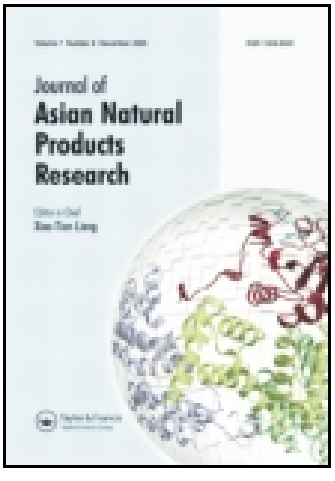


Journal of Asian Natural Products Research

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/ganp20

A new triterpenoid saponin from Ardisia gigantifolia

Li-Hua Mu[a], Xiao-Wu Huang[b], Dai-Hong Guo[a], Xian-Zhe Dong[a] & Ping Liu[a]

[a] Department of Clinical Pharmacology, General Hospital of PLA, Beijing, 100853, China

[b] Medical Materials Center, General Hospital of PLA, Beijing, 100853, China

Published online: 22 Jul 2013.

To cite this article: Li-Hua Mu, Xiao-Wu Huang, Dai-Hong Guo, Xian-Zhe Dong & Ping Liu (2013) A new triterpenoid saponin from Ardisia gigantifolia, Journal of Asian Natural Products Research, 15:10, 1123-1129, DOI: 10.1080/10286020.2013.817390

To link to this article: http://dx.doi.org/10.1080/10286020.2013.817390

Journal of Asian Natural Products Research, 2013
Vol. 15, No. 10, 1123–1129, http://dx.doi.org/10.1080/10286020.2013.817390

A new triterpenoid saponin from *Ardisia gigantifolia*

Li-Hua Mu[a1], Xiao-Wu Huang[b1], Dai-Hong Guo[a], Xian-Zhe Dong[a] and Ping Liu[a]*

[a]*Department of Clinical Pharmacology, General Hospital of PLA, Beijing 100853, China;* [b]*Medical Materials Center, General Hospital of PLA, Beijing 100853, China*

(*Received 28 November 2012; final version received 16 June 2013*)

A new triterpenoid saponin, named 3-*O*-β-D-glucopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 2)-[α-L-rhamnopyranosyl-(1 → 3)]-β-D-glucopyranosyl-(1 → 4)-[β-D-glucopyranosyl-(1 → 2)]-α-L-arabinopyranosyl-3β,16α,28,30-tetrahydroxy-olean-12-ene (**1**), along with four known triterpenoids (**2**–**5**), was isolated from the rhizomes of *Ardisia gigantifolia*. Their structures were elucidated by spectroscopic methods. Compounds **1**–**4** showed cytotoxic activity against Hela, EJ, BCG, and HepG-2 cell lines. The percentage of early apoptotic cells after treatment with **1** was significantly increased compared with control cells ($p < 0.05$).

Keywords: *Ardisia gigantifolia*; triterpenoid saponin; cytotoxic activity

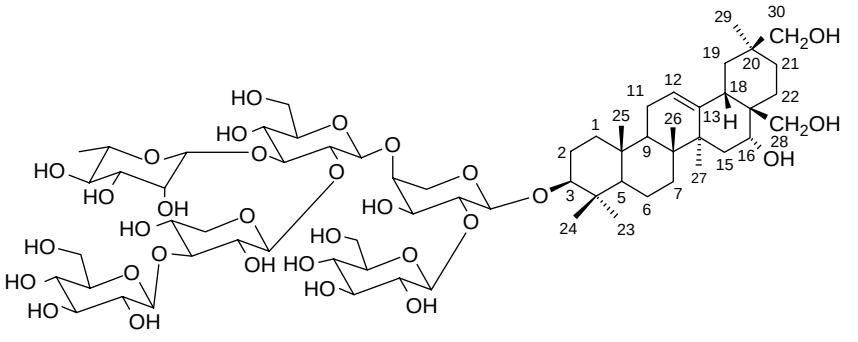
1. Introduction

Various triterpenoid saponins have been isolated from *Ardisia gigantifolia*. Many triterpenoid saponins have been previously reported to possess antitumor activities [1,2]. The triterpenoid saponins from *A. gigantifolia* are particularly interesting because most of them showed good antitumor activity. We recently isolated seven new oleanane-type triterpenoid saponins from the rhizomes of *A. gigantifolia*, and some of them exhibit cytotoxicity against human cancer cell lines [3,4]. Recently, we have also found one new saponin, along with four known saponins (Figure 1). This article reports the isolation and structural elucidation of these saponins, as well as their potent cytotoxicities against Hela human cervical carcinoma cells, EJ human bladder tumor cells, HepG-2 human hepatoma cells, and BCG human gastric carcinoma cells.

