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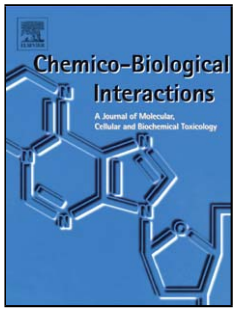

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Cajanol, a novel anticancer agent from Pigeonpea [*Cajanus cajan* (L.) Millsp.] roots, induces apoptosis in human breast cancer cells through a ROS-mediated mitochondrial pathway

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Abstract

Cajanol (5-hydroxy-3-(4-hydroxy-2-methoxyphenyl)-7-methoxychroman-4-one) is an isoflavanone from Pigeonpea [*Cajanus cajan* (L.) Millsp.] roots. As the most effective phytoalexin in pigeonpea, the cytotoxic activity of cajanol towards cancer cells has not been report as yet. In the present study, the anticancer activity of cajanol towards MCF-7 human breast cancer cells was investigated. In order to explore the underlying mechanism of cell growth inhibition of cajanol, cell cycle distribution, DNA fragmentation assay and morphological assessment of nuclear change, ROS generation, mitochondrial membrane potential (ΔΨm) disruption, and–expression of caspase-3 -9, Bax, Bcl-2, PARP and cytochrome c were measured in MCF-7 cells. Cajanol inhibited the growth of MCF-7 cells in a time and dose-dependent manner. The IC_{50} value was 54.05 μM after 72 h treatment, 58.32 μM after 48 h; and 83.42 μM after 24 h. Cajanol arrested the cell cycle in the G2/M phase and induced apoptosis via a ROS-mediated mitochondria-dependent pathway. Western blot analysis showed that cajanol inhibited Bcl-2 expression and induced Bax expression to desintegrate the outer mitochondrial membrane and causing cytochrome c release. Mitochondrial cytochrome c release was associated with the activation of caspase-9 and caspase-3 cascade, and active-caspase-3 was involved in PARP cleavage. All of these signal transduction pathways are involved in initiating apoptosis. To the best of our knowledge, this is the first report demonstrating the cytotoxic activity of cajanol towards cancer cells *in vitro*.

Keywords: Cajanol; Anti-cancer activity; MCF-7; Apoptosis; Cell cycle arrest

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1. Introduction

Cancer is the second leading cause of death in industrialized countries, and breast cancer is the second most deadly cancer among women [1, 2]. Cyclophosphamide, methotrexate, and 5-fluorouracile (termed CMF regimen) as well as anthracyclines and paclitaxel belong to the chemotherapeutical drugs for breast cancer. However, the development of drug resistance and severe side effects of standard anticancer drugs necessitates the search for novel treatment options for this disease [3]. The discovery of new natural and synthetic products for cancer treatment is of great urgency to improve prospects of affected women for cure from their disease [4].

Apoptosis is a form of programmed cell death which occurs through activation of cell-intrinsic suicide machinery [5] and is a hallmark of action of many anticancer drugs [6-8]. Activation of the apoptotic cascade results from a complex interaction of molecular events [9]. ROS are free radicals such as superoxide (O^{2-}), hydroxy radical (OH^{-}), and non-radical derivatives of oxygen such as H_2O_2 mainly derived from the respiratory chain in mitochondria [10, 11]. ROS generation and disruption of the mitochondrial membrane potential contributed to drugs-induced apoptosis [12, 13]. The mitochondria-dependent pathway for apoptosis is governed by Bcl-2-family proteins [14]. Bax/Bcl-2 regulates the release of cytochrome c from mitochondria into the cytosol, and the cytochrome c in the cytosol initiates caspases cascade which terminates cells to apoptosis [15].

Cajanol is an isoflavone from pigeonpea [*Cajanus cajan* (L.) Millsp.] roots [16]. The chemical structure of cajanol is shown in Fig. 1. The bioactivities of cajanol have sparsely been elucidated with the exception of their antiplasmodial and antifungal activity [16, 17]. In the present study, we first examined the growth inhibitory effect of cajanol on human

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breast cancer MCF-7 cells by the MTT (3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide) assay. Cell cycle, apoptosis analysis, generation of ROS, and mitochondrial membrane potential were studied by flow cytometry (Partec). DNA agarose electrophoresis, morphological assessment of nuclear changes and measurement of caspase-3 and -9 activities were used to assess apoptosis. The expression of cytochrome c, PARP, Bax and Bcl-2 proteins were further assayed by Western blotting.

To the best of our knowledge, the cytotoxic activity of cajanol towards cancer cells was studied for the first time in the present study. Our studies encourage the development of novel, efficient and less toxic plant derived molecules for cancer chemotherapy.

2. Material and methods

2.1. Growth of cell and chemicals

The human breast cancer MCF-7 cell line was purchased from Harbin Medical University, China. All the cells were maintained in RPMI 1640 medium supplemented with 10 % fetal bovine serum and 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were kept at 37 °C in a humidified atmosphere containing 5 % CO_2.

Cajanol (purity≥98%) was isolated from Pigeonpea [*Cajanus cajan* (L.) Millsp.] roots, and the chemical structure identified in our lab [18]. A 10 mg/mL stock solution of cajanol was prepared in dimethyl sulfoxide (DMSO) and stored at -80 °C. MTT, rhodamine 123 (Rh123) and propidium iodide (PI) were obtained from Sigma-Aldrich Inc. (St. Louis, MO). Deionized water was used in all experiments.

2.2. Cytotoxicity assays

Inhibition of cell proliferation by cajanol was measured by the MTT assay [19]. Briefly, MCF-7 cells were plated in 96-well culture plates (1×10^5 cells/well) separately. After 24 h

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incubation, cells were treated with cajanol (0, 9.88, 19.75, 39.5, 79, 158 and 316 μM, eight wells per concentration) for 24h, 48 h; or 72 h, MTT solution (5 mg/mL) was then added to each well. After 4 h incubation, the formazan precipitate was dissolved in 100 μL dimethyl sulfoxide, and then the absorbance was measured in an ELISA reader (Thermo Molecular Devices Co., Union City, USA) at 570 nm. The cell viability ratio was calculated by the following formula: Inhibitory ratio (%) = $\frac{OD_{control} - OD_{treated}}{OD_{control}}$ x 100 %. Cytotoxicity was expressed as the concentration of cajanol inhibiting cell growth by 50 % (IC_{50} value).

2.3. Flow cytometric analysis of cell cycle and apoptosis

Cell cycle was assayed with CyStain [20]. Briefly, 1×10^6 cells/well MCF-7 cells were seeded in six-well plate and left for 24 h in incubator to resume exponential growth. Cells were exposed to cajanol (0, 16, 32 and 64 μM) and incubated for 48 h. Then, the cells were harvested and washed with PBS. After suspension in 800 μL PBS, 200 μL CyStain (Partec GmbH, Germany). The cell cycle distribution of 10,000 cells was recorded by flow cytometry (Partec), and the percentage of cells at G0/G1, S, and G2/M phases was analyzed with FloMax software. The extent of apoptosis was measured through annexinV-FITC apoptosis detection kit (Beyotime Institute of Biotechnology, China) as described by the manufacture's instruction [20]. After exposure to cajanol (0, 16, 32 and 64 μM) for 48 h, cells were collected, washed twice with PBS, gently resuspended in annexin-V binding buffer and incubated with annexinV-FITC/PI in dark for 15 min and analyzed by flow cytometry using FloMax software. The fraction of cell population in different quadrants was analyzed using quadrant statistics. The lower left quadrant contained intact cells; lower right quadrant apoptotic and in the upper right quadrant

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necrotic or post-apoptotic cells.

2.4. DNA fragmentation assay

DNA fragmentation was assayed by agarose gel electrophoresis [21]. MCF-7 cells (1×10^6 cells/mL) were seeded in 6-well plates, exposed to cajanol (0, 16, 32 and 64 μM) for 48 h, and collected by centrifugation. Total DNA was purified with a DNA isolation kit (Waston Biotechnologies Inc, Shanghai, China) according to the manufacturer's instructions. The DNA was separated in 1% agarose gel and visualized by ultraviolet illumination (Image Master VDS-CL, Tokyo, Japan) after staining with ethidium bromide.

2.5. Morphological observation of nuclear change

Morphological observation of nuclear change was assayed with Hoechst 33258 [22]. MCF-7 cells (1×10^6 cells/mL) were seeded in 6-well plates and treated with 64 μM cajanol for 48 h at 37°C. Cells were collected, washed, fixed in 4 % paraformaldehyde for 30 min and stained with 5 μg/mL Hoechst 33258 for 5 min at room temperature. The apoptotic cells were visualized using inverted fluorescence microscope (Nikon TE2000, Tokyo, Japan).

2.6. Measurement of ROS generation

ROS generation was monitored by flow cytometry-using DCFH-DA [23]. Single-cell suspensions of cells treated with cajanol (0, 16, 32 and 64 μM) for 48 h were prepared in PBS supplemented with 50 mM glucose, and incubated with 10 μM DCFH-DA at 37°C for 30 min. Fluorescence generation due to the hydrolysis of DCFH-DA to dichlorodihydrofluorescein (DCFH) by non-specific cellular esterases and the subsequent oxidation of DCFH by peroxides was measured by means of flow cytometry.

2.7. The changes of mitochondrial membrane potential ($\Delta\Psi m$)

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The uptake of the cationic fluorescent dye rhodamine 123 has been used for the estimation of mitochondrial membrane potential [24]. MCF-7 cells were seeded at 1×10^6 cells/well into 6-well plates. After 24 h incubation, cells were treated with serial dilutions of cajanol (0, 16, 32 and 64 μM) for 48 h. Untreated controls and treated cells were harvested and washed twice with PBS. The cell pellets were then re-suspended in 2 mL of fresh incubation medium containing 1.0 μM rhodamine 123 and incubated at 37°C in a thermostatic bath for 30 min with gentle shaking. MCF-7 cells were separated by centrifugation and washed twice with PBS, then stained with 2 μg/mL PI and analyzed by means of flow cytometry.

