)
3			3		3	3.90 brd (10.2)
4a	2.20 m	2.10 brd (3.9)	4a	1.87 m	4a	1.87 m
4b	2.21 m	2.10 d (3.9)	4b	2.03 m	4b	2.25 m
5	5.57 m	5.54 dd (7.2, 3.9)	5a	1.74 m	5	
6	5.58 m	5.53 d (7.2)	5b	1.83 m	6	
7			6		7a	2.06 m
8a	1.69 m	1.67 m	7		7b	2.15 m
8b	1.89 m	1.67 m	8	1.39 s	8a	2.44 m
9a	1.82 m	1.81 m	9	1.30 s	8b	2.44 m
9b	1.82 m	1.91 m	10	1.16 s	9	
10	3.86 t (6.6)	3.86 t (6.6)			10	2.07 s
11					11	0.98 s
12	1.26 s	1.09 s			12	0.98 s
13	1.31 s	1.16 s			13	1.54 s
14	1.21 s	1.16 s				
15	1.12 s	1.07 s				
1′		4.35 d (7.8)	1′	4.48 d (7.2)	1′	4.24 d (8.1)
2′		2.84 t (7.8)	2′	3.38 m	2′	2.98 m
3′		3.10 t (7.8)	3′	3.66 brd (8.4)	3′	3.54 m
4′		3.00 m	4′	3.14 m	4′	3.68 m
5′		3.02 m	5′	3.41 m	5′	3.69 m
6′a		3.35 brd (10.8)	6′a	2.95 m	6′a	3.05 m
6′b		3.62 brd (10.8)	6′b	3.65 m	6′b	3.05 m
1″			1″	4.14 d (7.5)	1″	4.20 d (7.5)
2″			2″	3.52 m	2″	3.28 m
3″			3″	3.06 m	3″	3.30 m
4″			4″	3.04 m	4″	3.31 m
5″			5″	3.41 m	5″a	3.15 m
6″a			6″a	3.05 brd (10.2)	5″b	3.87 m
6″b			6″b	4.03 brd (10.2)		

[a] Recorded at 600 MHz.

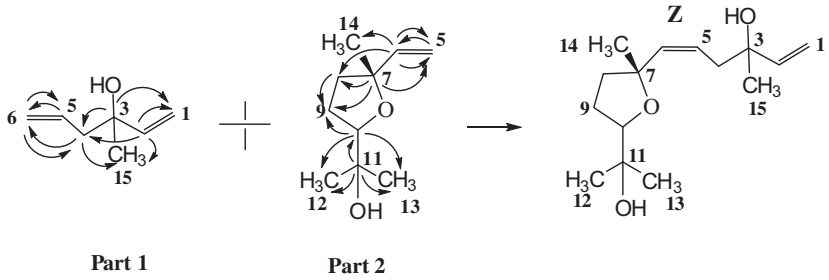

Fig. 2. Key HMBC correlations (C → H) of **1**.

Table 2
^{13}C NMR data for **1**–**12**.

