

Discovery of pentacyclic triterpenoid saponins with anti-proliferative activities from *Ardisia lindleyana*

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Research Article

Keywords: *Ardisia lindleyana*, Saponin, Triterpenoid, Anti-proliferative activity, Apoptosis

Posted Date: November 15th, 2022

DOI: <https://doi.org/10.21203/rs.3.rs-2251495/v1>

Abstract

One new pentacyclic triterpenoid saponin, ardisiapunine E (**1**), along with two known compounds were isolated from the root of *Ardisia lindleyana* D.Dietr. Their structures were elucidated by ^1H and ^{13}C NMR, DEPT, HMBC, HSQC, ^1H - ^1H COSY and NOESY spectroscopic analyses, ESI-MS, and literature. Compounds **1-3** exhibited obvious anti-proliferative activities against HeLa cell line in a dose- and time-dependent manner through inducing G2/M phase arrest and apoptosis *in vitro*, both consisting of pentacyclic triterpenes and sugar. Hence, this study identified a new and two known pentacyclic triterpenoid saponin promoting apoptosis as a potential therapeutic option for cancer.

Introduction

Ardisia lindleyana D.Dietr, an evergreen dwarf shrub, mainly distributes in areas of the south China [1] and is known for its use as an important traditional Miao Minority medicine [2] and an ornamental shrub. It is a representative of genus *Ardisia* which is the largest member in the family Primulaceae. Previous chemical studies of this plant have reported the isolation of several triterpenoids [3], triterpenoid saponins [4], quinones [5, 6] and isocoumarins [3], and other such bioactive compounds. Additionally, the biological studies on its constituents have demonstrated their anti-proliferative and anti-inflammatory activities. They are a very large and diverse class of plant products with a wide distribution, occurring naturally in many plant systems [7], and they act as potential anti-neoplastic pharmacophores, antiviral, antioxidants, or antibacterial agents [8]. In particular, Triterpenoid saponins play a crucial role in inhibiting tumor cell proliferation. It was reported that seventeen 13,28-epoxy triterpenoid saponins obtained from *Ardisia gigantifolia* STAPF could inhibit proliferation of MDA-MB-231 Cells [9]. Four new triterpenoid saponins isolated from the rhizome of *Ardisia gigantifolia* STAPF exhibited anti-carcinogenic activity against three human cancer cell lines [10]. Two new triterpenoid saponins, ardisicrenoside R and S, isolated from roots of *Ardisia crenata* had also been shown manifest cytotoxic activities against A549 (human pulmonary carcinoma cell line), MCF-7 (human breast cancer cell line), HepG2 (human hepatocarcinoma cell line) and MDA-MB-231 (human breast cancer cell MDA-MB-231) cell lines [11]. Therefore, we attempted further separation of the constituents from the plant to isolate more novel anti-proliferative agents.

In a continuation of our phytochemical investigations of *Ardisia lindleyana* D.Dietr [12], we are interested to its triterpenoid saponins. As a result, three pentacyclic triterpenoid saponins had been isolated and identified, consisting of one new compound and two known compounds (Fig. 1). In addition, compounds **1-3** were tested for their anti-proliferative activities using HeLa cell line (human cervical cancer cell line) *in vitro*. To further explore the mechanism of cytotoxic activity of compounds **1-3**, they were evaluated for the effects on cell morphology, apoptosis and cycle of HeLa.

Material And Methods

General experimental procedures

Column chromatography (CC) was performed using silica gel (Qingdao Marine Chemical Inc., Qingdao, China), Sephadex LH-20 (Pharmacia Co., Sweden), MCI gel (CHP20P-Wharton, Nanjing Yuanbaofeng Pharmaceutical Technology Co., Ltd, Nanjing, China), and ODS (Bona Ajar Technology Co., Ltd, Tianjin, China). Thin-layer chromatography (TLC) was performed with silica gel GF254 (Yantai Huayang New Material Technology Co., Ltd, Yantai, China). Analytical HPLC with an Alltech 2000ES evaporative light scattering detector (Alltech Co., KY, US) were performed on a Surveyor high performance liquid chromatography (Thermo Fisher Co., MA, US). Preparative HPLC with refractive index detector were carried out on a KNAUER apparatus (Knauer Co., Berlin, Germany). High-resolution electrospray ionization mass spectra (HR-ESI-MS) were performed on the Agilent G6230A TOF LC/MS (Agilent Technologies Co. Ltd., MA, US) using ESI ion source. NMR spectra were recorded on a Bruker ECA-400 MHz (Bruker Co. Billerica, MA, US) spectrometer with TMS as an internal standard. Chemical shifts were in ppm (δ), and coupling constants (J) were reported in Hertz (Hz).

Plant material

The roots of *Ardisia lindleyana* D. Dietr were collected in Guangxi (geographical coordinates: 104°26'-112°04' E, 20°54'-26°24' N with an altitude of about 300 meters) in China. The plant material was identified by Prof. Bin Li, Beijing Institute of Radiation Medicine. A voucher Specimen (Voucher # 2018-0808) is deposited in specimen cabinet of Beijing Institute of Radiation Medicine.

