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3S,4S-Disenecioylkhellactone from the Roots of *Peucedanum Japonicum* Induces Apoptosis in HL-60 Cells

Kyung-Yun Kang
Suncheon Research Center for Natural Medicines
Sung-Ju Lee
Suncheon Research Center for Natural Medicines
Hyun-Ji Kim
Laboratory of marine drugs, school of Earth and Environmental sciences, Seoul National University
Heonjoong Kang
Research institute of oceanography, Seoul National University / Laboratory of marine drugs, school of earth and environmental sciences, seoul national university
Sang-Jip Nam
Department of chemistry and nanoscience, Global Top5 program, Ewha womans university
Sung-Tae Yee (sungtae@sunchon.ac.kr)
Sunchon National University https://orcid.org/0000-0002-5108-8140

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Abstract

Background: The root of *Peucedanum japonicum* is used in traditional medicine in Japan, the Philippines, China, and Korea, and it has been reported to have a variety of biological activities, including anticancer activity. Inducing apoptosis in tumor cells has become a major focus of antitumor drug development; therefore, we studied the apoptotic effects of the MeOH/CH_2Cl_2 extract of the roots of *P. japonicum* and its components on HL-60 cells (a human leukemia cell line).

Methods: Compounds were purified using solvents with varying polarities followed by column chromatography (reverse phase), and the structures were confirmed using nuclear magnetic resonance spectroscopy. The viabilities of HL-60, A549, and MCF-7 cells were determined using the cell counting kit-8 (CCK-8) assay. Analysis of apoptosis signaling was performed only with HL-60 cells, and cell cycle progression, Annexin V/propidium iodide (PI) staining (analyzed by flow cytometry), the mitochondrial membrane potential (MMP, as analyzed by flow cytometry), and caspase-3, 8, and 9 activity (determined using the caspase-3, -8, and -9 activity kit according to the manufacturer's protocol) were evaluated.

Results: Two coumarin molecules, (-)-isosamidin (**1**) and 3′S,4′S disenecioylkhellactone (**2**), were isolated by bioactivity-guided fractionation. Only compound **2** showed cytotoxicity in HL-60 cells, which occurred due to increases in the sub-G1 population and the initiation of early and late apoptosis as determined by Annexin V/PI staining. In addition, decreases in the MMP were observed in HL-60 cells treated with compound 2. Several apoptotic features were observed, including increased cleavage of caspase-3, -8, and -9. Moreover, treatment with Z-DEVD-FMK (a caspase-3 inhibitor), Z-IETD-FMK (a caspase-8 inhibitor), or Z-LEHD-FMK (a caspase-9 inhibitor) significantly inhibited cell cytotoxicity.

Conclusions: We provide evidence that compound **2** induces apoptosis in HL-60 cells.

Background

Cancer is caused by many factors, such as uncontrolled cellular proliferation, invasion, and metastasis. The incidence of cancer continues to increase worldwide, and despite the continuous development of new drugs, many cancer types still have high mortality rates. Cancer chemotherapy relies on chemical substances derived from natural or synthetic products [1] and can exert powerful anticancer effects, though many chemotherapy drugs may also elicit substantial adverse side effects. Chemotherapy works by killing dividing cells and thus can negatively impact healthy cells that undergo high rates of mitosis. Furthermore, the toxic effects of synthetic chemicals can either lead to passive cell death or necrosis or result in activation of apoptosis [2]. In the quest to develop new anticancer agents with fewer side effects, herbal medicines derived from plants continue to be investigated. Plant-derived natural products such as flavonoids, terpenoids, and alkaloids have received considerable attention in recent years due to their diverse pharmacological properties, which include cytotoxic and anticancer effects [3, 4].

Peucedanum japonicum (Umbelliferae) is a coastal plant that is widely distributed in Japan, the Philippines, China, and Korea and mainly grows in sandy soil or sandy loam. Its root is used as a folk

medicine for the treatment of coughs, colds, and headaches as well as an anodyne [5]. The representative natural products previously isolated from *P. japonicumin* include pyranocoumarins, phenylcoumarins, and other coumarin derivatives, which possess diverse biological effects, including inhibition of Ca^{2+} channel, monoamine oxidase, and nitric oxide synthase activities; antidiabetic, anti-inflammatory, anticonvulsant, and blood-pressure-lowering effects and ameliorative effects on bronchial asthma [6–20].

HL-60 cells are derived from peripheral blood leukocytes in a background of acute promyelocytic leukemia. The cell line has been well characterized as an *in vitro* model of apoptosis, cell proliferation, and differentiation [21]. Apoptotic cell death is a complex process involving biochemical events and well-defined morphological changes, such as cell shrinkage, cytoplasm vacuolization, chromatin condensation, DNA fragmentation, and dissipation of the mitochondrial membrane potential (MMP) [2, 22, 23]. Recent studies have documented that two major pathways are involved in the regulation of apoptosis: extrinsic pathways related to cell receptors and intrinsic pathways mediated by mitochondria [22]. These studies have also demonstrated that mitochondria play a critical role in apoptosis progression [24–26]. Mitochondrial permeability transition leads to loss of the MMP, which causes the translocation of the proapoptotic protein Bax to the mitochondria and of cytochrome C from the mitochondria to the cytosol, which in turn results in the activation of apoptotic caspase cascades [25]. As such, apoptosis is mediated by mitochondrial genes and is an active process that can be adjusted [24–26].

In the present study, we isolated *P. japonicum* roots and investigated the anticancer effect of compound **2** on the apoptosis of the human leukemia cell line HL-60 via various assays.

Materials And Methods

Plant materials

P. japonicum roots (harvested from Geumo-do, Nam-myeon, Yeosu-si, Jeollanam-do, Korea) were purchased from the Dongbu Herbal Farming Cooperative. (Suncheon, Korea) in January 2015.

Chemicals and reagents

RPMI 1640 was purchased from Thermo Scientific (Waltham, MA, USA); Dulbecco's modified Eagle medium (DMEM) was obtained from Gendepot (Baker, TX, USA); cell counting kit-8 (CCK-8) was procured from Dojindo Laboratories (Kumamto, Japan); dimethyl sulfoxide (DMSO) and propidium iodide (PI) were acquired from Sigma Aldrich (St. Louis, MO, USA); Annexin V-FITC was purchased from BD Biosciences (Franklin Lakes, NJ, USA), and the JC-1 Assay Kit was obtained from Invitrogen (Carlsbad, CA, USA). Methanol, dichloromethane, hexane (Duksan Co., Ansan, Korea), HPLC grade acetonitrile, water, methanol (J. T Baker, Phillipsburg, NJ, USA), chloroform-*d* (Cambridge Isotope Laboratories, Inc., Tewksbury, MA, USA), silica gel 60 (70-230 mesh, Merck Co., Kenilworth, NJ, USA), Luna 5u (C18 100A 250×10 mm, Phenomenex Inc., Torrance, CA, USA), and YMC-actus (Triart C18 250×20 mm, YMC, Kyoto, Japan) were also used.

Extraction and isolation of compounds from *P. japonicum*

Roots of *P. japonicum* (dry wt. 1.0 kg) were extracted three times with MeOH in CH_2Cl_2 (1:1, v/v) at room temperature. These extracts were combined and partitioned three times between MeOH and *n*-hexane. The MeOH layer was further partitioned between H_2O and ethyl acetate to establish an H_2O fraction and an ethyl acetate fraction (8.41 g). The ethyl acetate-soluble fraction was subjected to silica gel flash column chromatography and eluted with a step gradient solvent system of 100% to 0% CH_2Cl_2 in MeOH, which resulted in 11 fractions (1-11). Compounds **1** (1.0 mg, t_R 23 min) and **2** (1.2 mg, t_R 37 min) were obtained by reverse-phase HPLC (Shiseido CAPCELL C_{18} 5 μm, 250×10 mm, 2.0 mL/min, UV detection at 210 nm) using 70% CH_3CN in H_2O as the eluant. Compounds **1** and **2** were identified as (-)-isosamidin and 3′*S*,4′*S* disenecioylkhellactone, respectively, by comparing their spectroscopic data and optical rotations with previously reported data [27, 28]. For **(-)-isosamidin(1)**; ^{1}H NMR ($CDCl_3$, 700 MHz) δ 7.61 (1H, d, *J* = 9.5), 7.36 (1H, d, *J* = 8.7), 6.81 (1H, d, *J* = 8.5), 6.57 (1H, d, *J* = 4.8), 6.23 (1H, d, *J* = 9.5), 5.69 (1H, s), 5.35 (1H, d, *J* = 4.8), 2.17 (3H, d, *J* = 1.2), 2.11 (3H, s), 1.90 (3H, d, *J* = 1.2), 1.45 (3H, s), 1.41 (3H, s); ^{13}C NMR ($CDCl_3$) δ169.5 (C), 164.9 (C), 159.5 (C), 158.4 (C), 156.4 (C), 153.6 (C), 128.7 (CH), 114.8 (CH), 114.1 (C), 112.8 (CH), 112.3 (C), 106.7 (C), 77.7 (C), 68.7 (CH), 27.4 (CH_3), 24.5 (CH_3), 23.1 (CH_3), 20.6 (CH_3), 20.3 (CH_3); LRESIMS m/z 286.4 $(M - 99 + H)^+$, 793.3 $(2M + Na)^+$ (^{13}C NMR chemical shifts were determined using 2D NMR spectroscopy data and confirmed by comparing them with previously reported data) (Fig. 1). For **3′*S*, 4′*S* disenecioylkhellactone(2)**; ^{1}H NMR ($CDCl_3$, 700 MHz) δ7.60 (1H, d, *J* = 9.2), 7.37 (1H, d, *J* = 8.7), 6.82 (1H, d, *J* = 8.7), 6.65 (1H, d, *J* = 4.8), 6.24 (1H, d, *J* = 9.5), 5.69 (1H, s), 5.64 (1H, s), 5.37 (1H, d, *J* = 4.8) 2.22 (3H, s), 2.17 (3H, s), 1.91 (3H, s), 1.90 (3H, s); ^{13}C NMR (CDCl3) δ 167.5 (C), 167.3 (C), 162.7 (C), 160.8 (C), 160 (C), 158.8 (C), 155.9 (C), 146.6 (CH), 131.7 (CH), 117 (CH), 117 (CH), 114.8 (CH), 114.4 (CH), 113.6 (C), 109.4 (C), 79.4 (C), 72 (CH), 61.8 (CH), 28.3 (CH_3), 28.2 (CH_3), 26.5 (CH_3), 23.2 (CH_3), 21.3 (CH_3), 21.2 (CH_3); LRESIMS m/z 327.1 $(M - 100 + H)^+$, 876.3 $(2M + Na)^+$ (^{13}C NMR chemical shifts were determined using 2D NMR spectroscopic data and confirmed by comparing them with previously reported data) (Fig. 2).