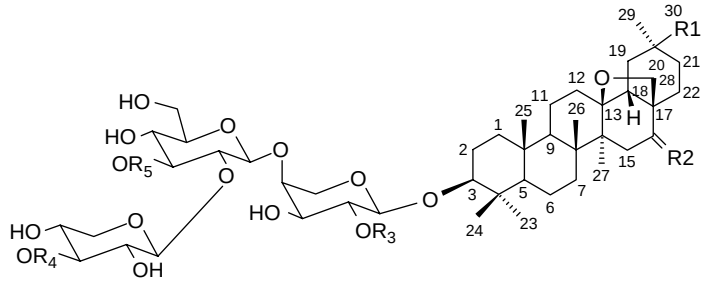
2. Results and discussion

Compound **1** was obtained as a white powder. HR-ESI-MS of **1** showed the quasi-molecular ion at *m/z* 1377.6661 $[M + Na]^+$, establishing the molecular formula of $C_{64}H_{106}O_{30}$. The ^{13}C NMR spectrum of **1** exhibited six sp^3 carbon signals at δ 15.8, 16.9, 17.1, 24.9, 27.4, and 28.2 and two sp^2 hybrid carbons at δ 122.4 and 145.3, which indicated that the aglycone of **1** was an olean-12-en skeleton, similar to the aglycone of ardisimamilloside C [5]. The existence of hydroxyl groups at C-3 and C-16 was deduced from the ^{13}C NMR resonances at δ 89.1 and 74.3. The configuration of hydroxyl at C-3 was determined using NOESY correlations. The NOESY correlation of Hax-3 (δ 3.14) with H-5 (δ 0.71) indicated that the hydroxyl at C-3 should be β-orientation. In the NOESY spectrum, the spatial proximity between H-16 and H-28 indicated an α-orientation of 16-OH. The α-orientation of 16-OH was further determined by comparing C-16 (δ 74.3) chemical shift with that in the literature (16α-OH: ca. δ 77.0; 16β-OH: ca. δ 64.0) [6]. The hydroxymethyl for C-30 was confirmed by the HMBC correlation

*Corresponding author. Email: liuping301@126.com



1



	R_1	R_2	R_3	R_4	R_5
2	CH_3	α-OH	β-D-glucopyranosyl	β-D-glucopyranosyl	α-L-rhamnopyranosyl
3	CH_3	α-OH	β-D-glucopyranosyl	H	H
4	CHO	α-OH	β-D-glucopyranosyl	β-D-glucopyranosyl	α-L-rhamnopyranosyl
5	CHO	α-OH	H	H	H

Figure 1. The structures of compounds **1–5**.

between H-29 at δ_H 1.11 and C-30 at δc 66.4. The existence of hydroxyl at C-28 was deduced by comparing the chemical shifts with those of ardisimamilloside C. These assignments could be confirmed through long-range correlations in the HMBC spectrum. Therefore, the aglycon moiety of **1** was identified as 3β,16α, 28,30-tetrahydroxy-olean-12-ene.

