

*Full Length Research Paper*

# Artabotryside A, a constituent from *Descurainia sophia* (L.) induces cell death in U87 glioma cells through apoptosis and cell cycle arrest at G2/M phase

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*Descurainia sophia* (L.) commonly known as Webb ex Prantl (flixweed) is a Chinese traditional medicinal plant. Seeds of flixweed are used in the treatment of some cancers. However, the anticancer constituents from the seeds have not been investigated. The present study was aimed to identify bioactive compound with anti-glioma activity from the seeds of *D. sophia* (L.) and to examine the growth inhibitory effect on U87 glioblastoma cells and possible mechanism of action. From bioactive fraction, a flavonol glycoside, artabotryside A, has been isolated and evaluated for *in vitro* cytotoxicity against U87 glioma cells by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and live/dead assay. Cellular and nuclear morphological changes were observed by phase contrast and fluorescence microscopy, respectively. Cell cycle analysis, apoptotic rate, mitochondrial membrane potential and level of reactive oxygen species (ROS) were detected by flow cytometry. Our results demonstrated that artabotryside A significantly reduced the proliferation of U87 cells in a dose- and time- dependent manner. By flow cytometric analysis, we found that artabotryside A treatment resulted in an increased apoptosis, mitotic arrest, level of ROS and decreased mitochondrial membrane potential in U87 cells in a time-dependent manner. Furthermore, caspase-3 inhibitor, Ac-DEVD-CHO, significantly inhibited the apoptotic effect of artabotryside A, indicating that artabotryside A induced caspase-dependent apoptosis in U87 cells. In addition, artabotryside A is less toxic to normal mouse splenocytes and glial cells. This selective cytotoxicity of artabotryside A against U87 glioma cells as compared to mouse splenocytes and glial cells is advantageous. Thus, artabotryside A might be a safe candidate for prevention and treatment of gliomas.

**Key words:** Flixweed, artabotryside A, anticancer, U87 cell, apoptosis.

## INTRODUCTION

*Descurainia sophia* (L.) Webb ex Prantl (flixweed) is an annual dicot weed belonging to family Brassicaceae and is widely distributed in northeast of China (Blackshaw, 1990; Sun et al., 2005b). In the middle Asia, the extract of aerial parts is used in folk medicines for the treatment of throat diseases, measles and smallpox. The tincture is

used as a diuretic, antihelmintic and hemostatic for internal hemorrhages (Bekker et al., 2005). In China, it is also used to prepare health cigarette in combination with other Chinese medicinal herbs (Fan, 2002). Seeds of flixweed are used as traditional Chinese medicines (TCM) to relieve cough, prevent asthma, reduce edema, promote urination and have a cardiostimulant effect. The seeds of *D. sophia* (L.) have been shown to inhibit the growth of various cancer cell lines *in vitro*. In one previous study, different extracts of flixweed seeds were tested for their anticancer activity against NCI-H460, SF-

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268 and SGC-7901 cell lines. However, n-butanol extract has shown better anticancer activity. The 50% growth inhibition values of n-butanol extract on NCI-H460, SF-268 and SGC-7901 cell lines were 93.44, 23.21 and 144.5  $\mu\text{g/mL}$ , respectively. However, the bioactive compounds responsible for growth inhibition and their molecular mechanism had not been identified (Li et al., 2010; Qian, 2006; Sun et al., 2005b).

As a medicinal material, this plant has got enormous attention for its bioactive chemical constituents. So far, numerous phytochemical studies have been conducted and identified the presence of cardiac glycosides (Chen et al., 1981), flavonoids and phenols (Wang et al., 2004), some new lactones and arylidihydronaphthoic acid (Sun et al., 2004; Sun et al., 2005a; Sun et al., 2006) in the seeds of flaxweed. Although the seeds of *D. sophia* (L.) have long been used to treat some cancers but anti-tumor constituents in the seeds of *D. sophia* (L.) has not yet been explored thoroughly.

In the present study, we performed high throughput screening with ethanolic extract of seeds against U87 glioma cells to identify the anticancer components in the seeds of *D. sophia* (L.). Based on high throughput screening method, we have identified, extracted, isolated and determined the molecular structure of "artabotryside A" from the seeds of *D. sophia* (L.) for the first time. Furthermore, we examined the anticancer activity of "artabotryside A" through cell cycle arrest and apoptosis. To the best of our knowledge, this is the first report representing the isolation of artabotryside A from *D. sophia* (L.) and its anticancer activity.

## MATERIALS AND METHODS

### Materials

The dried semen of *D. sophia* (L.) Webb ex Prantl were purchased from Yunnan Qiancaoyuan Medicinal Plant Company (Yunnan, China) and identified by associate Prof. Diao Yunpeng (Dalian Medical University, Dalian, China). Artabotryside A was isolated with >99% purity from seeds of *D. sophia* (L.). All the chemicals for extraction process were purchased from Honeywell (Beijing, China). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), propidium iodide (PI), RNase A, Rhodamine 123, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO) and calcein AM were purchased from Sigma (Beijing, China). Annexin V-FITC Apoptosis Detection Kit, reactive oxygen species (ROS) assay kit and caspase-3 inhibitor Ac-DEVD-CHO were purchased from Beyotime (Shanghai, China).

### Bioactivity guided identification of target compound

Dried seeds of *D. sophia* (L.) were extracted with 95% ethanol. The ethanol extract was evaporated to get crude extract. The crude extract was then dissolved in 80% methanol and fractionated into 80 fractions by using 0 to 90% methanol gradient on preparative high performance liquid chromatography (HPLC). Each fraction was dried and dissolved in DMSO (10 mg/ml). All fractions were then screened against U87 glioma cells at final concentration of 100

whole crude extract, two samples from negative fraction (not toxic), two samples from bioactive fractions and one sample from whole crude extract were run on analytical HPLC. The peak that was common in both bioactive fraction and whole crude extract but absent in negative fraction was identified as target peak. The crude extract was then extracted with two phase solvent system, petroleum and water (1:3) to get maximum solubility of target peak.

### Extraction and isolation

One kilogram (1 kg) seeds of *D. sophia* (L.) were dried, pulverized and solubilized with 95% ethanol for 10 h in a Soxhlet extractor at 95°C. The ethanol extract was evaporated to dryness by using a rotor evaporator machine at 90°C under reduced pressure to get crude extract (31.8 g). The crude extract was then mixed with petroleum and water (1:3) and poured into separation funnel, shaken vigorously to mix well and left for 30 min for the solution to be separated into two layers. Our target peak was found in aqueous layer. Both layers were separated in a conical flask and separation was repeated three times. Aqueous layer was collected in a round bottom flask and evaporated to dryness by using rotor evaporator at 90°C under reduced pressure to get aqueous extract.

### Separation of compound using preparative HPLC

Preparative HPLC (Waters) equipped with 2489 UV/visible detector, 2495 separation module and 2767 collector controlled with MassLynx TM software, was used to separate the target compound. Separation was performed with XTerra Prep MS C18 OBD (5  $\mu\text{m}$ , 30x100 mm) column at a column temperature of 25°C. The mobile phase composed of methanol (A) and 0.2% acetic acid in water (B) was eluted with linear gradient elution (A: 0 to 20 min, 10 to 70%; 20 to 30 min, 70 to 100%; 30 to 40 min, 100 to 100%). The flow rate was 30 mL/min and injection volume was 2500  $\mu\text{L}$ . The effluent was monitored at 254 nm and peak fraction (60 mL) was collected according to the elution profile.