Cells and cell culture

Human acute promyelocytic leukemia cells (HL-60; KCLB 10240), human lung adenocarcinoma cells (A549; KCLB 10185) and human breast adenocarcinoma cells (MCF-7; KCLB 30022) were obtained from the Korean Cell Line Bank (Seoul, Korea) and cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/mL) and streptomycin (100 μg/mL). Madin-Darby canine kidney (MDCK; KCLB 10034) cells were also obtained from the Korean Cell Line Bank and cultured in DMEM containing 10% FBS, 100 U/mL penicillin G, and 100 μg/mL streptomycin sulfate. The purities of all compounds tested by HPLC were confirmed to be >95%. Samples of test compounds were dissolved in DMSO and added to the medium to a final concentration of 0.03%. Cultures were maintained at 37°C under 5% CO_2/95% air, and media were changed every two days.

Cytotoxicity assay

Cell viabilities were determined using the cell counting kit-8 (CCK-8) assay. First, MCF-7, A549 and HL-60 cells were resuspended in RPMI 1640 medium at densities of 3×10^4 cells/mL, 1×10^4 cells/mL, 2×10^4 cells/mL, and 1×10^5 cells/mL, respectively. Next, 100 μL of the cell suspensions was added per well to 96-well flat-bottomed microtiter plates, followed by 100 μL of compounds **1** and **2** to achieve final concentrations of 1, 3, 10, and 30 μM. The plates were then incubated for 24 h at 37°C in 5% CO_2/95% air. Second, HL-60 cells were resuspended in RPMI 1640 medium at 1×10^5 cells/mL whereas MDCK cells were suspended in DMEM at 1×10^4 cells/mL. Next, 100 μL of cell suspension was added to the wells of 96-well flat-bottomed microtiter plates followed by 100 μL of compounds **1** and **2** (to final concentrations of 1, 10, and 30 μM), and the plates were incubated for 24 h at 37°C in 5% CO_2/95% air. Three replicates per condition were used in the experiments. After 24 h, 100 μL of medium was replaced with an equal volume of fresh medium containing 10 μL of CCK8, after which cell viability was measured.

Cell cycle assay

To determine the cell cycle distribution, HL-60 cells (5×10^5/well) were seeded and treated with compound **1** or **2** at 30 μM for 12 h, 18 h, and 24 h. After incubation, the cells were harvested, fixed in 70% ethanol, and treated with ribonuclease A (to increase cell permeability). Cell pellets were then incubated with PI for 2 h at 4°C in the dark before they were analyzed by flow cytometry. The percentages of cells in the G0/G1, S and G2/M phases of the cell cycle and the sub-G1 peak were determined after excluding cell debris and aggregates.

Annexin V/PI assay of apoptotic cells

Apoptosis induced by compounds **1** and **2** was quantified by flow cytometry using an Annexin V-FITC and propidium iodide (PI) solution according to the manufacturer's instructions. Briefly, HL-60 cells (5×10^5/well) were seeded into 24-well plates and treated to compounds **1** or **2** (30 μM for both) for 12 h, 18 h, and 24 h. Apoptosis was analyzed by staining with Annexin V-FITC/PI and performing flow cytometry, and the apoptosis percentages were calculated by counting the number of Annexin V- and PI-positive cells.

Detection of changes in mitochondrial membrane potentials (MMPs)

HL-60 cells were seeded in 24-well plates at a density of 5×10^5 cells/mL and treated with compounds **1** or **2** at 30 μM for different periods (12 h, 18 h, and 24 h). Cells were then harvested and stained with the lipophilic dye JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazole-carbocyanine iodide), which was used to measure the MMP by flow cytometry. When JC-1 enters the mitochondria, it aggregates and fluoresces red. Upon loss of the MMP, the dye diffuses throughout the cytoplasm and fluoresces green. The histogram shows the population of cells showing green (JC-1 monomers) and red (JC-1 aggregates) fluorescence. Frequency plots were prepared with FITC and PE gating to determine the percentages of mitochondria stained green (loss of the MMP) and red (normal MMP) [29].

Activation of caspase-3, -8, and -9

The activities of caspase-3, -8, and -9 were determined using the caspase-3, -8, -9 activity kit according the manufacturer's protocol. HL-60 cells were seeded into 24-well plates and treated with compounds **1** and **2** at 25 μM for 12 h, 18 h, and 24 h. Then, the cells were collected, washed, fixed, and stained with FITC before they were analyzed by flow cytometry.

Statistical analysis

Differences between groups are presented as the means±S.D. of 3 replicates. Statistical differences were analyzed using Student's t-test. Probability values less than 0.05 were considered significant (P values * <0.05, **<0.01, ***<0.001 vs control, $^{\#}$<0.05, $^{\#\#}$<0.01, $^{\#\#\#}$<0.001 vs 24 h sample).

Results

Isolation of compounds from the roots of P. japonicum

Dried and pulverized roots of *P. japonicum* were submerged in MeOH/CH_2Cl_2 (1:1, v/v) at room temperature to obtain an extract that was then fractioned into *n*-hexane, ethyl acetate, and H_2O fractions as described in the Materials and Methods. The ethyl acetate fraction was further subjected to repeated column chromatography followed by RP-HPLC, which yielded two coumarins: (-)-isosamidin **(1)** and 3′*S*,4′*S* disenecioylkhellactone **(2)**. The structures of these compounds were unequivocally determined by 1D and 2D NMR and by comparing spectroscopic and data with literature values (Figs. 1, 2).

Cytotoxic effect of compounds 1 and 2 on HL-60 cells

To evaluate the cytotoxicity of compounds **1** and **2**, three different cancer cell lines were treated for 24 h. In two of the three cancer cell lines (MCF-7, A549), cell viabilities after treatment with compounds **1** or **2** were either unchanged or slightly decreased versus those of corresponding untreated controls. However, compound **2** (30 μM) reduced the viability of HL-60 cells (treated, 11.077 ± 0.520%) (Fig. 3). Various anticancer studies have focused on reducing cytotoxic effects on normal cells; therefore, we examined the effects of compounds **1** and **2** on MDCK cells. HL-60 cells and MDCK cells were treated with compounds **1** or **2** (1, 10, and 30 μM) for 24 h, and cell viability was assessed. As shown in Fig. 4, concentration-dependent inhibition (30 μM) of cell survival was observed only in HL-60 cells treated with compound **2**. Interestingly, compound **2** did not exert any cytotoxic effects on MDCK cells (treated, 97.229 ± 3.818%). On the other hand, compound **1** hardly showed any significant cytotoxicity in either HL-60 or MDCK cells (HL-60 treated, 92.909 ± 7.244%; MDCK treated, 102.480 ± 3.319%). Further studies on the cytotoxic effects of compound **2** were carried out at 30 μM.

Compound 2 increased the proportion of cells in the sub-G1 phase

To determine whether the cytotoxic effect of compound **2** on HL-60 cells was associated with apoptosis, we investigated time-dependent changes in the proportions of cells in sub-G1 phase by flow cytometry

(Fig. 5). Compound **2** induced apoptosis in HL-60 cells, and 24 h of treatment shifted more cells to sub-G1 phase (untreated, 1.9 ± 0.0%; compound **2** (30 μM), 55.7 ± 1.84%). Furthermore, this significant increase occurred in a time-dependent manner (12 h, 43.05 ± 0.07%; 18 h, 45.65 ± 0.07%; 24 h, 55.7 ± 1.84%) and was accompanied by decreases in the percentages of cells in the G1 (untreated, 65.55 ± 0.92%; compound **2** (30 μM): 12 h, 41.45 ± 0.35%; 18 h, 42.15 ± 0.07%; 24 h, 36.25 ± 1.34%), S (untreated, 18.5 ± 0.42%; compound **2** (30 μM): 12 h, 9.05 ± 0.21%; 18 h, 8.55 ± 0.21%; 24 h, 6.35 ± 0.49%), and G2/M (untreated, 13.75 ± 0.35%; compound **2** (30 μM): 12 h, 6.2 ± 0.00%; 18 h, 3.45 ± 0.21%; 24 h, 1.3 ± 0.14%) phases (Fig. 5). However, the cell cycle distribution of HL-60 cells was not altered by treatment with compound **1**. These results suggest that compound **2** strongly induced HL-60 cell cycle arrest at sub-G1 phase.

