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# Anti-Proliferative Effect of Wogonin on Ovary Cancer Cells Involves Activation of Apoptosis and Cell Cycle Arrest

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Data Interpretation D  
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**Background:** The present study was designed to investigate the effect of wogonin on Caov-3 and A2780 ovary cancer cell proliferation and the mechanisms involved.

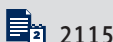
**Material/Methods:** Cell viability changes and apoptosis induction by wogonin were assessed by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and fluorescence microscopy. Morphological examination of cells was performed using transmission electron microscopy.

**Results:** Wogonin exhibited inhibitory effect on Caov-3 and A2780 cancer cell proliferation in a concentration-based manner. Caov-3 and A2780 cell proliferation was reduced to 18% and 21%, respectively on treatment with 200  $\mu$ M concentration of wogonin. Treatment with wogonin significantly enhanced the percentage of A2780 cells showing apoptosis. The nuclear membrane degradation and condensation of chromatin material was evident in A2780 cells on treatment with wogonin. Treatment of A2780 cells with wogonin suppressed the migration potential significantly. The proportion of A2780 cells in G1/G0 phase was markedly raised on exposure to wogonin for 48 hours.

**Conclusions:** In summary, this study demonstrated that wogonin acts as an ovary cancer cell proliferation inhibiting agent through activation of apoptosis. Wogonin, therefore, can be investigated further for the development of ovary cancer treatment.

**MeSH Keywords:** **Anti-Inflammatory Agents • Antioxidants • Flavonoids**

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## Background

Ovarian cancer has the highest rate of mortality among all the gynecological disorders and has very poor prognosis [1]. Throughout the world, ovarian cancer ranks as the fourth highest cause of death associated with cancer in women and is mostly detected at a very late stage [1,2]. Type I ovarian cancers usually grow slowly and represent an initial stage of tumor, whereas, type II cancers grow very fast and represent the advanced stage [3]. The treatment strategy generally used for ovarian carcinoma involves surgical resection followed by the administration of Taxol-platinum combination therapy [3]. There is around an 80% response rate to this treatment strategy [3]; however, over a period of 2 years, cancer relapse has been reported in more than 50% of patients [3,4]. Ovarian tumors develop resistance to first-line drugs like platinum and Taxol, and in such cases more potent chemotherapeutic agents including topotecan and doxorubicin are recommended [4–6]. Contrary to the other types of cancers, ovarian carcinoma cells largely remain in the dormant stage and escape the inhibitory action of these drugs [7,8]. Tumor relapse is caused by these non-proliferating cells after the cytotoxicity effect of the drug subsides [7,8]. The development of effective and novel chemotherapeutic agents is therefore highly desired for the treatment of ovarian cancer.

*Scutellaria* belongs to the *Labiatae* family of the plant kingdom and has been used for the treatment of allergies, hepatitis, inflammation, thrombosis, bacterial infections, and oxidative disorders [9,10]. The plants of this genus have wide-spread distribution throughout the tropical mountain range of North America, Europe, and Asia [11]. Phytochemical investigation of the *Scutellaria* genus has led to the isolation of more than 300 terpenoid and phenolic compounds [12,13]. The 3 main flavonoids characterized from this genus are baicalin, baicalein, and wogonin which act as anti-inflammatory, anti-human immunodeficiency virus, antioxidant, and anti-cancer agents [14,15]. The present study was designed to investigate the effect of wogonin on Caov-3 and A2780 ovary cancer cell proliferation. Moreover, the study also focused on understanding the mechanisms associated with the anti-proliferative effect of wogonin on ovary cancer cells.

## Material and Methods

### Cell lines and culture conditions

Caov-3 and A2780 ovary carcinoma cell lines were provided by the Chinese Academy of Sciences (Shanghai, China). Both cell lines were maintained in RPMI-1640 medium. The medium also contained 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. The conditions used

for cell culture consisted of 37°C in an incubator under humidified atmosphere of 5% CO<sub>2</sub> and 95% air.

### MTT cell proliferation assay

The cytotoxicity effect of wogonin on Caov-3 and A2780 cells was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for cell viability. In brief, Caov-3 and A2780 cell lines were put into 96-well plates at 2×10<sup>5</sup> cells per well density at 37°C for 12 hours. The cells were incubated with 10, 20, 50, 100, 150, and 200 µM of wogonin or dimethyl sulfoxide (control) for 72 hours. Then cells were treated for 4 hours with 20 µL of 0.5 mg/mL solution of MTT at 37°C followed by addition of 300 µL dimethyl sulfoxide to dissolve formazan crystals. The absorbance measurements were performed in microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at a wavelength of 482 nm.