### HPLC analysis and identification of preparative HPLC peak fraction

The peak fraction obtained by preparative HPLC was analyzed by analytical HPLC (Waters). The column used was XTerra MS C18 (5  $\mu\text{m}$ , 4.6 x 150 mm). The mobile phase composed of methanol (A) and 0.2% acetic acid (B) was eluted with linear gradient elution (A: 0 to 20 min, 10 to 70%; 20 to 30 min, 70 to 100%; 30 to 40 min, 100 to 100%). The injection volume was 10  $\mu\text{L}$  and flow rate was 0.2 mL/min. The temperature of column was 30°C. The effluent was monitored at 254 nm. Identification of the peak was carried out by mass spectrometry (MS) and nuclear magnetic resonance (NMR). MS was performed using a Quattro Micro (Waters Company, American) scanning from 50 to 1000 m/z.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were recorded on Bruker Avance 500 spectrometer operating at 500 MHz for  $^1\text{H}$  and 100 MHz for  $^{13}\text{C}$ .

### Cell culture

U87 cell line was purchased from American Type Culture Collection (ATCC) and was cultured in DMEM supplemented with 10% FBS while mouse splenocytes were freshly isolated from C57 mice and cultured in Roswell Park Memorial Institute medium (RPMI 1640) supplemented with 10% FBS and maintained at 37°C with 5%  $\text{CO}_2$  in humidified atmosphere. Normal glial cells (mixture of Astrocytes, microglia and fibroblasts etc.) were isolated from 2 weeks old C57 mice. Briefly, skin was opened at the mid line of the head from the

base of the skull to the mid-eye area using micro dissecting scissors. The skull was cut at the mid line fissure and skull cap was removed carefully without disturbing brain tissues. The brain was then transferred to 60 mm tissue culture plate and washed with DMEM/F12 culture medium containing 10% FBS and streptomycin and penicillin antibiotics. After separating cerebrum from cerebellum and brain stem, cerebral hemispheres were separated from each other using forceps. The grey matter of the cortex was peeled away leaving behind the white matter. The white matter was transferred to 60 mm cell culture plate and dissociated into cell suspension using mechanical digestion. The semi-digested tissue was treated with 500  $\mu$ L trypsin at 37°C with 5% CO<sub>2</sub> in humidified atmosphere for 10 min. The cells were then mixed with 500  $\mu$ L DMEM/F12 medium and pipette gently. After centrifugation at 1000 rpm for 10 min, the cell pellet was resuspended in DMEM/F12 medium supplemented with 10% FBS and transferred to 30 mm tissue culture plate coated with 0.1% gelatin. The cells were maintained at 37°C with 5% CO<sub>2</sub> in humidified atmosphere. The medium was changed after every 3 days. After 2 weeks, the cells were used for further experiments.

### Cell proliferation assay

The effect of artabotryside A on cell proliferation was measured by MTT assay as described previously (Yuan et al., 2011). Briefly, 5000 cells were seeded into 96-well tissue culture plates. After 24 h incubation at 37°C, cells were treated with different dosages of artabotryside A (50 to 800  $\mu$ M) for 24 and 48 h. The MTT reagent was then added to each well and the cells were further incubated for 4 h. Subsequently, 150  $\mu$ L DMSO was added to dissolve farmazan crystals and absorbance was measured at 570 nm in a microplate reader (Thermo Scientific).

### Live/dead assay

Live and dead cells were quantified using the fluorescent probes calcein acetoxymethylester (calcein AM) and PI as described previously (Khan et al., 2012). Calcein AM is highly lipophilic and cell membrane permeable. In viable cells, it is converted into calcein by esterases and emits strong green fluorescence. Thus, it stains only alive cells. PI is cell membrane impermeable and stains only dead cells. U87 cells were incubated with or without 350  $\mu$ M artabotryside A for 24 and 48 h or mouse glial cells were incubated with or without 350  $\mu$ M artabotryside A for 24 h. After incubation, floating and adherent cells were collected, washed with phosphate buffered saline (PBS) and incubated with PBS solution containing 2  $\mu$ M calcein AM and 4  $\mu$ M PI in the dark for 20 min at room temperature. After washing, the cells were resuspended in PBS and analyzed for the fluorescence of calcein and PI by flow cytometry.

### Observation of morphological changes by light microscopy

U87 cells were treated with artabotryside A (0 and 350  $\mu$ M) for 24 h. Morphological changes were observed by phase contrast microscopy (Olympus 1x71).

Freshly isolated mouse splenocytes were treated with or without artabotryside A (350  $\mu$ M) for 24 h. After 24 h incubation at 37°C, cells were stained with 0.4% trypan blue and observed dead and live cells microscopically.

### Fluorescent microscopy

Treated and untreated U87 cells were collected by centrifugation at

1000 rpm for 5 min, washed twice with PBS and fixed with 4% paraformaldehyde at room temperature for 30 min. After centrifugation, cells were washed with PBS, stained with PI (50  $\mu$ g/mL) and incubated at 37°C for 30 min. At the end of incubation, the cells were washed and resuspended in PBS for the observation of nuclear morphology under fluorescence microscope (Olympus 1x71).

### Flow cytometry (FCM) for cell cycle analysis

Cell cycle profile was analyzed as described previously (Liu et al., 2011). Briefly, U87 cells were treated with or without 350  $\mu$ M of artabotryside A for 24 and 48 h. The cells were then washed with PBS and fixed with 70% ice cold ethanol at 4°C overnight. After washing twice with PBS, cells were stained with a solution containing 50  $\mu$ g/mL of PI and 100/mL RNase A for 30 min in the dark at room temperature. The stained cells were analyzed by FCM (Beckman Coulter, Epics XL).

### FCM for apoptosis analysis

U87 cells were treated with or without 350  $\mu$ M of artabotryside A for 24 and 48 h. In the inhibitory assay, 100  $\mu$ M Ac-DEVD-CHO, a caspase-3 inhibitor was added 30 min before the treatment with artabotryside A. The cells were harvested, rinsed twice with PBS and labeled with 5  $\mu$ L FITC-conjugated annexin V according to the manufacturer's instructions (Beyotime, Shanghai, China). After incubation in dark for 10 min and then labeled with PI, the samples were immediately analyzed on a flow cytometer (Beckman Coulter, Epics XL).

### Measurement of Intracellular accumulation of ROS using FCM

Levels of ROS in cells of the control and treatment groups were determined by staining cells with 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) as described previously (Ji et al., 2009). The fluorescent dye DCFH-DA is a cell membrane permeable and is converted into cell membrane impermeable non-fluorescent compound DCFH by intracellular esterases. Oxidation of DCFH by ROS produces a highly fluorescent DCF. The fluorescence intensity of DCF inside the cells is proportional to the amount of peroxide produced. Briefly, U87 cells were incubated in 6-well plate for overnight. After treating the cells with or without 350  $\mu$ M of artabotryside A for 24 and 48 h, cells were further incubated with 10  $\mu$ mol/L DCFH-DA at 37°C for 30 min according to manufacturer's instruction (Beyotime). In the positive control group, cells labeled with DCFH-DA, were treated with 1  $\mu$ L rose up for 30 min. Subsequently, cells were removed, washed, re-suspended in PBS, filtered with 300 apertures and analyzed for DCF fluorescence by FCM. Approximately 10,000 cells were evaluated in each sample.