Compound 2 Induced Apoptosis Via Cell Membrane Collapse

Next, we evaluated the induction of early and late apoptosis by flow cytometry (Fig. 6). To quantitate apoptosis, we performed Annexin V/PI staining, which stains phosphatidylserines on the external layer of the cell membrane (a common feature of apoptosis). As shown in Fig. 6, the percentages of early and late apoptotic cells increased in a time-dependent manner in response to compound **2** (30 μM). By contrast, compound **2** induced a time-dependent decrease in the live cell populations. On the other hand, HL-60 cells showed no significant changes after treatment with compound **1** for any amount of time.

Compound 2 Induced Apoptosis Via Mitochondrial Membrane Potential Collapse

To confirm the induction of apoptosis by compound **2**, we evaluated the MMP using the JC-1 kit and running samples through a flow cytometer. Live cells with functional mitochondria showed red JC-1 aggregates, whereas apoptotic cells with impaired mitochondria contained green JC-1 monomers. Green and red fluorescence intensities were measured in HL-60 cells treated with compounds **1** or **2** for different amounts of time (12 h, 18 h, and 24 h). Compared with no treatment, compound **2** simultaneously increased JC-1 green fluorescence and decreased JC-1 red fluorescence in a time-dependent manner (Fig. 7). On the other hand, compound **1** caused no significant changes at any time. Thus, compound **2** was found to increase the permeability of the mitochondrial membrane in HL-60 cells, which might initiate apoptosis via the mitochondria-mediated pathway.

Two compounds of P. japonicum **induced apoptosis in HL-60 cells via activation of the caspase family**

Caspases, a family of cysteine proteases, are known to form an integral part of the apoptotic pathway. Therefore, to research whether the apoptosis induced by the two compounds in HL-60 cells is dependent on the activation of the caspase family, we investigated the effect of the expression of activated caspase-

3, -8, and − 9. As shown in Fig. 8, the percentages of activated caspases increased in a time-dependent manner in response to compound **2**. In addition, these percentages were affected by Z-DEVD-FMK (a caspase-3 inhibitor), Z-IETD-FMK (a caspase-8 inhibitor), and Z-LEHD-FMK (a caspase-9 inhibitor), which further confirmed the participation of caspase-3, -8, and − 9 in the mechanism of compound **2**.. On the other hand, compound 1 caused no significant changes at any time (Fig. 8).

Discussion

Despite massive research efforts, the fundamental cause of cancer has not been identified, although it has been established that some cancers are associated with gene mutations. Nonetheless, cancer in its various guises remains the most common cause of death. Cancer treatments involve the surgical resection of tumors or the use of antitumor agents, which are administered as a first-line therapy to induce the apoptosis of cancer cells. However, most synthetic agents also induce the apoptosis of normal cells and often cause distressing adverse effects. Recently, research on the development of new antitumor agents has focused on the use of herbal medicines because they are prone to exerting few side effects.

P. japonicum is a perennial herb distributed in Japan, the Philippines, China, and Korea, and its roots are used to treat coughs, colds, and headaches [5]. However, the cytotoxic effects of its roots have not been explored to date. The active compounds **1** and **2** isolated from *P. japonicum* in the present study possess the same core structure but differ with respect to the side chain and stereochemistry at C-3′ and C-4′, As the current study shows, these compounds exhibit different cytotoxicities against HL-60 cells. The senecioyl group, which is a practical functional group, can be identified in the structure of compound **2** and has been shown to inhibit inflammation [30]. In addition, there was a research report confirming that the core structure in the root of *Angelica keiskei* is the same, but apoptotic death in HL-60 cells is caused by the functional senecioyl group [31]. In the present study, at a concentration of 30 μM, compounds **1** and **2** were found to reduce the survival rates of four cancer cell lines (Fig. 3). Interestingly, only compound **2** reduced HL-60 cell viability dramatically, and in HL-60 cells, compound **2** at 30 μM decreased the survival rate more than did compound **1** (Fig. 4). Furthermore, we focused on the effects of both compounds on the apoptosis of HL-60 cells mediated via cellular mechanisms involving activated caspase-3, -8, and − 9 and observed morphological characteristics of apoptosis, such as apoptotic body formation and nuclear condensation of apoptosis, through sub-G1 DNA accumulation in HL-60 cells treated with compounds **1** and **2**. Compound **2** increased the proportion of cells in the sub-G1 phase while decreasing the proportions of cells in the G1/S/G2/M phases in a time-dependent manner. The number of living cells decreased in a time-dependent manner, and the percentages of cells in early apoptosis and late apoptosis also significantly increased upon treatment with compound **2** (Fig. 6). Based on morphological changes, cell death may be classified as apoptotic or necrotic [32]. Apoptosis driven by the mitochondrial pathway is characterized by plasma membrane blebbing, condensation, cell fragmentation, and the extensive degradation of chromosomal DNA [32]. Furthermore, when treated with compound **2**, HL-60 cells showed increases in the percentage of green JC-1 monomers whereas the percentages of red JC-1 aggregates decreased time-dependently because of the collapse of the MMP.

Conclusion

In this study, we isolated compounds **1** and **2** from the roots of *P. japonicum* and demonstrated that compound **2** induces apoptosis specifically in HL-60 cells exert its anticancer effects. Additionally, these results indicate that compound **2** induces cell death via the mitochondrial-mediated pathway (by activating the caspase family) in human leukemia HL-60 cells. Compound **2** specifically induced apoptosis in HL-60 cells compared with compound **1** because of structural differences.

Abbreviations

HL-60 cells : Human acute promyelocytic leukemia cells, MCF-7 : human breast adenocarcinoma cells, A549 : human lung adenocarcinoma cells, MDCK : Madin-Darby canine kidney, MMP : mitochondrial membrane potential, DMEM : Dulbecco's modified Eagle medium, RPMI 1640 medium : Rosewell Park Menorial Institute 1640 medium, CCK-8 : cell counting kit-8, DMSO : dimethyl sulfoxide , PI : propidium iodide, MeOH : methanol, H2O : water, CH_2Cl_2 : dichloromethane, HPLC : High Pressure Liquid Chromatography, NMR : nuclear magnetic resonance, $CDCl_3$: Chloroform, FBS : fetal bovine serum

Declarations

Acknowledgements

Not applicable.

Authors' contributions

KYK, SJL, SJN and STY conceived and designed the study. KYK, SJL, and HJK conducted the experiments. HK, SJN and STY provided the technical support and advices for the study. KYK and SJL wrote the manuscript, KYK, SJN and STY revised the manuscript. All authors contributed to the review and the approval of the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Not applicable.

Consent for publication

All of authors consent to publication of this study in Journal of Chinese Medicine.

Competing interests

The authors declare that they have no competing interests.

Author details

[1] Suncheon Research Center for Natural Medicines, Suncheon 540-742, Republic of Korea

[2] Laboratory of Marine Drugs, School of Earth and Environmental Sciences, Seoul National University, NS-80, Seoul 08826, Korea

[3] Research Institute of Oceanography, Seoul National University, NS-80, Seoul 151-747, Republic of Korea

[4] Department of Chemistry and Nanoscience, Global Top5 Program, Ewha Womans University, Seoul 120-750, Republic of Korea

[5] Department of Pharmacy, Sunchon National University, Suncheon 540-742, Republic of Korea

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Figures

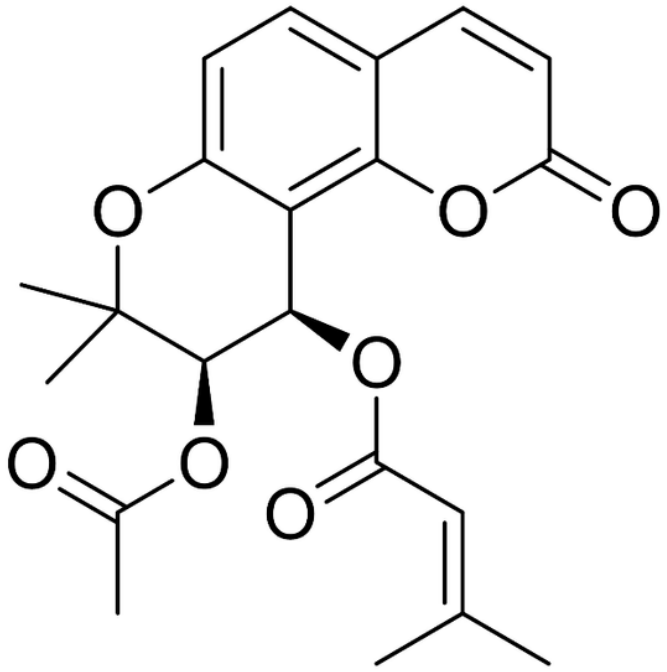
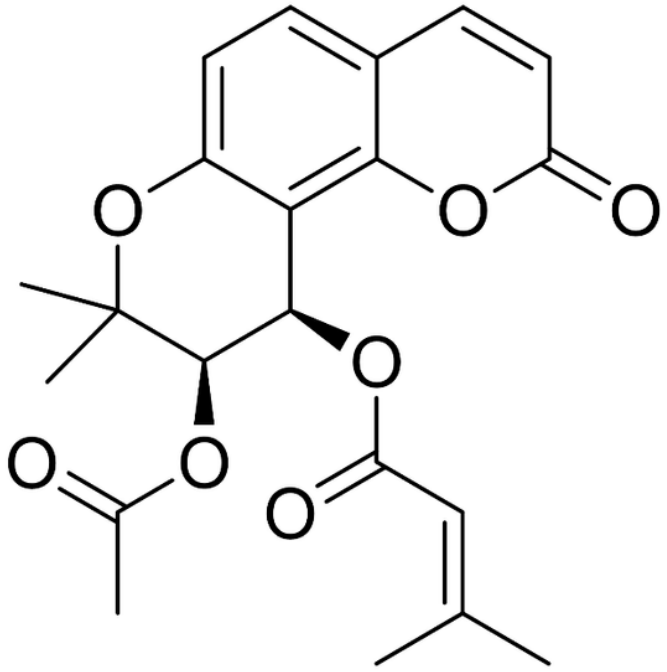


Figure 1

Structure of compound 1.