### Fluorescent microscopic evaluation of apoptosis

A2780 cancer cells were distributed in 12-well plates at 2×10<sup>5</sup> cells per well density. The cells were exposed to 100, 150, and 200 µM of wogonin for 72 hours at 37°C. The cells were trypsinized, washed with phosphate-buffered saline (PBS) and subsequently subjected to acridine orange (5 mg/mL solution)/ethidium bromide (3 mg/mL solution) staining. The cells, after washing with PBS, were fixed with 10% formaldehyde and then analyzed by fluorescence microscope (Nikon Corporation, Tokyo, Japan).

### Transmission electron microscopy

A2780 carcinoma cells were put into the flasks at 1.5×10<sup>6</sup> cells per well concentration and exposed to 100, 150, and 200 µM of wogonin for 72 hours. The harvested cells were 2 times washed with PBS for 5 minutes and then treated at 37°C with 3% glutaraldehyde for 3.5 hours. After embedding in LR White resin for 45 minutes the blocks were sliced into 60 nm thick sections using ultra-microtome (JEOL, Ltd., Tokyo, Japan). The changes in cellular ultra-structural features were examined by the transmission electron microscope (JEOL, Ltd.) to determine apoptosis.

### Determination of cell migration

The A2780 cells in 6-well plates at 1.5×10<sup>6</sup> cells/mL density were allowed to form a 100% monolayer at 37°C. A 100 mL pipette tip was used to draw a straight line through the middle of plate to create the wound. The cells were 3 times washed with PBS and then exposed to 100, 150, and 200 µM of wogonin for 72 hours. The fixing of cells was performed in 2% solution of crystal violet dye in 4% ethanol for 35 minutes. The cell

migration was examined under inverted light microscope (Nikon Corporation) randomly in 5 fields.

### Cell cycle analysis

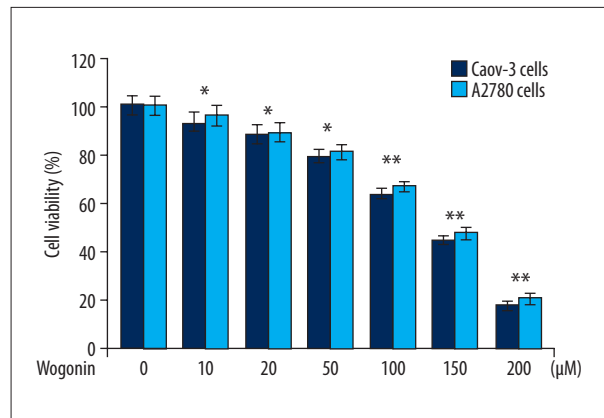
A2780 cells were distributed in 60 mm plates at  $2.5 \times 10^5$  cells per well density. Wogonin was mixed with the medium at 100, 150, and 200  $\mu\text{M}$  and cells were cultured in it for 72 hours followed by trypsinization and washing in PBS, 3 times. The cells were subjected to fixing in 70% ethyl alcohol for overnight and subsequently put into 20  $\mu\text{g}/\text{mL}$  solution of RNase A. The cells were then stained with 10  $\mu\text{g}/\text{mL}$  solution of propidium iodide (PI) at 37°C for 3 hours. The flow cytometry connected to FACSCalibur instrument (BD Biosciences, San Jose, CA, USA) was used for examination of DNA content to determine cell cycle distribution.

### Western blot

A2780 cells after incubation with 10, 20, 50, 100, 150, and 200  $\mu\text{M}$  of wogonin were put into the lysis buffer (ice-cold) for 3 hours. The confluent cells after PBS washing were treated with Nonidet P-40 (2%), Triton X-100 (0.2%),  $\text{NaPO}_4$  (35 mM; pH 7.6) mixed with sodium orthovanadate (2 mM), tris-hydrochloric acid (3 mM; pH 7.6), sodium chloride, and (120 mM) leupeptin and aprotinin (12  $\mu\text{g}/\text{mL}$ ). The lysate was centrifuged for 35 minutes at 11 000 x g at 4°C to collect the supernatant and protein concentration was determined by Lowry protein assay. The proteins were separated on 12% SDS-PAGE gels by electrophoresis and then transferred to the polyvinylidene fluoride membrane. Non-specific sites in the membranes were blocked on treatment for 1 hour at 37°C with non-fat milk powder (0.05%) in TBST. The incubation with primary anti-bodies against Bax (cat. no. ab7923; dilution 1: 1000) and Bcl-2 (cat. no. ab77566; dilution 1: 1000; both from Abcam, Cambridge, UK) was performed for overnight at 4°C. The membrane washing was followed by treatment for 2 hours with horseradish peroxidase-labeled goat anti-mouse secondary at room temperature. The immunoblots were visualized using chemiluminescence and autoradiography and quantification was carried out by LabWorks 4.5 analysis software (UVP, Inc., Upland, CA, USA).