2.8. Measurement of caspase-3 and caspase-9 activities

The activation of caspase-3 and caspase-9 were determined with the colorimetric kit (Nanjing kaiji Bio-Tek Corporation, China) [25]. MCF-7 cells (1×10^6 cells/mL) were harvested and washed once with PBS. After the MCF-7 cells were lysed, reaction buffer was added to the MCF-7 cells followed by the additional 5 μL of caspase-3 or caspase-9 colorimetric substrate (DEVD-pNA) and incubated in a 96-well plate for 4 h at 37°C in a CO_2 incubator. The plate was then read with a microplate reader at 405 nm. Activities of caspase-3 and caspase-9 were expressed relative to theoretical density value (OD).

2.9. Protein extraction and Western blot assays

To further evaluate the expression levels of various intracellular proteins related to apoptosis, MCF-7 cells were treated with cajanol (0, 16, 32 and 64 μM) for 48 h, respectively. For isolation of total protein fractions, cells were collected, washed twice with ice-cold PBS, and lysed using cell lysis buffer [20 mM Tris pH 7.5, 150 mM NaCl, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM EDTA, 1% Na_3CO_4, 0.5 μg/mL

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leupeptin, 1 mM phenylmethanesulfonyl fluoride (PMSF)]. The lysates were collected by scraping from the plates and then centrifuged at 10,000 rpm at 4°C for 5 min.

Total protein samples (20 μg) were loaded on a 12 % of SDS-polyacrylamide gel for electrophoresis, and transferred onto PVDF transfer membranes (Millipore, Billerica, USA) at 0.8 mA/cm^2 for 2 h. Membranes were blocked at room temperature for 2 h with blocking solution (1% BSA in PBS plus 0.05% Tween-20). Membranes were incubated overnight at 4°C with primary antibodies (anti-β-actin, anti-Bax anti-Cytochrome c were mouse polyclonal antibodies; anti-Bcl-2 and anti-PARP were rabbit polyclonal antibodies) at a 1:1000 dilution (Biosynthesis Biotechnology Company, Beijing, China) in blocking solution. After thrice washings in TBST for each 5 min, membranes were incubated for 1 h at room temperature with an alkaline phosphatase peroxidase-conjugated anti-mouse secondary antibody at a dilution of 1:500 in blocking solution. Detection was performed by the BCIP/NBT Alkaline Phosphatase Color Development Kit (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Bands were recorded by a digital camera (Canon, EOS 350D, Tokyo, Japan).

2.10. Statistical analysis

The data were expressed as a mean±S.D. An analysis of ANOVA variance with a Tukey post hoc test was used for multiple comparisons. Correlation was calculated using function ReglinP and inverted Student's *t*-test. All statistics were calculated using the STATISTICA program (StatSoft, Tulsa, OK, USA). A *P*-value of <0.05 was considered as significant.

3. Results

3.1. Cytotoxicity assays

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The anti-proliferative activity of cajanol on MCF-7 cells was determined by using the MTT reduction assay. Fig. 2 shows the effects of 0-316 μM cajanol on viability of MCF-7 cell lines after exposure for 24 h, 48 h and 72 h. The IC_{50} value was 54.05 μM after 72 h treatment, 58.32 μM after 48 h, and 83.42 μM after 24 h. Cajanol inhibited the growth of MCF-7 cells in a time- and dose-dependent manner.

3.2. Flow cytometric analysis of MCF-7 cell cycle distribution and apoptosis

To evaluate the cell cycle distribution of MCF-7 cells with or without cajanol treatment, the DNA content was measured by flow cytometry. As shown in concentration kinetic measurements (Fig.3), exposure to 16-64 μM cajanol caused an increase of the G2/M phase population from 19.24 to 47.87 %, as compared to 7.55 % ~~of~~ G2/M phase cells in untreated control samples. Hence, cajanol exerted growth-inhibitory effects via G2/M phase arrest in a concentration-dependent manner. Apoptosis of MCF-7 cells were analyzed by flow cytometry using annexinV-FITC/PI (Fig. 4). A small percentage of untreated MCF-7 (2.28±0.56%) cells bound annexin V-FITC (Fig. 4a). In contrast, the percentage of annexin V-FITC binding MCF-7 cells significantly increased in a concentration-dependent manner after treatment with 16-64 μM cajanol (12.84±3.86%-78.03±3.28%, $p < 0.01$) (Fig.4).

3.3. Assessment of apoptosis by the DNA fragmentation assay

Apoptosis is an active process that ultimately leads to the activation of endonucleases and cleavage of DNA into fragments of about 180-200 base pairs [26]. Treatment of MCF-7 cells with cajanol resulted in the formation of a DNA ladder (Fig. 4). Apoptosis-related DNA laddering was visible after treatment with 32 or 64 μM cajanol for 48 h (Fig. 4, lane 4 and lane 5), but not after treatment with 16 μM cajanol (lane 3).

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Untreated control cells did not induce apoptosis (lanes 2).

3.4. Nuclear morphology changes

To investigate the affection of cajanol on nuclear morphology during cell apoptosis, we used Hoechst 33258 staining showed considerable morphological changes in nuclear chromatin (Fig. 6). The nuclei of untreated control MCF-7 cells were stained in less bright blue and homogeneous color. By contrast, after treatment with 64 μM cajanol for 48 h, most cells exhibited very intense staining of condensed and fragmented chromatin. The white arrows pointed at the condensed chromatin, while the blue arrow pointed at the fragmented chromatin. Some of them formed typical apoptotic bodies. Only a few nuclei still displayed normal morphology.

3.5. Measurement of reactive oxygen species (ROS)

Generation of ROS upon cajanol treatment was measured by means of DCFH-DA and flow cytomery as an indicator of peroxides and superoxide accumulation. Upon challenge of MCF-7 cells for 48 h with cajanol, a concentration-dependent increase of ROS production was observed (Fig. 7). Mean fluorescence intensity of untreated cells was 11.62±0.22, and the mean values changed to 14.48±0.71, 19.81±1.09 and 22.26±0.68, after treatment with 16 μM, 32 μM and 64 μM cajanol, respectively. Fluorescence intensities of cajanol-treated cells MCF-7 were higher than those of untreated controls ($p < 0.01$).

3.6. Disruption of mitochondrial membrane potential (ΔΨm)

To analyze the change of mitochondrial membrane potential, rhodamine 123 was used as fluorescent dye. In Fig. 8A, four histograms of untreated MCF-7 cells (upper left histogram) and cells treated with 16 μM (upper right histogram), 32 μM (lower left

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histogram), and 64 μM cajanol (lower right histogram) are depicted. Quadrants 1 and 2 of the histograms show late apoptotic cells, stained with PI. Quadrants 1 and 3 contain cells with disrupted mitochondrial membrane potential (ΔΨm) as visualized by rhodamine 123 staining. Quadrant 4 shows intact, living cells without apoptosis and disrupted mitochondrial membrane potential. The percentages of ΔΨm disrupted cells are shown in Fig. 8B. The addition of cajanol at doses of 16, 32, 64 μM led to increasing percentages of ΔΨm disruption from 29.00±2.93 % and 46.72±7.58 % to 94.88±4.32 %.

3.7. Caspase-3 and caspase-9 activities

Caspases are important regulators of apoptosis [27]. Therefore, we investigated the involvement of caspase-3 and caspase-9 in cajanol-induced apoptosis. In the untreated MCF-7 cells, OD value of caspase-3 was 0.021 ± 0.008, and the OD value of caspase-9 was 0.015 ± 0.004 (Fig.9). After treatment with cajanol (16-64 μM) for 48 h, a dose-dependent increase of caspase-3 and caspase-9 activities were observed. The highest activities of caspase-3 and caspase-9 were found upon exposure to 64 μM cajanol. The OD values were 0.189 ± 0.013 and 0.174 ± 0.021, respectively, and were significantly higher than those in the control group ($p < 0.01$, Fig. 9).

3.8. Cajanol-mediated up-regulation of Bax and down-regulation of Bcl-2

Bcl-2 inhibits apoptosis by preventing cytochrome c release from the mitochondria and inhibiting caspase activation [28]. Bax induces apoptosis by desintegrating the outer mitochondrial membrane and causing cytochrome c release [29]. Therefore, we analyzed Bcl-2 and Bax expression upon treatment of MCF-7 cells with cajanol. After treatment with cajanol (16-64 μM) for 48 h, the relative Bcl-2 expression decreased from 66.89±4.16% to 47.20±5.85%, and the relative Bax expression increased from

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186.36±8.74% to 341.36±7.87%. Western analysis revealed that MCF-7 cells treated with cajanol-exhibited a significant dose-dependent increase in Bax expression compared with control cells (Fig. 10). By contrast, Bcl-2 expression decreased in a dose-dependent manner.

3.9. Cajanol induced the release of cytotchrome c

With treatment of cajanol (16-64 μM) for 48 h, the relative mitochondria cytochrome c expression decreased from 87.93±3.75% to 19.40±3.03%, and the relative cytosolic cytochrome c expression increased from 190.04±7.63% to 503.91±8.75% (Fig. 11). A significant increase in cytosolic cytochrome c was observed in MCF-7 cells, indicating cytochrome c release from mitochondria to cytoplasm as initiating for downstream caspase activation.

3.10. Cajanol involved in PARP cleaving

Induction of PARP cleavage is important for apoptosis induction [30]. As shown in Fig. 11, after treatment with cajanol (16-64 μM) for 48 h, the relative PARP expression decreased from 95.64±5.04% to 38.89±4.66%, meanwhile, the cleavage PARP expression was increased. Cajanol induced PARP cleavage, which is visible by the appearance of a protein band of 85 kDa band and the disappearance of a 116 kDa band.

Discussion

In the present study, we found that cajanol was cytotoxic towards human breast cancer MCF-7 cells. Cajanol is an isoflavone with three phenolic hydroxyl and a methoxyl groups. Structure-activity relationship of flavonoids showed that at least two hydroxylations in positions 3, 5, and 7 of the A ring were needed to induce apoptosis [31]. Furthermore, Joseph et al. (2009) reported that methylated isoflavones may have greater anti-cancer

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activity than those without methyl groups [32]. Accordingly, we concluded that two hydroxylations in positions 5 and 7 of the A ring and a methoxyl group in the B ring may contribute to cajanol's antitumor properties.