### Measurement of mitochondrial membrane potential using FCM

Rhodamine 123 was used to evaluate the changes in mitochondrial membrane potential as described previously (Ji et al., 2009). Briefly, U87 cells were incubated in 6-well plate for overnight. After treating the cells with or without 350  $\mu$ M of artabotryside A, for 24 and 48 h, cells were collected in 10 mL centrifuge tube. Cells were then resuspended in 1 mL PBS solution containing 10  $\mu$ g Rhodamine 123 and incubated in the dark for 30 min. After incubation, cells were centrifuged at 1500 rpm for 5 min, supernatant was removed, pellet was gently rinsed with PBS once and then resuspended in 500  $\mu$ L PBS. After filtration (300 apertures), the suspension was

analyzed by FCM.

### Statistical analysis

The results were expressed as the Mean  $\pm$  SEM from three independent experiments and statistically compared with the control group using one-way analysis of variance (ANOVA) and \*P-value < 0.05, was considered statistically significant.

## RESULTS

### Extraction, isolation and purification of artabotryside A

Dried seeds of *D. sophia* (L.) were extracted with 95% ethanol. The ethanol extract was dried and further extracted with two phase solvent system, petroleum and water (1:3) as described in Materials and Methods section. Our target compound was found in aqueous layers as assessed by analytical HPLC (Figure 1B, peak T). To separate and purify target peak from aqueous extract, various kinds of elution systems with different volume ratios were tried in HPLC separation. Finally, when the mobile phase of methanol and 0.2% acetic acid was eluted in a linear gradient mode (Methanol: 0 to 20 min, 10 to 70%; 20 to 30 min, 70 to 100%; 30 to 40 min, 100 to 100%), a good separation was achieved. The target peak was collected and dried. The volume of collected fraction was 60 ml and weight of pure dried compound was 6.8 mg.

In order to evaluate the purity, dried material was dissolved in 80% methanol and analyzed by analytical HPLC. HPLC analysis of sample solution showed only one single peak with a retention time of 16 min (Figure 1C). This single peak guarantees purity of the compound isolated, which elucidate further that the compound extracted and isolated from crude herb extract consisted mainly as one single compound. The HPLC analysis revealed that the purity of isolated compound is >99%. Our results implied that "artabotryside A" with purity >99% could be obtained successfully by preparative HPLC separation under the above optimized conditions. The structural elucidation of HPLC pure fraction was carried out by ESI-MS and NMR spectra as follows:

Artabotryside A: Yellow powder, mp 175–177°C, ESI-MS m/z(%):579[M](100);

<sup>1</sup>HNMR(500MHz,MeOD) $\delta$ :6.19(1H,d,J=2.0,6-H),6.38(1H,d,J=2.0,8-H),7.61(1H,d,J=2.1,2'-H)6.89(1H,d,J=8.4,5H),7.56(1H,dd,J=2.1,8.4,6-H),5.08(1H,d,J=1.3,rha-1),1.07(3H,d,J=8.2,rha-6),5.53(1H,d,J=5.1,ara-1);

<sup>13</sup>CNMR(100MHz,MeOD) $\delta$ :158.4(C-2),135.2(C-3),179.4(C-4),163.2(C-5),101.2(C-6),165.8(C-7),94.6(C-8),158.6(C-9),102.2(C-10),123.2(C-1',6'),117.2(C-2'),146.1(C-3'),149.8(C-4'),116.3(C-5'),99.8(rha-1),72.4(rha-2),72.3(rha-3),70.1(rha-4),68.4(rha-

5),17.6(rha-6),105.8(ara-1),77.2(ara-2),72.8(ara-3),74.0(ara-4),65.2(ara-5).

The MS and NMR spectral data were in consistent with those reported (Li and Yu., 1998). The structure of artabotryside A is shown in (Figure 1D).

### Artabotryside A inhibited U87-cells proliferation

MTT assay was used to test growth inhibitory effect of artabotryside A on U87 cells. Our results showed that artabotryside A inhibited growth of U87 cells in a dose- and time-dependent manner (Figure 2H). At low concentration, only a minor growth inhibition was observed; however, at 200  $\mu$ M, a statistically significant growth inhibition was observed after 24 and 48 h. The growth inhibition at 800  $\mu$ M concentration was more than 70 and 80% after 24 and 48 h, respectively. The half maximal inhibitory concentration (IC<sub>50</sub>) values were calculated as 480 and 370  $\mu$ M after 24 and 48 h, respectively.

Live and dead cells were further quantified by live/dead assay using flow cytometry. The cells stained and retained calcein are alive and scattered in region B4. The region B3 and B1 showed dead cells. The results showed that artabotryside A treatment reduced the viability of U87 cells from 96.99 $\pm$ 1.32% to 71.03 $\pm$ 2.51% and 49.66 $\pm$ 3.19% after 24 and 48 h, respectively as shown in Figures 2A, B, C and G). However, artabotryside A is less toxic to normal mouse glial cells as shown in Figure 2F.