Extraction and Isolation

The roots (60 kg) were extracted with 80% ethanol solution. After removing the solvent of combined extracts under reduced pressure, the dried (approximately 11.8 kg) was suspended in deionized water and partitioned with petroleum ether, acetic ether (EtOAc) and n-butyl alcohol (n-BuOH) successively. Then, the residue was The EtOAc extract (290 g) was subjected to a silica gel column using Dichloromethane–MeOH (200–300 mesh, 100:0, 100:1, 50:1, 25:1, 10:1, 5:1, 2:1 and 1:1, v/v) to produce 12 fractions (Fr.1–Fr.12). The remaining Fr.9 (7 g) after crystallization was fractionated and subjected to a silica gel column (200–300 mesh, Petroleum ether–ethyl acetate, 100:0, 100:1, 50:1, 25:1, 10:1, 5:1, 2:1 and 1:1, v/v) to afford 13 fractions (Fr.9-a–Fr.9-m), Fr.9-c (200 mg) was further separated by preparative HPLC using MeOH-H₂O (70:30) to yield compound **1** (6 mg) and compound **2** (23 mg).

The n-BuOH extract (4000 g) was subjected to an AB-8 Macroporous Adsorption Resin column using ethanol solution at different concentrations of 0%, 25%, 50%, 75% and 95% to produce 5 fractions (Fr.0%, Fr.25%, Fr.50%, Fr.75% and Fr.95%). A large amount of white precipitation (approximately 1200 g) was precipitated from Fr.50%, and a small fraction of the precipitation (1.2 g) was further separated by preparative HPLC using MeOH-H₂O (85:15) to yield compound **3** (1 g).

Cytotoxicity Assay

HeLa cells were cultured in 96-well plates at a density of 5×10^4 cells/mL in Dulbecco's modified Eagle's medium (DMEM, Gibco, US) with 10% fetal calf serum (Gibco, US), 100 U/mL penicillin, and 100 μ g/mL

streptomycin, Plates were incubated in an atmosphere containing 5% CO₂ at 37 °C. Cells were treated with different concentrations of test compounds for 48 h. The cytotoxicity assay was performed using the Cell Counting Kit-8 (CCK-8, Applygen technologies Co., Ltd, Beijing, China) assay.

Cell morphology, cycle, and apoptosis assay

HeLa cells were seeded in 12-well tissue culture plate at a density of 5×10^4 cells/mL incubated at 37 °C for 24 h incubation, and treated with different concentrations of compounds **1-3** for 48 h. **Cell morphology assay:** the cells were collected, washed with PBS, and fixed with 4% Paraformaldehyde fix solution (Shanghai Biyuntian Biotechnology Co., Ltd, Shanghai, China) for 2 h. Then, fixed cells were washed with PBS, and incubated with Dio (Shanghai Biyuntian Biotechnology Co., Ltd, Shanghai, China) at 37 °C in the dark. After 20 min, the cells were washed with PBS, stained with Hoechst 33342 staining solution (Shanghai Biyuntian Biotechnology Co., Ltd, Shanghai, China) for 10 min, and washed with PBS. Finally, the stained cells in PBS were observed and photographed using a fluorescence microscope. **Cell apoptosis and cycle assay:** In cell apoptosis assay, all the cells were collected, and 1×10^5 cells were taken to incubate with Annexin V-FITC and PI at room temperature for 20 min in the dark. In cell cycle assay, all the cells were collected and fixed with precooled 70% ethanol at 4 °C for 12 h. Then the cells were washed with PBS, and incubated with PI at 37 °C for 30 min in the dark. Finally, all the cells were detected using a flow cytometer.

Results And Discussions

Ardisiapunine E (**1**) was obtained as a white amorphous powder. It showed positive Liebermann-Barchard reaction and negative Molish reaction, and it was purplish red when developed with alcohol reagent containing 10% sulfuric acid. The HR-ESI-MS gave a molecular formula of C₃₅H₅₄O₈Cl at m/z 637.3512 [$M + Cl$]⁻ (calcd. for C₃₅H₅₄O₈Cl⁻, 637.3507) with 9 degrees of unsaturation. The ¹³C NMR spectrum (150 MHz, Pyridine-*d*₅) of **1** showed 35 carbon signals (Table 1). Combined with the ¹³C NMR and DEPT (135 degrees) experiment, there were two sp² carbonyl carbons at δ_C 205.9 and 212.3 ppm, and the two carbonyl carbons belonged to the quaternary carbon and tertiary carbon, namely, ketone group and aldehyde group respectively; other 33 carbon signals can be categorized into seven sp³ quaternary carbons at δ_C 36.5 (C-4), 39.5 (C-10), 42.8 (C-8), 47.6 (C-20), 49.9 (C-14), 55.1 (C-17) and 85.9 (C-13) ppm, five sp³ oxymethine carbons at δ_C 69.3 (C-4'), 72.7 (C-2'), 74.4 (C-3'), 88.3 (C-3) and 107.3 (C-1') ppm, three sp³ methine carbons at δ_C 49.8 (C-9), 55.3 (C-5) and 55.7 (C-18) ppm, two oxymethylene carbons at δ_C 66.6 (C-5') and 74.7 (C-28) ppm, ten sp³ methylene carbons at δ_C 17.5 (C-6), 18.6 (C-11), 25.7 (C-19), 26.6 (C-2), 29.4 (C-22), 29.8 (C-12), 31.4 (C-21), 33.6 (C-7), 38.9 (C-1) and 45.5 (C-15) ppm, and six methyl carbons at δ_C 15.9 (C-25), 16.4 (C-24), 18.6 (C-26), 21.7 (C-27) 23.6 (C-29) and 27.8(C-23) ppm. ¹H-NMR (600 MHz, Pyridine-*d*₅) spectrum (Table 1) of **1** displayed six methyl singlets at δ_H 0.83 (6H, s, H-25, H-29), 0.96 (3H, s, H-24), 1.12 (3H, s, H-27) and 1.27 (6H, s, H-23, H-26), an aldehyde signal at δ_H 9.52 (1H, d, J = 18.48 Hz, H-30), five oxymethine signals at δ_H 3.34 (1H, s, H-3), 4.78 (1H, s, H-1'), 4.45 (1H, s, H-2'),

4.18 (1H, s, H-3') and 4.34 (1H, s, H-4'), three oxymethine signals at δ_{H} 4.34 (1H, s, H-5'a), 4.18 (1H, s, H-28a) and 3.83 (2H, s, H-5'b, H-28b).