Cell cycle control represents a major regulatory mechanism of cell growth [33]. Blockade of the cell cycle is regarded as an effective strategy for the development of novel cancer therapies [34, 35].. Cell cycle analysis of the treated culture revealed that cajanol induced a concentration-dependent G2/M phase cell cycle arrest with an accompaniment decrease in G1 and S phase. This confirmed that cajanol inhibited DNA synthesis and induced a block at the G2/M boundary. The cdc2/cyclin B complex is involved in regulating the G2/M phase of the cell cycles [36]. We may presume that cajanol inhibit the complex formation or its phosphorylation. The cell cycle arrest may partly explain cajanol's apoptosis inducing and anti-proliferative effects.

Commonly known, apoptosis is a highly regulated death process by which cells undergo inducible non-necrotic cellular suicide. It plays an important role in anti-carcinogenesis [37]. Data obtained from DNA laddering, the appearance of apoptotic bodies and flow cytometric annexinV-FITC/PI staining showed that cajanol induced apoptosis in MCF-7 cells. Therefore, we assessed the changes of ROS and $\Delta\Psi m$ in MCF-7 cells and expression of apoptosis-related proteins. Cajanol generated ROS in a dose-dependent manner in MCF-7 cells. Increased levels of ROS are known to cause mitochondrial membrane depolarization [38]. The mitochondrial membrane depolarization has been reported to be one of the earliest intracellular events of apoptosis [39, 40]. Indeed, we also found $\Delta\Psi m$ decreased in cajanol-treated MCF-7cells. We conclude that ROS generation by cajanol was responsible for disruption of the

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mitochondrial membrane potential. These results suggested that cajanol-induced intracellular ROS plays an important role in eliciting early signals for triggering apoptosis.

Decreased mitochondrial membrane potential regulates mitochondrial permeability transition pore (MPT) opening [41], and it is associated with cytochrome c release [42]. High Bax/Bcl-2 ratio also resulted in cytochrome c release [43]. In this study, we found that cajanol decreased $\Delta\Psi m$ and increased Bax/Bcl-2 ratio, both of which could explain cajanol-induce cytochrome c release. Moreover, in the cytosol, cytochrome c forms an apoptosome together with Apaf-1 and pro-caspase-9, resulting in the activation of caspase-9. Caspase-9 activates the effector pro-caspases, including pro-caspase-3, an effector caspase of apoptosis [44]. PARP represents an intrinsic substrate for caspase-3 [45, 46] and is cleaved upon caspase-3 activation. PARP is a highly conserved nuclear enzyme tightly binding to DNA with importance for DNA repair, recombination, proliferation and genomic stability [47]. Cleavage of PARP is an early and critical event required for tumour cells apoptosis [48]. The activated caspase-3, caspase-9 and the cleavage of PARP detected in the results further explained clearly the signaling pathway of cajanol-induced apoptosis in human breast cancer cells (showed in Fig.12)

Many flavonoids possess anti-tumor activity towards various human cancer cell lines and xenograft systems of human tumors, suggesting that they may be promising candidates for novel anticancer agents [49, 50]. Genistein is another isoflavone from pigeonpea. It has a potential for breast cancer chemoprevention [51]. At high concentrations, genistein is reported to induce apoptosis through mitochondrial-dependent pathways [52]. Constantinou et al. (1998) showed that 150 μM genistein treatment for 48 h resulted in 57.5% apoptotic MCF-7 cells [53]. Cajanol has a similar chemical structure,

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but lower concentrations (64 μM) induced higher percentages of apoptosis in MCF-7 cells (78.03±3.28%) in the present investigation. Therefore, we have reason to believe that the potential of cajanol in cancer therapy is more promising than that of genistein.

In conclusion, the present study showed that cajanol inhibited the growth of MCF-7 cells in a dose-dependent manner and that this reduction in cell viability resulted from cell cycle arrest at G2/M phase, accompanied by apoptotic cell death. Cajanol induced apoptosis by a mitochondria-dependent pathway, involving inhibition of Bcl-2 expression and induction of Bax expression to desintegrate the outer mitochondrial membrane and to cause cytochrome c release. Further downstream of the apoptosis cascade, cajanol activated caspase-9 and caspase-3 leading to PARP cleavage. Further studies on the *in vivo* activity of cajanol towards MCF-7 xenograft tumors in nude mice are in progress.

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Conflict of Interest Statement

The authors declare that they have no conflict of interest.

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Legends to Figures

Fig. 1. Molecular structure of cajanol

Fig. 2. Effect of cajanol towards MCF-7 cells as determined by the MTT assay. The values for each cajanol concentration tested represent the average (mean±S.D.) from eight replicate wells and are representative of three separate experiments.

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Fig. 3. Cell cycle distribution of MCF-7 cells after treatment with different concentrations of cajanol for 48 h. (a) treatment with 0 μM cajanol; (b) treatment with 16 μM cajanol; (c) treatment with 32 μM cajanol; (d) treatment with 64 μM cajanol. Blue=G1; red=S; green=G2/M. (B) Columns, mean of three experiments, data are presented as mean±S.D. *$p < 0.01$; p value compared with the control group (0 μM).

Fig. 4. Cajanol -induced apoptosis in MCF-7 cells using annexinV-FITC/PI (A) Flow cytometric histograms. (a) treatment with 0 μM cajanol; (b) treatment with 16 μM cajanol; (c) treatment with 32 μM cajanol; (d) treatment with 64 μM cajanol. (B) Columns show mean values of three experiments (±S.D.). *$p < 0.01$; p value compared with the control group (0 μM).

Fig. 5. Assessment of apoptosis in MCF-7 cells by the DNA fragmentation assay. Lane 1, DNA size marker; Lane 2, treatment with 0 μM cajanol; Lane 3, treatment with 16 μM cajanol; Lane 4, treatment with 32 μM cajanol; Lanes 5, treatment with 64 μM cajanol. The experiment was repeated three times and representative photographs are shown.

Fig. 6. Morphological observation of MCF-7 cells treated with 64 μM cajanol for 48 h by inverted fluorescence microscopy. Cells undergoing apoptosis and nuclear fragmentation are indicated by arrows. (a) Untreated cells; (b) cajanol-treated cells. The experiments were repeated three times and representative photographs are shown.

Fig. 7. Effect of cajanol on intracellular ROS formation in MCF-7 cells. (A) Red curve, treatment with 0 μM cajanol; black curve, treatment with 16 μM cajanol; green curve, treatment with 32 μM cajanol; blue curve, treatment with 64 μM cajanol. (B) Columns, mean of three experiments, data are presented as mean±S.D. *$p < 0.01$; p value compared with the control group (0 μM).

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Fig. 8. Mitochondrial membrane potential of MCF-7 cells after treatment with cajanol. (A) Flow cytometric analysis of cajanol-induced ΔΨm disruption in MCF-7 cells using Rh123/PI staining. (a) treatment with 0 μM cajanol; (b) treatment with 16 μM cajanol; (c) treatment with 32 μM cajanol; (d) treatment with 64 μM cajanol. Quadrants: Q4: live cells; Q1 and Q3: ΔΨm disrupted cells. (B) Columns, mean of three experiments, data are presented as mean±S.D. *$p < 0.01$; p value compared with the control group (0 μM).

Fig. 9. Effect of cajanol on caspase-3 and caspase-9 activities. Data are presented as mean±S.D. *$p < 0.01$; p value compared with the control group (0 μM).

Fig. 10. Cajanol-mediated upregulation of Bax and downregulation of Bcl-2 by Western blotting assay, MCF-7 cells were treated with cajanol (0, 16, 32, 64 μM) for 48 h, respectively. The test was repeated three times and representative blots are shown. Data are presented as mean±S.D. *$p < 0.01$; p value compared with the control group (0 μM).

Fig.11. Cajanol induce cytochrome c release and PARP cleave by Western blotting assay, MCF-7 cells were treated with cajanol (0, 16, 32, 64 μM) for 48 h, respectively. The test was repeated three times and representative blots are shown. Data are presented as mean±S.D. *$p < 0.01$; p value compared with the control group (0 μM).

Fig. 12. Signaling pathway of cajanol -induced apoptosis in MCF-7 cells.