In ^{1}H NMR spectrum, six anomeric signals appeared at δ 5.89 (1H, brs, Rha-1), 5.37 (1H, d, $J = 7.8$ Hz, GlcI-1), 5.18 (1H, d, $J = 7.8$ Hz, GlcIII-1), 5.00 (1H, d, $J = 6.6$ Hz, Xyl-1), 4.86 (1H, d, $J = 7.8$ Hz, GlcII-1), and 4.80 (1H, d, $J = 6.0$ Hz, Ara-1). On acid hydrolysis with 2 M HCl, **1** afforded sugar moieties that were identified as L-rhamnose, L-arabinose, D-xylose, and D-glucose in the relative proportions of 1:1:1:3 based on the GC–MS analysis of their chiral derivatives [7]. All proton signals due to sugars were assigned by careful analysis of HMQC–TOCSY and NOESY spectra, and the carbon signals were assigned by HMQC and HMQC–TOCSY spectra. The attachment points of the sugar chain and interglycosidic linkage were established by HMBC and NOESY correlations (Figure 2). HMBC correlations were observed between H-1 (δ 4.80) of arabinosyl and C-3 (δ 89.1) of the aglycone, H-1 (δ 5.37) of glucosyl-I and C-2 (δ 79.7) of arabinosyl, H-1 (δ 4.86) of glucosyl-II and C-4 (δ 78.1) of arabinosyl, H-1 (δ 5.00) of xylosyl and C-2 (δ 81.3) of glucosyl-II, and

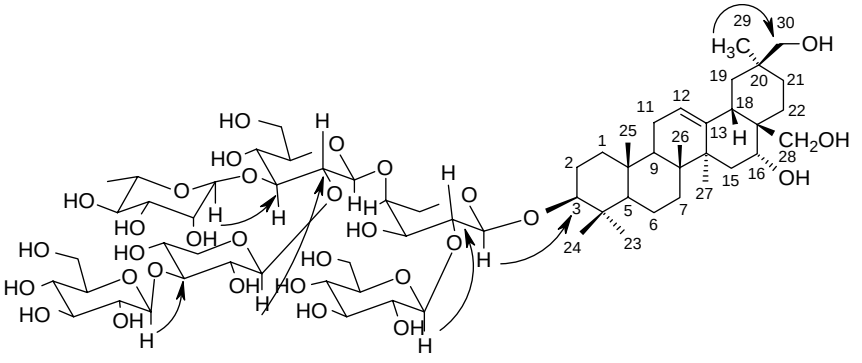


Figure 2. Selected HMBC correlations for compound **1**.

H-1 (δ 5.89) of rhamnosyl and C-3 (δ 85.5) of glucosyl-II and GlcIII-H-1 at δ_H 5.18 and Xyl-C-3 at δ_C 87.1. NOESY correlations were observed between GlcI-H-1 at δ_H 5.37 and Ara-H-2 at δ_H 4.52, GlcII-H-1 at δ_H 4.86 and Ara-H-4 at δ_H 4.19, Xyl-H-1 at δ_H 5.00 and GlcII-H-2 at δ_H 3.95, Rha-H-1 at δ_H 5.89 and GlcII-H-3 at δ_H 4.12, and GlcIII-H-1 at δ_H 5.18 and Xyl-H-3 at δ_H 4.05. In the ^{1}H NMR spectrum of **1**, the relatively large $^3J_{H\text{-}1,H\text{-}2}$ coupling constant of the anomeric protons for the Glc, Xyl, and Ara (between 5.6 and 8.0 Hz) moieties indicated a β-configuration for D-Glc and D-Xyl and an α-configuration for L-Ara. In the HMBC spectrum, the CH coupling constant of C-1 (δ 103.6) was 174 Hz according to the satellite peaks, indicating that the glycosidic bond of rhamnose was linked in the α-orientation [8]. The three-bond HMBC correlations from the anomeric proton to C-3 and C-5 of the rhamnose also indicated that the anomeric proton is equatorial, thus possessing an α-configuration in the $^1C_4$ form. On the basis of the above data, the structure of **1** was established as 3-*O*-β-D-glucopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 2)-[α-L-rhamnopyranosyl-(1 → 3)]-β-D-glucopyranosyl-(1 → 4)-[β-D-glucopyranosyl-(1 → 2)]-α-L-arabinopyranosyl-3β,16α,28,30-tetrahydroxy-olean-12-ene.