Table 1 NMR data of compound **1** in pyridine

NO	Compound 1		NO	Compound 1	
	(600 MHz for ¹ H; 150 MHz for ¹³ C)			(600 MHz for ¹ H; 150 MHz for ¹³ C)	
	δ_{C} , mult.	δ_{H} (<i>J</i> in Hz)		δ_{C} , mult.	δ_{H} (<i>J</i> in Hz)
1	38.9, CH ₂	overlapped, Ha	17	55.1, C	—
		overlapped, Hb	18	55.7, CH	—
2	26.6, CH ₂	overlapped, Ha	19	25.7, CH ₂	2.45 (s, 1H), Ha
		overlapped, Hb			overlapped, Hb
3	88.3, CH	3.34 (s, 1H)	20	47.6, C	—
4	36.5, C	—	21	31.4, CH ₂	overlapped, Ha
5	55.3, CH	0.73 (s, 1H)			overlapped, Hb
6	17.5, CH ₂	overlapped, Ha	22	29.4, CH ₂	overlapped, Ha
		overlapped, Hb			overlapped, Hb
7	33.6, CH ₂	overlapped, Ha	23	27.8, CH ₃	1.27 (s, 3H)
		overlapped, Hb	24	16.4, CH ₃	0.96 (s, 3H)
8	42.8, C	—	25	15.9, CH ₃	0.83 (s, 3H)
9	49.8, CH	overlapped	26	18.6, CH ₃	1.27 (s, 3H)
10	39.5, C	—	27	21.7, CH ₃	1.12 (s, 3H)
11	18.6, CH ₂	overlapped, Ha	28	74.7, CH ₂	4.18 (s, 1H), Ha
		overlapped, Hb			3.83 (s, 1H), Hb
12	29.8, CH ₂	overlapped, Ha	29	23.6, CH ₃	0.83 (s, 3H)
		overlapped, Hb	30	205.9, CH	9.52 (d, <i>J</i> = 18.48 Hz, 1H)
13	85.9, C	—	1'	107.3, CH	4.78 (s, 1H)
14	49.9, C	—	2'	72.7, CH	4.45 (s, 1H)
15	45.5, CH ₂	2.83 (s, 1H), Ha	3'	74.4, CH	4.18 (s, 1H)
		overlapped, Hb	4'	69.3, CH	4.34 (s, 1H)
16	212.3, C	overlapped	5'	66.6, CH ₂	4.34 (s, 1H), Ha

Three spin coupling systems ($H_{1-1'}-H_{1-2'}-H_{1-3'}$, $H_{2-2'}-H_{1-3}$, and $H_{1-5}-H_{2-6}$) were revealed by the analysis of $^1H-^1H$ COSY data of **1** (Fig. 2). Combined with the $^1H-^1H$ COSY data, the HMBC correlations (Fig. 2) from $H_{1-1'}$ to $C_{3-3'}/C_{2-2'}/C_{3-3'}/C_{5-5'}$, from $H_{2-2'}$ to $C_{1-1'}/C_{3-3'}$, from $H_{3-3'}$ to $C_{2-2'}$, from $H_{5-5'}$ to $C_{1-1'}/C_{3-3'}/C_{4-4'}$, from $H_{3-3'}$ to $C_{1-1'}/C_{5-5'}/C_{23-23'}/C_{24-24'}$, from $H_{23-23'}$ to $C_{3-3'}$, from $H_{24-24'}$ to $C_{3-3'}/C_{5-5'}$, from $H_{30-30'}$ to $C_{20-20'}$, from $H_{30-30'}$ to $C_{20-20'}$, from $H_{15-15'}$ to $C_{8-8'}/C_{14-14'}/C_{16-16'}$, from $H_{28-28'}$ to $C_{16-16'}/C_{17-17'}/C_{18-18'}$, from $H_{27-27'}$ to $C_{8-8'}/C_{13-13'}/C_{14-14'}$, from $H_{5-5'}$ to $C_{1-1'}/C_{4-4'}/C_{6-6'}/C_{7-7'}/C_{10-10'}/C_{24-24'}/C_{25-25'}$ revealed the partial structure of **1**. In addition, acid hydrolysis of **1** with 2N HCl released L-arabinose. Based on the above observations, the molecular formula, degrees of the unsaturation, and the chemical shift characteristics, the whole planar structure of **1** was assigned as shown in Fig. 1.

The relative stereochemistry of **1** was ascertained from the NOE interactions (Fig. 3) observed in the NOESY spectrum. Significant NOE correlations were observed between $H_{30-30'}$ and $H_{21-21'}$, $H_{22-22'}$, $H_{18-18'}$ and $H_{19-19'}$, these NOE interactions suggested that $C_{18-18'}$ and $C_{30-30'}$ were on the axial positions. In addition, there were obvious correlations between $H_{1-1'}$ and $H_{5-5'}$, $H_{23-23'}$, $H_{3-3'}$ and $H_{23-23'}$, indicating that the glycosyl was on the axial positions as $C_{23-23'}$. Thus, the structure of compound **1** was established and named as ardisiapunine E.

By comparing their NMR data with those published in literatures, the two known compounds **2** and **3** were 3-*O*- α -L-arabinopyranosyl cyclamiretin A [13] and ardisiacrispin B [14], respectively.