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Figure 1

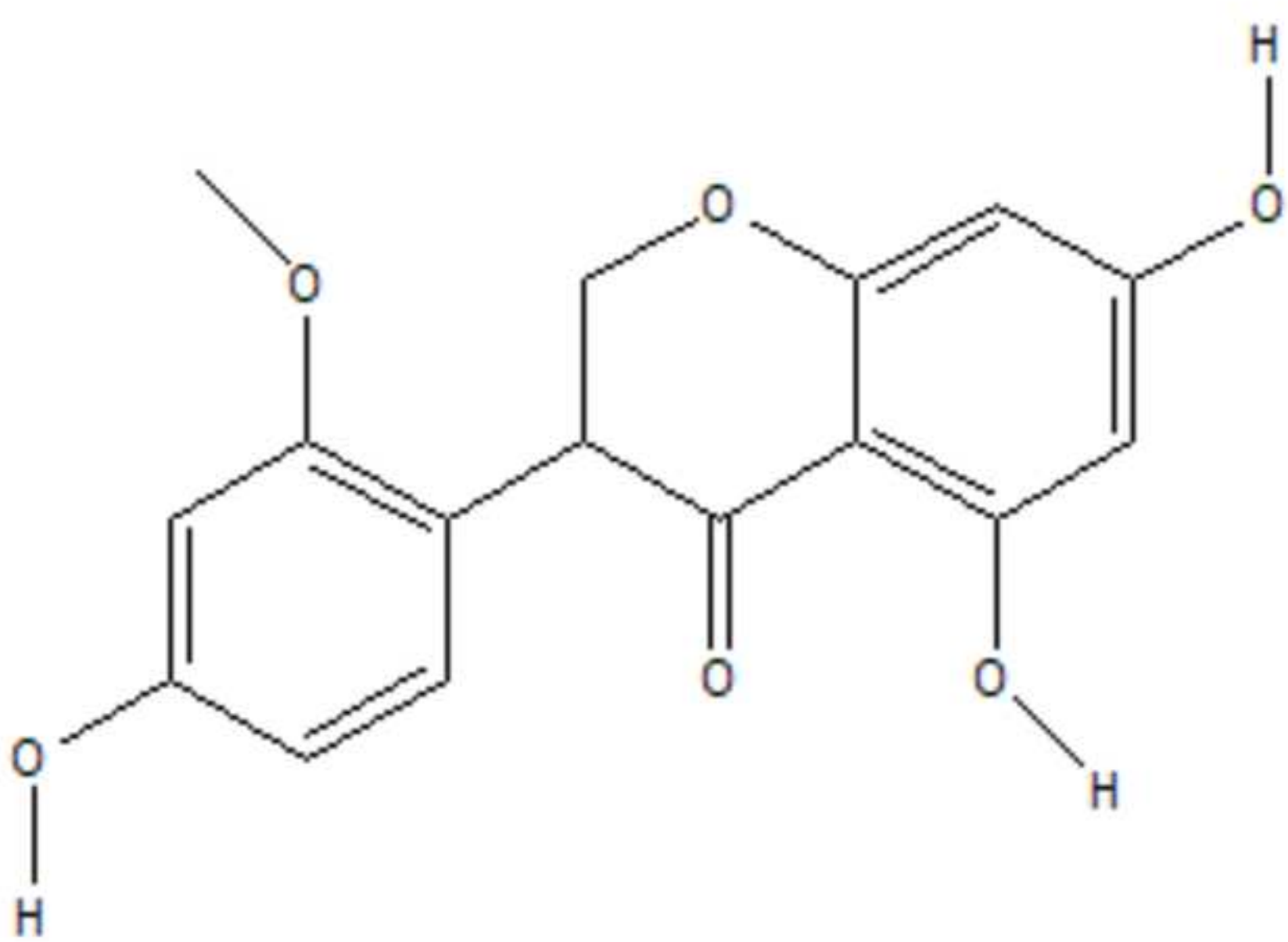
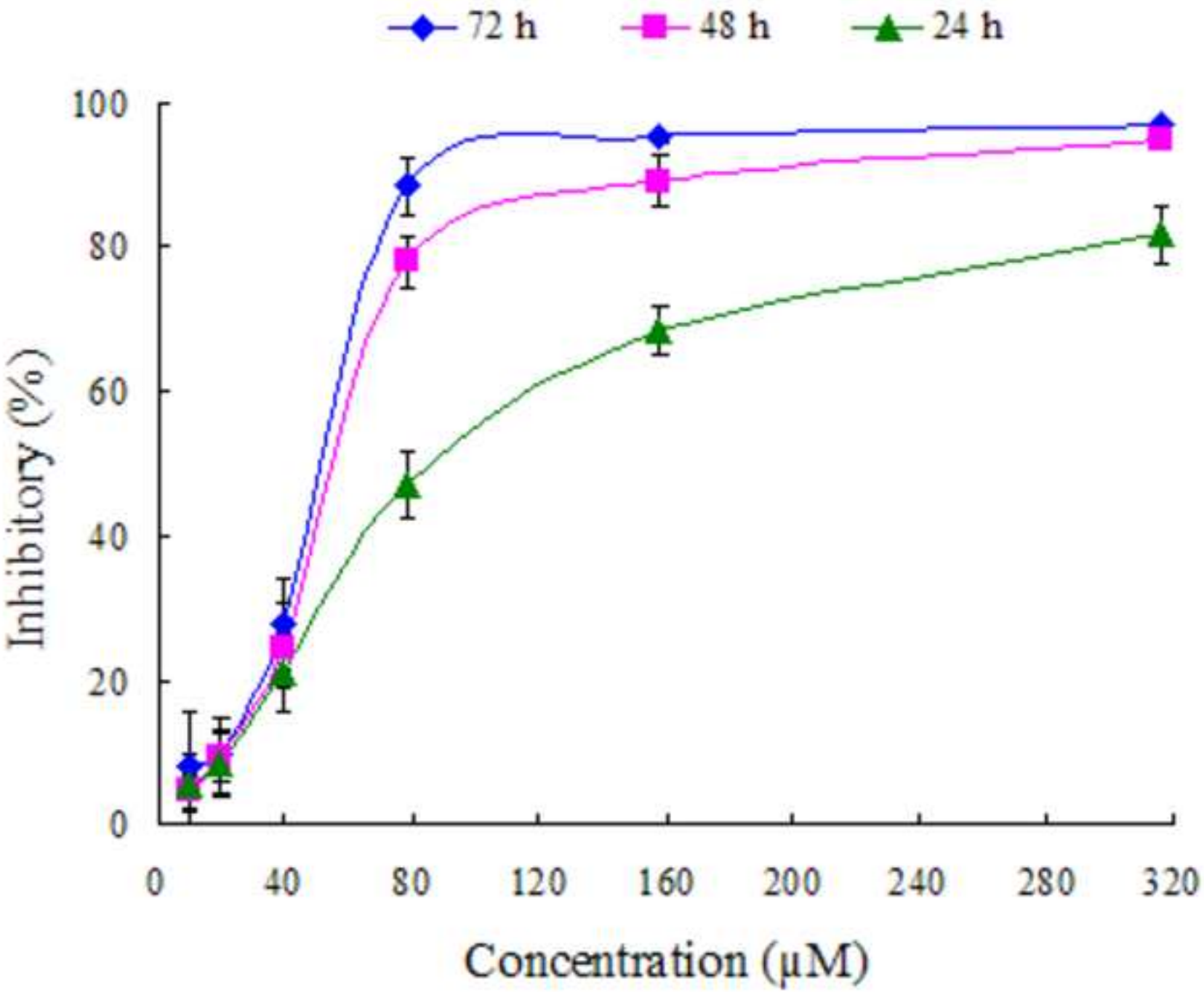


Figure 2


72 h
48 h
24 h
Inhibitory (%)
100
80
60
40
20
0
0
40
80
120
160
200
240
280
320
Concentration (μM)

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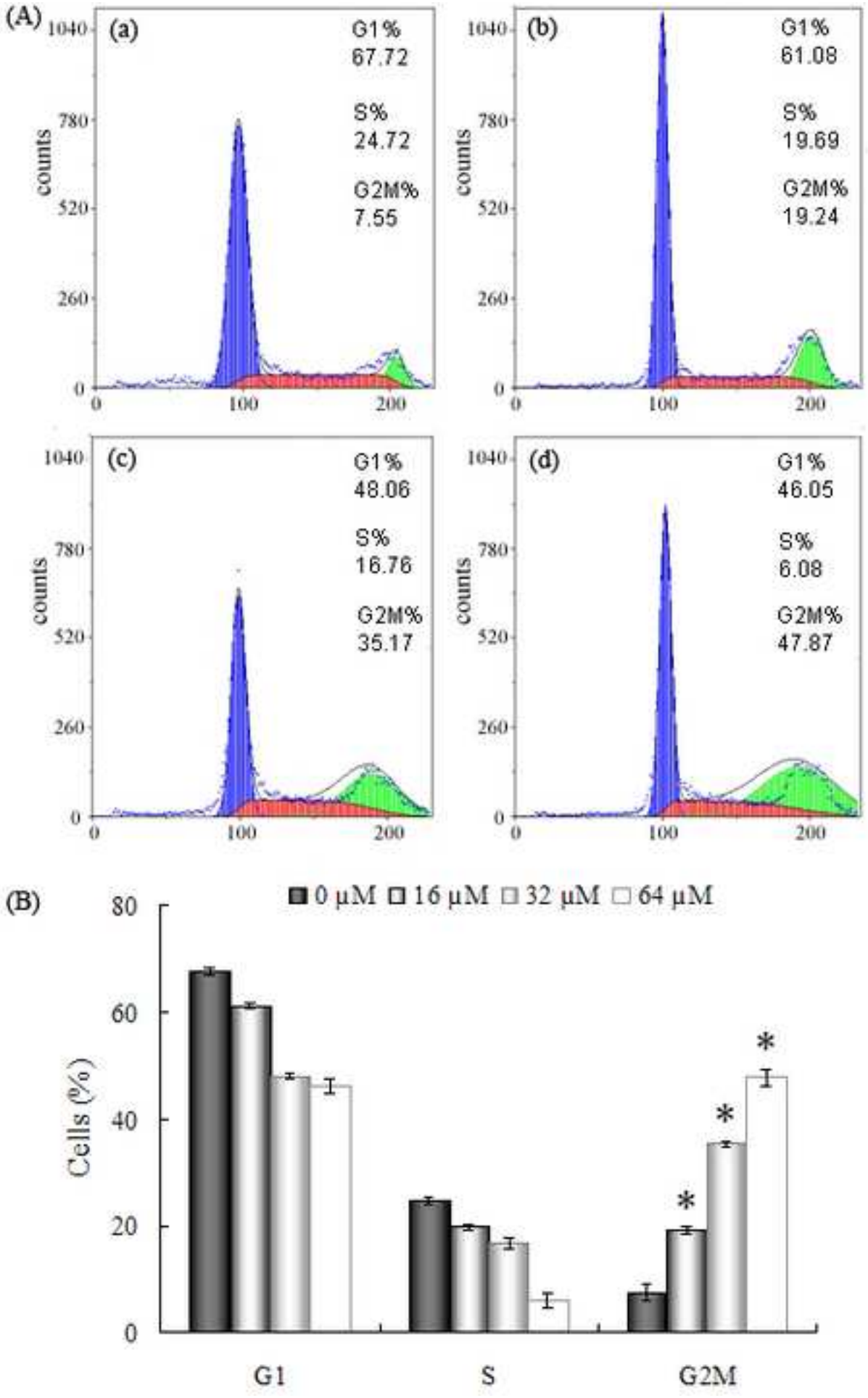