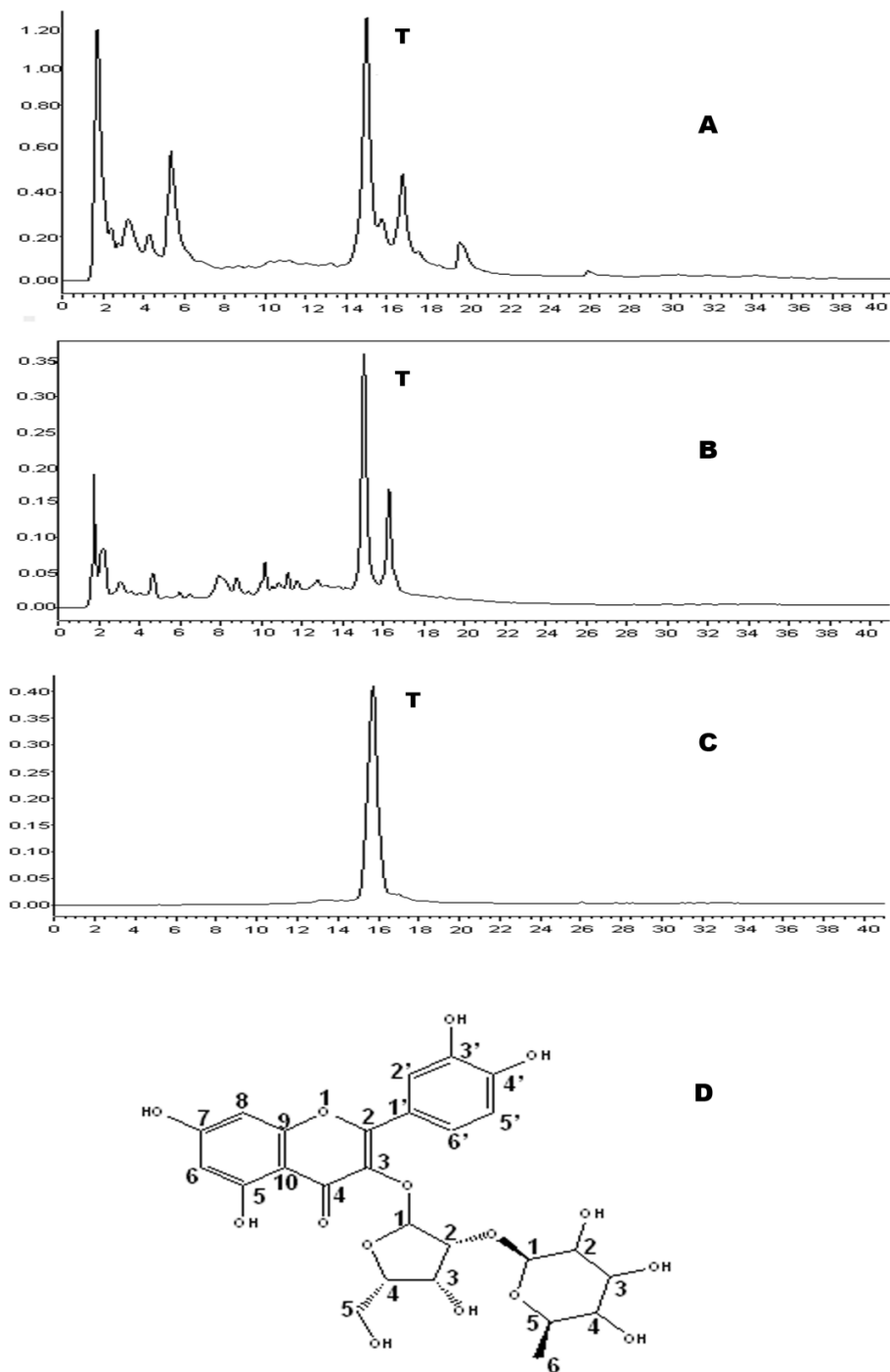
### Effect of artabotryside A on morphological changes and DNA fragmentation

Morphological changes were observed by phase contrast microscopy. After 24 h treatment with 350  $\mu$ M artabotryside A, a decrease in total number of cells and an increase in floating cells was observed in culture medium indicating artabotryside A induced cell death (Figure 3B). However, artabotryside A revealed less toxic effect on mouse splenocytes as assessed by trypan blue dye-exclusion method. Dead cells (satined blue) and live cells (excluded trypan blue dye) were observed microscopically (Figures 3C and D).

Nuclear morphological changes were also detected by PI staining. After 48 h treatment with artabotryside A at 350  $\mu$ M concentration, an obvious change in nuclear morphology including nuclear shrinkage and DNA fragmentation was observed, designating artabotryside A induced cell death through apoptosis ( Figure 3F)

### Artabotryside A induces G2/M phase arrest in U87 cells

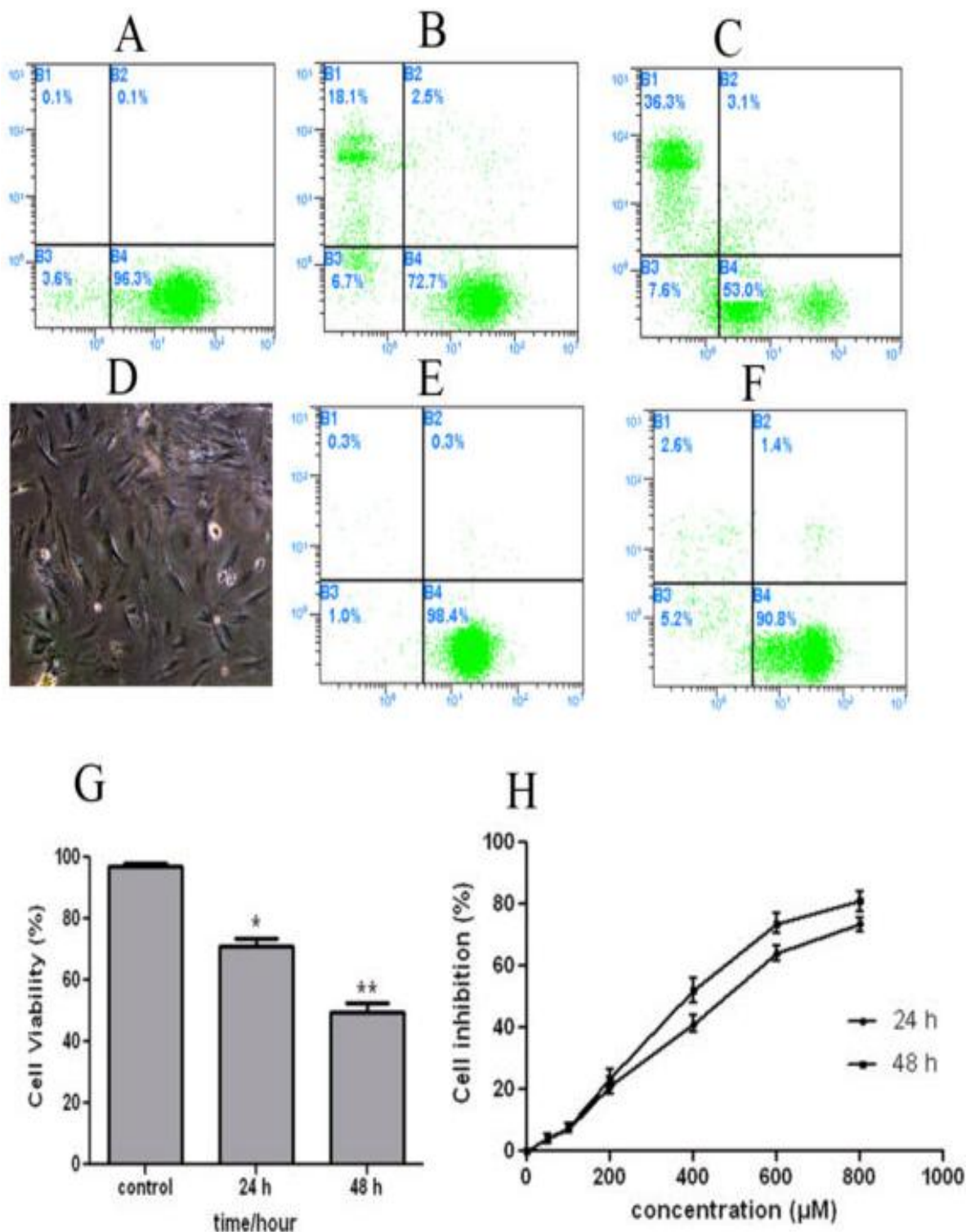
Cell cycle arrest and cell death are the main causes of