Compounds **1-3** were screened for their inhibitory activities against HeLa cell line. However, the known compound **3** showed better cytotoxic activity than cisplatin (the IC_{50} of 1.81 μ M and 8.26 μ M for compound **3** and cisplatin [12], respectively), while the new compound **1** and the known compound **2** exhibited moderate cytotoxic activities with IC_{50} value for 13.23 and 20.27 μ M, respectively.

Ardisiacrispin B is a triterpenoid saponin derived from the plants of *Ardisia*, its cytotoxic activity has attracted considerable interest [15]. However, the mechanism of cytotoxic activity of Ardisiacrispin B has not been further explored. Compounds **1-2** equally belongs to triterpenoid saponin, and the mechanism by which they suppress tumor cell proliferation also needs to be further explored. Therefore, the cell morphology, apoptosis and cycle assays were carried out. Since the cell cycle and cell apoptosis regulation are of vital importance in cancers, researchers have given sufficient weight to the control of cell cycle and cell apoptosis [16-18]. It was shown that compounds **1-3** disrupted cell morphology in a dose-dependent manner (Fig. 4). The HeLa cells in control group performed a status of regular appearance, intensive growth, integral cell membrane, and clear nucleolus. After the intervention of compounds **1-3**, apoptotic bodies gradually increased, cells appeared cytomorphosis and nuclear shrinkage, and even rupture, which revealed that compounds **1-3** could induce apoptosis in HeLa cells *in vitro*.

CCK-8 assay demonstrated that compounds **1-3** could inhibit cell proliferation on HeLa cells, and the inhibitory rate dose and time dependently increased following those compounds treatment. As presented in Fig. 5, the inhibitory effects of compounds **1-3** on HeLa cell line proliferation increased with increasing

concentrations ranging from 6.25 to 100 μ M, 6.25 to 100 μ M and 0.20 to 100 μ M during 12, 24 and 48 h action time, respectively.

To further verify whether compounds **1-3** inhibits HeLa cell proliferation by inducing apoptosis, we used the annexin V and PI double staining kit to quantify HeLa cell apoptosis.

The percentage of specific cell populations at various stages of apoptosis was shown in Fig. 6. The apoptosis rate in the control group was 13.62%. Significantly, the apoptosis rate after treated with compound **1** dose-dependently elevated to 25.99% (12.5 μ M) and 45.37% (25 μ M), respectively; compound **2** treatment dose dependently increased the apoptosis rate of 21.93% (12.5 μ M) and 48.73% (25 μ M); the apoptosis rate dose dependently increased following compound **3** treatment of 32.92% (1.56 μ M) and 54.03% (3.13 μ M). Thus, the treatment with compounds **1-3** could significantly enhance the apoptosis in a dose-dependent manner ($P < 0.001$).

To explore whether those compounds-induced apoptosis was associated with cell cycle arrest, we detected the cell cycle distribution of HeLa cells using flow cytometry to analyze cellular DNA content. As shown in Fig. 7, there was a significant increase in the percentage of HeLa cells with compound **1** (25 μ M), or compound **2** (25 μ M), or compound **3** (3.13 μ M) treatment in the G2/M phase versus control ($P < 0.001$). However, the number of G0/G1 phase cells decreased with the concentration of compounds **1-3** ($P < 0.01$ or $P < 0.001$). In brief, all that results inferred that compounds **1-3** inhibited the proliferation of HeLa cells and induced G2/M phase arrest.

Conclusions

As a part of ongoing investigations on the cytotoxic agents of *Ardisia lindleyana* D.Dietr, one new pentacyclic triterpenoid saponin, ardisiapunine E (**1**), along with two known compounds were isolated from *Ardisia lindleyana* D.Dietr. The structure of the new compound **1** were elucidated by ^1H and ^{13}C NMR, DEPT, HMBC, HSQC, ^1H - ^1H COSY and NOESY spectroscopic analyses, and HR-ESI-MS. The known compounds were identified by comparison with data reported in the literature. Compounds **1-3** exhibited obvious anti-proliferative activities on HeLa cell line *in vitro* through inducing G2/M phase arrest and apoptosis in a dose- and time-dependent manner. Hence, this study identified a new pentacyclic triterpenoid saponin promoting apoptosis as a potential therapeutic option for cancer.