### Statistical analysis

The values are expressed as the mean  $\pm$  standard error of triplicate experiments. Analysis of the statistically significant differences was made by Student-*t* test and one-way analysis of variance. The data were analyzed by GraphPad Prism 7 software (GraphPad Software Inc., La Jolla, CA, USA). The differences were taken significant statistically at  $P < 0.05$ .



**Figure 1.** The viability inhibitory effect of wogonin on Caov-3 and A2780 carcinoma cells. The cells following 72 hours of exposure to different concentrations of wogonin were assessed by MTT assay. The presented values are mean  $\pm$  standard deviation of triplicate measurements. \*  $P < 0.05$  and \*\*  $P < 0.02$  versus control cells. MTT – (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).

## Results

### Wogonin exhibits cytotoxic effect on Caov-3 and A2780 cancer cells

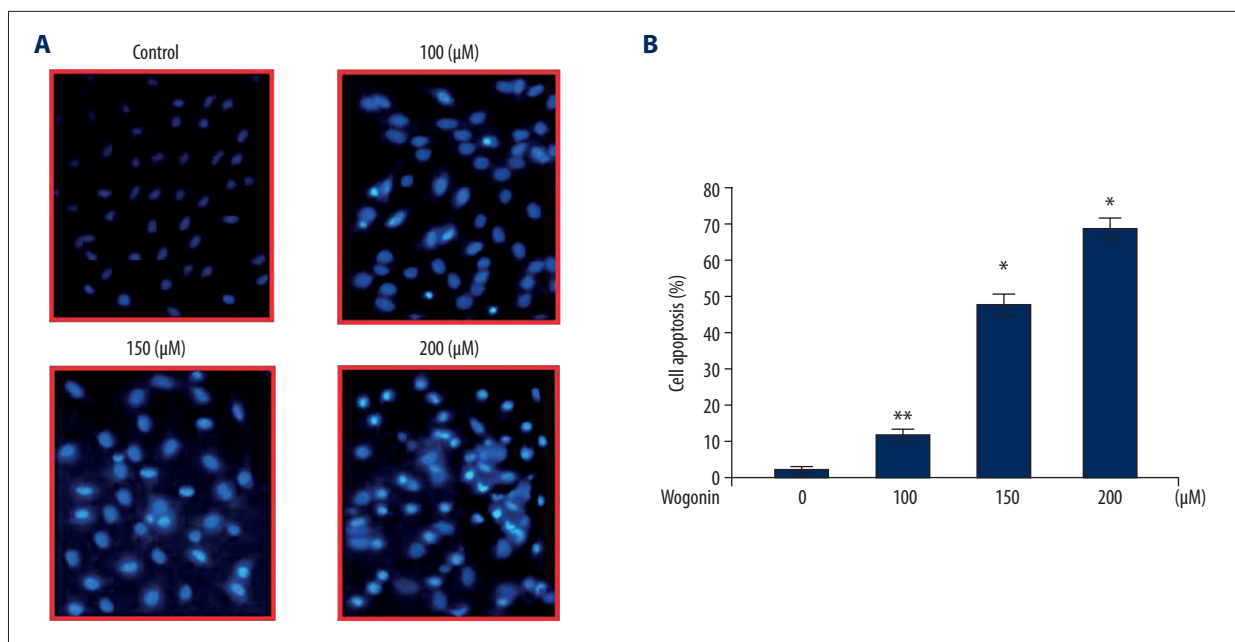
The cytotoxicity of wogonin against Caov-3 and A2780 carcinoma cells was investigated at 10, 20, 50, 100, 150, and 200  $\mu\text{M}$  by MTT assay (Figure 1). Wogonin reduced Caov-3 and A2780 cell viability with increases in the concentration. Treatment with 10, 20, 50, 100, 150, and 200  $\mu\text{M}$  of wogonin reduced Caov-3 cell viability to 93%, 88%, 79%, 64%, 45%, and 18%, respectively ( $P < 0.05$ ). The viability of A2780 cells was reduced to 96%, 89%, 81%, 67%, 48%, and 21%, respectively on treatment with 10, 20, 50, 100, 150, and 200  $\mu\text{M}$  of wogonin ( $P < 0.05$ ).