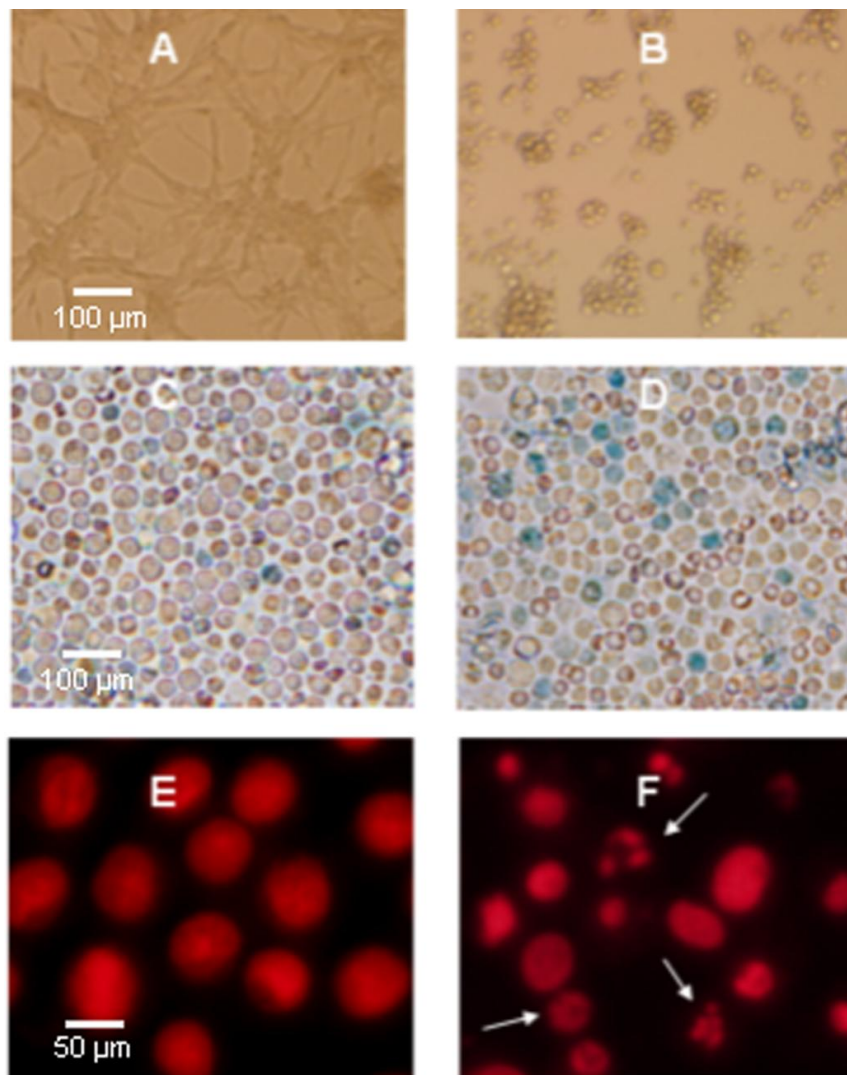
**Figure 1.** Analytical HPLC chromatogram of ethanol extracts. A, Water extract; B, isolated single compound; C, structure of artabotryside A; D, the letter T represent the target peak.

cell growth inhibition. To determine whether the cell growth inhibition involved growth arrest at specific phase of cell cycle, flow cytometric analysis of cell cycle distribution phases was performed. Our results showed that artabotryside A arrested the cell cycle at G2/M phase in a time-dependent manner. Treatment with artabotryside A at 350  $\mu\text{M}$  showed a significant increase

in G2/M phase from  $20.6 \pm 2.05\%$  to  $34.6 \pm 1.7\%$  and  $40.3 \pm 2.04\%$ , with a corresponding decrease in G0/G1 phase from  $63 \pm 3.26\%$  to  $49.6 \pm 2.9\%$  and  $43.6 \pm 2.86\%$ , without any change in S phase after 24 and 48 h, respectively (Figure 4). These results revealed that artabotryside A remained the cell cycle at G2/M phase and thereby induced apoptosis.



**Figure 2.** Cell growth inhibition of artabotryside A. U87 cells were treated with various doses of artabotryside A for 24 and 48 h. Growth inhibition was measured by live/dead assay and MTT assay. A, Control; (B, C), U87 cells treated with 350  $\mu$ M artabotryside A for 24 and 48 h, respectively and analyzed with FCM for the percentage of live and dead cells. The region B4 shows live cells (stained with calcein) while regions B3 and B1 show dead cells (stained with PI); D, primary glial cells isolated from C57 mice; (E, F), primary glial cells treated with 350  $\mu$ M artabotryside A for 0 and 24 h, respectively and analyzed for the percentage of live and dead cells using FCM; G, quantification of live and dead U87 cells from three independent experiments; H, cell growth inhibition analysis by MTT assay. Data are expressed as Mean  $\pm$  SEM. of three independent experiments. \* $p$ <0.05, \*\* $p$ <0.01 compared with control.

