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# Juglone exerts antitumor effect in ovarian cancer cells

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ABSTRACT

***Objective(s)***: Juglone is isolated from many species of the Juglandaceae family and used as an anti-viral, anti-bacterial, and anti-tumor therapeutic. Here, we evaluated juglone-induced antitumor effect in ovarian cancer SKOV3 cells.

***Materials and Methods:*** MTT assay was performed to examine juglone anti-proliferative effect. Cell cycle and apoptosis were studied using flow cytometry in juglone-treated SKOV3 cells. To investigate molecular mechanism of cell cycle and apoptosis, protein expression levels were measured by Western blot analysis of cyclin D1, Bcl-2, Bax, cytochrome c, caspase-9 and caspase-3. To investigate the motility of juglone-treated SKOV3 cell, Matrigel invasion assay was employed to characterize cell invasion. Also, matrix metalloproteinase-2 (MMP-2) expression levels were detected by western blot.

***Results:*** Juglone significantly inhibited SKOV3 cell proliferation as shown by G0/G1 phase arrest, and this effect was mediated by inactivation of cyclin D1 protein ($P$<0.05). Juglone induced apoptosis in SKOV3 cell which was accompanied by caspase-9 and caspase-3 activation ($P$<0.05). Juglone decreased Bcl-2 levels and increased Bax and cytochrome c (Cyt c) levels ($P$<0.05). Juglone sufficiently inhibited invasion while evidently decreased MMP-2 expression ($P$<0.05).

***Conclusion:*** The results suggest that juglone could probably induce apoptosis through mitochondrial pathway and restrained cell invasiveness by decreasing MMP expression.

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## Introduction

Ovarian cancer is the sixth most common cancer and the leading cause of female death. It is typically treated in its advanced stages with surgery and platinum-based chemotherapy. Although platinum-based agents remain the core of primary treatment, the disease progresses during this treatment with a median overall survival rate of less than 12 months. Many platinum-containing regimens are effective in this setting and include carboplatin with paclitaxel and carboplatin with gemcitabine (1). Despite chemotherapeutic improvements, the mortality rate among most women with relapsed ovarian cancer remains high and treatments need to be improved.

Juglone (5-hydroxy-1, 4-napthoquinone) is a natural compound that is isolated from many plants. In living plants, juglone is in a non-toxic glycosylated form, but when exposed to soil or air, this allelochempound is immediately transformed into an oxidized and highly toxic form (2). Therefore, juglone has various pharmacological effects and has been used in folk medicine for many years in China for its anti-viral and anti-bacterial activities (3). Recent studies showed that juglone is a potent cytotoxic agent and induces cell apoptosis through the mitochondria-dependent pathway in human cancer cell lines such as human lung cancer (A549) cells, human leukemia (HL-60) cells, and human cervical carcinoma (HeLa) cells (4-6). However, the mechanisms through which juglone induces anticancer effects in human ovarian cancer remain unknown. Hence, the current study investigated juglone anticancer potential and its underlying mechanisms in human ovarian cancer SKOV3 cells.

## Materials and Methods

### *Cell lines and cell culture*

SKOV3 cells were kindly provided by Dr Xiu-hong Zhong (Department of Basic Medical Jilin Medical College). Cells were cultured in Iscove's Modified Dulbecco's Media (IMDM) (Gibo-BRL, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Gibo-BRL, Carlsbad, CA, USA) in a humidified 5% $CO_2$

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incubator at 37 ˚C. The medium was changed every day.

### *MTT assay*

Cell viability was assessed by measuring cells ability to metabolize 3-(2,5-dimethydiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Briefly, cells (2×104/well) were seeded in triplicate in 96-well plates and cultured at 37 ˚C with 5% $CO_2$ for 24 hr. Juglone was added at the final concentrations of 0, 6.25, 12.5, 25, 50 or 100 μM. Three replicate wells were used for each data point in the experiments. After incubation for 24 hr, 20 μl MTT solution (Sigma-Aldrich, 5 mg/ml in PBS) was added to each well and cells were incubated for 4 hr. The plates were centrifuged and supernatants were removed. Intracellular formazan crystals were dissolved in 150 μl dimethyl sulfoxide (DMSO). The absorbance was measured at 570 nm with a microplate reader (Bio-Rad, USA). The percentage of cell viability was calculated as follows: Cell viability (%)= (OD of treatment/OD of control) ×100. The half-maximal inhibitory concentration (IC50) was obtained from the dose-response curve using original 6.0 software.

