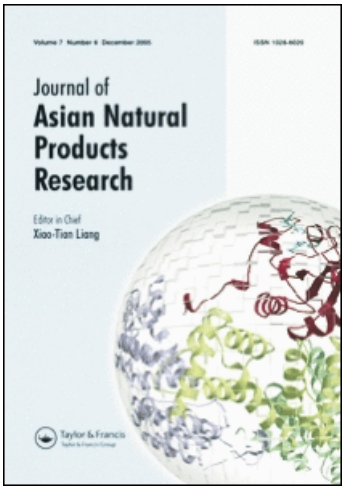

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Cytotoxic triterpenoid saponins from the roots of *Ardisia crenata*

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Ardisiacrenoside I (**1**), a new triterpenoid pentasaccharide with an unusual glycosyl glycerol side chain, was isolated from *Ardisia crenata* together with five closely related triterpenoid saponins. Their structures were elucidated by a combination of mass spectrometry, IR, 1D, and 2D NMR spectroscopy. Their cytotoxic activities were evaluated against several different human tumor cell lines by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) method.

Keywords: Myrsinaceae; *Ardisia crenata*; triterpenoid saponin; ardisiacrenoside I

1. Introduction

Ardisia crenata Sims (Myrsinaceae) is widely distributed in the southern part of China. Its roots have been used for the treatment of respiratory tract infections and menstrual disorders in traditional Chinese medicine, and showed significant anti-fertility effects in modern pharmacological studies. Earlier investigations on this plant resulted in the isolation of more than 20 triterpenoid saponins[1–3]. In our search for biologically active compounds from this plant, a new triterpenoid pentasaccharide, ardisiacrenoside I (**1**), was isolated, together with five known saponins (**2**–**6**) from the roots of this plant (Figure 1). Cytotoxic activities of compounds **1**–**6** were evaluated against human tumor cell lines HCT-8, Bel7402, BGC-823, A549, A2780, and KETR3.

2. Results and discussion

Compound **1** was obtained as a white amorphous powder, mp 276–278°C, $[\alpha]_D^{25}$ +20.0 (*c* 0.05, H_2O), and showed a positive reaction to the Liebermann–Burchard reagent. The IR spectrum showed absorption bands for hydroxyl (3417 cm^{-1}), two C=O groups (1707 and 1727 cm^{-1}), and a C=C bond (1612 cm^{-1}). Its HR-ESI-MS data showed a pseudo-molecular ion peak $[M + NH_4]^+$ at *m/z* 1358.6515 corresponding to molecular formula $C_{62}H_{100}O_{31}$. The ^1H NMR spectrum of **1** revealed the presence of six methyl singlets at δ 0.82, 0.88, 0.94, 1.10, 1.24, 1.66 (each s, 3H) and one olefinic proton at δ 5.54 (1H, br s). The ^{13}C NMR spectrum showed 62 carbon signals including two olefinic carbon signals at δ 122.8 and 144.3. These data suggested that the aglycone of **1** might possess an olean-12-ene skeleton. A further comparison with the ^{13}C NMR spectral data of **2** suggested that **1** has the same aglycone as **2**. Additionally, its ^1H and ^{13}C NMR spectral data also displayed five anomeric sugar signals at δ 4.86 (obscured by another signal), 4.97 (d, $J = 6.5$ Hz), 5.16 (d, $J = 7.5$ Hz), 5.58 (br s), and 6.03 (s) and δ 103.8, 102.8, 104.5, 100.5, and 101.2,

