JBUON 2018; 23(1): 111-116
ISSN: 1107-0625, online ISSN: 2241-6293 • www.jbuon.com
E-mail: editorial_office@jbuon.com

ORIGINAL ARTICLE

Swerchirin exerts anticancer activity on SKOV3 human ovarian cancer cells via induction of mitochondrial apoptosis, G2/M cell cycle arrest and inhibition of Raf/MEK/ERK cascade

Lan Luo[1], Haibo Zhao[2], Qian Luo[3]

[1]Department of Gynecology, [2]Department of Oncology, [3]Department of Radiology, Jining No.1 People's Hospital, Jining, Shandong 272100, China

Summary

Purpose: *Ovarian cancer is one of the major causes of death in females around the world. There are several drug regimens available for this type of cancer, but despite initial response to chemotherapy, the disease consistently relapses, showing that there is a need to find more efficient and novel anticancer agents. Plant-derived natural products may prove beneficial in this case due to their lower cytotoxicity. In the present study we evaluated the anticancer activity of swerchirin against human SKOV3 ovarian cancer cell line.*

Methods: *Cell viability was evaluated by MTT colorimetric assay. Effects on the cell cycle phase distribution as well as mitochondrial membrane potential (MMP) were evaluated by flow cytometry. Effects on cell apoptosis were evaluated by annexin-V/propidium iodide (PI) staining assay while effects on apoptosis related protein expressions were evaluated by Western blot assay.*

Results: *The results indicated that swerchirin reduced the cell viability in a dose-dependent manner and exhibited an IC_{50} of 20 μM at 48 hrs of incubation. The anticancer activity of swerchirin was found to be due mitochondrial apoptosis and G2/M cell cycle arrest. This anticancer activity of swerchirin against SKOV3 was found to be concentration-dependent and increased with increase in the concentration of swerchirin. Additionally, swerchirin inhibited the expression of phospho-MEK and phospho-ERK in a dose-dependent manner.*

Conclusion: *In summary, we conclude that swerchirin significantly increased SKOV3 cell death due to cell cycle arrest, loss of MMP, apoptosis and inhibition of Raf/MEK/ERK pathway.*

Key words: *ERK pathway, ovarian cancer, protein expression, swerchirin*

Introduction

Ovarian cancer is one of the most deadly malignancies across the globe, while chemotherapy still remains the backbone for its management [1,2]. According to an estimate, more than 20,000 new cases of ovarian cancer and 14,000 new deaths have been recorded in United States only in 2013 [2]. Although surgical interventions may prove useful in the treatment of ovarian cancer, most of the cases are diagnosed at advanced disease stages. Additionally, despite frequent preliminary responses to chemotherapy, the disease often relapses. Moreover, there are limited chemotherapeutic agents available for the management of ovarian cancer [3,4]. So far, bevacizumab is the only approved therapy in ovarian cancer for which consistent analytical markers are yet to be established. Xanthones are secondary metabolites commonly occurring in higher plant families, fungi, and lichens. Their pharmacological properties have raised great interest. Structures of xanthones

Correspondence to: Qian Luo, MD. Department of Radiology, Jining No.1 People's Hospital, No.6 Jiankang Rd, Jining, Shandong 272100, China.
Tel/Fax: +86 0537 6056666, E-mail: qianluo772@gmail.com
Received: 21/08/2017; Accepted: 07/09/2017

are related to that of flavonoids and their chromatographic behaviors are also similar [5]. Xanthones have reported to exhibit anticancer activities against a range of cancers [6]. Swerchirin is an important xanthone generally isolated from the plants of Gentianaceae family. Plants belonging to this family are well known for their bitter taste owing to the presence of xanthones and are used in traditional remedies against fever, loss of appetite and are still used in many "tonic" formulations [7]. The present study was designed to evaluate the anticancer properties of swerchirin against the ovarian cancer cell line SKOV3.

Methods

Chemicals, reagents, cell line and culture conditions

Swerchirin (purity ≥98%), annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit, and MTT were procured from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), penicillin, streptomycin and phosphate buffered saline (PBS), supplemented with calcium chloride and magnesium chloride, were procured from Gibco Life Technologies, Grand Island, NY, USA. All other chemicals and solvents used were of the highest purity grade. Ovarian cancer cell line cell SKOV3 was procured from Cancer Research Institute of Beijing, China, and it was maintained in DMEM supplemented with 10% FBS and antibiotics (100 µg/ml streptomycin and 100 U/ml penicillin G) in a incubator at 37°C (5% CO_2 and 95% air).