The structure of the known compound was identified by spectral data as 3β-*O*-β-D-glucopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 2)-[α-L-rhamnopyranosyl-(1 → 3)]-β-D-glucopyranosyl-(1 → 4)-[β-D-glucopyranosyl-(1 → 2)]-α-L-arabinopyranosyl-16α-hydroxy-13,28-epoxy-oleanane (**2**) [3], lysikoianoside (**3**) [9], cyclamiretin A 3β-*O*-β-D-glucopyranosyl-(1 → 3)-β-D-xylopyranosyl-(1 → 2)-[α-L-rhamnopyranosyl-(1 → 3)]-β-D-glucopyranosyl-(1 → 4)-[β-D-glucopyranosyl-(1 → 2)]-α-L-arabinopyranosyl (**4**) [10], and primulanin (**5**) [11]. The cytotoxicities of **1–4** against four human cancer cell lines, namely Hela human cervical carcinoma cells, EJ human bladder tumor cells, HepG-2 human hepatoma cells, and BCG human gastric carcinoma cells, were evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay described in previous papers [3]. The IC_{50} value of each compound was measured on the basis of cell viability after a 48-h treatment (Table 2).

Compound **1** was examined for its cytotoxic activities against HepG-2 cells. HepG-2 cells were treated with compound **1** at 0, 2, 4, 8, 16, and 32 μM for 48 h. We found that compound **1** inhibited cell proliferation in HepG-2 cells, and the IC_{50} value was 8.7 μM. Next, we examined the apoptosis by flow cytometry using fluorescein isothiocyanate-labelled Annexin V (Annexin V-FITC) and propidium iodide (PI) double staining. The results revealed

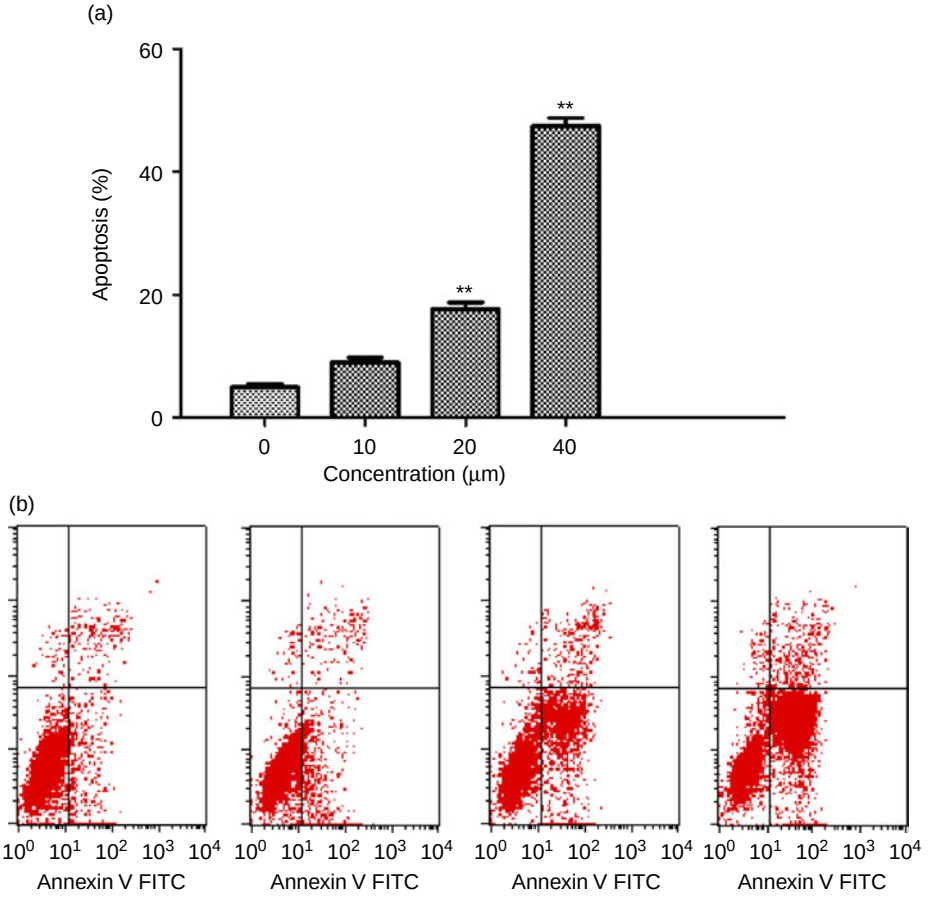


Figure 3. Apoptosis induced by compound **1** in HEPG-2 cells. (a) Cells were treated by 0, 10, 20, and 40 μM compound **1** and collected after incubation for 24 h. And the cells were stained with Annexin V-FITC and PI and detected for apoptosis by flow cytometry. $^{**}p < 0.01$. (b) Representative flow cytometry scans of apoptosis induced by compound **1** with Annexin V-FITC and PI staining.

that the percentage of early apoptotic cells (Annexin V-FITC staining) after compound **1** treatment significantly increased compared with the control cells [$p < 0.05$; Figure 3 (a),(b)].