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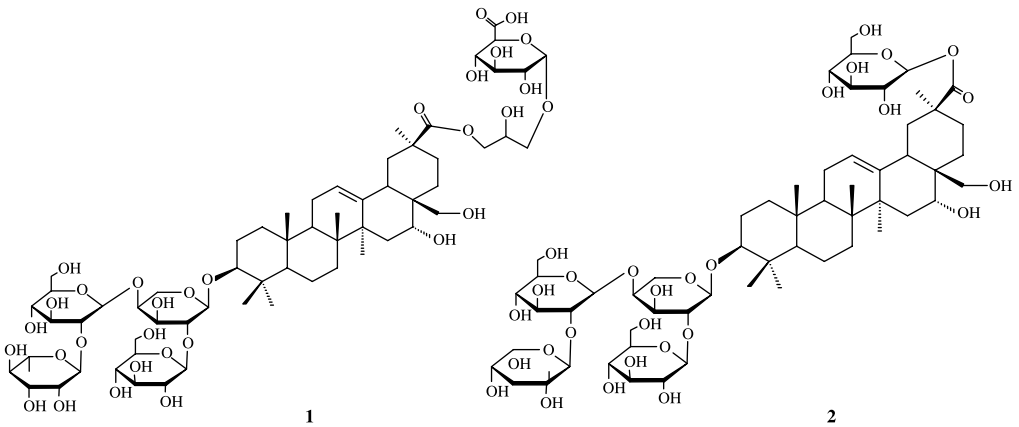
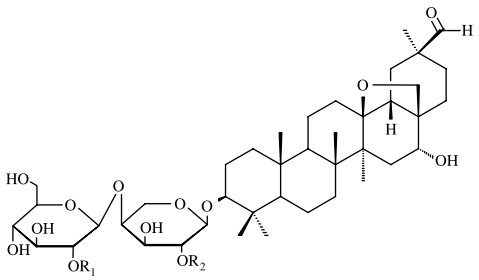


1 **2**



	R1	R2
3	α-L-rham	β-D-glc
4	β-D-xyl	H
5	H	β-D-glc
6	α-L-rham	H

Figure 1. Structures of compounds **1**–**6**.

respectively, which were confirmed by an HSQC spectrum. The protons within each spin system unit were identified by the interpretation of a TOCSY spectrum, while the carbons attached to these protons were determined by an HSQC spectrum. The sequence of protons of each sugar unit was determined by the use of $^{1}H-^{1}H$ COSY spectrum. After mapping all of the spin system for each individual sugar, a five-proton network ascribed to a glycerol moiety emerged from the $^{1}H-^{1}H$ COSY spectrum in the region of δ 3.90–4.60 and the corresponding carbons of this residue were detected at δ 65.7 (CH_2), 69.0 (CH), 70.8 (CH_2) via an HSQC experiment. These arrangements were further sustained by the HMBC spectrum (Figure 2). The monosaccharides were determined to be arabinose, glucose, rhamnose, and glucuronic acid by comparing their ^{13}C NMR spectral dada with those of authentic samples [4] and known compounds from this plant [1–3,5]. The above evidence suggested that **1** was a triterpenoid saponin with pentasaccharide and a glycerol moiety. The MS data at *m/z* 1339.4, 1321.4, 1089.5, 943.5, 781.4, 619.5, and 487.4 were fully in accordance with the above proposal (Figure 3).

All monosaccharides were determined to be in the pyranose forms by ^{13}C NMR evidence. The β-anomeric configurations for the glucose unit were derived from their large $^{3}J_{H1,H2}$ coupling constants (7–8 Hz).

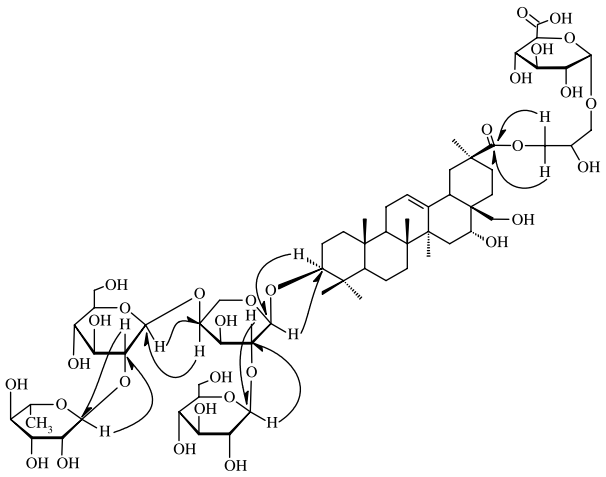


Figure 2. Key HMBC correlations of compound **1**.