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Figures

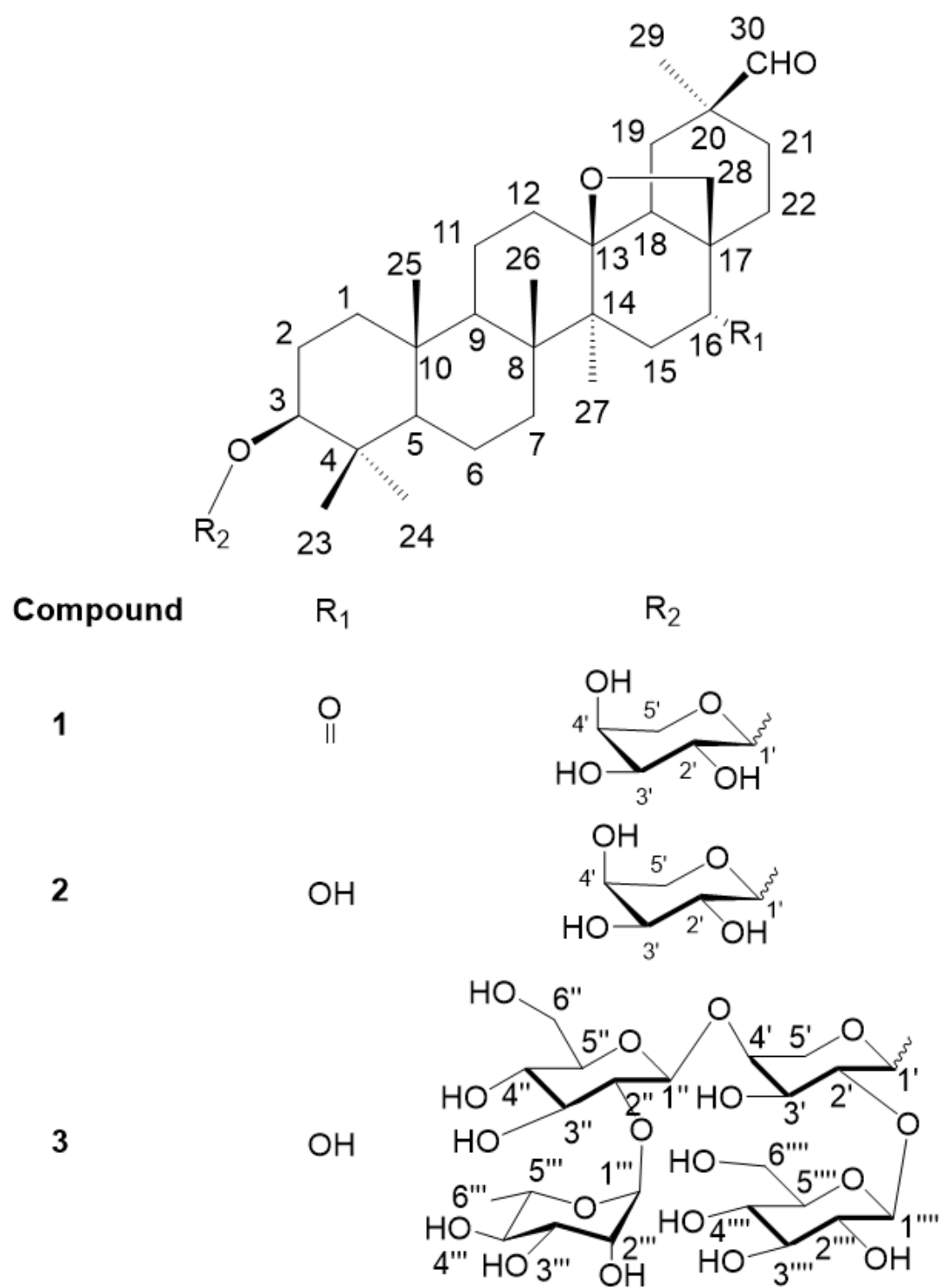


Figure 1

Chemical structures of **1-3**.

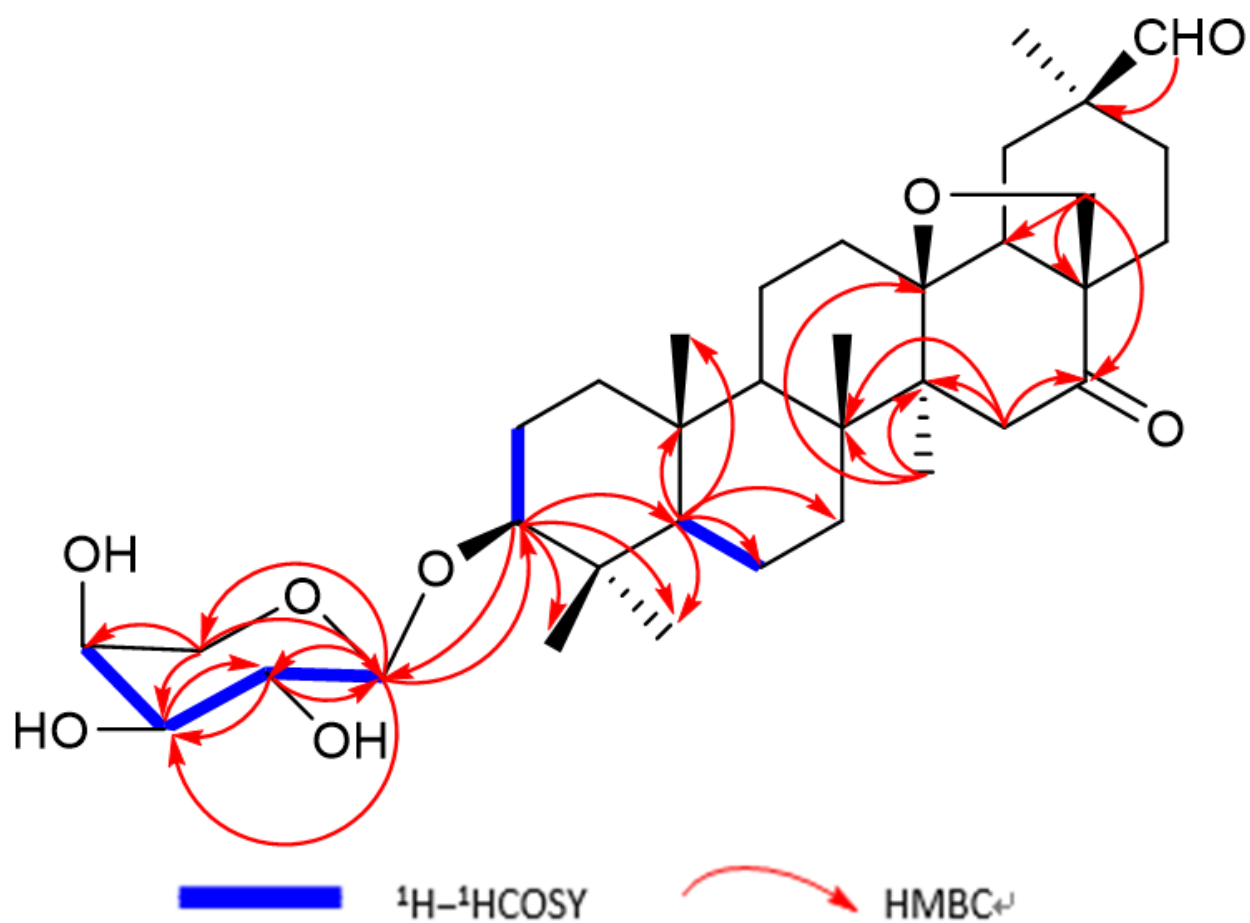


Figure 2

Key ^1H - ^1H COSY and HMBC correlations of compound 1.