3. Experimental

3.1 *General experimental procedures*

Optical rotations were measured with a PolAAr 3005 polarimeter (Optical Activity Ltd, Huntingdon, UK). IR spectra were obtained on a Nicolet 6700 FT-IR (by a KBr disk method) spectrometer (Thermo-Fisher, Wultham, MA, USA). 1D NMR and 2D NMR spectra were recorded on a VARIAN INOVA 600 (Palo Alto, CA, USA) spectrometer in pyridine-d_5. Chemical shifts were expressed in δ from internal tetramethylsila; coupling constants were reported in hertz. HR-ESI mass spectra were recorded using Waters SYNAPT (ESI-Q-TOF) mass spectrometer (Milford, MA, USA). The extract was purified using macroporous resin D101 (Shenyang Chemical, Inc., Shenyang, China) and column chromatography with silica gel (Qingdao Haiyang Chemical Co. Ltd,

Qingdao, China). The compounds were finally isolated with the help of a Hanbon Scicr&Tech NP7000 preparative HPLC system equipped with Shodex RI-102 detector using an ODS (octadecyl-bonded silica) column (YMC-ODS, 20 mm × 250 mm, 5 μm, Kyoto, Japan).

3.2 Plant material

The dry rhizomes of *A. gigantifolia* Stapf were obtained from Guangzhou, Guangdong, China, in 2007 and were authenticated by Prof. Ping Liu. The voucher specimen (collection no. 029) was deposited in the Traditional Chinese Medicine Pharmacy of General Hospital of PLA, Beijng, China.

3.3 Extraction and isolation

The dried and cut rhizome parts of *A. gigantifolia* (13.5 kg) were percolated with 60% ethanol. The 60% ethanol extract (1.54 kg) was partitioned successively between water and petroleum ether, ethyl acetate, and *n*-butanol. After removing the solvent, the *n*-butanol layer (600 g) was subjected to a macroporous resin D101 column (5 kg, 11 cm × 82.5 cm) and eluted with H_2O and 30%, 50%, 70%, and 95% EtOH. The 50% EtOH eluate (10 g) was further separated by ODS column chromatography (MeOH:H_2O, 45:55 → 55:45 → 60:40 → 65:35). The 65% MeOH eluate fraction (281 mg) was further separated, respectively, by preparative HPLC at a flow rate of 15.0 ml/min to afford **1** (9.5 mg, t_R 7.1 min, 42% CH_3CN), **2** (24 mg, t_R 18.9 min, 42% CH_3CN), and **4** (9.5 mg, t_R 7.1 min, 42% CH_3CN). The 70% EtOH eluate (52 g) was chromatographed over a silica gel column with $CHCl_3$:MeOH (3:1) to give six fractions. Fraction 3 (0.4 g) was further separated by preparative HPLC at a flow rate of 15.2 ml/min to afford **5** (7.3 mg, t_R 8.9 min, 60% MeOH). Fraction 7 (10 g) was subjected to a silica gel column with $CHCl_3$:MeOH:H_2O (65:35:10), which gave three fractions 5-1, 5-2, and 5-3. Fraction 5-2 (2 g) was further separated by preparative HPLC at a flow rate of 15.2 ml/min to afford **3** (52 mg, t_R 10.6 min, 73% MeOH).

3.3.1 Compound *1*

White amorphous powder: $[\alpha]_D^{25}$ −2.0 (c = 0.05, MeOH); IR (KBr) ν_{max}: 3422, 2923, 1636, 1384, 1125, and 1075 cm^{-1}; for ^1H NMR (600 MHz, pyridine-d_5) and ^{13}C NMR (150 MHz, pyridine-d_5) spectral data, see Table 1. HR-ESI-MS (positive) m/z: 1377.6661 $[M + Na]^+$ (calcd for $C_{64}H_{106}O_{30}Na$, 1377.6643).