### Wogonin induces apoptosis in A2780 cells

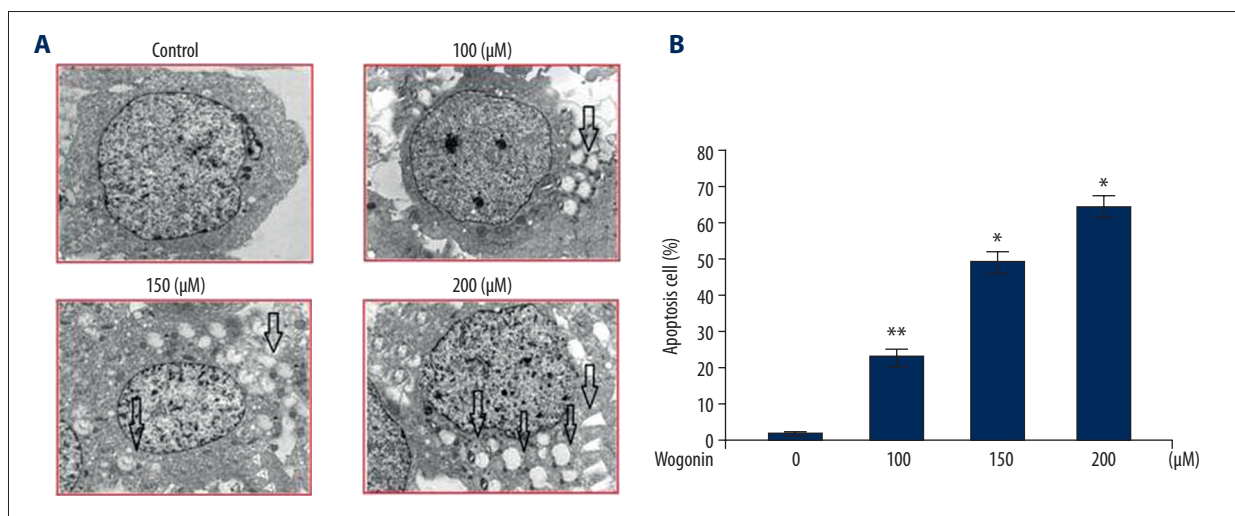
The fluorescence microscopy was used for assessment of apoptosis in A2780 cells on treatment with 50, 100, 150, and 200  $\mu\text{M}$  of wogonin. Wogonin treatment of A2780 cells increased blue fluorescence on staining with Hoechst 33342 in concentration-based manner (Figure 2). On increasing the wogonin concentration from 50 to 200  $\mu\text{M}$  the blue fluorescence increased markedly.

### Apoptosis analysis by wogonin using transmission electron microscopy

Apoptotic changes by wogonin treatment were analyzed morphologically in A2780 cells using transmission electron



**Figure 2.** Effect of wogonin on apoptosis induction in A2780 cells. (A) The fluorescence microscopy following Hoechst 33342 staining was used for determination of apoptosis in A2780 cells. Magnification 300×. (B) Quantification of apoptotic cells. \*  $P < 0.05$  and \*\*  $P < 0.02$  versus control cells.