No.	1[a]	2[a]	3[a]	4[a]	5[a]	6[a]	7[a]	8[a]	9[a]	10[a]	11[a]	12[a]
1	111.9	111.3	111.1	37.3	69.2	69.0	68.8	41.2	41.3	36.5	38.1	37.8
2	144.7	146.4	144.6	45.9	28.4	28.3	28.0	46.7	46.6	48.0	47.2	46.5
3	72.6	71.9	84.9	70.0	126.1	126.0	125.7	63.6	63.5	199.5	74.4	71.0
4	45.5	45.5	35.5	38.0	133.7	133.7	133.4	46.2	46.0	125.7	39.5	39.6
5	122.1	123.0	33.7	124.3	21.0	20.9	20.6	76.7	77.0	163.0	124.5	123.7
6	139.5	139.4	107.9	136.2	15.0	15.0	14.6	77.8	77.5	55.3	138.2	137.6
7	82.6	82.5	78.0	21.4				129.9	133.5	128.1	24.5	24.6
8	37.8	38.1	28.4	43.6				135.7	132.6	137.4	39.0	40.1
9	26.3	26.7	24.9	208.2				67.8	76.6	76.5	77.6	66.8
10	85.4	83.9	22.6	29.7				25.5	22.3	21.5	20.2	23.9
11	71.1	78.3		28.4				28.0	28.0	27.6	29.1	28.8
12	27.1	27.3		29.4				26.6	26.6	28.3	30.4	30.1
13	27.1	26.4		19.5				27.8	27.9	23.8	20.4	20.0
14	27.1	24.0										
15	24.1	22.2										
1′		97.6	93.9	103.9	103.5	103.5	103.3		101.7	101.6	101.6	101.1
2′		74.0	72.2	73.4	74.4	74.1	73.8		74.5	74.4	74.4	73.9
3′		77.5	73.9	76.7	77.7	77.4	77.1		77.7	77.5	77.6	77.1
4′		70.6	70.4	69.6	70.9	70.4	70.7		71.0	70.6	71.1	70.5
5′		77.0	77.3	75.8	76.7	76.6	75.8		77.7	75.3	77.6	76.1
6′		61.6	69.0	68.3	69.1	69.1	68.8		61.9	69.0	62.1	68.8
1″			103.6	100.5	104.1	100.7	101.3			104.8	101.6	104.4
2″			73.0	73.4	74.2	74.1	71.1			74.2	74.4	73.9
3″			77.3	76.6	77.6	77.4	71.0			77.5	77.6	77.0
4″			71.0	70.3	70.9	70.4	72.4			70.4	71.0	70.1
5″			77.3	65.7	77.7	66.4	67.8			66.5	77.6	66.1
6″			61.4		61.9		18.4				62.1	

[a] Recorded at 150 MHz.

at δ_H 4.35 showed correlation with the carbon signal at δ_C 78.3 (C-11), indicating the occurrence of a glucopyranosyl at C-11 (Fig. 3).

The relative stereochemistry of **2** was completely established by the NOESY spectrum (Fig 3), the significant NOESY correlation signals were observed at 12, 13-CH_3/H-1′ and 10-CH_3/12, 13, 14-CH_3, indicating that the H-10 and 14-CH_3 were all on the same side of furan-ring (Meng et al., 2010). From the anomeric carbon (δ_C 97.6) and the anomeric proton [δ_H 4.35 (1H, d, J = 7.8 Hz)] and other characteristic NMR resonances, the sugar unit was identified as β-glucopyranose, which was further confirmed by strong NOE signals between H-1$_{glc}$ and H-3$_{glc}$, H-5$_{glc}$ (Agrawal, 1992). Therefore, compound 2 was concluded to be (5Z)-6-[5-(2-O-β-D-glucopyranosyl-propan-2-yl)-2-methyltetrahydrofuran-2-yl]-3-methylhexa-1,5-dien-3-ol (**2**), and given the trivial name shanyeside B.

Compound **3** was obtained as a colourless oil. $[\alpha]_D^{20}$ = +13.6 (c = 0.18, methanol). The molecular formula was established as $C_{22}H_{36}O_{12}$ by HRESI-MS, which showed a sodium adduct ion peak $[M+Na]^+$ at m/z 515.2093 (calculated 515.2099). The ^{1}H NMR spectrum (Table 1), showed the proton signals of a terminal olefin group at δ_H 5.16 (1H, J = 17.1, 1.5 Hz), 4.94 (1H, J = 10.5, 1.5 Hz) and 5.91 (1H, J = 17.1, 10.5 Hz), as well as three methyl groups attached to the quaternary carbon at δ_H 1.39 (3H, s), 1.30 (3H, s) and 1.16 (3H, s). The ^{13}C NMR spectrum exhibited three methyls (δ_C 28.4, 24.9, 22.6), three methylenes (δ_C 111.1, 35.5, 33.7), one methine (δ_C 144.6), and three quarternary carbons (δ_C 107.9, 84.9, 78.0), which suggested that **3** has a monoterpene skeleton. Further demonstrating the planar skeleton structure of **3** was obtained from the HMBC experiment (Fig. 3). The HMBC spectrum exhibited correlations from δ_C 84.9 (C-3) to H-1a, H-1b, H-4a, H-4b, H-10; δ_C 35.5 (C-4) to H-5a, H-5b, H-10; δ_C 33.7 (C-5) to H-4a, H-4b; δ_C 107.9 (C-6) to H-5a, H-5b, H-8, H-9, H-2′; and δ_C 78.0 (C-7) to H-5a, H-5b, H-8, H-9, H-1′, indicating the presence of monoterpene skeleton. In the sugar part, two anomeric proton signals at δ_H 4.48 (1H, J = 7.2 Hz), 4.14 (1H, J = 7.5 Hz) and the ^{13}C NMR signals of sugars showed the existence of two glucopyranosyl moieties (Tables 1 and 2). $^3J_{C-H}$ correlations of genin and sugars were observed not only between the anomeric proton at δ_H 4.48 and the carbon at δ_C 78.0, but also between δ_H 3.38 and δ_C 107.9, which indicated the double linkage. The high-field shifting of δ_C 93.9 (C-1′) and 73.9 (C-3′) compared with usual values could