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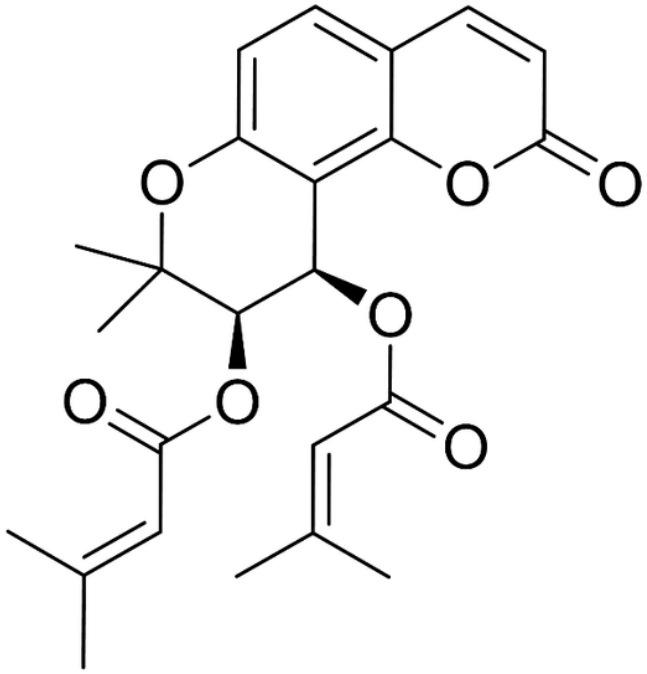
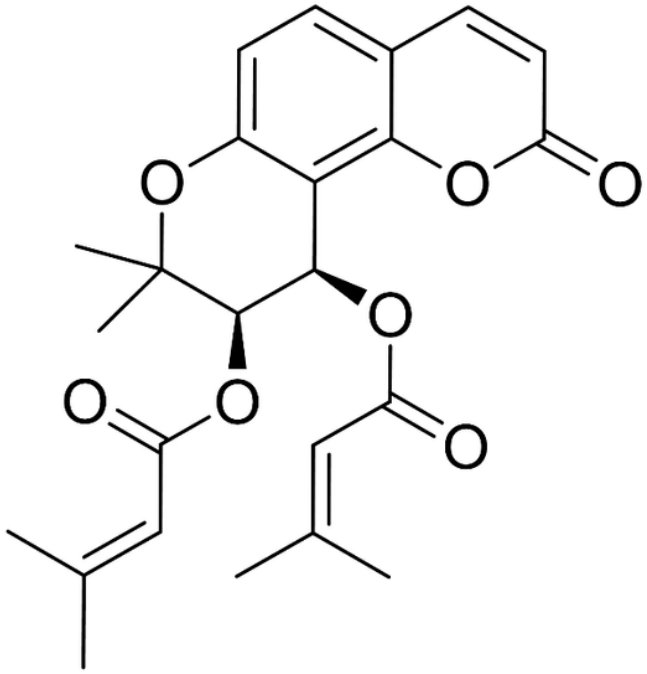


Figure 2

Structure of compound 2.



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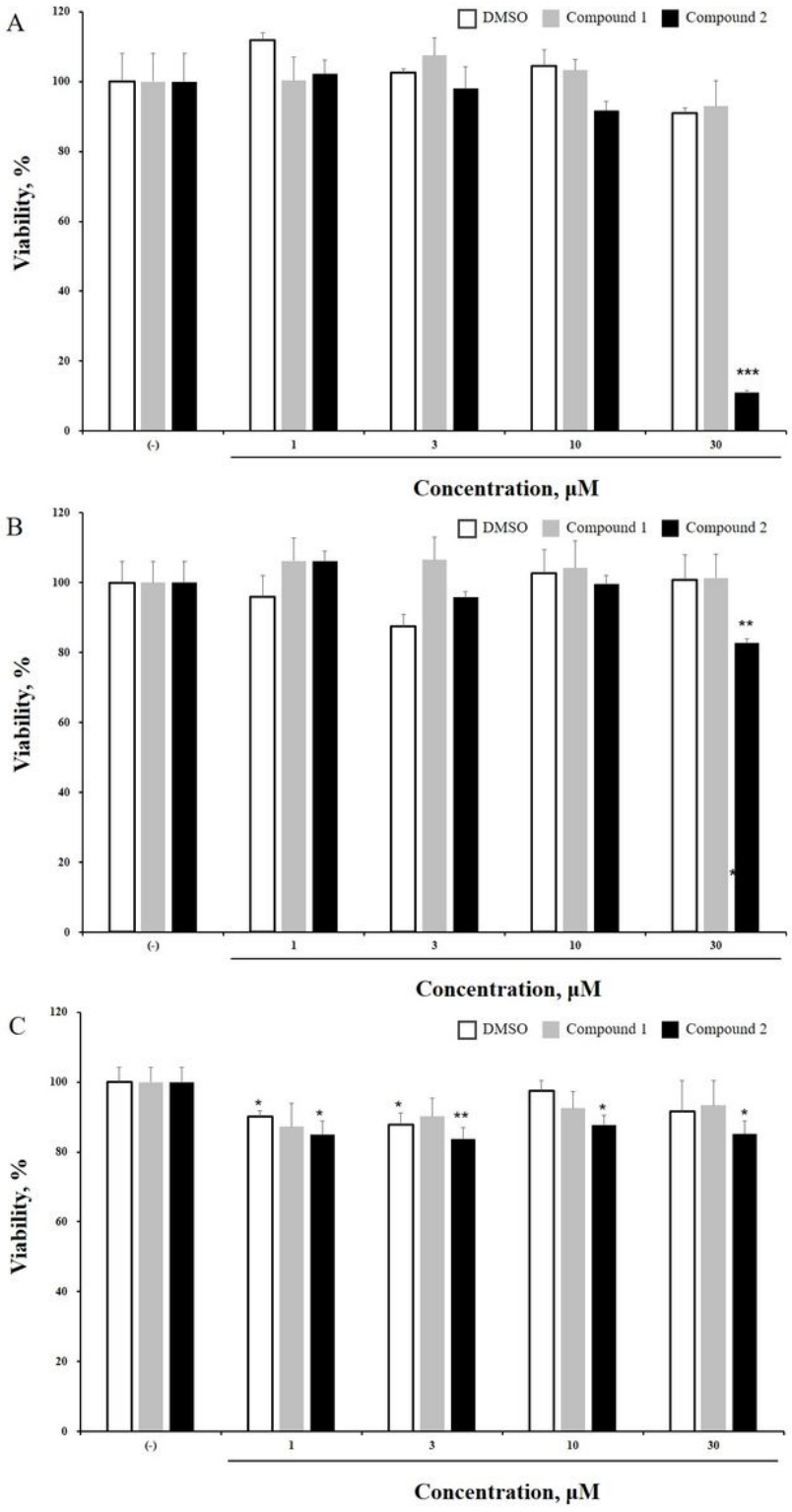

Figure 3

The effect of P. japonicum root extracts on the viabilities of cancer cell lines. Three cancer cell lines (A: human acute promyelocytic leukemia cells, HL-60; B: human lung adenocarcinoma cells, A549; C: human breast adenocarcinoma cells, MCF-7) were treated with P. japonicum extracts for 24 h. Culture supernatants were removed, and cell counting kit-8 (CCK-8) was added to the cells. Viability was

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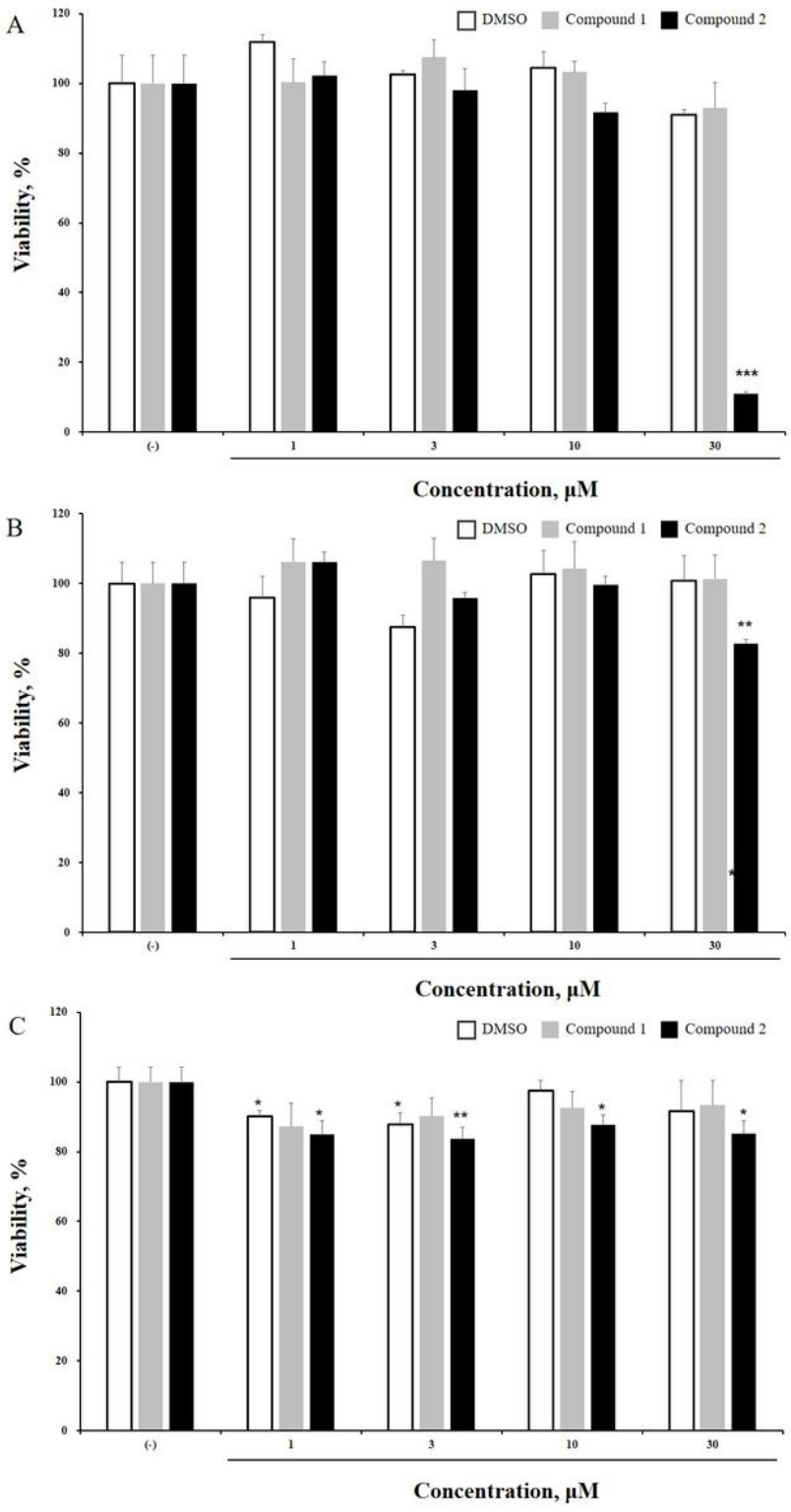