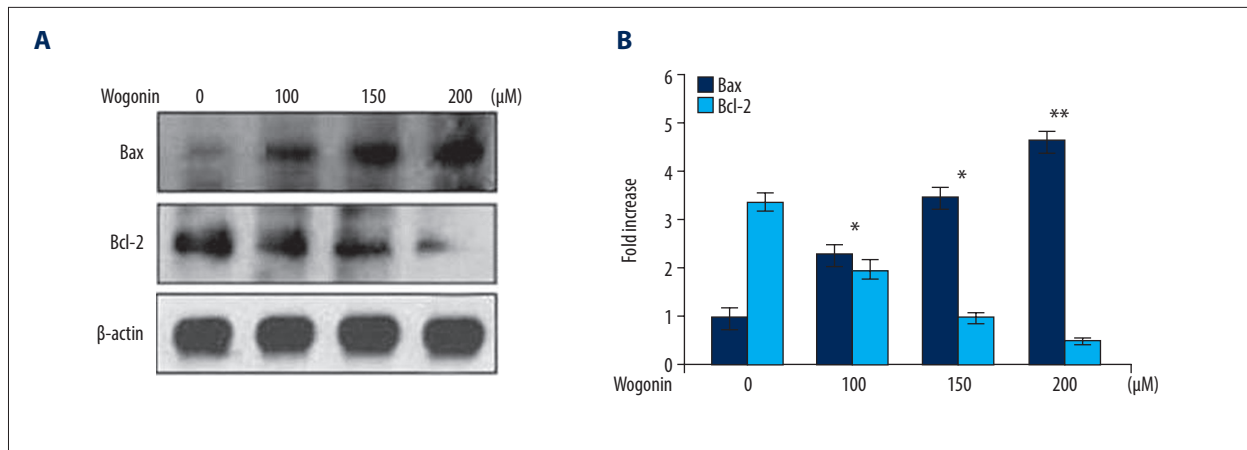


**Figure 3.** Effect of wogonin on morphology of A2780 cells by transmission electron microscopy (TEM). (A) The cells on exposure to 100, 150, and 200 μM of wogonin were examined by TEM. Magnification 250×. Arrows indicate condensed chromatin and apoptotic bodies in wogonin-treated cells. (B) Quantified data. \*  $P < 0.05$  and \*\*  $P < 0.02$  versus control cells.

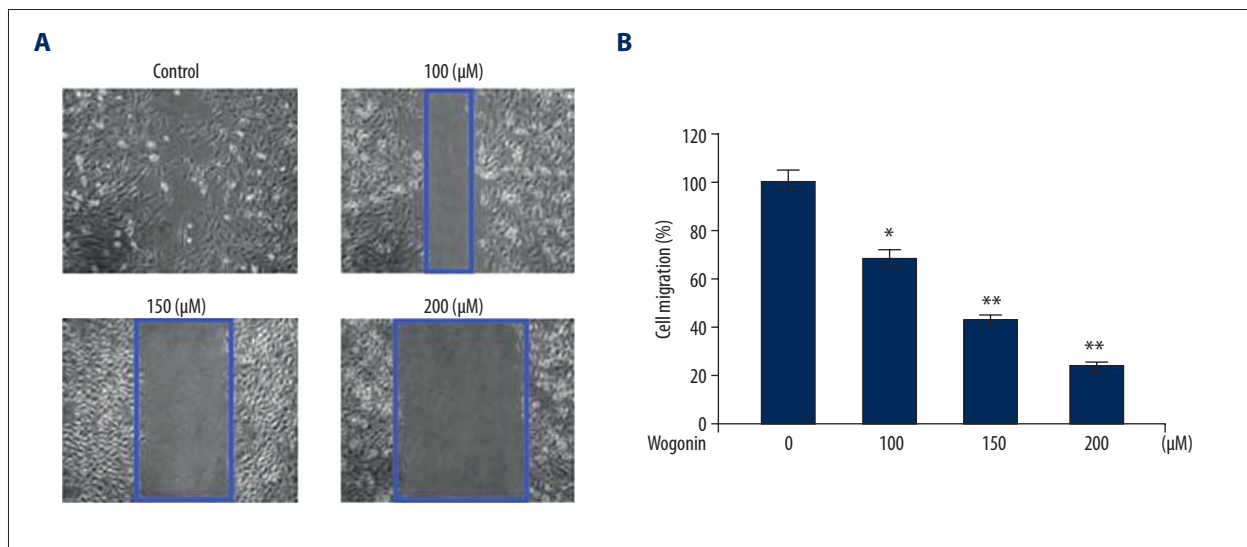
microscopy (Figure 3). Treatment of A2780 cells with wogonin lead to breakdown of nuclear membrane and condensed chromatin material at 72 hours. There was no membrane breaking and chromatin condensation in the control A2780 cell cultures. The chromatin condensation, formation of apoptotic bodies and breakdown of nuclear membrane was more common in A2780 cell cultures with increase in wogonin concentration from 100–200 μM.