3.4 Acid hydrolysis of compound 1 and determination of absolute configuration of monosaccharides

Compound **1** (5 mg) was heated in 2 mol/l HCl (5 ml) at 90°C for 4 h. The reaction mixture was extracted with $CHCl_3$ (5 ml × 3). The remaining aqueous layer was concentrated to dryness to give a residue. The residue was dissolved in pyridine (0.1 ml), to which 0.08 M D-cysteine methyl ester hydrochloride in pyridine (0.15 ml) was added. The mixture was kept at 60°C for 1.5 h. After the reaction mixture was dried *in vacuo*, the residue was trimethylsilylated with 1-trimethylsilylimidazole (0.1 ml) for 2 h. The mixture was partitioned between hexane and H_2O (0.3 ml each), and the hexane extract was analyzed by GC–MS under the following conditions: capillary column, DB-17 MS (30 m × 0.25 mm inner diameter, 0.25 μm film), ion source temperature, 250°C; interface temperature, 300°C; and carrier, helium gas (1.16 ml/min). In the acid hydrolysate of **1**, L-rhamnose, D-glucose, L-arabinose, and D-xylose were confirmed by comparison of the retention times of their derivatives with those of the derivatives of L-rhamnose, D-glucose, L-arabinose, and D-xylose prepared in a similar way, which showed retention times of 5.25, 7.15, 5.08, and 6.11 min, respectively.

Table 1. ^{13}C NMR and ^{1}H NMR spectral data of compound **1** (600 MHz, pyridine-d_5).

Position	^{1}H	^{13}C	Position	^{1}H	^{13}C
1	0.82, 1.47	39.0	Ara-1	4.80 (d, 6.0)	104.5
2	1.75, 1.91	26.4	2	4.52	79.7
3	3.14 (dd, 11.4, 4.0)	89.1	3	4.49	72.7
4	–	39.6	4	4.19	78.1
5	0.71 (d, 11.4)	55.9	5	3.61, 4.52	63.7
6	1.25,1.42	18.6	GluI-1	5.37 (d, 7.8)	105.0
7	1.31, 1.54	33.3	2	4.05	76.2
8	–	40.1	3	4.26	78.2
9	1.71	47.2	4	4.10	71.8
10	–	37.0	5	4.02	78.1
11	1.88	23.9	6	4.30, 4.39	63.0
12	5.37 (br s)	122.4	GlcII-1	4.86 (d, 7.8)	104.0
13	–	145.3	2	3.95	81.3
14	–	42.1	3	4.12	85.5
15	1.63, 2.21	34.8	4	4.10	69.8
16	4.64	74.3	5	3.68	78.0
17	–	41.1	6	4.30, 4.39	62.2
18	2.49	42.6	Xyl-1	5.00 (d, 6.6)	105.1
19	1.30, 2.72 (t, 13.2)	48.4	2	4.04	75.3
20	–	37.2	3	4.05	87.1
21	2.19, 2.28	30.6	4	4.14	69.3
22	2.21, 2.30	31.4	5	3.61, 3.75	70.3
23	1.17 (s)	28.2	Rha-1	5.89 (brs)	103.6
24	1.05 (s)	16.9	2	4.95	72.2
25	0.83 (s)	15.8	3	4.54	72.8
26	0.93 (s)	17.1	4	4.33	73.9
27	1.80 (s)	27.4	5	4.80	70.5
28	3.61 (d, 10.8), 3.75 (d, 10.8)	69.8	6	1.65 (d, 6.0)	18.6
29	1.11 (s)	24.9	GlcIII-1	5.18 (d, 7.8)	104.9
30	3.56 (t, 11.4), 4.47	66.4	2	4.04	74.2
			3	4.04	78.0
			4	4.22	71.9
			5	3.95	78.5
			6	4.19, 4.52	62.6

Notes: Assignments based on TOCSY, HMQC, and HMBC experiments. Overlapped signals are reported without designating multiplicity.