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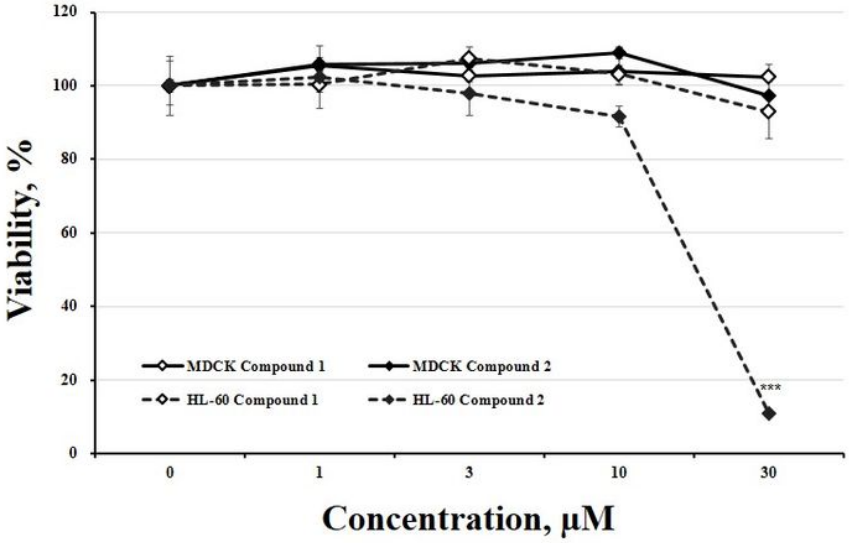
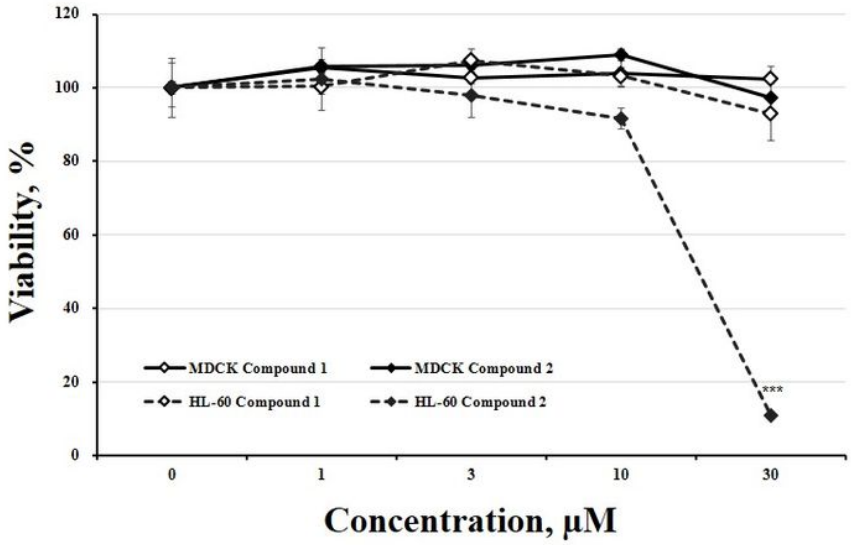


Figure 4

HL-60 cell viability versus MDCK cell viability. HL-60 and MDCK cells were treated with each dose of compounds 1 and 2 for 24 h. The culture supernatant was removed, and CCK-8 was added. Cell viabilities were determined by ELISA. The results are presented as the means±S.D. of experiments performed in triplicate.



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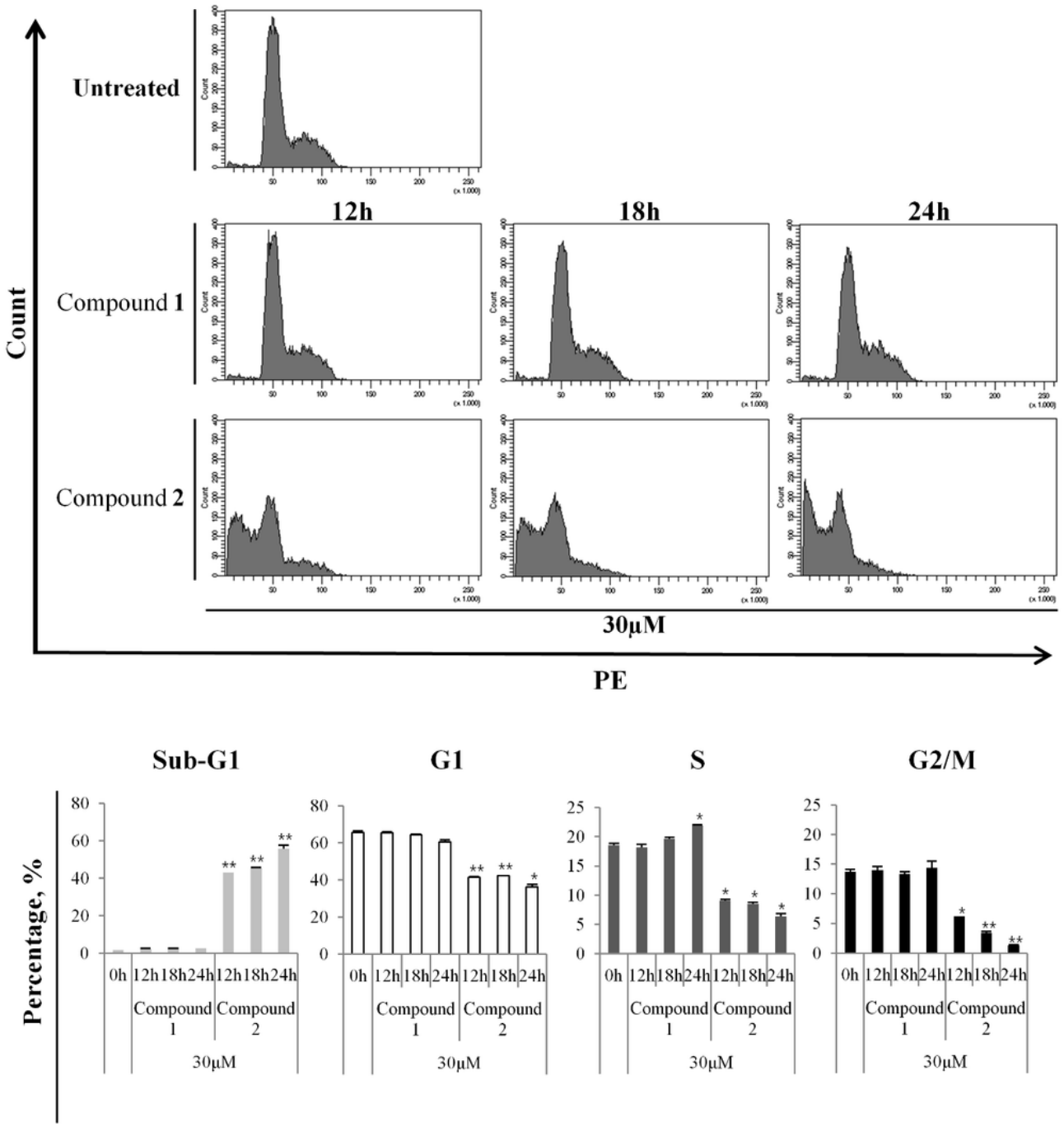
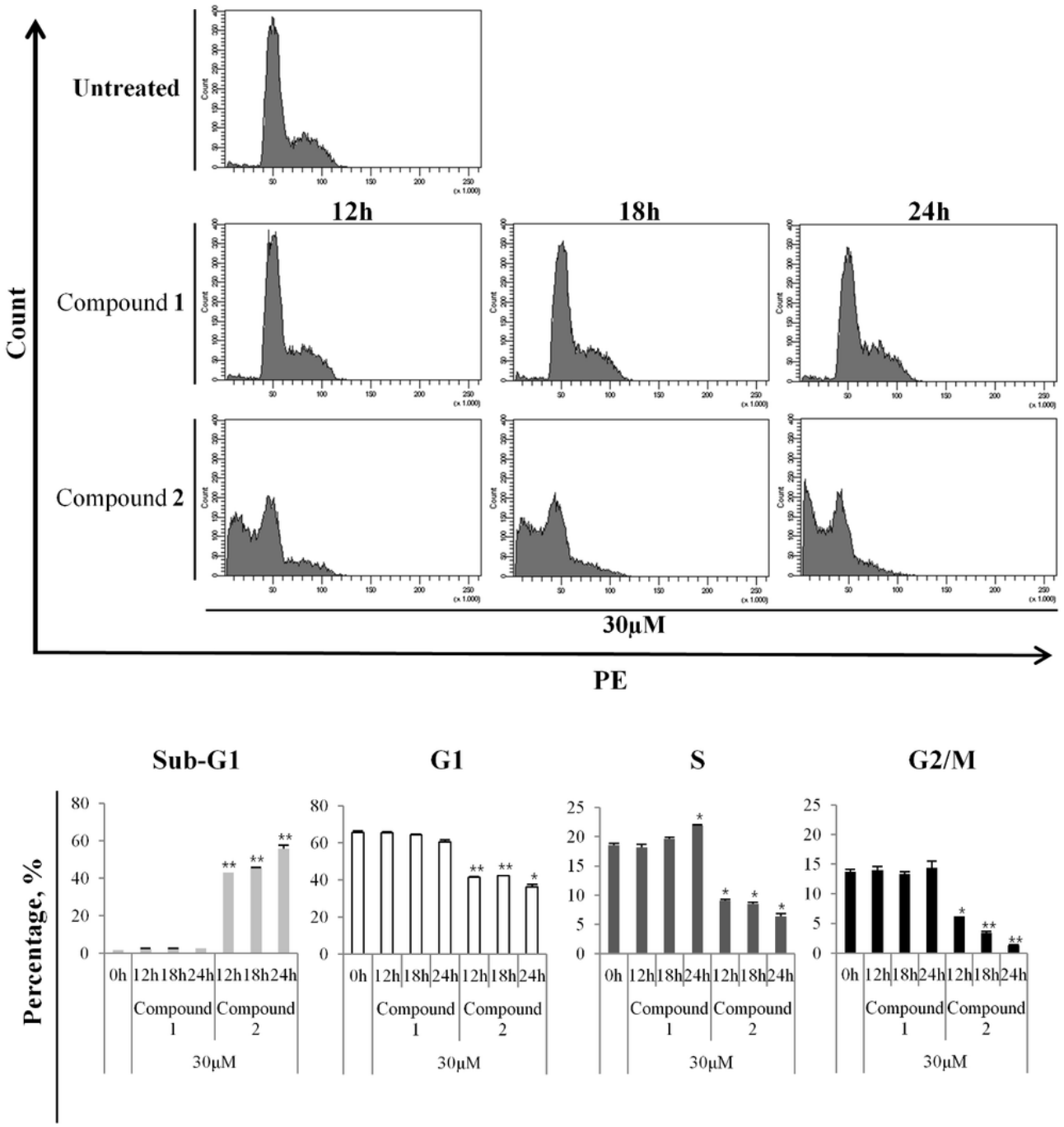