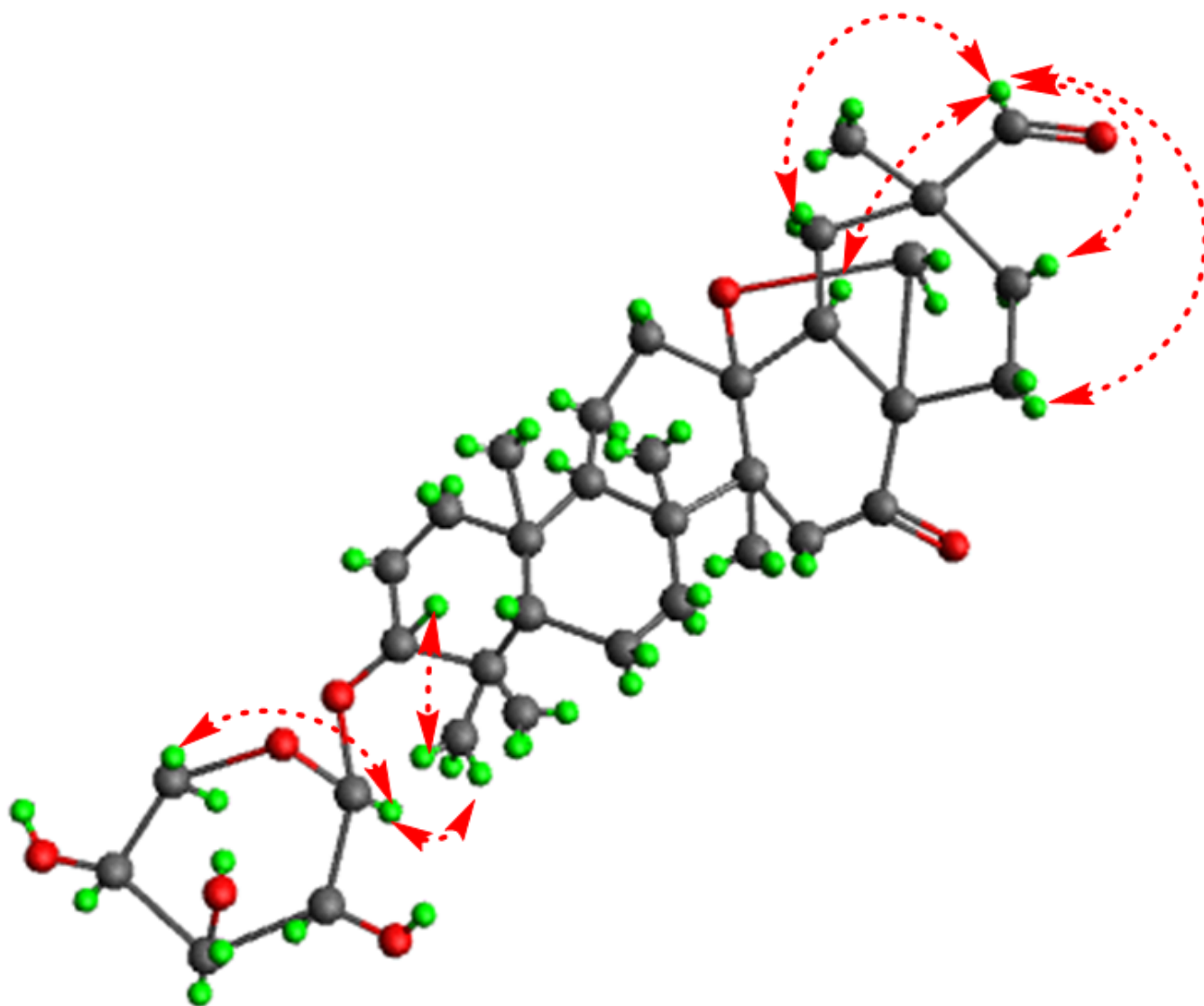


Figure 3

Key NOESY correlations of compound **1**.