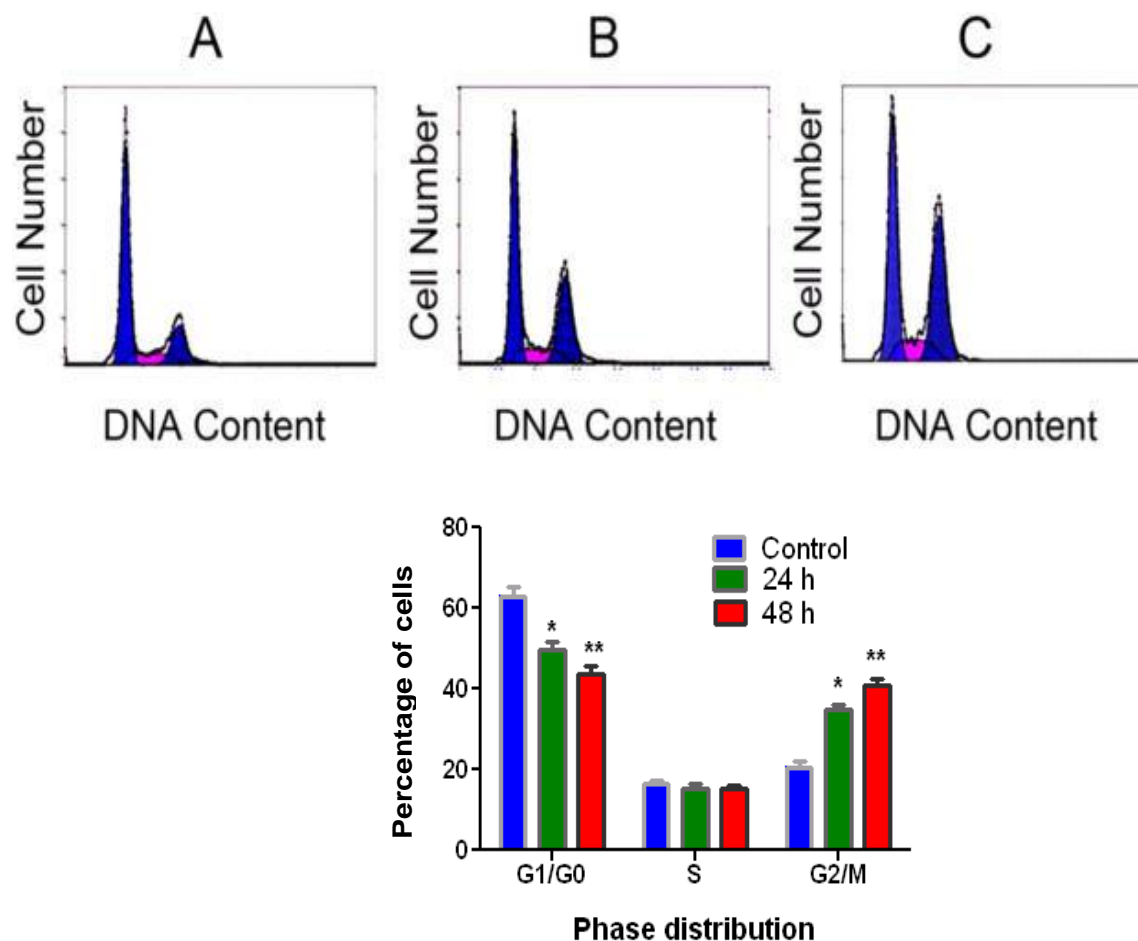


**Figure 3.** Morphological changes of U87 glioblastoma cells (A, B, E and F) and splenocytes (C and D) by phase contrast microscopy and fluorescence microscopy. (A and E) Control U87 cells; (B and F) U87 cells were treated with 350 $\mu$ M artabotryside A for 24 and 48 h, respectively. Arrows indicate condensed and fragmented DNA. C, control splenocytes; D, splenocytes were treated with 350  $\mu$ M artabotryside A for 24 h. Dead cells stained blue. The experiment was performed three times and one set of result is shown in Figure 3.

### Artabotryside A induces apoptosis in U87 cells

Artabotryside A induced cell death in U87-cells. However, cell death could either be resulted from apoptosis or necrosis. To determine the percentage of apoptosis and necrosis in U87 cells, cells were incubated with or without 350  $\mu$ M artabotryside A, in the presence or absence of 100  $\mu$ M Ac-DEVD-CHO, a caspase-3 inhibitor, for different time intervals and the percentages of cells undergoing apoptosis/necrosis were determined by flow cytometric analysis after staining with annexin V-FITC and PI. When the cells are double stained with annexin V-FITC and PI, four different populations of cells can be

observed. The cells that do not stain with either annexin V or PI are alive and reside in region B3; the cells that stain with only annexin V are in the stage of early apoptosis and reside in region B4; the cells that stain with both reagents are nonviable apoptotic/necrotic cells and scatter in region B2 while region B1 represents the nuclear fragments/necrotic cells stained with only PI. Flow cytometric analysis of apoptosis showed that there is significant increase in both early and late apoptosis after 24 and 48 h, respectively. The results revealed that early and late apoptosis process was time-dependent. The total number of cells undergoing apoptosis was relatively consistent. However, the rate of early apoptosis



**Figure 4.** Flow cytometric analysis of the cell cycle distribution in U87 cells treated with 350  $\mu$ M artabotryside A for various times. (A) Untreated control, (B) 24 h, (C) 48 h. Data are expressed as Mean  $\pm$  SEM. of three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$  compared with untreated control group.

was higher than late apoptosis after 24 h while the rate of late apoptosis increased after 48 h with a decrease in early apoptosis (Figures 5B and C).

Furthermore, there was significant decrease in both early and late apoptosis when the cells were treated with 100  $\mu$ M Ac-DEVD-CHO, a caspase-3 inhibitor (Figures 5D and E). The data suggested that artabotryside A induced caspase dependent apoptosis in U87 cells.

#### Generation of ROS by artabotryside A in U87 cells

ROS play an important role in depolarizing mitochondria and induction of apoptosis. Level of ROS in treated and untreated control groups were measured by staining cells with DCFH-DA, using FCM. The level of ROS was  $42.17 \pm 1.5\%$  and  $50.6 \pm 2.53\%$  after 24 and 48 h, respectively when cells treated with 350  $\mu$ M artabotryside A. These values are significantly higher than those of the control group ( $25.30 \pm 3.64\%$ ). The level of ROS in positive control group was  $39.7 \pm 1.95\%$  (Figure 6). These

results showed that artabotryside A increased ROS level in a time-dependent manner.

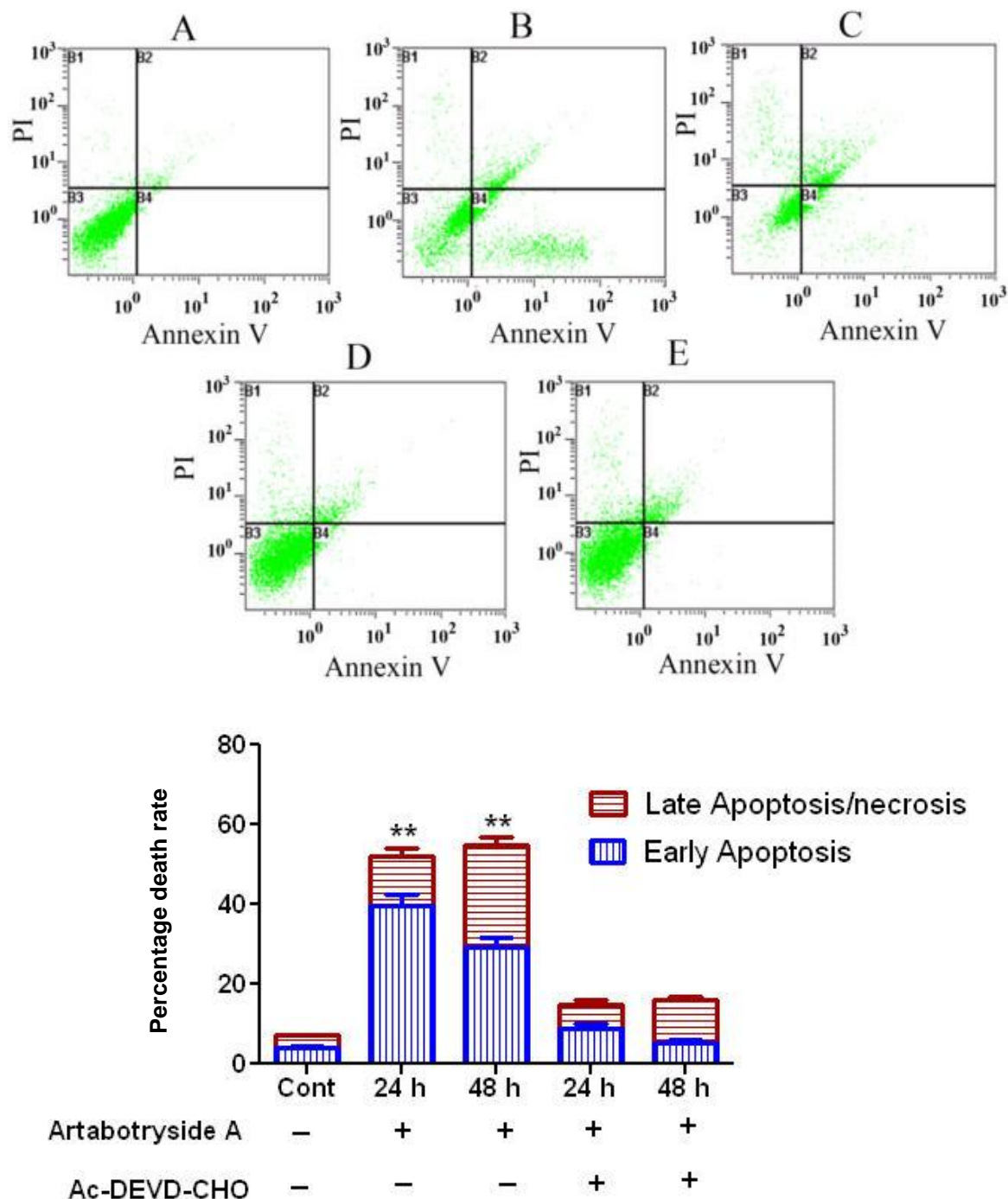
#### Changes in mitochondrial membrane potential by artabotryside A in U87 cells