Figure 5

Incremental increases in the percentage of HL-60 cells in sub-G1 phase after treatment with compound 2. HL-60 cells were treated with compound 2 for 12, 18, and 24 h. After the indicated incubation times, cells were harvested, stained with PI for 2 h, and analyzed by flow cytometry. The results are presented as the means±S.D. of experiments performed in duplicate.



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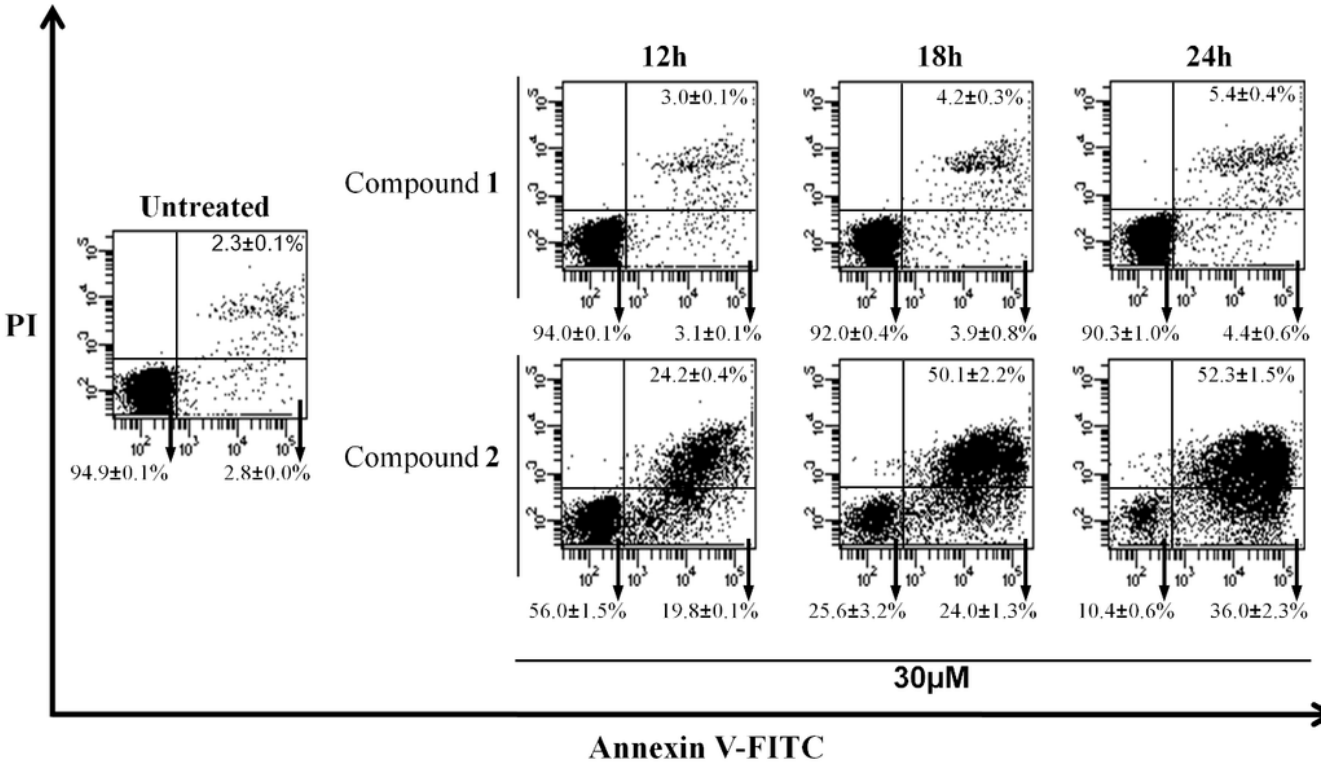