MTT assay for assessment of cell viability

The anti-proliferation effect of swerchirin on ovarian cancer SKOV3 cells was demonstrated by MTT ([3-(4, 5-Dimethylthiazole-2-yl)-2, 5-duphenyltetrazolium bromide]) assay. SKOV3 cells were grown at 1×10^6 cells per well in 96-well plates for 12 hrs and then exposed to doubling dilutions starting from 2.5 to 40 µM swerchirin dose for 24 and 48 hrs. To each well, MTT solution (20 µl) was added. Prior to the addition of 500 µl of DMSO, the medium was completely removed. To solubilize MTT formazan crystals, 500 µl DMSO was added. ELISA plate reader was used for the determination of optical density (OD).

Estimation of cell cycle distribution of SKOV3 cells

For estimation of cell cycle distribution, the cells were seeded in 6-well plates (2×10^5 cells/well) and swerchirin treatment was given to the cells at doses of 0, 10, 20 and 40 µM followed by 24-h incubation. For estimation DNA content, PBS was used to wash the cells and fixed in ethanol at -20°C. This was followed by re-suspension in PBS holding 40 µg/ml PI and RNase A (0.1 mg/ml) and Triton X-100 (0.1%) for 30 min in the dark at 37°C. Afterwards, analysis was carried out by flow cytometry as previously reported [8].

Determination of MMP

SKOV3 cells were seeded at a density of 2×10^5 cells/well in a 6-well plates, kept for 24 hrs and treated with 10 mM swerchirin for 6-72 hrs at 37°C in 5% CO_2 and 95% air. Thereafter, cells from all samples were collected, washed twice by PBS and re-suspended in 500 µl $DiOC_6$ (1 µmol/l) for MMP at 37°C in the dark for 30 min. The samples were then examined instantly using flow cytometer as previously described [9].

Assessment of apoptotic cell populations

SKOV3 cells at a density of 2×10^5 cells/well were seeded in 6-well plates and were administered 10 to 40 mM swerchirin for 48 hrs. The cells were then subjected to annexin V/PI treatment. Afterwards, the cell sample was studied by fluorescent microscopy as previously described [10].

Determination of protein expression by western blotting analysis

The swerchirin-administered cells were harvested and lysed. The protein concentrations of the lysates were quantified by bicinchoninic acid assay (BCA) using specific antibodies. β-actin was used as a control. From each sample equal amounts of protein were loaded and separated by electrophoresis on a 12% denaturing SDS gel. Afterwards, the proteins were then electroblotted on polyvinylidene difluoride membranes (0.45 m pore size).

Statistics

All experiments were carried out in triplicate. The values obtained were the mean ± standard deviation (SD) of the triple experiments and were considered significant at $p<0.05$.

Student's t-test using GraphPad7 software was employed for statistical analyses.

Results

Effect of swerchirin on cell viability of SKOV3 human ovarian cancer cells

To identify the anti-proliferative effect of swerchirin (Figure 1) on ovarian cancer SKOV3 cells, cells were treated with swerchirin concen-

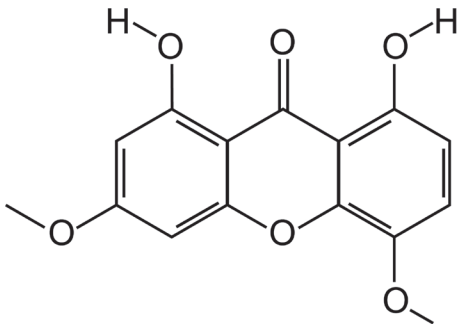

Figure 1. Chemical structure of swerchirin.

tration ranging from 0-40 μM for 48 hrs. Swerchirin displayed potent anti-proliferative effect against SKOV3 cells and significantly reduced the cell viability, exhibiting an IC_{50} 20 μM at 48 hrs of incubation (Figure 2). This anti-proliferative activity of swerchirin was found to be concentration-dependent and increased with increasing concentration of swerchirin.