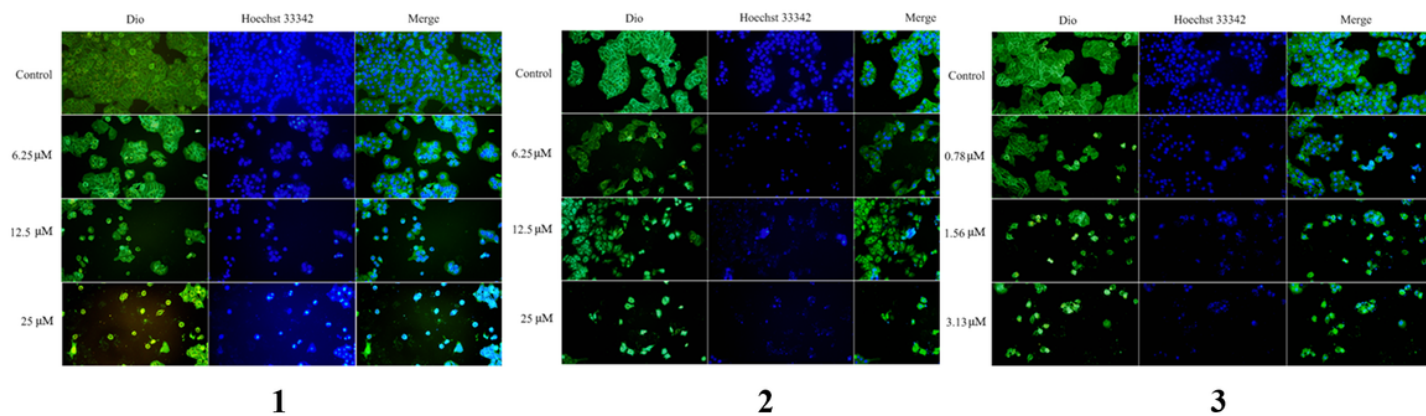


Figure 4

Compounds **1-3** induced morphological changes of HeLa cells.

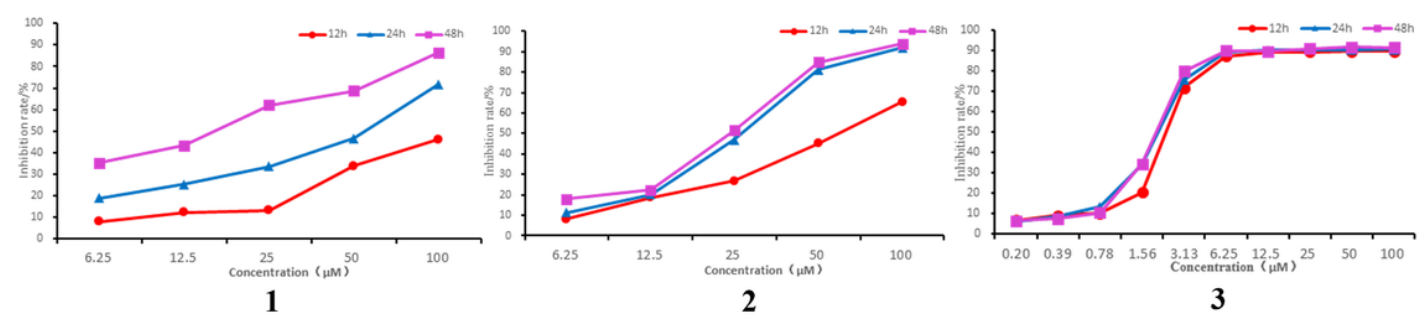


Figure 5

Effect of compounds **1-3** on the proliferation of HeLa cells, as determined by CCK-8 assay



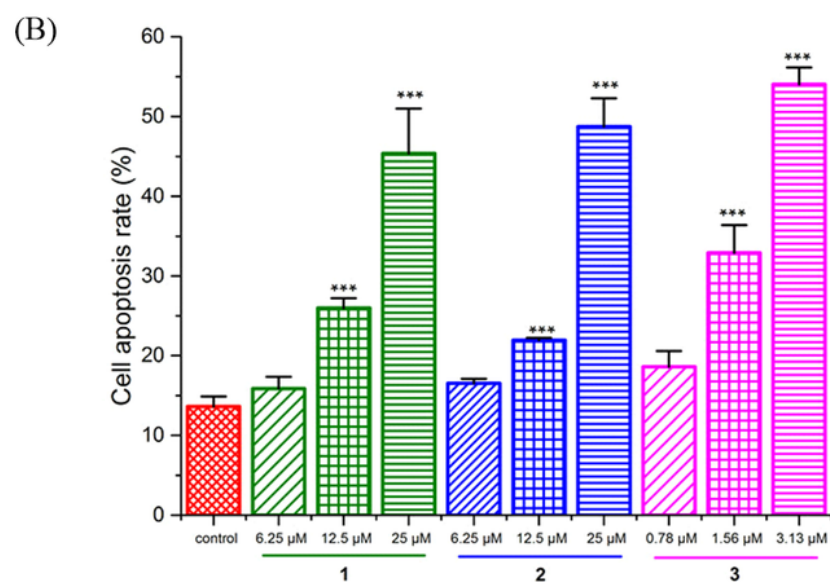
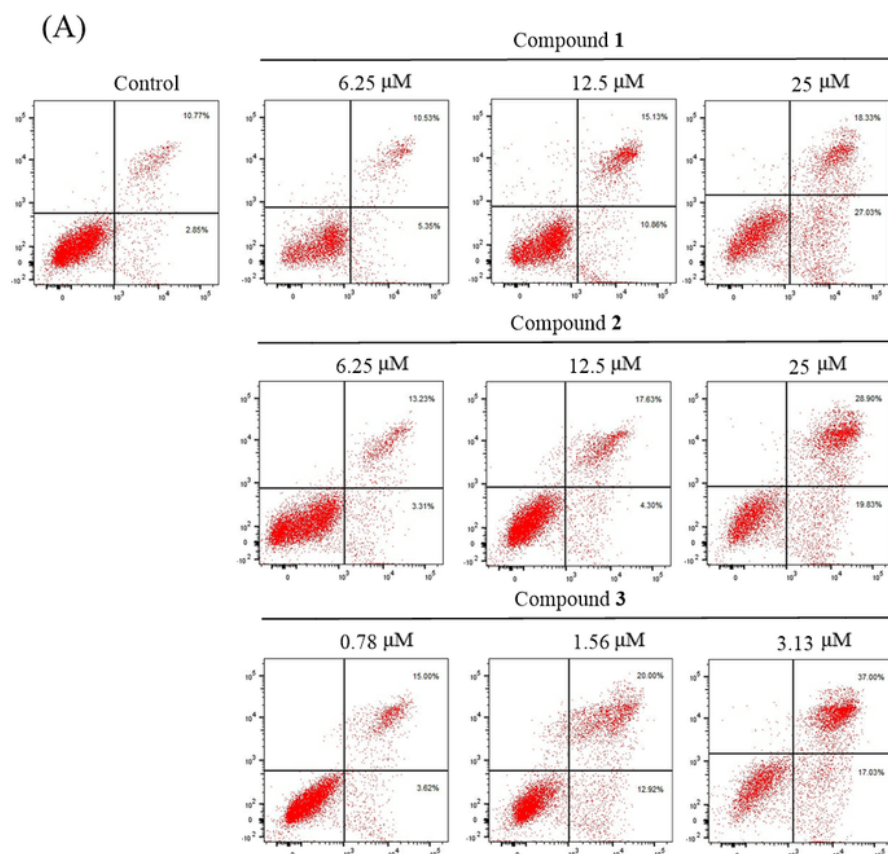