The α-anomeric configuration of the rhamnose was deduced from the anomeric proton appearing as a singlet, together with its remarkably high chemical shift (δ 6.03). In a similar way, the α-anomeric configuration of the glucuronic acid was established by the ^{1}H non-splitting pattern of anomeric proton along with the relatively high chemical shift of anomeric carbon. The anomeric proton of the arabinose unit was obscured by another signal, thus no useful information was available from the ^{1}H NMR spectrum. However, the α-orientation was determined by comparing its NMR spectral data with those of pertinent reference compounds [4,5].

The sequence of the oligosaccharide chain was deduced from HMBC experiments, which showed correlations of H-1 of rhamnose with C-2 of the inner glucose and H-2 of the inner glucose with C-1 of rhamnose, H-1 of the inner glucose with the C-4 of the arabinose and H-4 of the arabinose with C-1 of the inner glucose, H-1 of the terminal glucose with C-2 of the arabinose and H-2 of the arabinose with C-1 of the terminal glucose, and the H-1 of the arabinose with the C-3 of the aglycone and H-3 of the aglycone with the C-1 of the arabinose. The above information indicated that the linkage of the sugar chain can be determined as an α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranosyl-(1 → 4)-[β-D-glucopyranosyl-(1 → 2)]-α-L-arabinopyranosyl unit and was attached to the C-3 of the aglycone. Another chain having the glycerol fragment and glucuronic acid residue at C-30 was deduced from the HMBC correlations (Figure 4) and chemical shift principles together with the analysis of the MS. In the HMBC spectrum, H-1 of the glycerol was correlated with C-30. The glucuronic residue was determined at C-3 of the glycerol unit due to 8.8 ppm shift downfield. These deductions were corroborated by MS peaks at *m/z* 1339.4 and 1089.5 in the negative ESI-MS, which have an interval of 250 Da (glucuronic acid + glycerol moiety; Figure 3). From the foregoing evidence, and also a comparison of its data with those of ardisicrenoside E [6], the structure of compound **1** was elucidated to be 3-β-*O*-α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranosyl-(1 → 4)-[β-D-glucopyranosyl-(1 → 2)]-α-L-arabinopyranosyl-16α,28-dihydroxy-olean-12-*en*-30-oic acid-30-*O*-[3′-*O*-(α-D-glucopyranuronate) glycerol(1′ → 30)] ester and named ardisiacrenoside I.

Additionally, compounds **2**–**6** were identified as ardisicrenoside D (**2**) [7], ardisiacrispin B (**3**), [2] primulanin (**4**), [6] cyclaminorin

(**5**), [8] and 3β-*O*-{α-L-rhamnopyranosyl-(1 → 2)-β-D-glucopyranosyl-(1 → 4)-α-L-arabinopyanosyl}-cyclamiritin A (**6**)[6] by the detailed analysis of NMR spectral data and comparison with those of published results.

The inhibitory assay of compounds **1**–**6** toward tumor cells was carried out by the MIT method with taxol as a positive control (Table 2). Three triterpenoid trisaccharides **4**–**6** showed strong effects against A2780 with IC_{50} values (μg/ml) of 2.48, 1.51, and 6.40, respectively. Ardisiacrispin B (**3**) showed stronger cytotoxicities against HCT-8, Bel7402, BGC-823, A549, A2780, and