### *Cell cycle and apoptosis assay*

Briefly, cells (1×105 cells/ml) were treated with juglone (0, 12.5, 25, 50, or 100 μM) for 24 hr and then washed with PBS. After centrifugation, cells were fixed with 70% ethanol. Later, fixed cells were washed with PBS twice, and 50 μl RNAse and 25 μl propidium iodide (PI) were added to the cell suspension and incubated for 15 min. DNA content analysis was performed using Muse flow cytometry (Millipore, Billerica, MA, USA). SKOV3 cell apoptosis was analyzed using an AnnexinV-FITC/PI apoptosis detection kit (KeyGen Biotech Co. Ltd. Najing, China). The samples were stained with 5 μl Annexin V-FITC and 5 μl propidium iodide (PI) in the dark for 15 min at room temperature. Finally, samples were analyzed by Muse flow cytometry (Millipore, Billerica, MA, USA).

### *Cell invasion assay*

The Transwell chambers with 8 μm pore size filters (Corning Incorporated, Corning, NY, USA) were coated with Matrigel (BD Biosciences, San Jose, CA, USA). Cells that were treated with or without juglone (12.5, 25, 50, or 100 μM) for 24 hr were suspended in 0.1 % FBS. They were seeded in the upper chamber in 200 μl IMDM medium, and 600 μl IMDM medium containing 10 % FBS was added to the bottom chamber. Following incubation, the remaining cells in the upper chamber were gently removed with a cotton swab. Cells that migrated to the lower side of the inserts were stained with 0.1 % crystal violet and observed under light microscopy (Olympus, Tokyo, Japan). Then, crystal violet was completely dissolved in 33% acetic acid. The absorbance was detected at 570 nm by microplate reader (Bio-Rad, USA).

### *Western blot analysis*

SKOV3 cells were stimulated as previously described. Cells were lysed with RIPA lysis buffer (Sigma-Aldrich). Protein concentration was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology, China). Equal amounts of protein (30 μg) were separated by 10 % SDS-PAGE and transferred to PVDF membranes (Millipore, Billerica, MA, USA). Then, the membrane was incubated with primary antibodies against cyclin D1, MMP-2, cytochrome C (Cyt c) Bcl-2, Bax, pro-caspase-9, and activated caspase-3 (1:1000; Epitomics, Burlingame, CA, USA). β-actin, as a loading control (1:1000; Epitomics, Burlingame, CA, USA) was used overnight at 4°C. Primary antibody binding was detected with anti-rabbit IgG conjugated to HRP and visualized by an enhanced chemiluminescence (ECL) detection system (Pierce, Rockford, IL, USA).

### *Statistical analysis*

Results are expressed as mean±SD for three independent experiments. Statistical differences were evaluated using Student's test or one-way analysis of variance (ANOVA). The LSD method of multiple comparisons was used when the probability for ANOVA was statistically significant. Values were considered statistically significant at an alpha value of $P<0.05$. All statistical analyses were performed with the SPSS 13.0 software.

## Results

### *Effect of juglone on proliferation of human ovarian cancer SKOV3 cells*

We treated SKOV3 cells with increasing doses of juglone for 24 hr and examined cell growth using MTT assays. As shown in Figure 1, juglone treatment markedly inhibited SKOV3 cell survival in a dose-dependent manner. IC50 values were calculated as 30.13 μM at 24 hr.

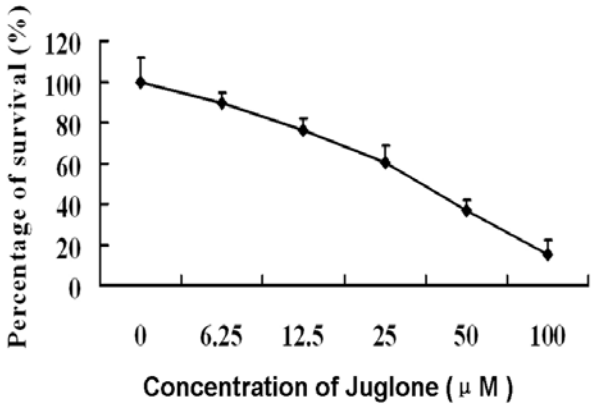


**Figure 1.** Juglone's effect on human ovarian cancer SKOV3 cell proliferation. SKOV3 cells were seeded in 96-well plates and incubated with different juglone concentrations for 24 hr. Cell viability was determined by MTT assay. Values are means±SE of three experiments