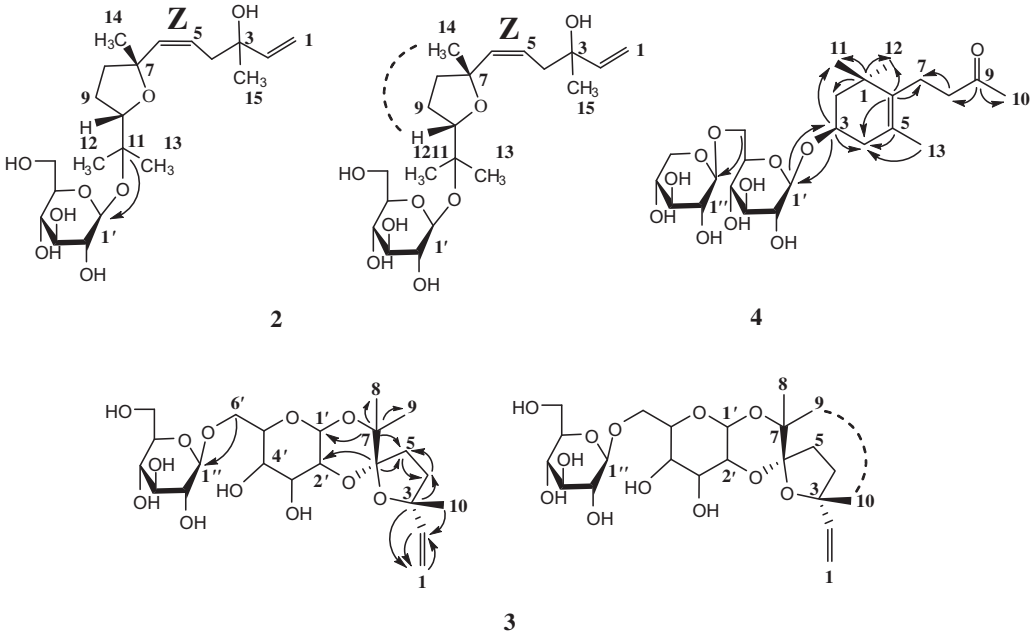

Fig. 3. Key HMBC correlations (C → H) of **2**, **3** and **4**. Key NOESY correlations (H···H) of **2** and **3**.

readily be explained by β-effects due to etherification of position 1′ and 2′ at the same time (Chang & Case, 2005). Except (β-effects), the C-2′ signal was also affected by steric hindrance and finally shifted to higher-field. The glucosyl moiety was identified by acid hydrolysis of **3** with comparison to an authentic sample.

