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ORIGINAL ARTICLE

Anticancer effects of juglone in OVCAR-3 human ovarian carcinoma are facilitated through programmed cell death, endogenous ROS production, inhibition of cell migration and invasion and cell cycle arrest

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Summary

Purpose: *Accumulating evidence suggests that Juglone is a potent anticancer molecule of plant origin. However, its anticancer effects have not been fully explored against human ovarian cancer cells. Therefore this study was undertaken to evaluate the anticancer effects of Juglone against the human OVCAR-3 ovarian cancer cells.*

Methods: *Cell viability was evaluated by WST-1 assay. Cellular apoptosis was studied using fluorescence microscopy with DAPI staining. The percentage of OVCAR-3 human ovarian cancer cells was examined by using flow cytometry in combination with annexin V-FITC/propidium iodide (PI) staining. Effects on cell cycle were studied by flow cytometer while effects on cell migration and invasion were evaluated using wound healing and transwell assay, respectively.*

Results: *Juglone inhibited the growth rate of OVCAR-3 ovarian cancer cells and showed an IC_{50} of 30 μM. However, Juglone showed very high IC_{50} (100 μM) against the normal SV40 ovarian cells. DAPI staining showed that Juglone caused nuclear fragmentation of the OVCAR-3 cells, suggestive of apoptosis. Annexin V/PI staining showed that the percentage of the apoptotic OVCAR-3 cells increased from 2.15 in control to 45.24% at 60 μM concentration of Juglone. The induction of apoptosis in the OVCAR-3 cells was also accompanied with activation caspase-3, upregulation of Bax and downregulation of Bcl-2. Juglone was also found to cause an upsurge in the ROS levels in the OVCAR-3 cells. Cell cycle analysis showed that Juglone caused accumulation of the OVCAR-3 cells in the G2/M phase of the cell cycle triggering G2/M cell cycle arrest. Wound healing assay and transwell assay showed that Juglone suppressed the migration as well as the invasion of the OVCAR-3 cells, suggestive of the antimetastatic potential of this molecule.*

Conclusions: *Juglone may prove advantageous in ovarian cancer treatment.*

Key words: *ovarian cancer, juglone, apoptosis, cell migration, cell cycle arrest*

Introduction

Ovarian cancer is one of the common gynaecological malignancies across the globe, it accounts for 2.5% of all malignancies in women and is responsible for 5% of all cancer-related deaths in women [1]. Although the incidence of ovarian cancer has declined over the last few decades, the clinical outcome is still far from satisfactory [2]. It has been reported that in United states alone approximately more than 22,000 new ovarian cancer cases and 14,000 ovarian cancer deaths are recorded annually [3]. The late diagnosis and lack of therapeutic targets pose hurdles in the treatment of this disease. Improvement of prevention through early detection and identification of therapeutic

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targets may prove beneficial to overcome ovarian cancer-related mortality [4].

Plants are highly sophisticated natural chemical factories and exhibit a remarkable potential to synthesize wide arrays of chemical entities [5]. This study was undertaken to examine the anticancer effects of Juglone against human ovarian cancer cells and also to ascertain its molecular mechanisms. Juglone is a naphthoquinone generally derived from walnut and has shown enormous pharmacological potential [6]. This molecule causes significant decrease in the growth of cervical carcinoma [7]. The growth of gastric cancer cells has also been reported to be suppressed by Juglone via induction of apoptotic cell death [8]. Another study has shown that the viability of the ovarian cancer cells is decreased by Juglone [9]. Moreover, Juglone has been shown to enhance the radiation sensitivity of cancer cells [10]. However, the anticancer effects of this molecule against the OVCAR-3 ovarian cancer cells have not been thoroughly explored.

The purpose of this study was to investigate the anticancer effects of juglone molecule in OVCAR-3 human ovarian cancer cells on apoptosis, endogenous ROS production, cell migration and invasion and cell cycle phase distribution.

Methods

Cell viability and colony formation assay

The viability of OVCAR-3 cancer cells and SV40 transfected normal ovarian surface epithelial cells was monitored by WST-1 assay. In brief, OVCAR-3 cells were cultured in 96-well plates at a density of 2×10^5 cells/well and treated with 0 to 200 µM concentrations of Juglone for 24 h at 37°C. This was followed by incubation of the cells with WST-1 at 37°C for another 4 h. The absorbance was then measured at 450 nm using a victor 3 microplate reader to determine the proliferation.