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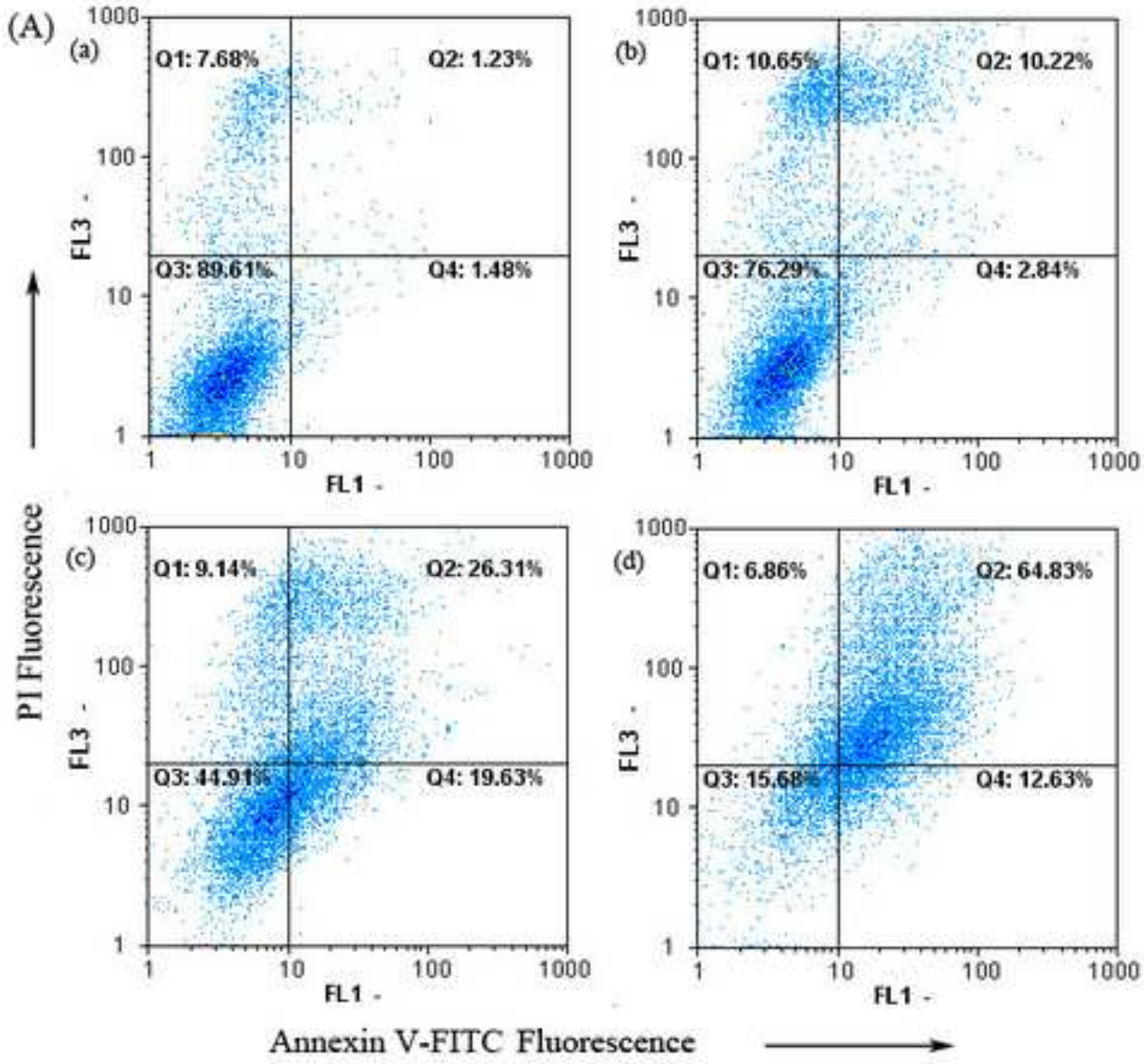

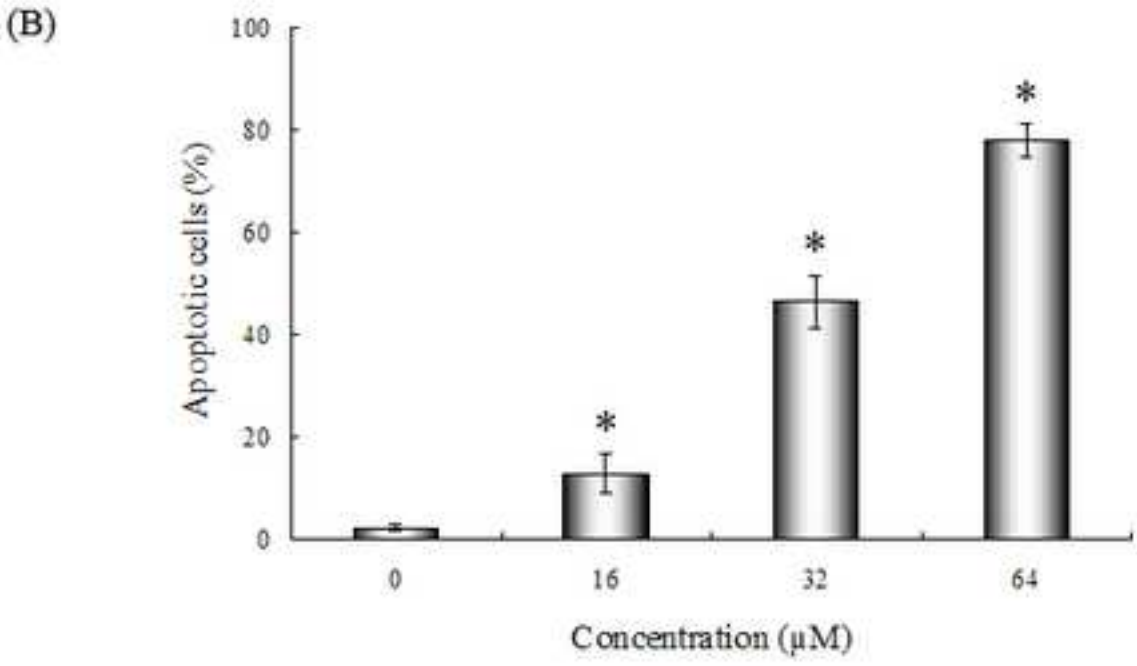

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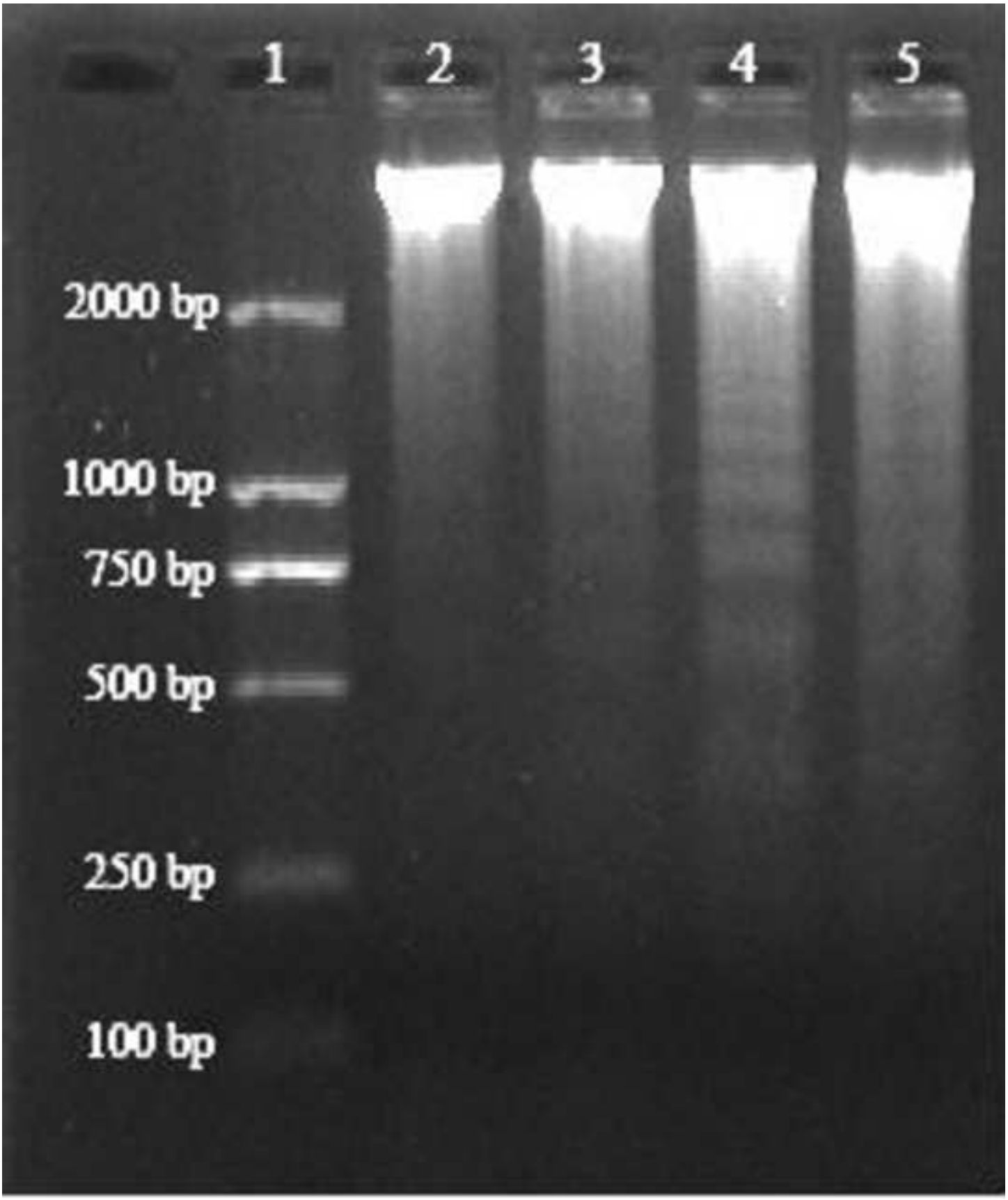