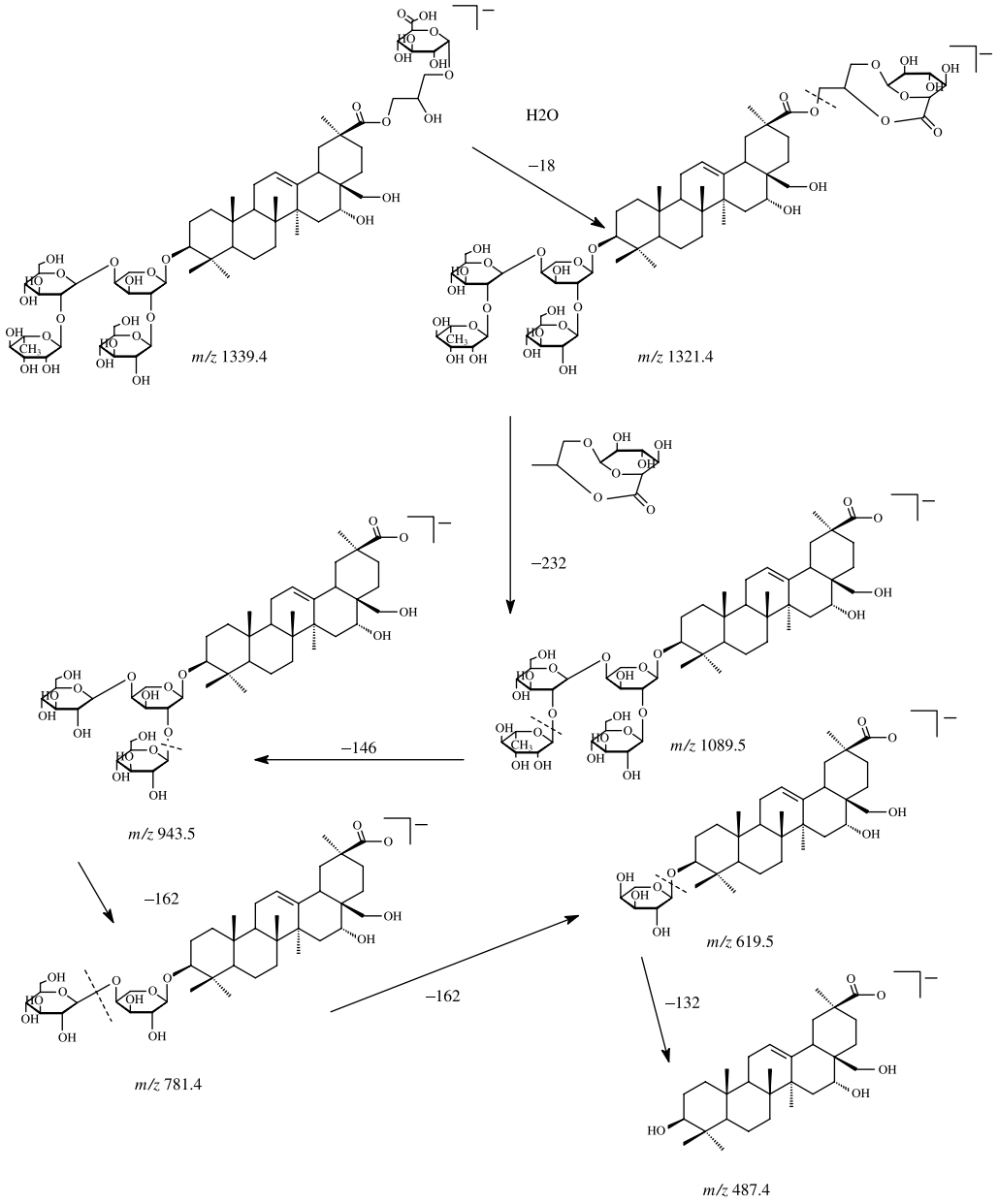


Figure 3. The major MS fragments of compound **1**.