Apoptosis assay and ROS determination

The OVCAR-3 cells (0.6×10^6) were cultured in 6-well plates and treated with Juglone at of 0, 9, 18 and 36 µM concentrations for 24h at 37°C. Subsequently, 25 µl of cell culture were put onto glass slides and stained with DAPI. The slides were then cover-slipped and examined under fluorescence microscope. ApoScan kit was used to determine the apoptotic OVCAR-3 cell percentage. In brief, Juglone-treated OVCAR-3 cells (5×10^5 cells/well) were incubated for 24 h. This was followed by staining of these cells with annexin V-FITC/PI. The percentage of apoptotic OVCAR-3 cells at each concentration was then determined by flow cytometry. The ROS levels were determined as described previously [12].

Cell cycle analysis

The cultured OVCAR-3 cells were firstly treated with varied concentrations of Juglone for 24h at 37°C. The cells were then washed with phosphate buffered saline (PBS) and afterwards the OVCAR-3 cells were stained with Annexin V/PI and the distribution of the cells in the cell cycle phases was assessed by FACS flow cytometer.

Wound healing assay

The Juglone-treated cells were cultured till 80% confluence. This was followed by the removal of RPMI-1640 medium and subsequent washing with PBS. Afterwards, a wound was scratched with a sterile pipette tip and a picture was taken. The plates were then incubated for about 24 h at 37°C and then a picture was taken again under an inverted microscope.

Cell invasion assay

The effects of Juglone on the invasion ability of OVCAR-3 cells were determined by transwell chamber assay with Matrigel. Around 200 ml cell culture were placed onto the upper chamber and only RPMI-1640 medium was placed in the bottom chamber. After 24 h of incubation, the cells were removed from the upper chamber and the cells that invaded via the chamber were fixed with methyl alcohol and subsequently stained with crystal violet. Inverted microscope was used to count the number of invaded cells at 200x magnification.

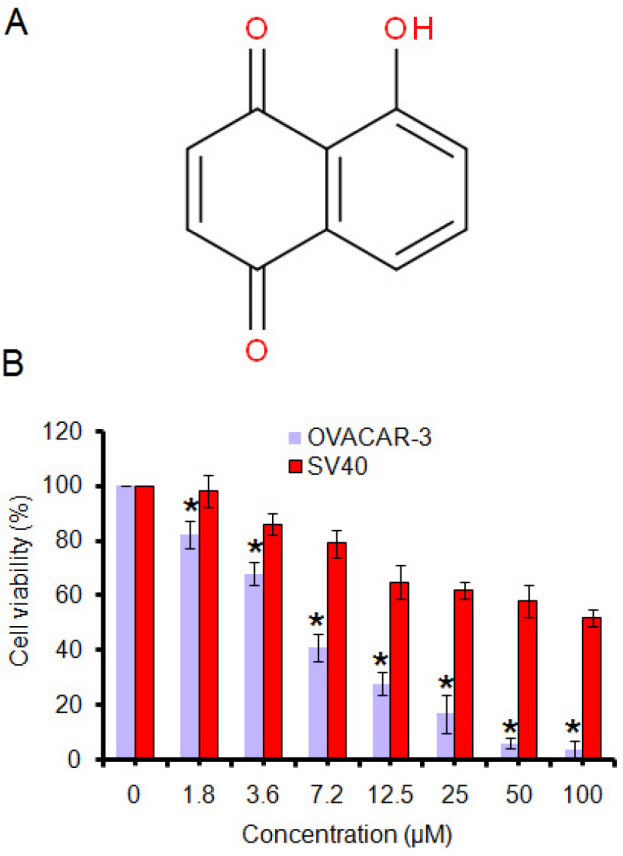

Figure 1. A: Chemical structure of Juglone. **B:** Effect of Juglone on the viability of the OVCAR-3 and SV40 cells. The experiments were performed in triplicate and expressed as mean ± SD (*p<0.05).

Western blot analysis

The OVCAR-3 cells were lysed in RIPA lysis buffer containing the protease inhibitor. Around 45 µg of proteins from each sample were separated through electrophoresis via SDS-PAGE gels which was followed by transferring the proteins to polyvinylidene difluoride (PVDF) membrane. Next, fat-free milk was used to block the membrane at room temperature for 1 h. Thereafter, the membranes were treated with primary antibodies at 4°C overnight. Subsequently, the membranes were incubated with secondary antibodies. Finally, the band signals were detected by Odyssey Infrared Imaging System. Actin was used as control for normalization.