Depolarization in mitochondrial membrane potential is a characteristic feature of apoptosis. We determined mitochondrial membrane potential in treated and control groups using FCM. Our results showed that mitochondrial membrane potential in U87 cells treated with 350  $\mu$ M artabotryside A, was  $86.46 \pm 1.46\%$  and  $75.46 \pm 1.35\%$  after 24 and 48 h, respectively (Figure 7). These values were significantly lower than those of control group ( $96 \pm 0.8\%$ ).

#### DISCUSSION

Seeds of *D. sophia* (L.) have long been used to treat some types of cancers in TCM (Sun et al., 2005b; Li et al.,



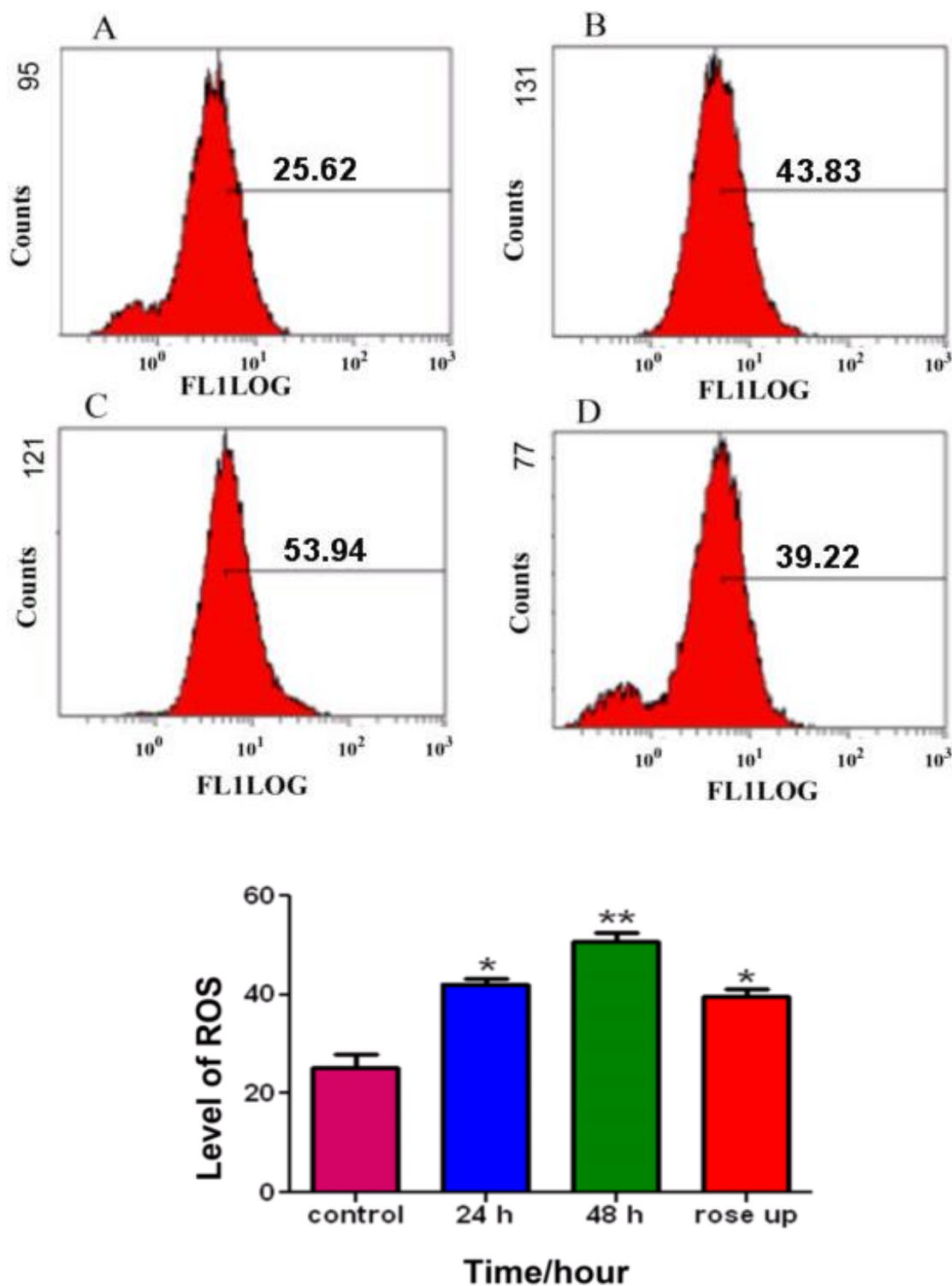


**Figure 5.** Flow cytometric analysis of apoptosis in U87 cells treated with 350 μM artabotryside A for various times. A, Untreated control; B, 24 h; C, 48 h; D and E, cells were treated with 350 μM Artabotryside A in the presence of 100 μM Ac-DEVD-CHO for 24 and 48 h, respectively. Data are expressed as Mean ± SEM. of three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$  compared with untreated control group.

2010; Qian 2006). In the present study, a bioactivity guided approach, based on MTT assay for cells growth inhibition was used to identify bioactive compound in ethanolic extract of flixweed seeds. From bioactive fraction, a flavonol glycoside "artabotryside A" was isolated. This compound has been reported the component

of *Artabotrys hexapetalus*' leaf (Li and Yu., 1998). However, the presence of artabotryside A in *D. sophia* (L.) and its anticancer activity has not yet been reported.