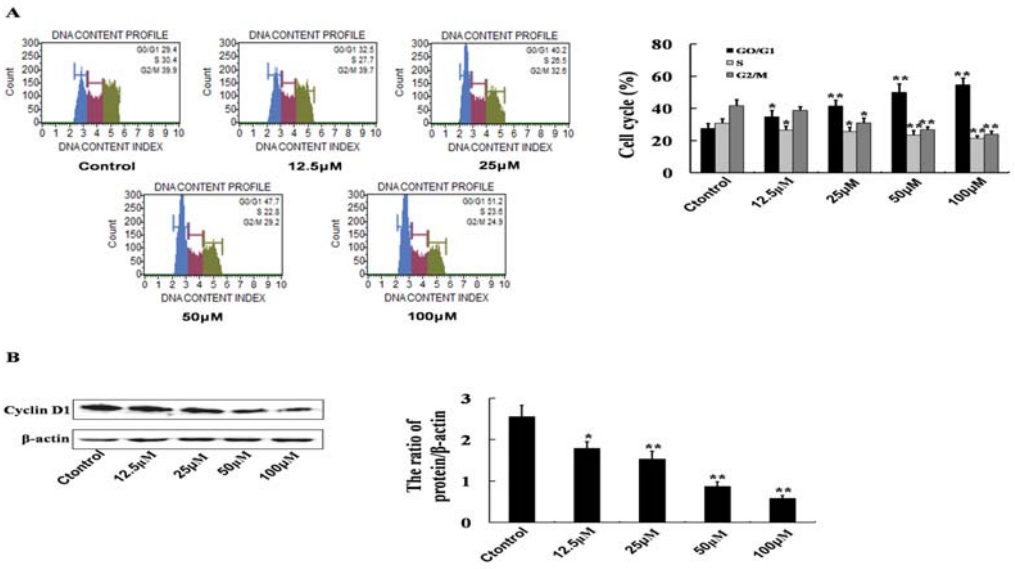


**Figure 2.** Analysis of the SKOV3 cells cycle after juglone treatment. A: The DNA content was measured by flow cytometry using PI staining. B: Western blot analysis was performed with antibodies against cyclin D1. β-actin was used as an internal reference. **P*<0.05, ***P*<0.01 compared to control

### *Juglone induce cell G0/G1 phase arrest in SKOV3 cells*

Cells were treated with juglone for 24 hr and cell cycle was studied using flow cytometry following PI staining. The results showed that juglone increased G0/G1 phase and decreased S and G2/M phase in a dose-dependent manner (Figure 2A). Juglone treatment resulted in a decrease in cyclin D1 protein levels in a concentration-dependent manner (Figure 2B).