### Wogonin promotes Bax/Bcl-2 ratio in A2780 cells

The expression of Bax and Bcl-2 in A2780 cells following wogonin treatment was determined by western blotting (Figure 4). Treatment of A2780 cells with 100, 150, and 200 μM of wogonin markedly promoted Bax expression and suppressed Bcl-2 level. On increasing wogonin concentration from 100 μM to 200 μM, the ratio of Bax/Bcl-2 increased significantly.



**Figure 4.** Effect of wogonin on apoptotic proteins. (A) The cells after exposure to 100, 150, and 200 μM of wogonin for 72 hours were examined by western blotting. (B) The protein expression was quantified with respect to β-actin as control. \*  $P < 0.05$  and \*\*  $P < 0.02$  versus control cells.



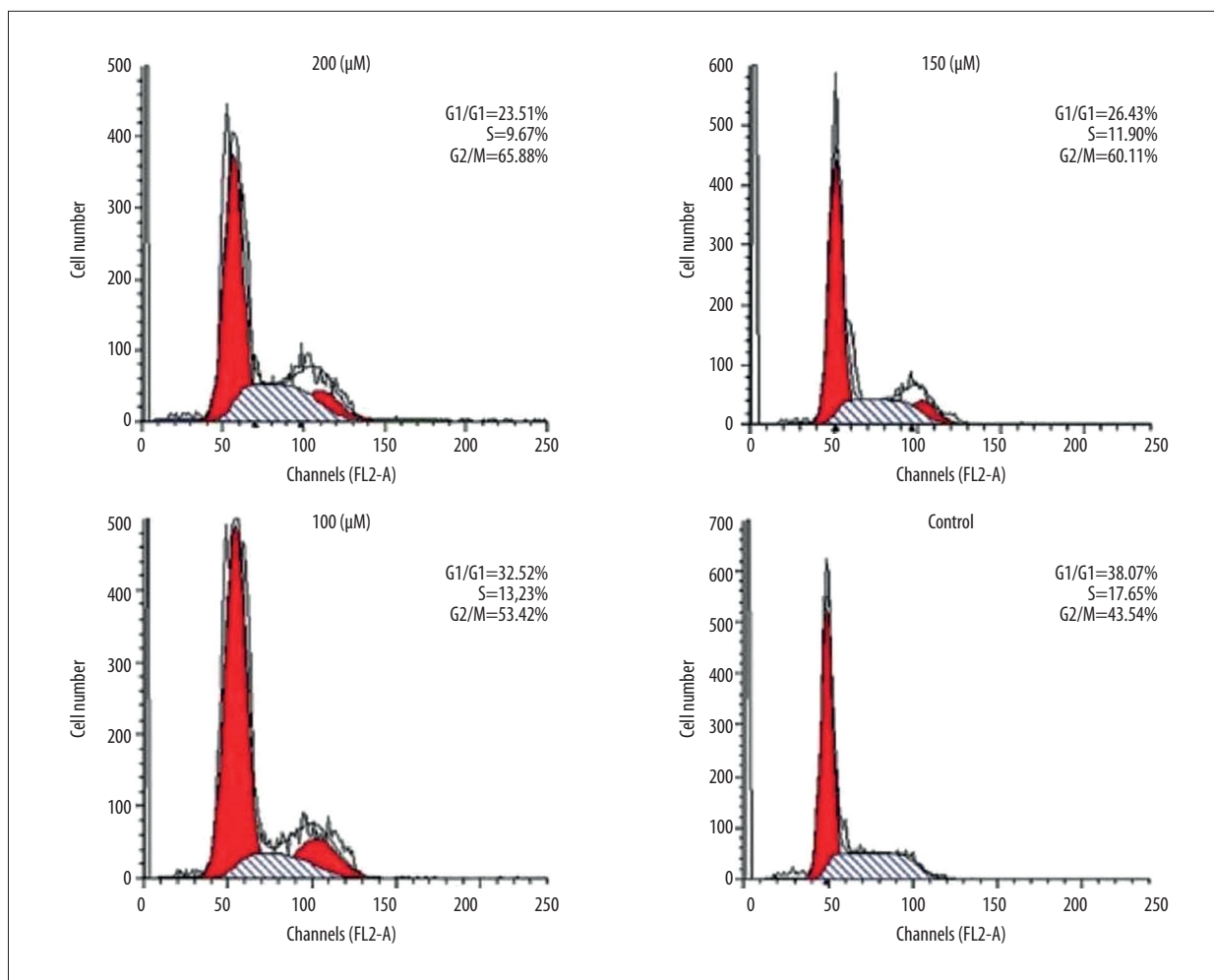
**Figure 5.** Effect of wogonin on A2780 cell migration. (A) The cells incubated with wogonin at 100, 150, and 200 μM for 72 hours were subjected to wound healing assay. Images were captured at 250× magnification. (B) Quantification of cell migration. \*  $P < 0.05$  and \*\*  $P < 0.01$  versus control A2780 cells.