Figure 6

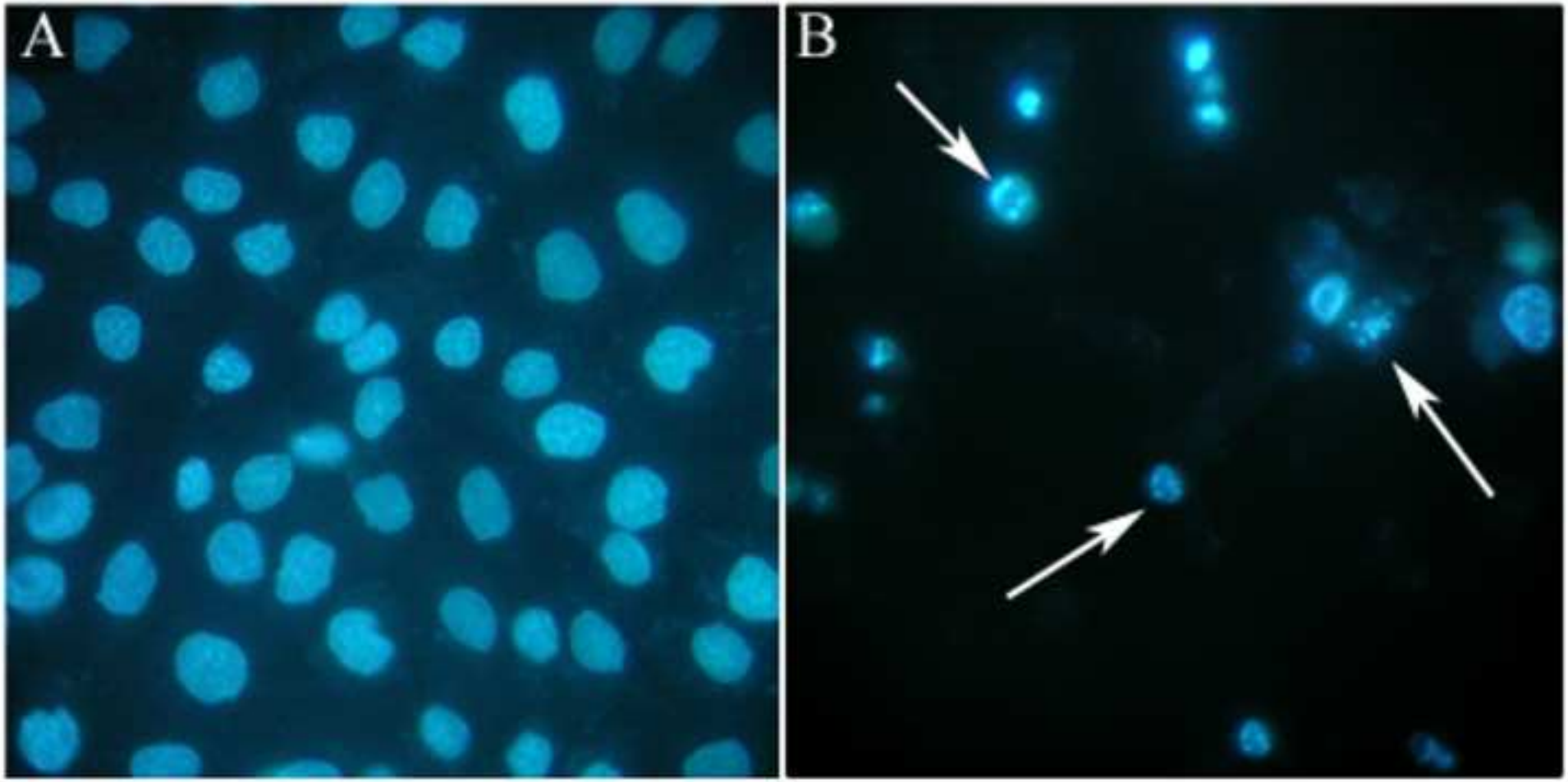
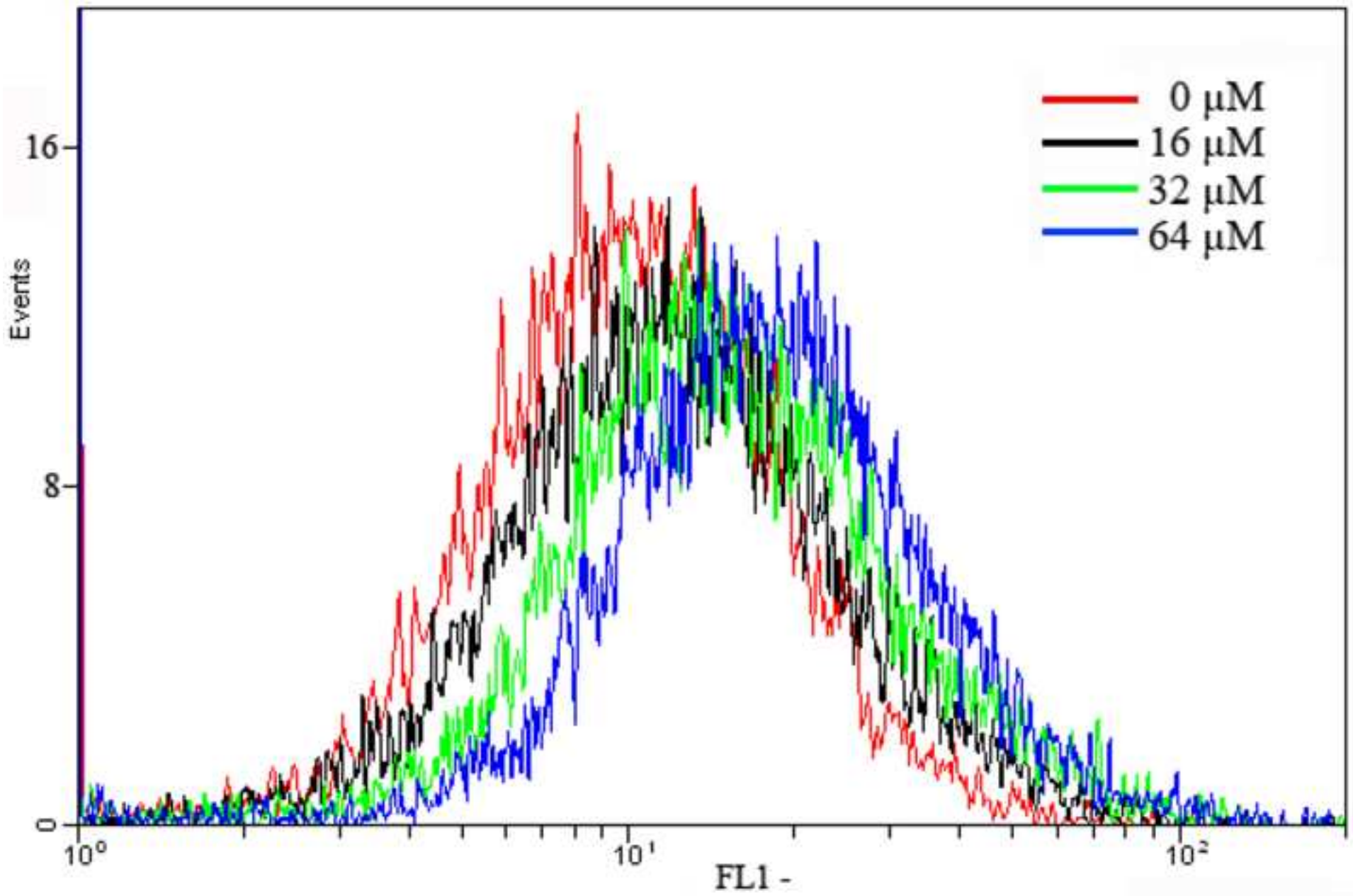


Figure 7



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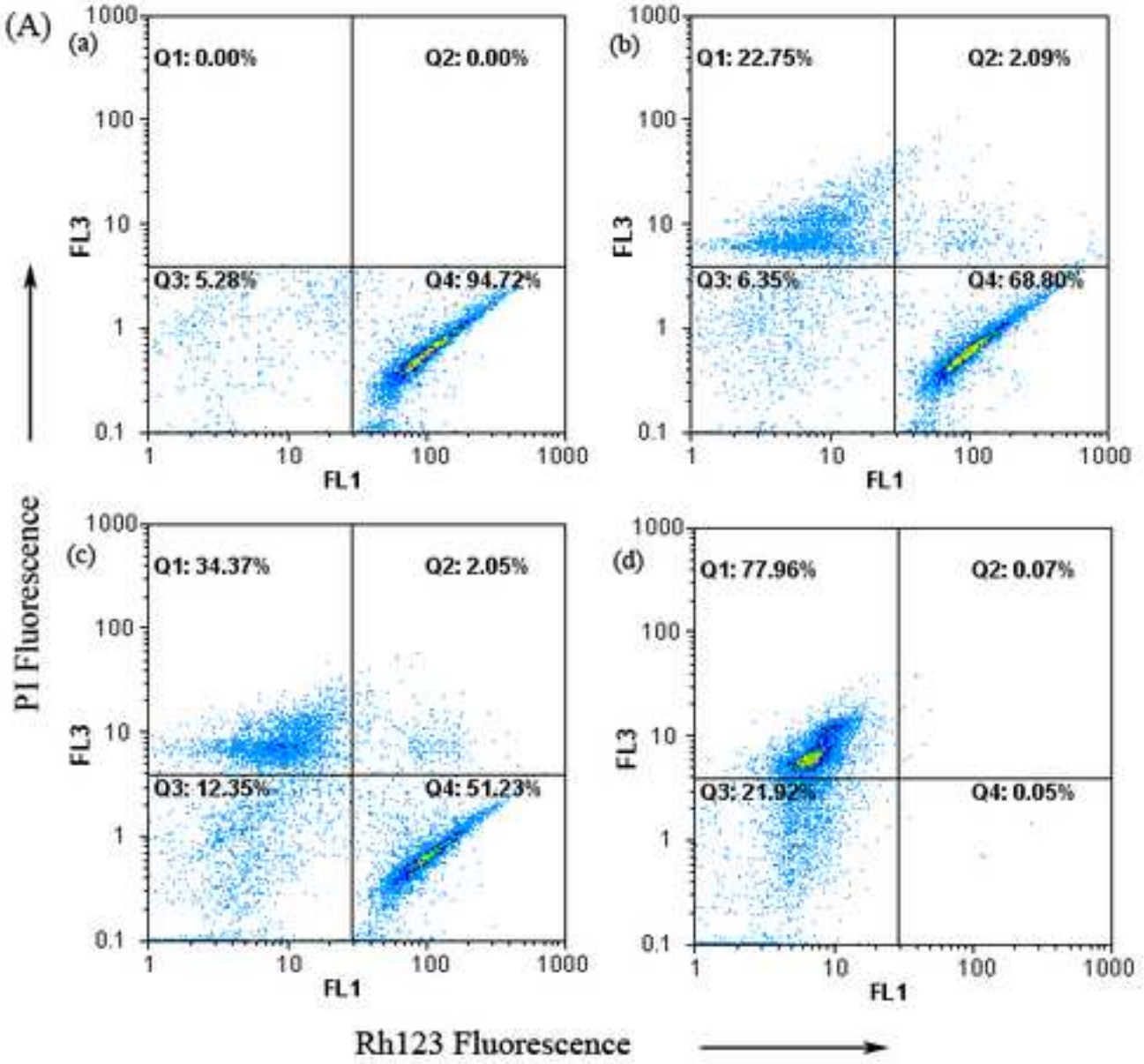