Statistics

The experiments were done in triplicate and the values presented as mean±SD. *p<0.05, **p<0.01 and ***p<0.001 were considered statistically significant. Student's t-test using GraphPad prism 7 software was employed for statistical analysis.

Results

Juglone inhibits the growth of ovarian cancer cells

The proliferation rate of the OVCAR-3 and the normal SV40 cells following treatment with various concentrations of Juglone were determined by WST-1 assay (Figure 1A). The results revealed that Juglone caused a significant decrease in the proliferation rate of the OVCAR-3 cells, which was concentration-dependent with an IC_{50} of 30µM (Figure 1B). Interestingly, the effects of Juglone on the normal SV40 cells were less and an IC_{50} of 100 µM was recorded for Juglone against these normal cells (Figure 1B).

Induction of both apoptosis by Juglone in OVCAR-3 ovarian cancer cells

OVCAR-3 cells were treated with different doses of Juglone and then stained with DAPI to ascertain if juglone causes apoptosis in the OVCAR-3 cells. The results of DAPI staining showed that Juglone caused nuclear fragmentation of the OVCAR-3 cells, characteristic of apoptosis (Figure 2). Annexin V/PI

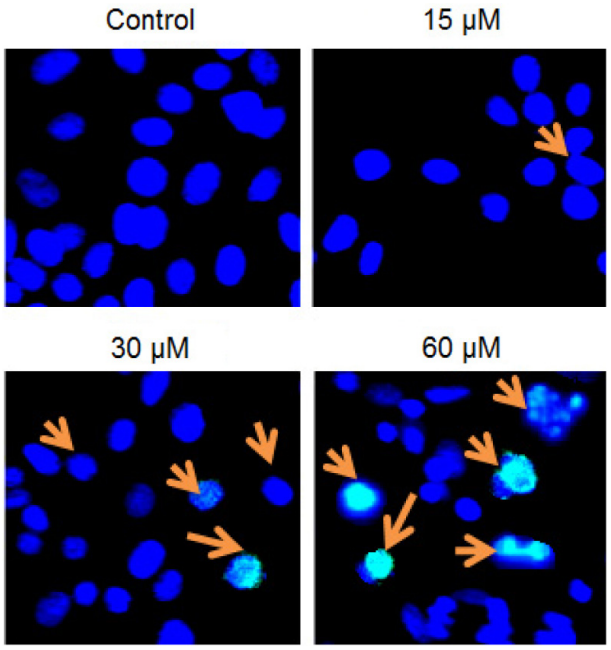

Figure 2. DAPI staining showing that Juglone induces apoptosis in OVCAR-3 cells in a concentration-dependent manner. Arrows indicate the apoptotic cells which increased with increasing dosage of the molecule. The experiments were performed in triplicate.

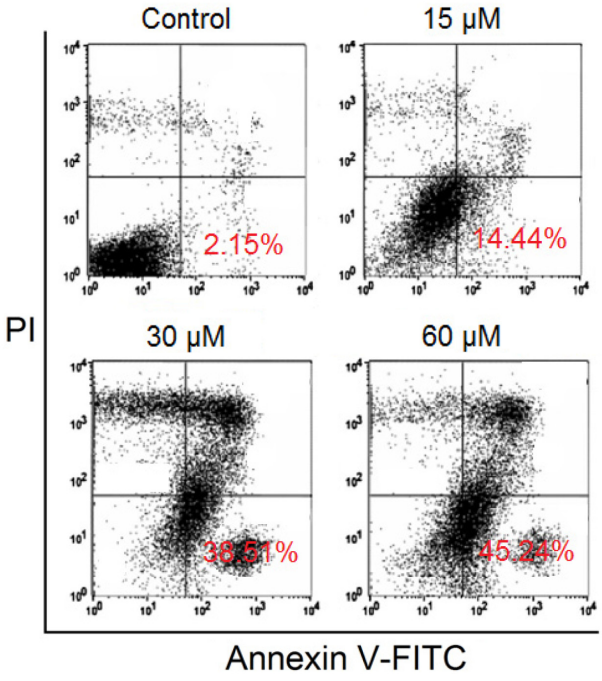

Figure 3. Effect of Juglone on the percentage of the apoptotic OVCAR-3 cells as depicted by annexin V/PI staining. The experiments were performed in triplicate.