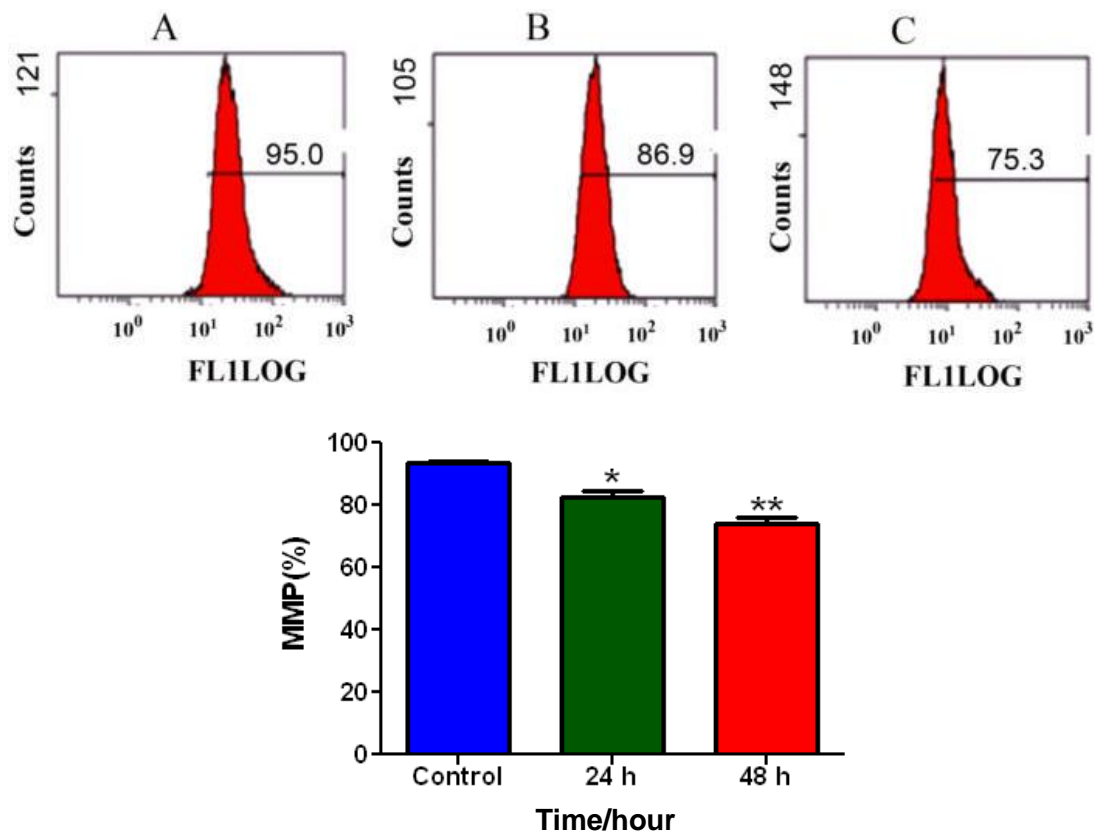
In the present study, we examined that artabotryside A inhibited the viability of U87 glioblastoma cells in a dose- and time-dependent manner. However, this growth



**Figure 6.** The level of ROS in U87 cells treated with 350  $\mu$ m artabotryside A for various times. A, untreated control; B, 24 h; C, 48 h; D, roseup. Data are expressed as Mean  $\pm$  SEM. of three independent experiments. \* $p$ <0.05, \*\* $p$ <0.01 compared with untreated control group.

inhibition could be the result of apoptosis, necrosis, cyclic block or pure growth suppression (Wu et al., 2006). To further investigate this effect, DNA fragmentation,

apoptosis assay and cell cycle assay were performed. Apoptosis is characterized by DNA fragmentation, chromatin condensation and caspases activation



**Figure 7.** The mitochondrial membrane potential in U87 cells treated with 350  $\mu$ m artabotryside A for various times. A, untreated control; B 24 h; C, 48 h. Data are expressed as Mean  $\pm$  SEM. of three independent experiments. \* $p < 0.05$ , \*\* $p < 0.01$  compared with untreated control group.

(Jin et al., 2006; Nagata, 1998;). Our results demonstrated that control group showed no fragment changes; however, cells treated with artabotryside A showed significant changes in nuclear morphology including DNA fragmentation and chromatin condensation. This result showed that artabotryside A induced cell death in U87 cells through apoptosis process.

These results were further confirmed by flow cytometric analysis of apoptosis. Flow cytometric results also demonstrated that induction of cell death by artabotryside A, is through apoptotic process. However, early and late apoptosis process was time-dependent. The percentage of cells undergoing early apoptosis after 24 h treatment was higher than cells undergoing late apoptosis. After 48 h treatment, this effect was vice versa.

Cell cycle control is one of the major regulatory mechanisms of cell growth. Many anticancer agents or DNA damaging agents arrest the cell cycle at G0/G1, S or G2/M phase and thereby induce apoptosis. Quercetin, a flavonoid, has been reported to induce G2/M phase arrest in glioma cells (Gamet-Payraastre et al., 2000; Lu et al., 2007; Murray, 2004). In agreement, our results showed that artabotryside A, a flavonol glycoside, induced cell cycle arrest at G2/M phase in U87 glioblastoma cells in a time-dependent manner.

Flavonoids, a group of phenolic compounds, have multiple biological, pharmacological and medicinal properties including anti-inflammatory, antiviral, antiallergic, antithrombotic and anticancer effect. The anticancer activity of flavonoids and flavonols is attributed by their ability to elevate ROS generation (Kim et al., 2008; Sharma et al., 2007; Wang et al., 1999). Kaempferol, another promising antiglioma flavonoid, has been reported to induce cell death in U87, LN229 and T98G glioblastoma cell lines through ROS production (Sharma et al., 2007). A recent study by Mohamed and Mahrous (2009) has shown that flixweed seeds contain kaempferol. Therefore, we determined the level of ROS in U87 glioblastoma cells treated with artabotryside A, a flavonol glycoside, by staining cells with DCFH-DA. The level of ROS significantly increased in a time-dependent manner after cells exposed to artabotryside A, compared with control group. The results are in agreement with previous studies (Kim et al., 2008; Mohamed and Mahrous, 2009; Sharma et al., 2007; Wang et al., 1999). Excessive production of ROS such as superoxide, hydrogen peroxide, anion radical and hydroxyl radical results in oxidative stress, loss of cell functions and ultimately cell death (Gamet-Payraastre et al., 2000; Ito et al., 2004). ROS can also induce lipid peroxidation and cross linking

of thiol groups in proteins. Both of these phenomena cause the opening of the mitochondrial permeability transition pore (PTP) (Ji et al., 2009). In the present study, a significant reduction in mitochondrial membrane potential was observed in cells of treatment groups suggesting opening of PTP. The opening of mitochondrial PTP can lead to a release of cytochrome c and other pro-apoptotic molecules from intermembrane space to cytosol where cytochrome c binds and activates caspase-9, which then results in the activation of other downstream caspases and ultimately caspase-3. Caspase-3 has been identified as a main executioner of apoptotic response inside the cells. Finally, activated caspase-3 induces the release of death substrate and lyses DNA in nucleus resulting in DNA fragmentation (Ji et al., 2009). In the present study, involvement of caspase-3 was investigated in cell death mediated by artabotryside A using a specific caspase-3 inhibitor, Ac-DEVD-CHO. Treatment of cells with caspase-3 inhibitor resulted in a significant decrease in apoptosis. These results provide a correlation between caspase-3 activity and artabotryside A-induced apoptosis in U87 glioblastoma cells. However, it should also be mentioned that pretreatment with caspase-3 inhibitor did not result in complete inhibition of apoptosis, indicating that some other pathways are also involved. Further investigations are needed to find out the detailed molecular mechanism of apoptosis in U87 cells induced by artabotryside A (Figure 7).

## Conclusion

We have isolated and demonstrated the anticancer activity of artabotryside A, from the seeds of *D. sophia* (L.) for the first time. The results showed that artabotryside A, inhibited the growth of U87 glioblastoma cells through G2/M phase arrest and induction of caspase-3-dependent apoptosis. However, it is less toxic to mouse splenocytes and glial cells. This aspect of selective cytotoxicity of artabotryside A, is of great interest and need to verify *in vivo*, in order to develop it into a novel chemotherapeutic agent against gliomas.

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