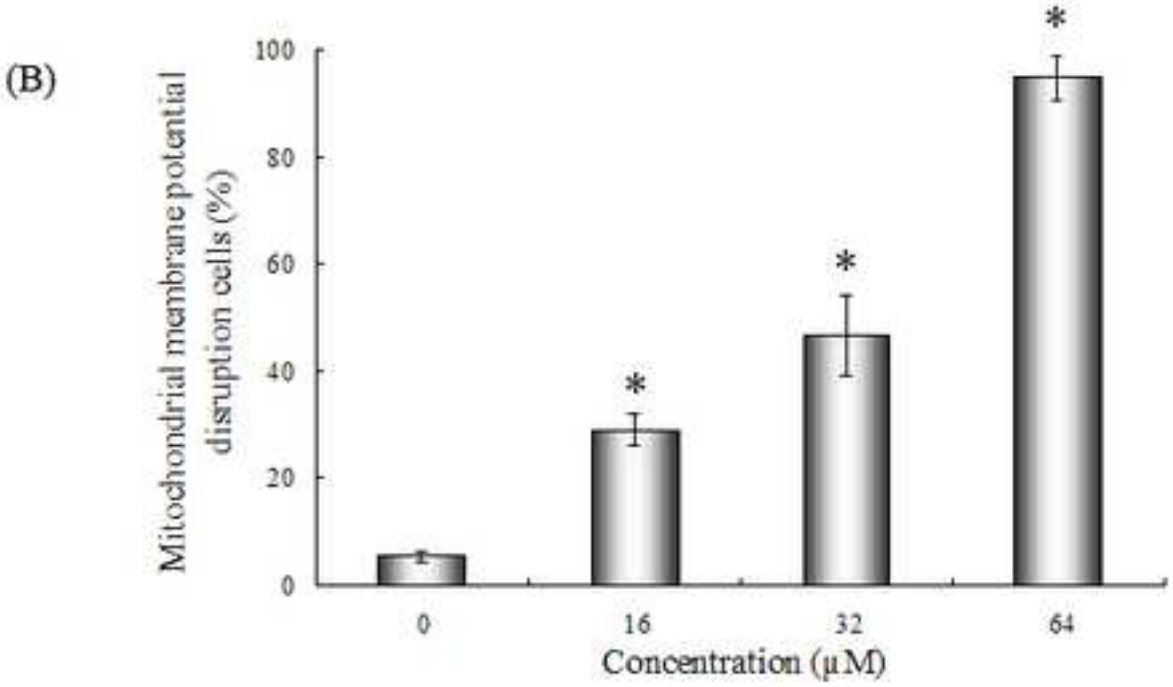
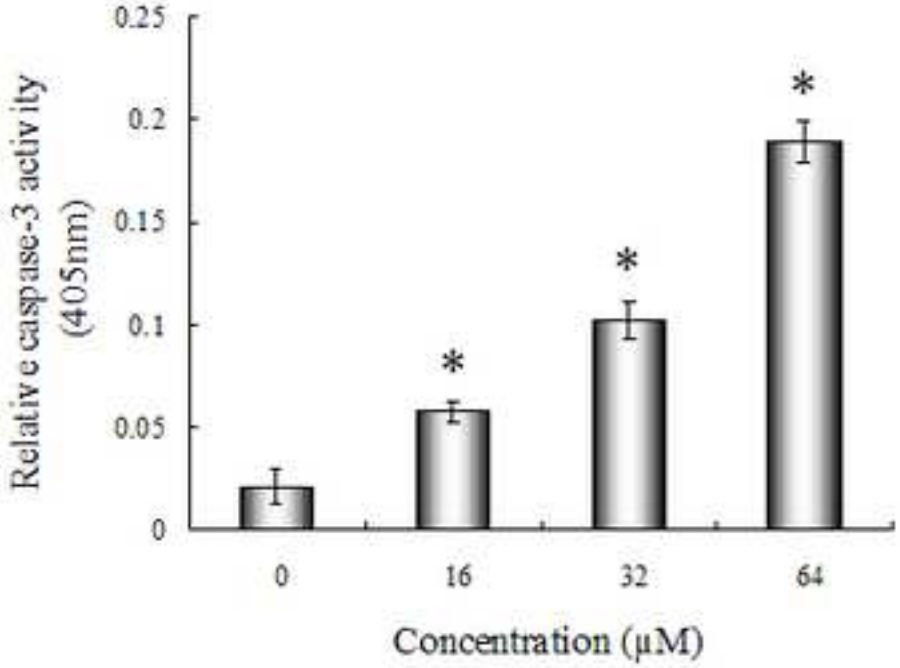