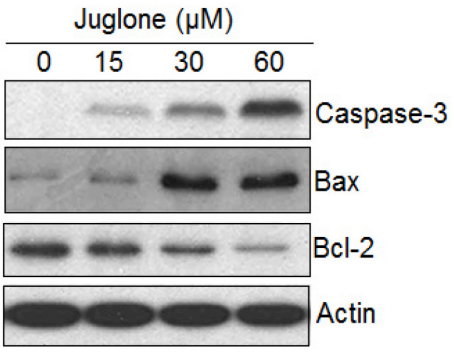

Figure 4. Western blot analysis showing the effects of indicated concentrations of Juglone on the expression of apoptosis-related proteins. The ROS levels increased with increasing dosage of the molecule. The experiments were performed in triplicate and expressed as mean±SD.

staining was also carried out and the apoptotic OVCAR-3 cell percentage was determined at different concentrations of the molecule. The apoptotic cell percentage was 2.15, 14.44, 38.51.53 and 45.24% at Juglone concentrations of 0, 15, 30 and 60 μM (Figure 3). Western blot analysis showed that Juglone caused increase of Bax and caspase-3 and 9 and decrease of Bcl-2 expression. Moreover, Juglone also promoted their cleavage (Figure 4).

Juglone causes increase of ROS in OVCAR-3 cells

The effects of Juglone were also examined on the ROS levels in the OVCAR-3 cells at 0, 15, 30 and 60 μM concentrations. The results showed that the molecule caused significant increase in the ROS levels of OVCAR-3 cells. The ROS levels increased to around 195% at 60 μM concentration relative to control.

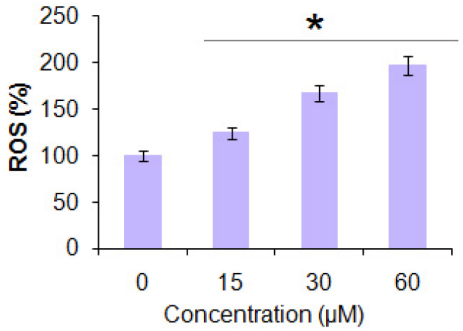

Figure 5. Effect of Juglone on the ROS levels in OVCAR-3 cells as presented by flow cytometry. The ROS levels increased with increasing dosage of the molecule. The experiments were performed in triplicate and expressed as mean±SD (*p<0.05).

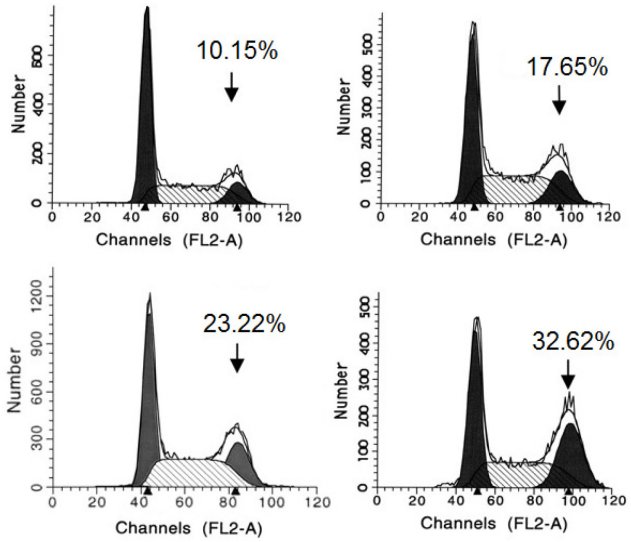

Figure 6. Effect of Juglone on the cell cycle distribution of the OVCAR-3 cells as presented by flow cytometry. The percentage of G2/GM phase cells with increased dosage of the molecule. The experiments were performed in triplicate.

Juglone causes G2/M cell cycle arrest of ovarian cancer cells

The OVCAR-3 ovarian cancer cells were treated with various concentrations of Juglone and the distribution of OVCAR-3 cells at each phase of cell cycle was determined by flow cytometry. The results showed that the G2/M phase cells increased considerably upon Juglone treatment. The percentage of G0/G1 phase cells were 10.15, 17.65, 23.22 and 32.62% at 0, 15, 30 and 60 μM concentrations of Juglone respectively, suggestive of G2/M arrest of the OVCAR-3 cells (Figure 6).