Finally, the relative stereochemistry of **3** was completely established by the NOESY spectrum and coupling constants. Significant NOESY correlation signals were observed at 9, 10-CH_3/H-4, 5 and 9-CH_3/10-CH_3 indicating, that the 9-CH_3 and 10-CH_3 were all on the same side of furan-ring (Fig. 3). Additionally, the β-stereochemistry of anomeric carbons were determined by the coupling constants (J = 7.2 and J = 7.5) of the anomeric protons (Agrawal, 1992). Therefore, the structure of **3** was identified as 5-ethenyl-2-[2-O-β-D-glucopyranosyl-(1′′86′)-β-D-glucopyranosyl-propan-2-yl]-5-methyltetrahydrofuran-2-ol, and given the trivial name shanyeside C.

Compound **4** was also obtained as a colourless oil. $[\alpha]_D^{20}$ = −14.0 (c = 0.21, methanol). The ESI-MS showed a sodium adduct ion peak at m/z 527 $[M+Na]^+$ and a quasimolecular ion 503 $[M-H]^-$, indicating a molecular weight of 504. According to the HRESI-MS, ^{1}H and ^{13}C NMR spectroscopic data, the molecular formula of **4** was determined to be $C_{24}H_{40}O_{11}$, $[M+Na]^+$ at m/z 527.2465 (calculated 527.2463). The ^{1}H NMR spectrum (Table 1), showed the presence of four methyl groups attached to the quaternary carbon at δ_H 2.07 (3H, s), 1.54 (3H, s), 0.98 (3H, s) and 1.01 (3H, s). The ^{13}C NMR spectrum exhibited four methyls (δ_C 29.7, 29.4, 28.4, 19.5), four methylenes (δ_C 24.0, 40.4, 46.0, 47.8), one methine (δ_C 70.0), and four quarternary carbons (δ_C 37.3, 124.3, 136.2, 208.2), in addition to the sugar part. Further analysis of 2D NMR spectra of **4** indicated that this compound was an ionone derivative (Miyase, Ueno, Takizawa, Kobayashi, & Oguchi, 1988). Two sugar linkages of the β-form were indicated by the coupling constants of the anomeric proton signals at δ_H 4.24 (1H, d, J = 8.1 Hz) and 4.20 (1H, d, J = 7.5 Hz) in the ^{1}H NMR data. In addition, the ^{13}C NMR signals showed the existence of glucopyranosyl and xylopyranosyl moieties as shown in Table 1. These observations were also confirmed by acid hydrolysis and comparison with an authentic sample. The down-field shift of the glucose C-6′ signal (δ_C 68.3) indicated that xylopyranose and glucopyranose were linked through a 1 → 6 glycosidic bond. The glycosidic site was established unambiguously by a HMBC experiment in which long-range correlation between H-1′ (δ_H 4.24) and C-3 (δ_C 70.0) was observed (Fig. 3). The relative configuration at C-3 was determined to be β-stereochemistry by the coupling constant ($J_{3\text{-}4\beta}$ = 10.1 and $J_{3\text{-}2\beta}$ = 12.0) (Chang & Case, 2005). Consequently, the structure of compound **4** was established as 4-[4β-O-β-D-xylopyranosyl-(1′′ → 6′)-β-D-glucopyranosyl-2,6,6-trimethyl-1-cyclohexen-1-yl]-butan-2-one, and given the trivial name shanyeside D.

The known compounds were identified by comparing their physical and spectroscopic data with values reported in the literature. They are (Z)-3-hexenyl O-β-D-glucopyranosyl-(1′′ → 6′)-β-D-glucopyranoside (**5**) (Noiarsa et al., 2007), (Z)-3-hexenyl O-β-D-xylopyranosyl-(1′′ → 6′)-β-D-glucopyranoside (**6**) (Kishida, Fujii, Ida, & Akita, 2005), (Z)-3-hexenyl O-β-D-rhamnopyranosyl-(1′′ → 6′)-β-D-glucopyranoside (**7**) (Kishida et al., 2005), (3R,5S,6S, 7E,9S)-megastiman-7-ene-3,5,6,9-tetrol (**8**) (Tung et al., 2009), (3R,5S,6S,7E,9S)-megastigman-7-ene-3,5,6,9-tetrol 9-O-β-D-glucopyranoside (**9**) (Yamamoto et al., 2008), (6S,7E,9R)-6,9-dihydroxy-4,7-megastigmadien-3-one 9-O-[β-D-xylopyranosyl-(1′′ → 6′)-β-D-glucopyranoside] (**10**) (Matsuda, Isawa, & Kikuchi, 1997), Linarionoside C (**11**) (Otsuka, 1994), and (3S,9R)-3,9-dihydroxy-megastigman-5-ene 3-O-primeveroside (**12**) (Otsuka & Tamaki, 2002).