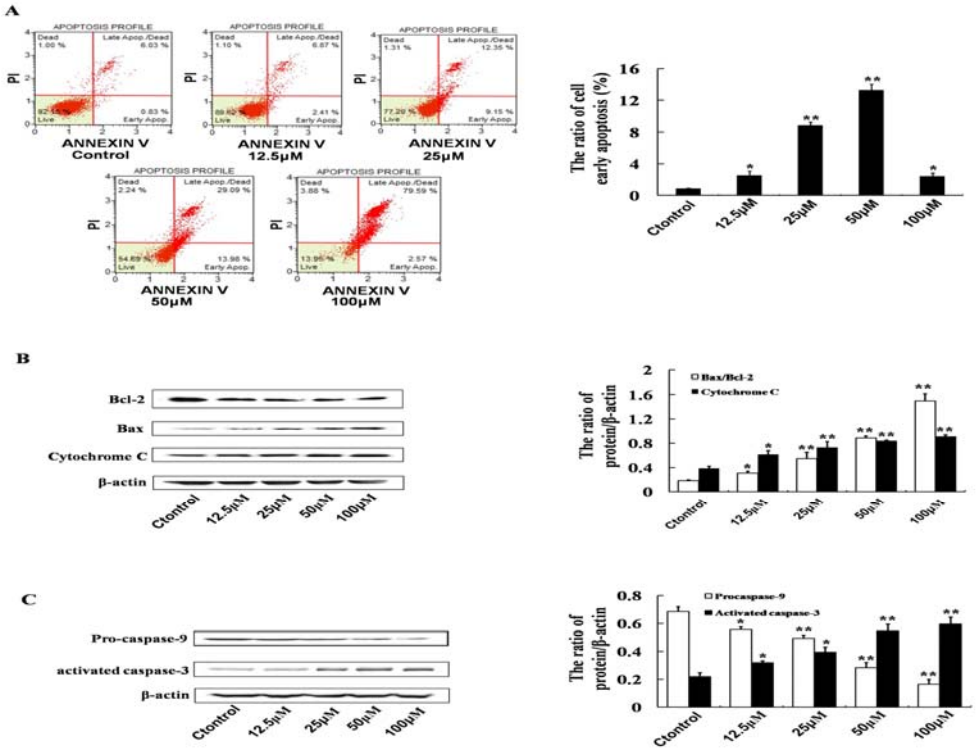


**Figure 3.** Analysis of juglone-treated SKOV3 cells apoptosis. A: Cells were treated with juglone for 24 hr, then stained with Annexin V-FITC and PI in the dark, and analyzed using flow cytometry. B and C: Western blot analysis was performed with antibodies against Cyt c, Bcl-2, Bax, procaspase-9, and activated caspase-3 in juglone-treated SKOV3 cells. β-actin was used as an internal reference. **P* <0.05, ***P*<0.01 compared to control

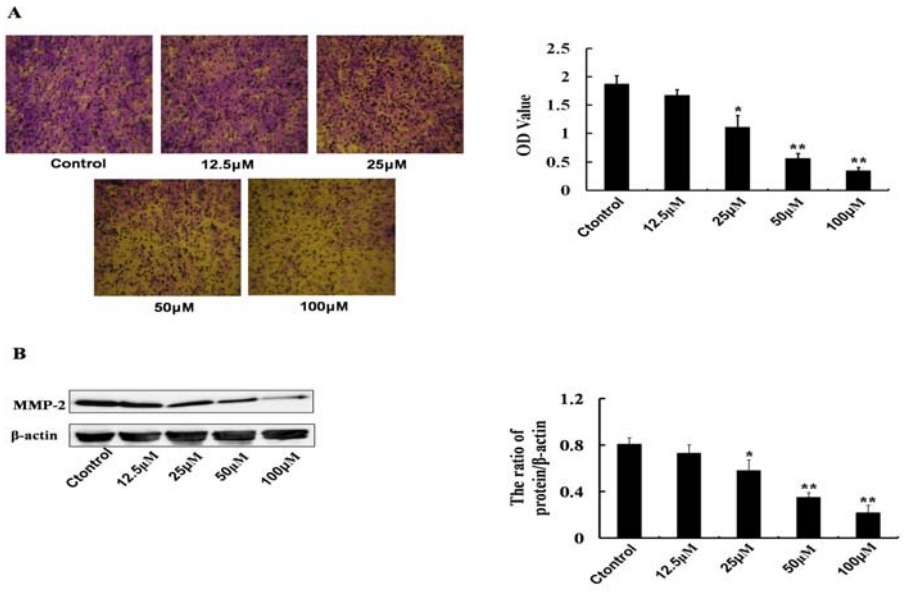


**Figure 4.** Analysis of juglone-treated SKOV3 cell invasion. A: The Transwell chambers and Matrigel were used to evaluate cell invasion. Cells were treated with juglone for 24 hr and those that migrated to the lower side of the inserts were stained with 0.1% crystal violet and observed under light microscopy. Then, crystal violet was completely dissolved in 33 % acetic acid, and the absorbance was detected at 570 nm. B: Western blot analysis was performed with antibodies against MMP-2 in juglone-treated SKOV3 cells. β-actin was used as an internal reference. **P*< 0.05, ***P*< 0.01 compared to control

### *Juglone induced cell apoptosis in SKOV3 cells*

To quantitate apoptosis, we performed Annexin V-FITC/PI double staining. The results showed significant early and late stage apoptotic cell accumulation in juglone-treated cells in dose-dependent manners (Figure 3A). Apoptosis is always accompanied by mitochondrial membrane disruption that leads to Cyt c release from the mitochondria into the cytoplasm (7). As shown in Figure 3B, we found that Cyt c protein increased following incubation with juglone. Bcl-2 and Bax belong to a multi-gene protein family that plays an important role in regulating apoptosis. Bcl-2 is an anti-apoptotic protein, whereas Bax is a pro-apoptotic protein and antagonizes Bcl-2 effects (7). As shown in Figure 3B, anti-apoptotic Bcl-2 protein levels declined and pro-apoptotic Bax protein levels increased in response to juglone in a dose-dependent manner. Thus, the Bax/Bcl-2 ratio increased. Caspase, a family of cysteine proteases, forms integral parts of the apoptosis pathway. Therefore, we detected caspase-9 and caspase-3 protein levels.
As shown in Figure 3C, juglone treatment led to a dose-dependent decrease of precursor procaspase-9 while increased activated caspase-3.