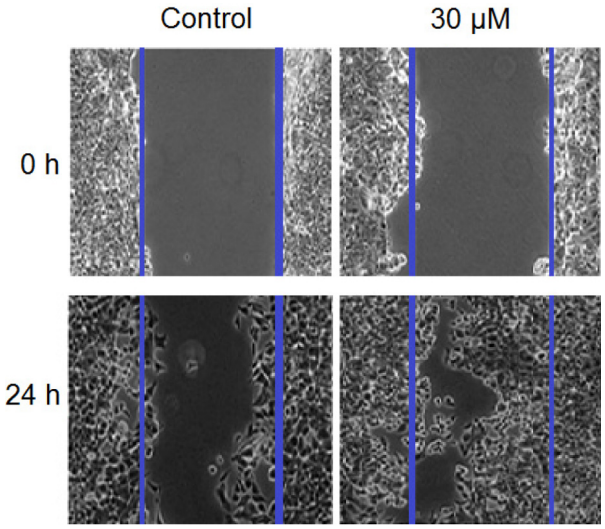

Figure 7. Effect of Juglone on the migration of the OVCAR-3 cells as presented by wound healing assay. Juglone treatment (30 μM) led to significant decrease in cell migration. The experiments were performed in triplicate.

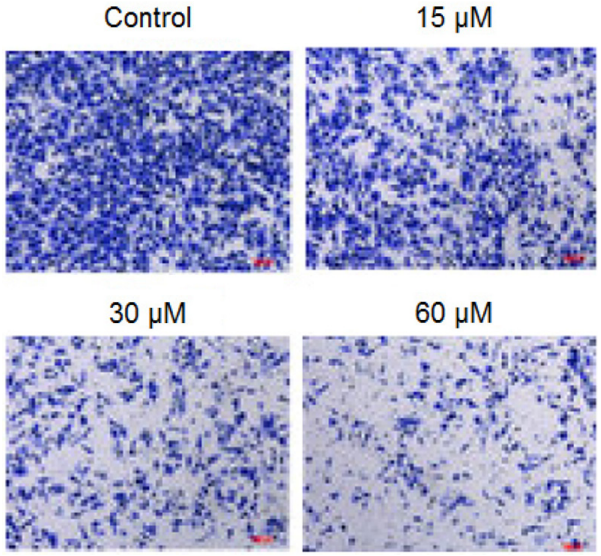

Figure 8. Effect of Juglone on the invasion of the OVCAR-3 cells as illustrated by transwell assay. Juglone treatment led to significant decrease in cell invasion. The experiments were performed in triplicate.

Juglone inhibits the migration and invasion of the ovarian cancer cells

The effects of Juglone were also investigated on the invasion and migration of the OVCAR-3 ovarian cancer cells by transwell and wound healing assay. The results showed that Juglone caused remarkable decrease in the migration of the OVCAR-3 cells in a concentration-dependent manner, as evidenced from the wound width (Figure 7). Moreover, transwell assay showed that the invasion of the OVCAR-3 cells was also decreased in a concentration-dependent manner (Figure 8).

Discussion

With advancements in science, there has been some improvements in overall survival rate of ovarian cancer but this disease still causes a significant number of deaths world over [13].

The treatment of ovarian cancer is mainly limited by the lack of newer chemotherapeutic agents and biomarkers for early detection [2,3]. Herein we investigated the anticancer effects of Juglone on ovarian cancer cells. The results showed that Juglone suppresses the proliferation rate of the human ovarian cancer cells. However, the anticancer effects of Juglone were significantly lower against the normal ovarian cells, suggesting that Juglone selectively targets cancer cells. These results are well supported by previous studies wherein Juglone has been shown to inhibit the growth of melanoma cells [14]. Similarly, in glioblastoma cells. Juglone suppressed the growth of cells by promoting apoptotic cell death [15]. Against this background, we sought to decipher the molecular mechanisms responsible for the anticancer effects of Juglone. Consistently, DAPI staining analysis of the Juglone-treated OVCAR-3 cells was performed and the results showed that the molecule caused growth inhibition of the OVCAR-3 cells via induction of apoptosis which was also accompanied with upregulation of caspase-3 and Bax and downregulation of Bcl-2 expression. These observations are in concordance with previous investigations wherein Juglone has been reported to induce apoptosis in human leukemia cells [16].

ROS has been shown to be involved in the promotion of the apoptotic cell death [12]. Herein we also examined if Juglone causes increase of ROS in ovarian cancer cells and the results showed that the molecule caused significant increase in the ROS levels of OVCAR-3 cells. These results are consistent with previous studies wherein in Juglone has been reported to prompt ROS-mediated apoptosis in human leukemia cells [16].