### Wogonin inhibits cancer cell migration

Effect of 100, 150 and 200 μM of wogonin on migration of A2780 cells was examined using wound healing assay (Figure 5). A significant decrease in A2780 cell migration was caused by wogonin at 72 hours in comparison to the control cultures. The migrated cell proportion decreased to 67.34%, 13.76%, and 4.54%, respectively on treatment with 100, 150, and 200 μM of wogonin. In control cultures the migration potential of A2780 cells was 96.65%.

### Wogonin treatment leads to A2780 cell cycle arrest in G2/M phase

The wogonin treatment of A2780 cells was followed by flow cytometry using PI staining to examine DNA content distribution (Figure 6). Wogonin treatment significantly increased A2780 cell proportion in G2/M phase in comparison to the control cells. The proportion of A2780 cells in G0/G1 and S phases was reduced significantly on treatment with 100, 150, and 200 μM of wogonin.



**Figure 6.** Effect of wogonin A2780 cell cycle distribution. The wogonin was added to A2780 cell cultures at 100, 150, and 200  $\mu\text{M}$  and then flow cytometry using propidium iodide was used for analysis of cell cycle distribution.

## Discussion

Cancer is a leading health problem accounting for around 8 million deaths every year throughout the world and the number is expected to reach 13 million by 2030 [1]. Apoptosis is one of the vital physiological process associated with the elimination of unwanted cells from the body [16]. The main characteristic feature of carcinoma cells is the tendency to escape apoptosis and undergo division rapidly [16]. In the present study wogonin treatment suppressed Caov-3 and A2780 ovary carcinoma cell viability markedly in concentration-based manner. To confirm whether wogonin treatment inhibits ovary carcinoma cell viability by induction of apoptosis flow cytometry was used. The results revealed that wogonin treatment of A2780 cells markedly enhanced rate of apoptosis in comparison to the control. These findings proved that wogonin exhibits cytotoxicity effect on ovary carcinoma cells by promoting apoptotic rate. The expression of Bcl-2 family proteins has vital role in controlling the process of cell apoptosis by regulating the

permeability of mitochondrial membrane [17]. The ratio of Bax and Bcl-2 expression is very low in the carcinoma cells. Most of the anti-cancer drugs induce apoptosis in carcinoma cells by promoting the ratio of Bax and Bcl-2 [18]. The results from current study showed that wogonin treatment of A2780 cells enhanced the Bax and suppressed Bcl-2 expression. Therefore, wogonin treatment induced apoptosis by increasing the ratio of Bax/Bcl-2 in A2780 cells.

The cell apoptosis is evident by various changes like shrinking, chromatin condensation, blebbing in the membrane and fragmentation of the genetic material [19–21]. All these changes are controlled in a well-regulated manner by various signaling pathways [19–21]. Although apoptosis can be initiated either by intrinsic or extrinsic pathways, the most common initial event involves damage to DNA [22,23]. The plant derived secondary metabolites have been found to inhibit carcinoma cell growth by inducing apoptosis without any cytotoxicity against normal cells [24–26]. In the present study, acridine

orange/ethidium bromide staining showed a significant increase in A2780 cell apoptosis by treatment with wogonin. The wogonin induced apoptosis of A2780 cells was also confirmed using transmission electron microscopy analysis. The breakdown of nuclear membrane, chromatin condensation, and formation of apoptotic bodies in A2780 cells was evident on treatment with wogonin. Metastasis of the cancer cells involves migration and invasion to distant organs through stroma, dissemination of the tumor cells, and growth of the cells at the site of metastasis [27]. In the present study, treatment of A2780 cells with wogonin suppressed the migration potential in concentration-based manner.

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## Conclusions

In summary, the current study demonstrated that wogonin acts as an ovary cancer growth inhibiting agent. In ovary cancer cells wogonin treatment causes induction of apoptotic changes, inhibits migration, and leads to arrest of cell cycle. Thus, wogonin can be of immense importance for the treatment of ovary cancer.