Table 2. Cytotoxic activities of compounds **1–4** against four human cancer cell lines.

	IC_{50} (μM)			
Sample	BCG	Hela	EJ	HepG-2
1	6.5 ± 0.21	7.7 ± 0.22	6.7 ± 0.15	8.7 ± 0.18
2	3.1 ± 0.12	3.3 ± 0.23	3.6 ± 0.15	3.2 ± 0.22
3	8.1 ± 0.32	7.3 ± 0.13	7.2 ± 0.21	5.8 ± 0.12
4	6.7 ± 0.23	8.1 ± 0.33	9.2 ± 0.19	8.7 ± 0.25
Cisplatin	14.8 ± 0.71	10.9 ± 0.52	12.4 ± 0.51	12.8 ± 0.45

Note: Values were expressed as mean ± SD of three independent determinations.

3.5 Cytotoxicity assay

Hela, EJ, BCG, and HepG-2 cells were seeded in 96-well plates at a density of 0.8×10^4 cells/well and incubated for 24 h. Test samples were dissolved in dimethyl sulfoxide (DMSO) and added to the medium. Following a 48-h incubation, the wells were incubated with MTT (100 μl/well concentrated at 5 mg/ml) at 37°C for 4 h. The supernatant was aspired, and 200 μl of DMSO was added to redissolve the formazan crystals. The optical density was measured by an enzyme-linked immunosorbent assay plate reader at 490 nm (PerkinElmer 1420-012, Fremont, CA, USA). The experiments were repeated three times, and the cytotoxicity was expressed as the IC_{50} value, which reduces the number of viable cells by 50% (Table 2).

3.6 Assessment of apoptosis

For the detection of early apoptosis and secondary necrosis, Annexin V-FITC kit purchased from Immunotech (Marseille, France) was used according to manufacturer's recommendations. Briefly, cells were stained simultaneously with PI and Annexin V-FITC and assessed with a flow cytometer (Beckman-Coulter EPICS XLe) [12].

3.7 Statistics

The results are expressed as the mean ± standard deviation (SD). Statistical significance ($p < 0.05$) was evaluated by one-way analysis of variance followed by Bonferroni post hoc test. All experiments presented were conducted three times.

Acknowledgments

This work was financially supported by the National Nature Science Foundation of China (No. 31000153). The authors are grateful to Mr Li-Ping Kang for the NMR and HR-ESI-MS measurement in the Instrumentation Center of the Academy of Military Medical Sciences.

Note

1. These authors contributed equally to this work.

References

[1] A. Yokosuka, T. Sano, K. Hashimoto, and H. Sakagami, *Chem. Pharm. Bull.* **57**, 1425 (2009).
[2] W. Li, X.Y. Bi, K. Wang, and D.X. Li, *Phytochemistry* **70**, 816 (2009).
[3] L.H. Mu, Q.Q. Gong, and H.X. Zhao, *Chem. Pharm. Bull.* **58**, 1248 (2010).
[4] L.H. Mu, N.Y. Wei, and P. Liu, *Planta Med.* **78**, 617 (2012).
[5] J. Huang, Y. Ogihara, and H. Zhang, *Chem. Pharm. Bull.* **48**, 1413 (2000).
[6] S.B. Mahato and A.P. Kundu, *Phytochemistry* **37**, 1517 (1994).
[7] J.G. Luo, L. Ma, and L.Y. Kong, *Bioorg. Med. Chem.* **16**, 2912 (2008).
[8] Y.H. Pei, H.M. Hua, Z.L. Li, and G. Chen, *Acta Pharm. Sin.* **46**, 127 (2011).
[9] H. Kohda, O. Takeda, and S. Tanaka, *Chem. Pharm. Bull.* **37**, 3304 (1989).
[10] P. Wen, X.M. Zhang, Z. Yang, N.L. Wang, and X.S. Yao, *J. Asian Nat. Prod. Res.* **10**, 873 (2008).
[11] C. Lavaud, G. Massiot, J.B. Barrera, and L. Menolivier, *Phytochemistry* **37**, 1671 (1994).
[12] Q.L. Zhao, Y. Fujiwara, and T. Kondo, *Free Rad. Biol. Med.* **40**, 1131 (2006).