Cell cycle arrest is another mechanism by which anticancer agents exert their effects on the cancer cells [17]. Herein we observed that Juglone inhibits the growth of the OVCAR-3 cells by also inducing G2/M cell cycle arrest. Juglone has been reported to inhibit the metastasis of the human pancreatic cancer cells [18]. Therefore, we also examined the anti-metastatic potential of Juglone on the OVCAR-3 ovarian cancer cells and found that the molecule inhibited the migration and invasion of the ovarian cancer cells, indicative of the potential of Juglone as anticancer agent.

Conclusion

The results of the current study revealed that Juglone inhibited the growth of the human ovarian cancer cells via induction of apoptosis and cell cycle arrest. Juglone also inhibited the migration and invasion of the human ovarian cancer cells, indicative of the potential of Juglone as lead molecule for ovarian cancer treatment.

Conflict of interests

The authors declare no conflict of interests.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: Cancer J Clin 2018;68:394-424.
2. Henderson JT, Webber EM, Sawaya GF. Screening for ovarian cancer: updated evidence report and systematic review for the US Preventive Services Task Force. JAMA 2018;319:595-606.
3. Mathieu KB, Bedi DG, Thrower SL, Qayyum A, Bast Jr RC. Screening for ovarian cancer: imaging challenges and opportunities for improvement. Ultrasound Obstetr Gynecol 2018 Mar;51:293-303.
4. La Vecchia C. Ovarian cancer: epidemiology and risk factors. Eur J Cancer Prevent 2017;26:55-62.
5. Torre LA, Trabert B, DeSantis CE et al. Ovarian cancer statistics, 2018. CA: Cancer J Clin 2018 Jul;68:284-96.
6. Auyong TK, Westfall BA, Russell RL. Pharmacological aspects of juglone. Toxicon 1963;1:235-9.
7. Zhang W, Liu A, Li Y et al. Anticancer activity and

mechanism of juglone on human cervical carcinoma HeLa cells. Can J Physiol Pharmacol 2012;90:1553-8.

8. Ji YB, Qu ZY, Zou X. Juglone-induced apoptosis in human gastric cancer SGC-7901 cells via the mitochondrial pathway. Experim Toxicol Pathol 2011 Jan 1;63:69-78.
9. Fang F, Qin Y, Qi L et al. Juglone exerts antitumor effect in ovarian cancer cells. Iran J Basic Medical Sci 2015;18:544.
10. Aithal KB, Kumar S, Rao BN, Udupa N, Rao SB. Tumor growth inhibitory effect of juglone and its radiation sensitizing potential: in vivo and in vitro studies. Integr Cancer Ther 2012;11:68-80.
11. Hazan RB, Phillips GR, Qiao RF, Norton L, Aaronson SA. Exogenous expression of N-cadherin in breast cancer cells induces cell migration, invasion, and metastasis. J Cell Biol 2000;148:779-90.
12. Hua F, Li CH, Chen XG, Liu XP. Daidzein exerts anticancer activity towards SKOV3 human ovarian cancer cells by inducing apoptosis and cell cycle arrest, and inhibiting the Raf/MEK/ERK cascade. Int J Molec Med 2018;41:3485-92.
13. Petricoin III EF, Ardekani AM, Hitt BA et al. Use of proteomic patterns in serum to identify ovarian cancer. Lancet 2002;359:572-7.
14. Aithal KB, Kumar SM, Rao NB, Udupa N, Rao SB. Juglone, a naphthoquinone from walnut, exerts cytotoxic and genotoxic effects against cultured melanoma tumor cells. Cell Biol Int 2009;33:1039-49.
15. Wu J, Zhang H, Xu Y et al. Juglone induces apoptosis of tumor stem-like cells through ROS-p38 pathway in glioblastoma. BMC Neurol 2017;17:70.
16. Xu HL, Yu XF, Qu SC, Qu XR, Jiang YF. Juglone, from Juglans mandshruica Maxim, inhibits growth and induces apoptosis in human leukemia cell HL-60 through a reactive oxygen species-dependent mechanism. Food Chem Toxicol 2012;50:590-6.
17. Seshadri P, Rajaram A, Rajaram R. Plumbagin and juglone induce caspase-3-dependent apoptosis involving the mitochondria through ROS generation in human peripheral blood lymphocytes. Free Radical Biol Medic 2011;51:2090-107.
18. Avci E, Arikoğlu H, Kaya DE. Investigation of juglone effects on metastasis and angiogenesis in pancreatic cancer cells. Gene 2016;588:74-8.