Figure 6

Cell apoptosis assay: all the cells were collected, and 1×10^5 cells were taken and incubated with Annexin V-FITC and PI for 20 min at room temperature without light. Finally, all the cells were detected using a flow cytometer. Data are presented as mean $\pm$ SD, $n = 3$. One way ANOVA, ** $p < 0.01$, *** $p < 0.001$, compared with control group.

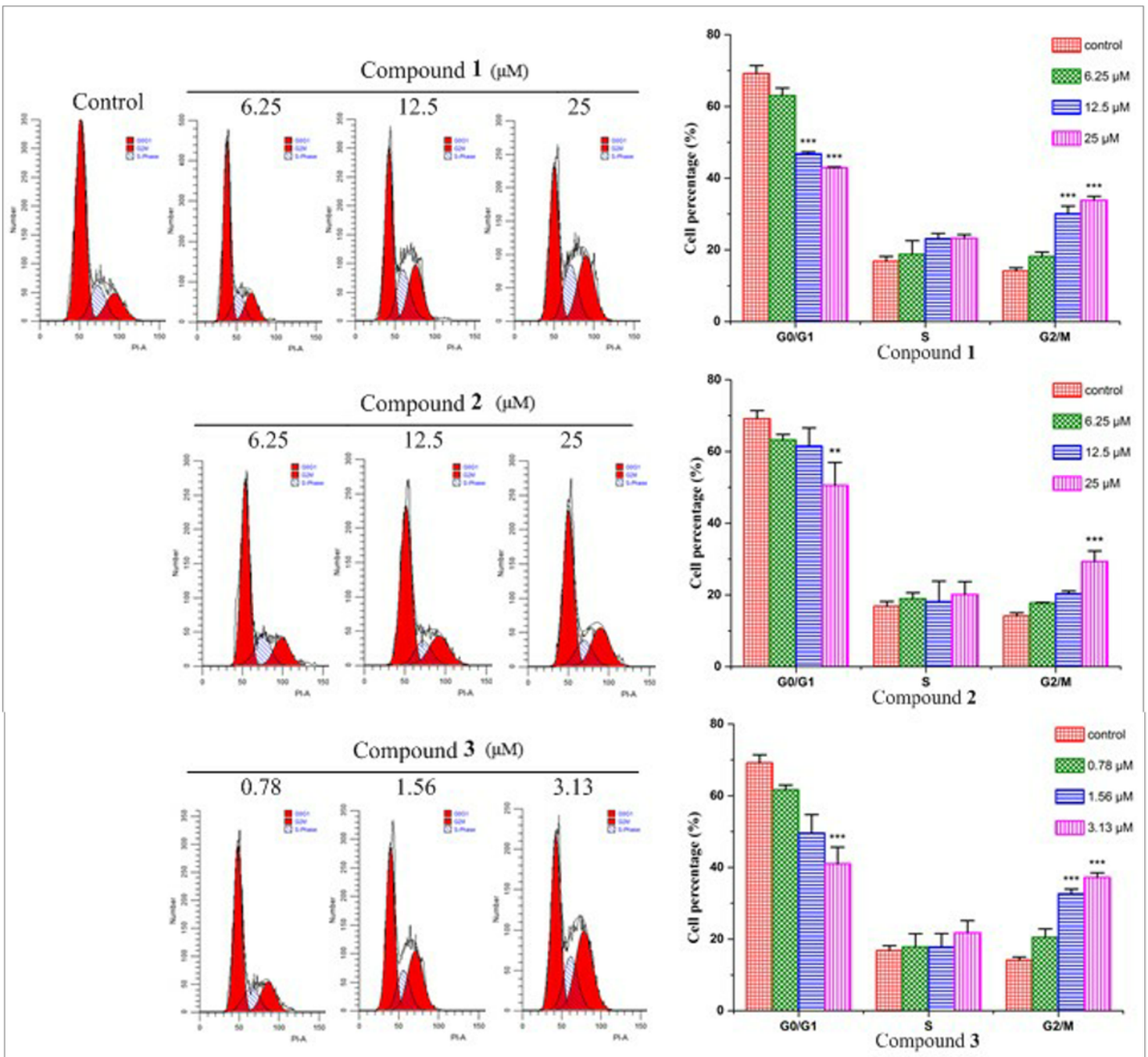


Figure 7

Cell cycle assay: all the cells were collected and fixed with precooled 70% ethanol for 12 h at 4 °C. The cells were then washed with PBS and incubated with PI for 30 min at 37 °C without light. Finally, all the cells were detected using a flow cytometer. Data are presented as mean ± SD, n = 3. One way ANOVA, ** $P < 0.01$, *** $P < 0.001$, compared with control group.

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