Figure 9



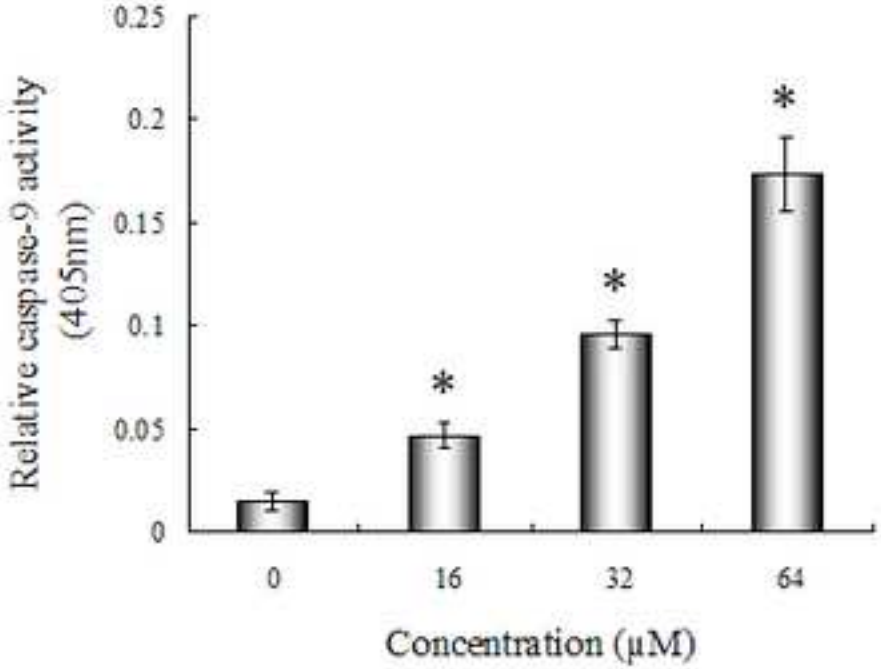


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Figure 10

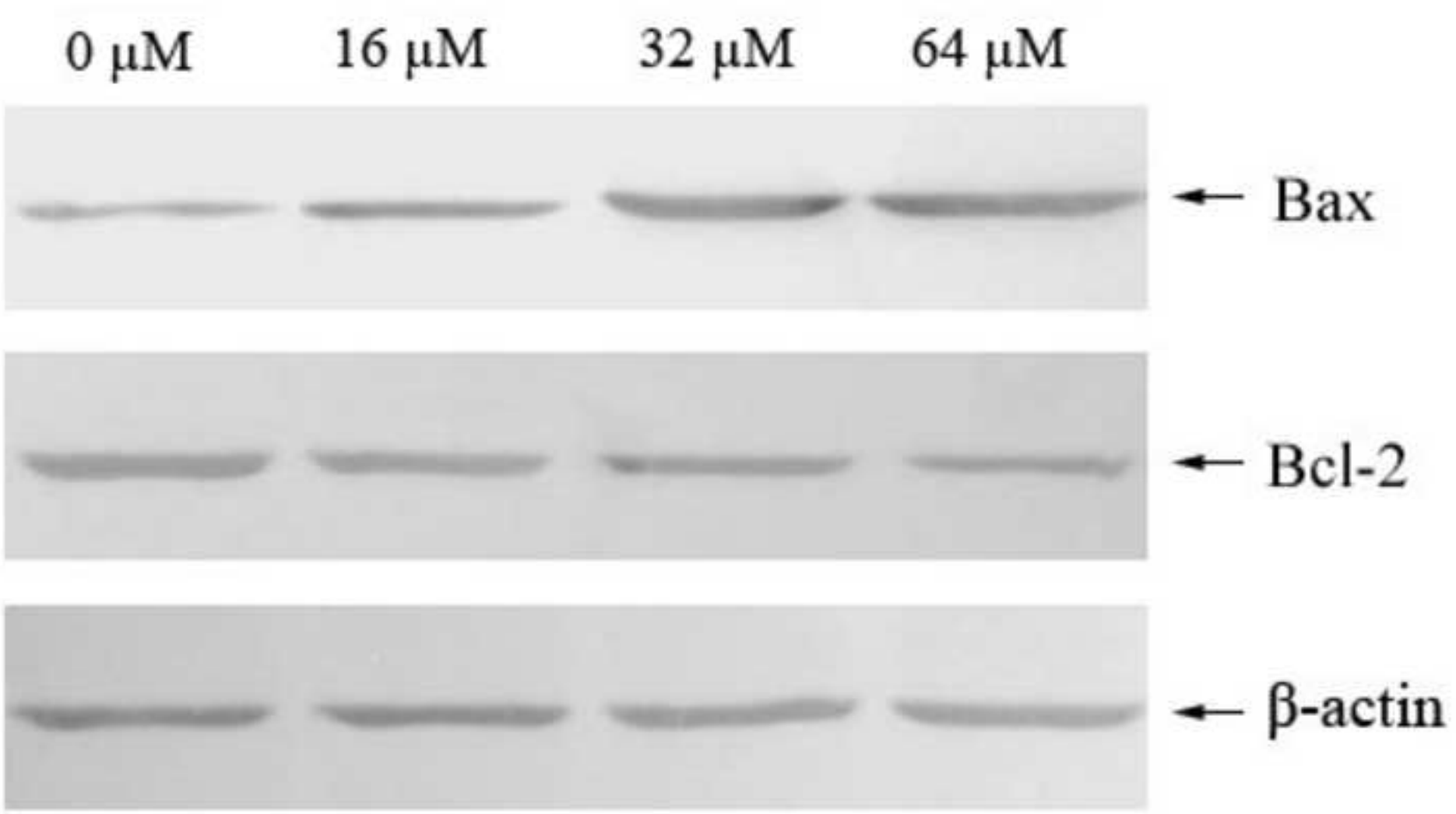


Figure 11

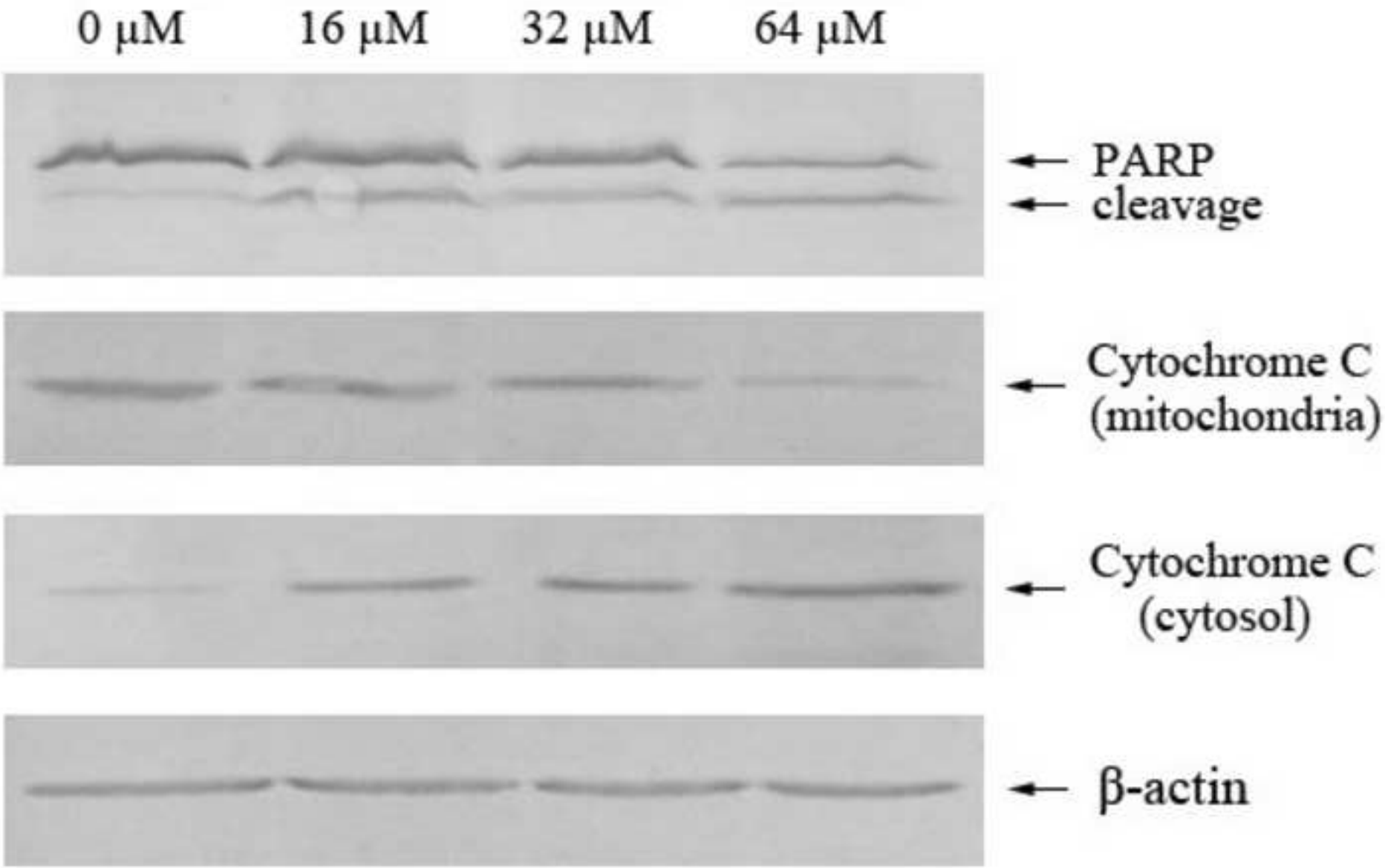
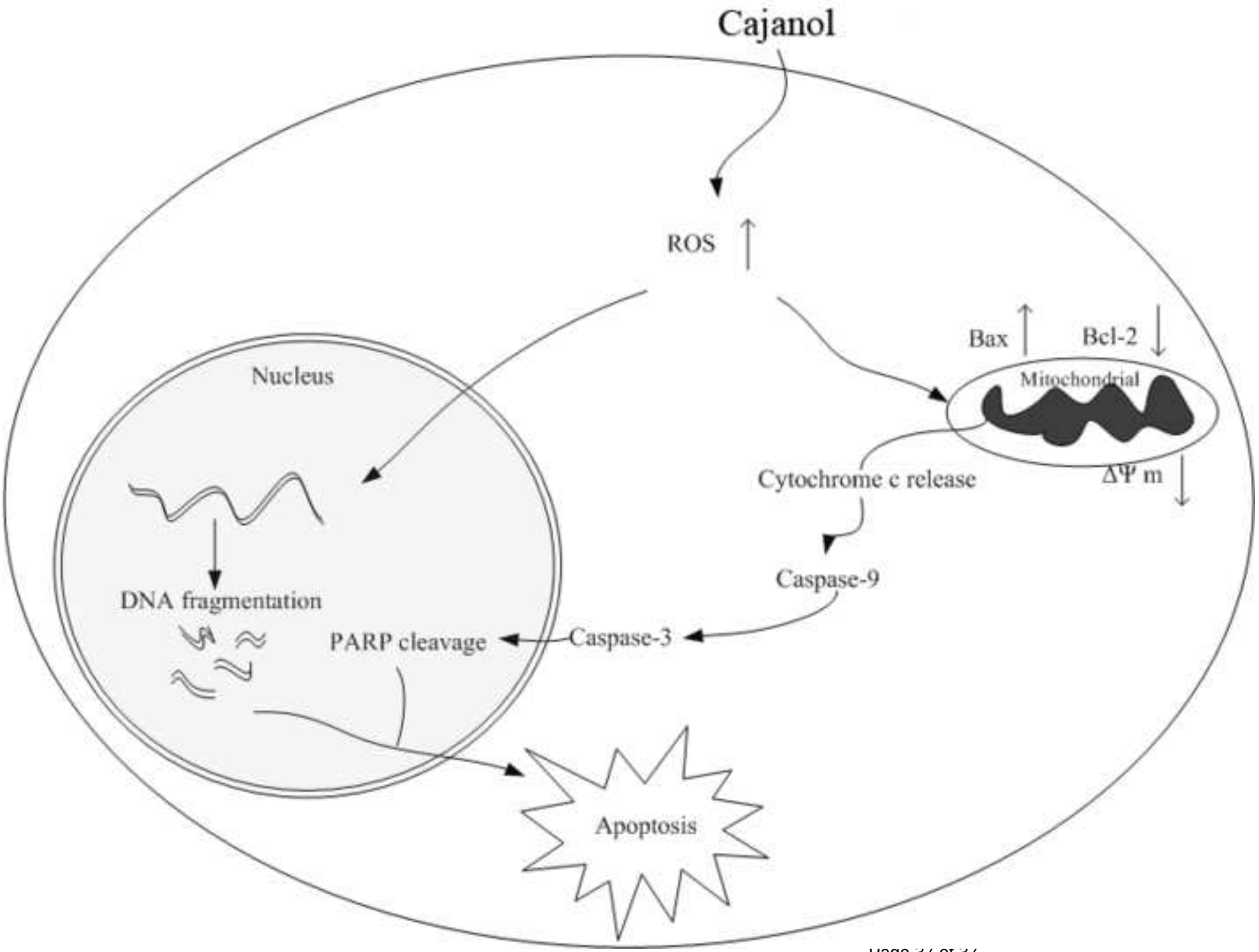

0 μM
16 μM
32 μM
64 μM
PARP
cleavage
Cytochrome C
(mitochondria)
Cytochrome C
(cytosol)
β-actin

Figure 12


Cajanol
ROS
Bax
Bcl-2
Mitochondrial
Nucleus
Cytochrome c release
ΔΨ m
Caspase-9
DNA fragmentation
PARP cleavage
Caspase-3
Apoptosis