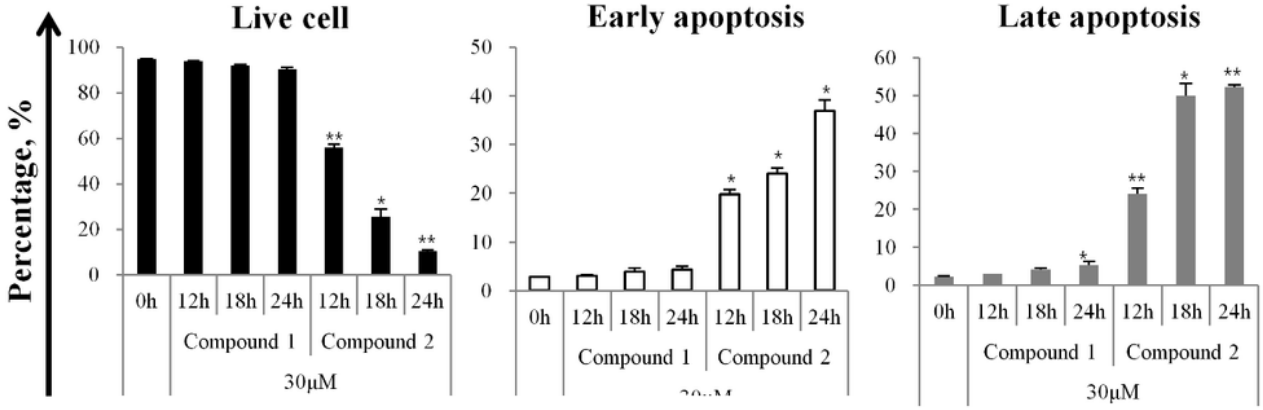
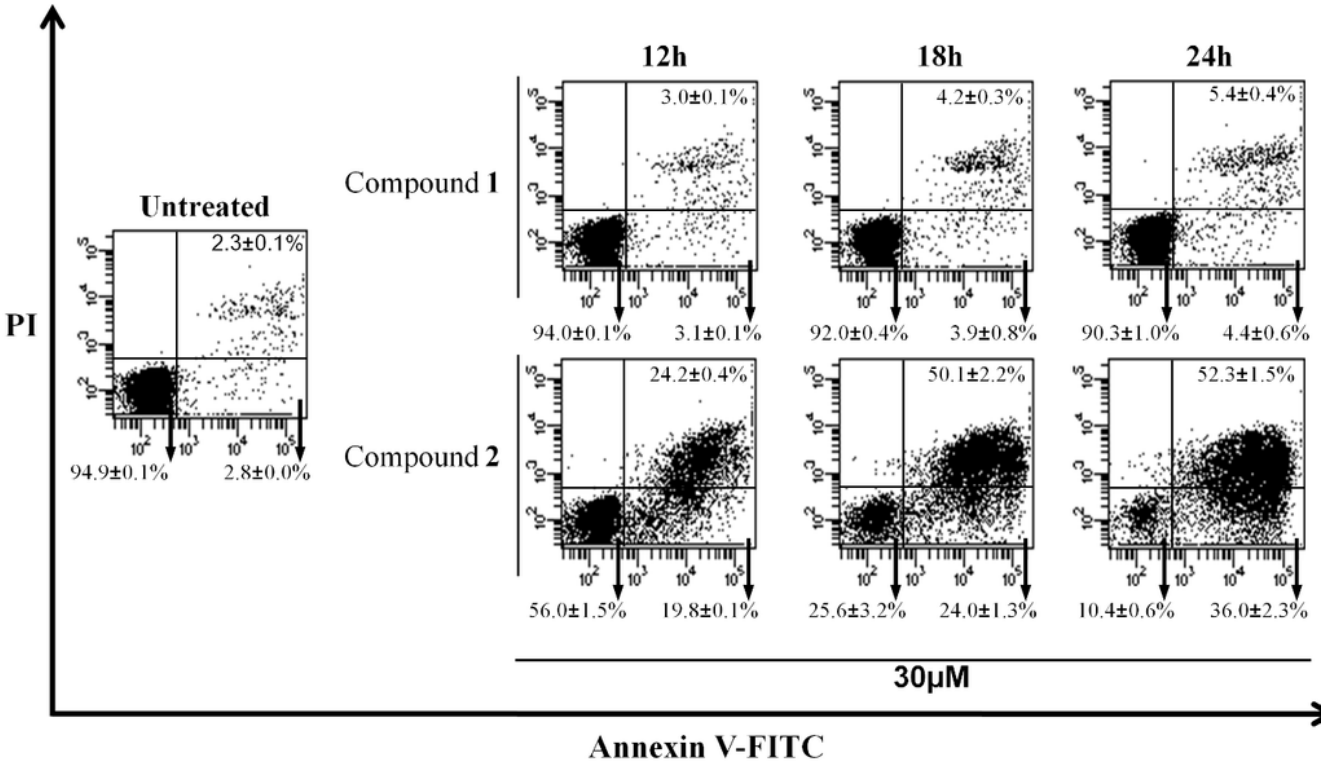
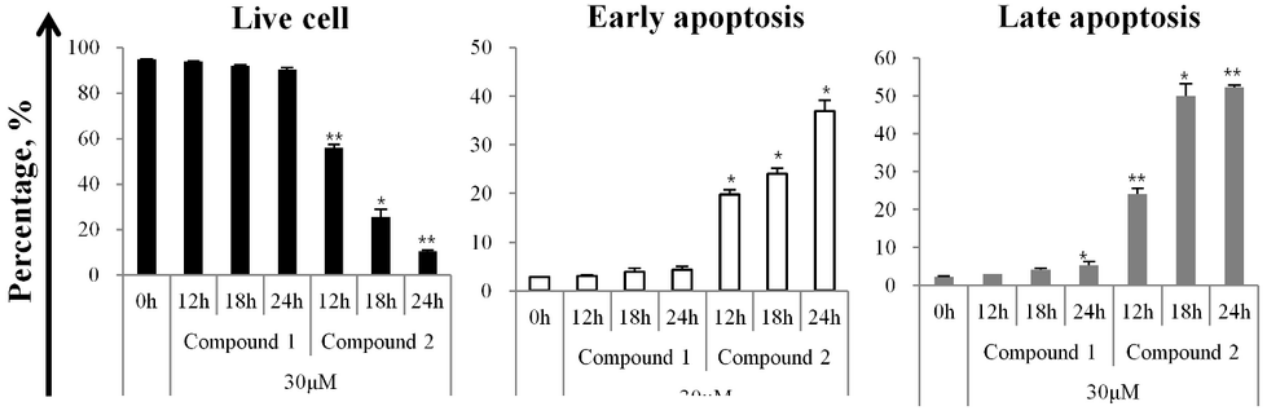


Figure 6

The effect of compound 2 on HL-60 cell membranes. HL-60 cells were treated with compound 2 for 12, 18, and 24 h. After the indicated times, cells were harvested, stained with Annexin V (FITC) and PI for 15 min, and analyzed on a flow cytometer. The results are presented as the means±S.D. of experiments performed in duplicate.





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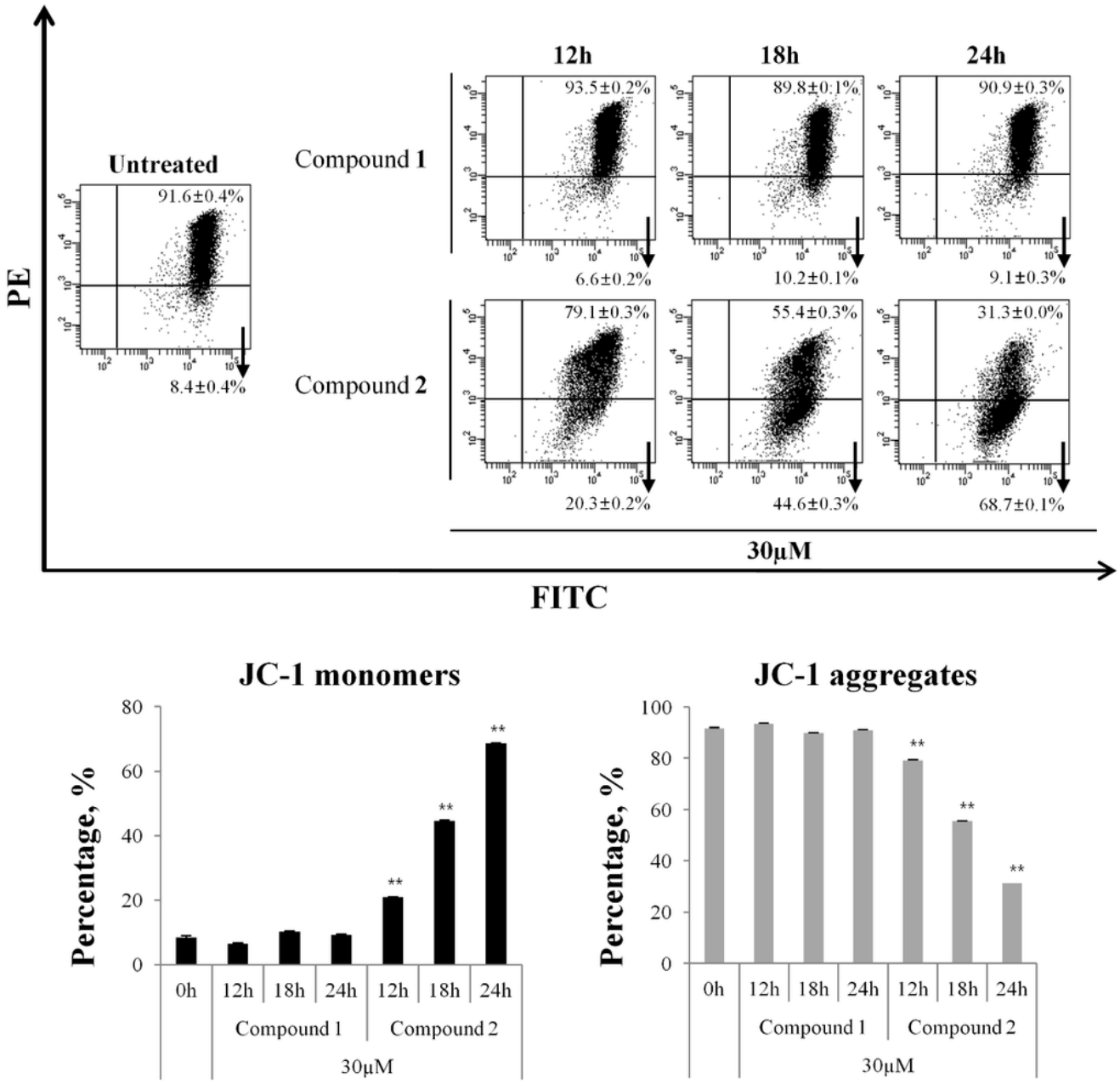
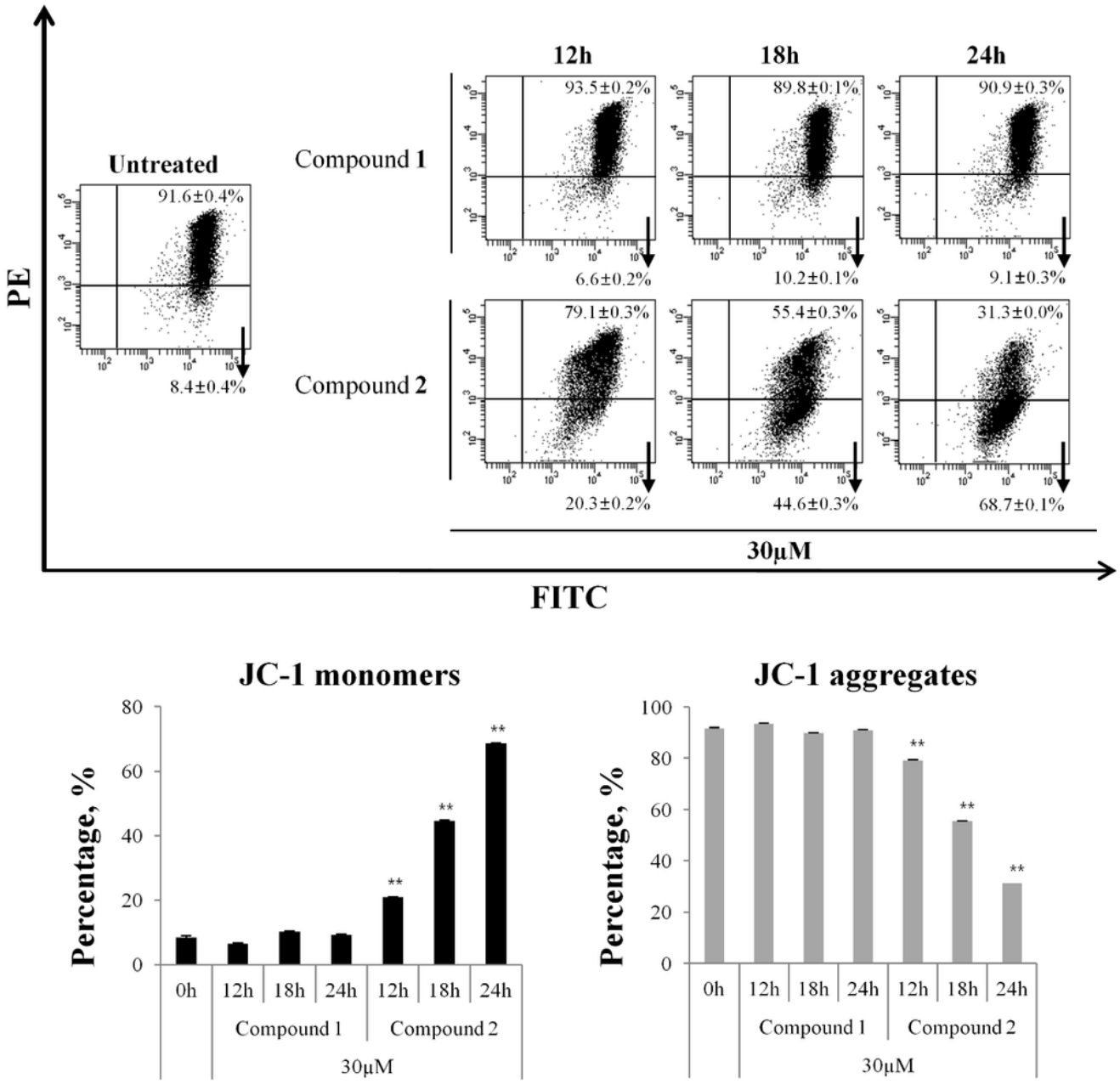