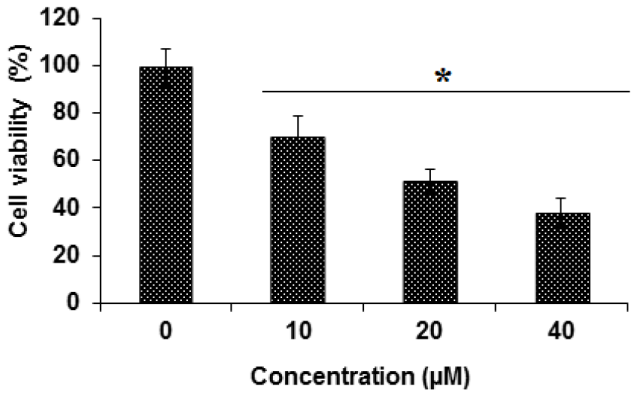

Figure 2. Effect of indicated doses swerchirin on ovarian cancer cell viability. The experiments were carried out in triplicate and expressed as mean ± SD (*p <0.05). The results indicate that swerchirin decreases significantly cell viability in a concentration-dependent manner.

Effect of swerchirin on cell cycle phase distribution of SKOV3 cells

Swerchirin caused alterations in cell cycle distribution of ovarian cancer SKOV3 cancer cell line. It was observed that the percentage of SKOV3 cells was considerably increased in G2 phase at swerchirin concentrations of 0 to 40 μM, causing G2/M cell cycle arrest (Figure 3). Additionally, the populations of SKOV3 cells in G2 phase were slightly but significantly increased at a dose of 10 μM concentration of swerchirin, however tremendous increase in G2 phase cells was observed at 40 μM. This swerchirin-induced G2 phase increase of SKOV3 cancer cells was found to exhibit a dose–dependent pattern.

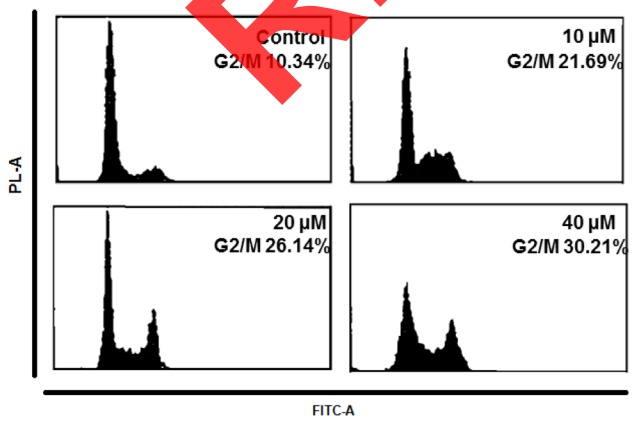

Figure 3. Effect of indicated doses swerchirin on cell cycle distribution as determined by flow cytometry. The experiments were carried out in triplicate and the images are representatives of three biological replicates. This Figure shows that swerchirin increases the cells at G2/M phase of the cell cycle in a concentration-dependent manner.

Effect of swerchirin on MMP

Since mitochondria play an important role in the normal functioning of cells and disruption of mitochondrial integrity may cause cell death, we speculated whether swerchirin reduces the MMP in SKOV3 cells treated with swerchirin at varied concentrations (0-40 μM). Our results indicated that swerchirin-treated SKOV3 cells showed a significant reduction in MMP in a dose-dependent manner. The MMP was reduced by 67% at 40 μM of swerchirin as compared to untreated control (Figure 4).

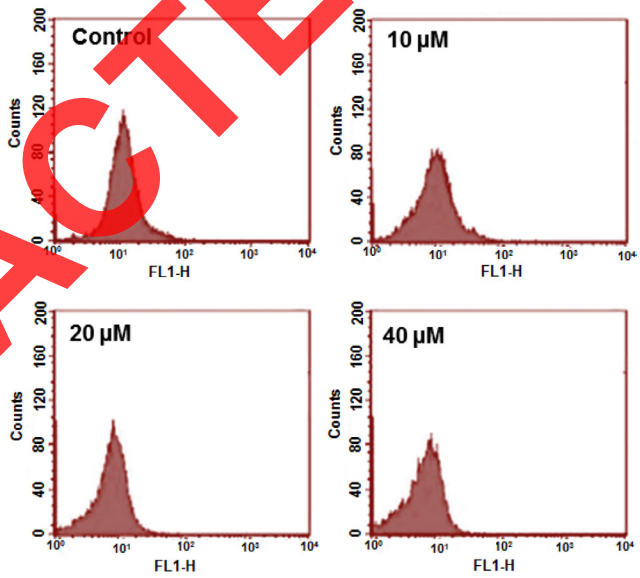

Figure 4. Effect of indicated swerchirin doses on mitochondrial membrane potential carried out by flow cytometry. The images are representatives of three biological experiments. The Figure shows that swerchirin decreases the mitochondrial membrane potential in a concentration-dependent manner.