### *Juglone inhibited cell invasion in SKOV3 cells*

To investigate juglone effect on SKOV3 cell motility, Matrigel invasion assay was employed to characterize juglone's role in cell invasion. Results showed that the number of juglone-treated cells (0, 25, 50, or 100 μM) that invaded through the Matrigel-coated membrane was also markedly less than the number of non-treated cells (Figure 4A). Thus, juglone inhibited cell invasion. MMP-2 plays a critical role in tumor cell invasion, so we examined juglone's effect on MMP-2 expression by Western blot. Results showed that juglone markedly reduced MMP-2 protein levels in a concentration-dependent manner compared with the control group (Figure 4B).

## Discussion

Recent data indicate that juglone may be a useful anti-proliferation agent for some cancer types (4-6). However, there is little evidence for its anti-proliferative effect on ovarian cancer SKOV3 cells. Furthermore, its effect on invasion has not been investigated in SKOV3 cells. Thus, in the initial in vitro study, we measured juglone inhibitory effect on the growth of SKOV3 cells. In the current study, we demonstrated that juglone inhibitory action correlated with cell cycle arrest and apoptosis induction in SKOV3 cells. We also revealed that juglone could inhibit cell invasion.

Here, we found that juglone decreased cells viability in a dose-dependent manner. To mechanistically explain our observation, we investigated juglone effects on cell cycle and apoptosis. We found that juglone prevented cell progression from G1 into S phase and blocked cell proliferation. To better understand the mechanism underlying G0/G1 arrest in juglone-treated cells, we examined cyclin D1 protein levels, which is a proto-oncogenic cell cycle determinant of the G1/S check-point (8). Our results showed that juglone down-

regulated cyclin D1 protein expression. Furthermore, our results demonstrated that juglone markedly induced apoptosis in SKOV3 cells. Apoptosis can be activated through two main pathways: the extrinsic dependent pathway and the intrinsic mitochondria-dependent pathway (9). Recent studies showed that juglone induces cell apoptosis through the mitochondria-dependent pathway in human lung cancer (A549) cells, human leukemia (HL-60) cells, and human cervical carcinoma (HeLa) cells (4-6). Our results disclosed that Juglone-induced apoptosis parallels a significant Cyt c up-regulation and an increase in Bax/Bcl-2 ratio. The procaspase-9 level was gradually reduced and caspase-3 activity was elevated in a dose-dependent manner in SKOV3 cells treated with juglone. When Bax/Bcl-2 ratio increases, the mitochondrial permeability transition pore opens which results in the efflux of pro-apoptotic proteins like Cyt c and caspase-9 (10). Perhaps juglone can activate the mitochondrial pathway to induce apoptosis in SKOV3 cells.

Metastasis is widely associated with poor prognosis and is the most common reason for therapy failure. Most ovarian cancer cases are highly invasive and metastatic, ultimately leading to the demise of the patient (11). In the present study, we used Transwell/Matrigel invasion assays to demonstrate that juglone inhibitory effect on ovarian cancer SKOV3 cell invasion. The extracellular matrix (ECM) is a barrier to prevent cellular invasion and metastasis, and its loss allows cancer cells to invade the blood or lymphatic system and spread to other tissues and organs. Matrix metalloproteinases (MMPs), specifically MMP-2, have been investigated with regard to tumor invasion and its critical roles in type IV collagen degradation, a major ECM constituent (12). Our results showed that juglone inhibited SKOV3 cell invasion with decreased MMP-2 expression, suggesting that juglone regulates MMP2 expression and inhibits SKOV3 cell invasion.

## Conclusion

In summary, the current study shows that juglone may have important antitumor effects in SKOV3 cells. Juglone led to cell cycle arrest and apoptosis in SKOV3 cells. Moreover, juglone restrained SKOV3 cell invasiveness. Therefore, it is reasonable to believe that juglone could be considered as a potential candidate for anti-cancer therapy.

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## Conflict of interests

All authors confirm that they have no conflict of interests.

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