KETR3 with IC_{50} values (μg/ml) of 1.59, 1.67, 1.78, 1.78, 2.05, and 1.64, respectively. Compounds **1** and **2** showed no cytotoxicities against cell lines mentioned above.

3. Experimental

3.1 General experimental procedures

Melting points were measured on a Boetius apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer 683 spectrometer. All ^1H and ^{13}C NMR spectra and 2D NMR data were recorded in C_5D_5N at 343 K on an Inova-500 spectrometer. ESI-MS were measured on an Agilent 1100 series LC/MSD Trap spectrometer. HR-ESI-MS

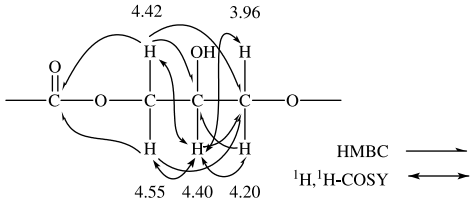


Figure 4. Key HMBC and $^1H-^1H$ COSY correlations of glycerol moiety.

Table 1. ^1H and ^{13}C NMR spectral data of compound **1** (in C_5D_5N, $t = 343$ K, δ in ppm, J in Hz).

No.	δ_C	δ_H	No.	δ_C	δ_H
1	38.8		Glucose (G) (Terminal)		
2	26.0				
3	89.1	3.12 (m)	G-1	104.5	5.16 (d, 7.5 Hz)
4	39.2		G-2	75.6	3.86
5	55.7	0.68 (d, 11.5 Hz)	G-3	77.4	4.14
6	18.3		G-4	71.4	4.02
7	33.1		G-5	77.4	3.87
8	39.9		G-6	62.4	4.19, 4.29
9	47.0		Glucose (G′)		
10	36.7		(Inner)		
11	23.6		G′-1	102.8	4.97 (d, 6.5 Hz)
12	122.8	5.54 (br s)	G′-2	77.8	4.01
13	144.3		G′-3	78.5	4.00
14	41.6		G′-4	71.6	3.88
15	34.5		G′-5	77.8	3.69
16	73.6	4.52 (br s)	G′-6	62.3	4.09, 4.29
17	40.1		Rhamnose (R)		
18	43.4		R-1	101.2	6.03
19	44.2		R-2	71.7	4.53
20	44.5		R-3	72.0	4.45
21	33.3		R-4	74.0	4.07
22	31.2		R-5	69.4	4.72
23	28.1	1.10 (s)	R-6	18.3	1.65 (d, 6.5 Hz)
24	16.5	0.94 (s)	30-*O*-Glycerol		
25	15.5	0.82 (s)	Gly-1	65.7	4.42, 4.55
26	16.9	0.88 (s)	Gly-2	69.0	4.40
27	27.2	1.66 (s)	Gly-3	70.8	3.96, 4.20
28	70.1	3.41, 3.59 (d, 10.5 Hz)	Glucuronic acid		
29	28.5	1.24 (s)	G_A-1	100.5	5.58 (br s)
30	178.1		G_A-2	70.9	4.09
3-*O*-Sugar			G_A-3	74.1	4.40
Arabinose (A)			G_A-4	73.3	4.16
A-1	103.8	4.86	G_A-5	72.0	4.64
A-2	79.6	4.38	G_A-6	177.9	
A-3	71.4	4.40			
A-4	74.9	4.47			
A-5	62.9	3.80, 4.39 (d, 9.5 Hz)			

was performed on a Finnigan LTQ FTMS. Optical rotations were recorded on a Perkin-Elmer 241 digital polarimeter. Column chromatography was performed with a DA101 macroporous resin (Shenyang Chemical Inc., Shenyang, China) and silica gel (200–300 mesh; Qingdao Marine Chemical Inc., Qingdao, China).