3.2. Characteristic data of compounds

(5Z)-6-[5-(2-Hydroxypropan-2-yl)-2-methyltetrahydrofuran-2-yl]-3-methylhexa-1,5-dien-3-ol (**1**): colourless oil; $[\alpha]_D^{20}$ = −12.0 (c = 0.14, methanol); HRESI-MS m/z: 277.1776 (calcd. for $C_{15}H_{26}O_3$ + Na^+, 277.1774); see Table 1 for ^{1}H NMR and see Table 2 for ^{13}C NMR.

(5Z)-6-[5-(2-O-β-D-glucopyranosyl-propan-2-yl)-2-methyltetrahydrofuran-2-yl]-3-methylhexa-1,5-dien-3-ol (**2**): colourless oil; $[\alpha]_D^{20}$ = −4.0 (c = 0.25, methanol); HRESI-MS m/z: 439.2307 (calcd. for $C_{21}H_{36}O_8$ + Na^+, calculated 439.2302); see Table 1 for ^{1}H NMR and see Table 2 for ^{13}C NMR..

5-Ethenyl-2-[2-O-β-D-glucopyranosyl-(1′′ → 6′)-β-D-glucopyranosyl-propan-2-yl]-5-methyltetrahydrofuran-2-ol(**3**): colourless

Table 3
Cytotoxicity of compounds **1–4** isolated from the leaves of *C. pinnatifida*.

Compound	Control	0.1 μM	1 μM	10 μM	100 μM	IC_{50} (μM)
1	0.00 ± 0.60	3.45 ± 0.64**	4.10 ± 0.70**	5.17 ± 1.22***	1.96 ± 0.53	>100
2	0.00 ± 0.96	8.52 ± 1.30***	1.88 ± 0.18*	5.78 ± 1.04**	3.20 ± 0.52*	>100
3	0.00 ± 0.63	2.57 ± 1.05*	2.14 ± 0.56	2.40 ± 0.49	3.19 ± 0.64*	>100
4	0.00 ± 0.73	−1.62 ± 0.96	−0.59 ± 0.67	1.18 ± 0.95	0.28 ± 0.75	>100
		1 μM	10 μM	100 μM	1000 μM	
5-Fu	0.00 ± 0.74	−6.25 ± 0.54***	10.79 ± 0.76***	30.41 ± 0.53***	52.29 ± 0.35***	727.14

* $P < 0.05$ vs control.
** $P < 0.01$ vs control.
*** $P < 0.001$ vs control.

oil; $[\alpha]_D^{20}$ = +13.6 (*c* = 0.18, methanol); HRESI-MS *m/z*: 515.2093 (calculated for $C_{22}H_{36}O_{12}$ + Na^+, 515.2099); see Table 1 for ^1H NMR and see Table 2 for ^{13}C NMR.

4-[4*β*-O-*β*-D-Xylopyranosyl-(1″ → 6′)-*β*-D-glucopyranosyl-2,6,6-trimethyl-1-cyclohexen-1-yl]-butan-2-one (**4**): colourless oil; $[\alpha]_D^{20}$ = −14.0 (*c* = 0.21, methanol); HRESI-MS *m/z*: 527.2465 (calculated for $C_{24}H_{40}O_{11}$ + Na^+, 527.2463); see Table 1 for ^1H NMR and see Table 2 for ^{13}C NMR.

(Z)-3-Hexenyl O-*β*-D-glucopyranosyl-(1″ → 6′)-*β*-D-glucopyranoside (**5**): ^1H NMR (DMSO-d_6, 300 MHz) δ_H: 0.92 (3H, t, *J* = 7.5 Hz, H-6), 2.03 (2H, m, *J* = 6.9 Hz, H-5), 2.27 (2H, q, *J* = 6.9 Hz, H-2), 3.49 (1H, m, H-1), 4.13 (1H, d, *J* = 7.8 Hz, H-1′), 4.25 (1H, d, *J* = 7.8 Hz, H-1″), 5.36 (2H, m, *J* = 10.8 Hz, H-3, 4).