Figure 7

The effect of compound 2 on mitochondrial membrane potential in HL-60 cells. HL-60 cells were treated with compound 2 for 12, 18, and 24 h. After the indicated incubation times, cells were harvested, stained with JC-1 for 30 min and then analyzed by flow cytometry. The results are presented as the means±S.D. of experiments performed in duplicate.



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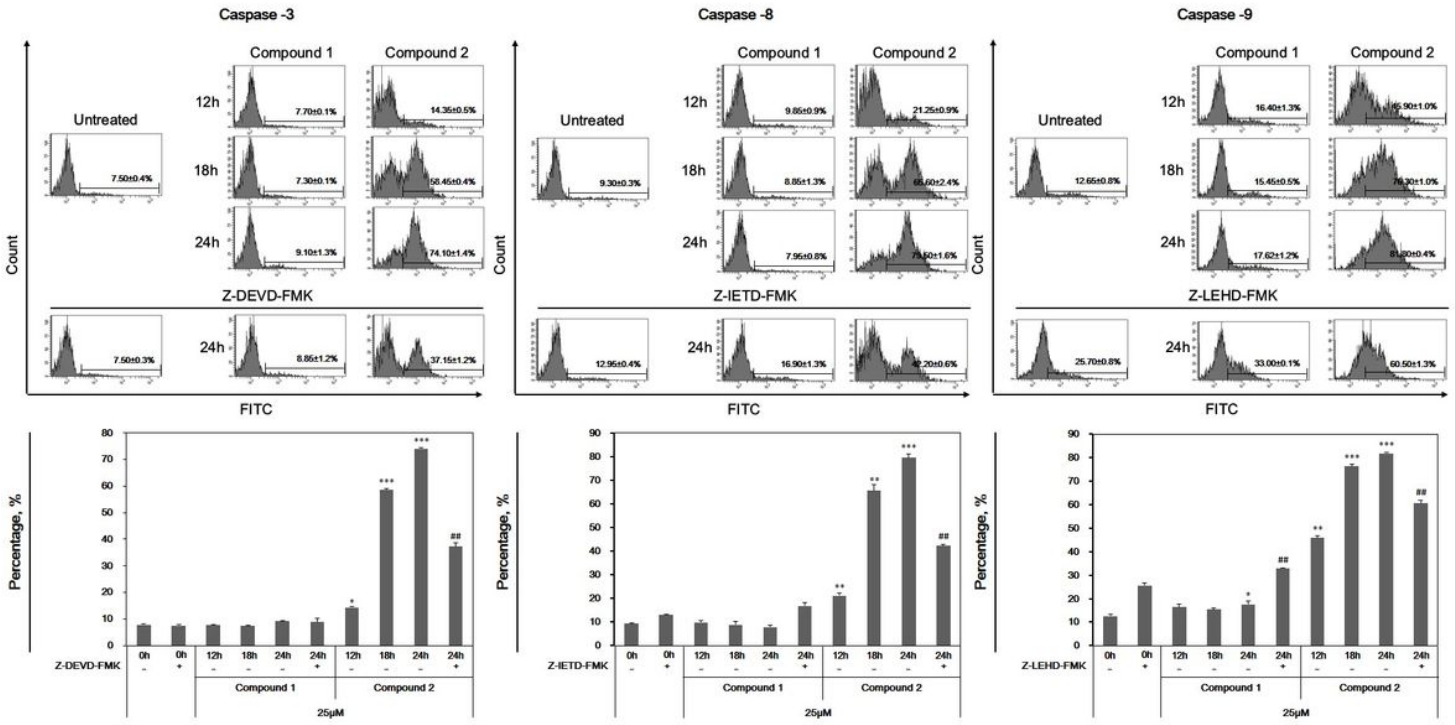
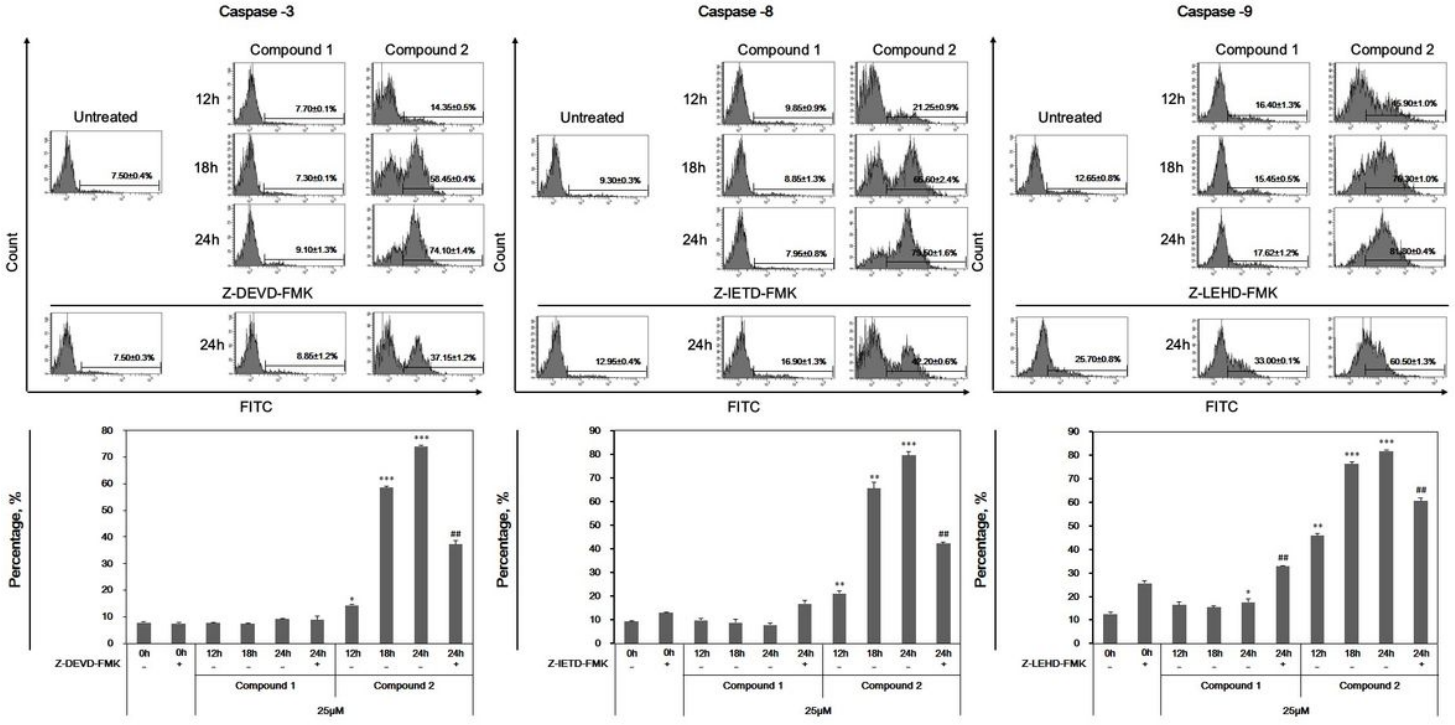


Figure 8

The effect of compounds 1 and 2 on the activation of caspase-3, -8, and -9. HL-60 cells were treated with the compounds for 12, 18, and 24 h. After the indicated incubation times, cells were harvested and stained with FITC for 1 h. Activated caspase-8 was analyzed by flow cytometry. The results are presented as the means ± S.D. of experiments performed in duplicate.



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