3.2 Plant material

The roots of *A. crenata* were collected in Jiujiang, Jiangxi province of China, and were identified by Professor Ceming Tan (Institute of Forest Resource, Jiujiang, Jiangxi province of China). The roots were harvested and air-dried at room temperature. A voucher specimen is deposited in the herbarium of the Institute of Materia Medica, Peking Union Medical College and Chinese Academy of Medical Sciences, Beijing, China.

3.3 Extraction and isolation

The roots (6.0 kg) of *A. crenata* were refluxed with 80% EtOH three times and the extract was concentrated under reduced pressure to give a residue (800 g). The residue was suspended in H_2O and partitioned successively with petroleum ether, EtOAc, and BuOH to yield petroleum ether (31.8 g), EtOAc (53.5 g), and BuOH (458 g) soluble fractions, respectively. The *n*-butanol fraction (450 g) was chromatographed on a DA101 macroporous resin eluted with H_2O and 15, 30, 50, 75, and 95% EtOH. The 30, 50, and 75% EtOH-eluted solutions were combined on the basis of TLC analyses and gave a residue (314 g). The residue (300 g) was chromatographed on a Si-gel column eluted with a gradient of $CHCl_3$–CH_3OH–H_2O and collected in different gradient fractions: fraction 5 (5:1:0), fraction 8 (2:1:0), fraction 10 (1:1:0), and fraction 14 (1:1:1). These fractions were chromatographed over reversed-phase silica gel, eluted with a gradient increasing MeOH in H_2O. As a result, fraction 5 yielded compounds **4** (80 mg), **5** (35 mg), and **6** (50 mg), fraction 8 yielded compound **3** (400 mg), fraction 10 yielded compound **2** (90 mg), and fraction 14 yielded compound **1** (30 mg).

Ardisiacrenoside I (**1**) An amorphous white powder; mp 276–278°C, $[\alpha]_D^{25}$ +20.0 (*c* 0.05, H_2O); IR (KBr) ν_{max} 3417, 1707, 1612 cm^{-1}; ^1H and ^{13}C NMR spectral data: see Table 1; ESI-MS *m/z* 1339.4 $[M - H]^-$, 1321.4, 1089.5, 943.5, 781.4, 619.5, 487.4; HR-ESI-MS *m/z* 1358.6515 $[M + NH_4]^+$ (calcd for $C_{62}H_{100}O_{31}NH_4$, 1358.6594).

Cytotoxicity against human tumor cell was measured in a 5-day MTT test for HCT-8 human ileocecal carcinoma, Bel7402 human hepatocellular cancer, BGC-823 stomach adenocarcinoma, A549 human lung carcinoma, A2780 ovary adenocarcinoma, and KETR3 human renal carcinoma [9]. Briefly, 1×10^3 cells/100 μl were seeded in 96-well microplates and preincubated for 24 h to allow cell attachment. This medium was then aspirated, and 100 μl of fresh medium containing various concentrations of the test

Table 2. Cytotoxic activities of compounds **1**–**6**.

	IC_{50} (μg/ml)					
Sample	HCT-8	Bel7402	BGC-823	A549	A2780	KETR3
1	>10	>10	>10	>10	>10	>10
2	>10	>10	>10	>10	>10	>10
3	1.59	1.67	1.78	1.78	2.05	1.64
4	>10	>10	>10	>10	2.48	>10
5	>10	>10	>10	>10	1.51	>10
6	>10	>10	>10	>10	6.40	>10
Taxol	0.0316	0.0871	0.0060	0.0166	0.0054	0.0055

drug were added to the cultures. The cells were incubated with each drug for 5 days. Cell survival was evaluated by adding 50 μl of MTT reagent (5 mg MTT/ml in RPMI 1640 medium) to each well. After 4 h reincubation at 37°C, 100 μl DMSO was added to dissolve the precipitate of reduced MTT. Microplates were agitated on a rotation platform at room temperature for 15 min, and the absorbance of the reaction mixtures was determined at 570 nm with a multiwell scanning spectrophotometer.

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