(Z)-3-Hexenyl O-*β*-D-xylopyranosyl-(1″ → 6′)-*β*-D-glucopyranoside (**6**): ^1H NMR (DMSO-d_6, 300 MHz) δ_H: 0.92 (3H, t, *J* = 7.5 Hz, H-6), 2.03 (2H, m, *J* = 6.9 Hz, H-5), 2.27 (2H, q, *J* = 6.9 Hz, H-2), 3.42 (1H, m, H-1), 4.14 (1H, d, *J* = 7.8 Hz, H-1′), 4.17 (1H, d, *J* = 7.5 Hz, H-1″), 5.39 (2H, m, *J* = 10.8 Hz, H-3, 4).

(Z)-3-Hexenyl O-*β*-D-rhamnopyranosyl-(1″ → 6′)-*β*-D-glucopyranoside (**7**): ^1H NMR (DMSO-d_6, 300 MHz) δ_H: 0.92 (3H, t, *J* = 7.5 Hz, H-6), 1.12 (3H, d, *J* = 6.0 Hz, H-6″), 2.01 (2H, m, *J* = 6.9 Hz, H-5), 2.27 (2H, q, *J* = 6.6 Hz, H-2), 3.41 (1H, m, H-1), 4.12 (1H, d, *J* = 7.8 Hz, H-1′), 4.57 (1H, d, *J* = 1.8 Hz, H-1″), 5.37 (2H, m, *J* = 10.8 Hz, H-3, 4).

(3R,5S,6S,7E,9S)-Megastiman-7-ene-3,5,6,9-tetrol (**8**): ^1H NMR (DMSO-d_6, 300 MHz) δ_H: 0.71 (3H, s, H-11), 0.99 (3H, s, H-13), 1.08 (3H, s, H-13), 1.12 (3H, s, *J* = 6.3 Hz, H-10), 1.25 (1H, dd, *J* = 12.3, 3.2 Hz, H-2 eq), 1.48 (1H, m, *J* = 12 Hz, H-2ax), 1.56 (2H, m, H-4), 3.85 (1H, m, H-3), 4.52 (1H, d, *J* = 7.5 Hz, H-9), 5.66 (1H, dd, *J* = 12.9, 6.0 Hz, H-8), 5.91 (1H, d, *J* = 12.9 Hz, H-7).

(3R,5S,6S,7E,9S)-Megastigman-7-ene-3,5,6,9-tetrol 9-O-*β*-D-glucopyranoside (**9**): ^1H NMR (DMSO-d_6, 300 MHz) δ_H: 0.72 (3H, s, H-11), 1.00 (3H, s, H-13), 1.05 (3H, s, H-13), 1.20 (3H, s, *J* = 6.3 Hz, H-10), 1.25 (1H, dd, *J* = 12.3, 3.2 Hz, H-2 eq), 1.48 (1H, m, *J* = 12 Hz, H-2ax), 1.56 (2H, m, H-4), 3.85 (1H, m, H-3), 4.14 (1H, d, *J* = 7.8 Hz, H-1′), 4.32 (1H, d, *J* = 7.5 Hz, H-9), 5.72 (1H, dd, *J* = 12.9, 6.0 Hz, H-8), 5.97 (1H, d, *J* = 12.9 Hz, H-7).

(6S,7E,9R)-6,9-Dihydroxy-4,7-megastigmadien-3-one 9-O-[*β*-D-xylopyranosyl-(1″ → 6′)-*β*-D-glucopyranoside] (**10**): ^1H NMR (DMSO-d_6, 300 MHz) δ_H: 0.90 (3H, s, H-12), 0.94 (3H, s, H-11), 1.18 (3H, s, *J* = 6.3 Hz, H-10), 1.84 (3H, s, H-13), 1.94 (1H, d, *J* = 16.5 Hz, H-2 eq), 2.35 (1H, d, *J* = 16.5 Hz, H-2ax), 2.63 (H, d, *J* = 8.7 Hz, H-6), 3.51 (1H, m, H-6′a), 3.68 (1H, m, H-5″b), 3.87 (1H, m, H-6′b), 4.18 (1H, d, *J* = 7.8 Hz, H-1′), 4.19 (1H, d, *J* = 7.8 Hz, H-1″), 4.28 (1H, d, *J* = 6.0 Hz, H-9), 5.56 (1H, dd, *J* = 15.9, 9.0 Hz, H-7), 5.68 (1H, dd, *J* = 15.9, 6.0 Hz, H-8), 5.79 (1H, s, H-4).

Linarionoside C (**11**): ^1H NMR (DMSO-d_6, 300 MHz) δ_H 1.03 (3H, s, H-11), 1.06 (3H, s, H-12), 1.10 (3H, s, *J* = 6.3 Hz, H-10), 1.57 (3H, s, H-13), 1.23–1.39 (3H, m, H-2, H-8), 1.72 (1H, m, H-4ax), 1.88 (2H, m, H-7), 2.25 (1H, m, H-4 eq), 3.43–3.67 (4H, m, H-6′, H-6″), 3.75 (H, m, H-9), 3.92(H, m, H-3), 4.17 (1H, d, *J* = 7.5 Hz, H-1′), 4.26 (1H, d, *J* = 7.8 Hz, H-1″).

(3S,9R)-3,9-Dihydroxy-megastigman-5-ene 3-O-primeveroside (**12**): ^1H NMR (DMSO-d_6, 300 MHz) δ_H: 0.98 (6H, s, H-11, 12), 1.05 (3H, s, *J* = 6.0 Hz, H-10), 1.57 (3H, s, H-13), 1.30–1.38 (3H, m, H-2, H-8), 1.74 (1H, m, H-4ax), 1.89 (2H, m, H-7), 2.23 (1H, m, H-4 eq), 3.55 (2H, m, H-5b, H-9), 3.68 (1H, m, H-5″b), 3.89 (2H, m, H-6′b, H-3), 4.20 (1H, d, *J* = 7.5 Hz, H-1′), 4.24 (1H, d, *J* = 7.8 Hz, H-1″).

See Table 2 for ^{13}C NMR of compounds **5–12**.

3.3. Cytotoxic activity

To the best of our knowledge, this is the first report of the presence of sesquiterpenes in hawthorn. Many investigations have shown that the highly bioactive group of sesquiterpenes with a wide spectrum of biological activities, particularly antitumour and cytotoxic activities, make this group of exceptional interest (Cordell, 1976). So far it has been reported that the farnesane-type sesquiterpenoid generally show modest *in vitro* inhibition of the proliferation of the HeLa human cervical carcinoma cell line (CCL-2), with an IC_{50} value of 4.5 ± 0.2 μm (Liu, Zhou, Zhang, & Xuan, 2009).

Compounds **1–4** were evaluated for their *in vitro* cytotoxicity against human glioma (U87) cell lines by the MTT method (Hussain et al., 1993). Unfortunately, the tested compounds exhibited no significant cytotoxicity in the cell model system used (Table 3). It is hard to explain why compound **1** and **2** are inactive in human glioma (U87) cell lines. Maybe it is selective for cell lines or the *cis*-olefin methylene group (Z) makes their stereoconfiguration unable to bind to target proteins.

5. Conclusions

Phytochemical study of *C. pinnatifida* has resulted in four new sesquiterpenes (**1–4**), together with eight known ones (**5–12**). As far as we know, this is the first report of sesquiterpene isolated from this plant. Taking it into consideration that this plant is both a medicine and food material, our work is clearly important, revealing its chemical constituents.

In this study we have shown that four new compounds from hawthorn leaves exhibited no significant cytotoxicity in the cell model system used, based on the *in vitro* assay (U87). Further studies should also be carried out to reveal more hawthorn leaves ingredients and other pharmaceutical functions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2011.05.049.

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