Effect of swerchirin on induction of apoptosis in SKOV3 cells

Analysis of apoptotic cells indicated that swerchirin administration caused cancer cells apoptosis in a concentration-dependent manner. The apoptotic cell populations increased from 5.2% in control to 67.8% at 40 μM concentration (Figure 5). Furthermore, the results showed that swerchirin induced cytosolic cytochrome c, Bax, cleaved caspase 3 and cleaved caspase 9 expressions with associated suppression of Bcl-2 expression as compared to the untreated cells taken as control

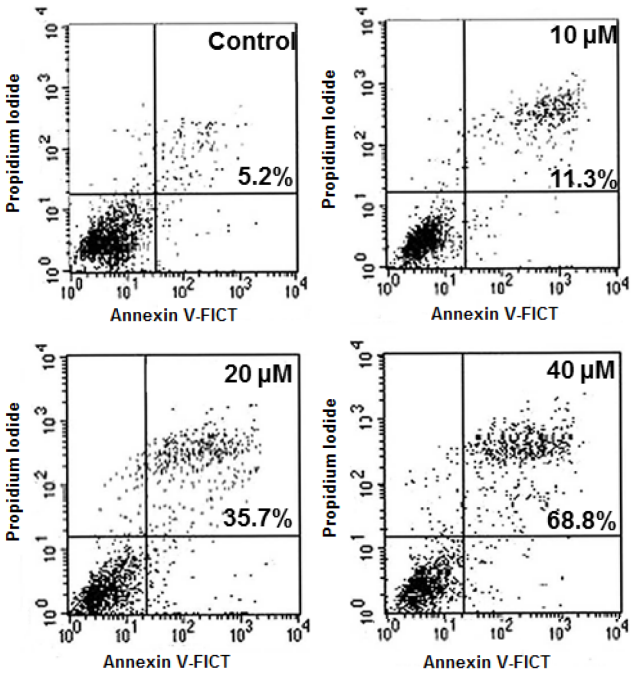

Figure 5. Effect of indicated doses of swerchirin on SKOV3 cell apoptosis as depicted by annexin V/PI staining. The images are representatives of three biological experiments. The results indicate that the apoptotic cell populations increase in a concentration-dependent manner.

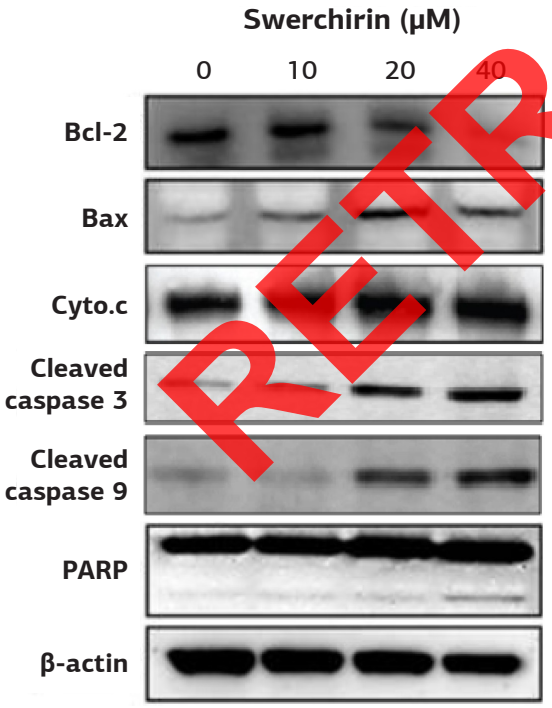

Figure 6. The effect of indicated doses of swerchirin on the expression of caspase-dependent mitochondrial apoptosis pathway proteins in SKOV3 cells. Representative images of cytochrome c, Bax, Bcl-2, PARP, cleaved caspase 9 and cleaved caspase 3 protein expression detected by western blot. β-actin was used as a control. The results indicate that swerchirin enhances the expression of Cytochrome c, Bax, PAPRP, cleaved caspase 9 and 3 but downregulates the expression of Bax.

(Figure 6). Additionally, treatment of SKOV3 cells with swerchirin also caused cleavage of PARP fragment (Figure 6), an endogenous substrate of activated caspase-3 and its cleavage is known to be characteristic of cell apoptosis. Taken together, the results indicate that mitochondria and Bcl-2 family members were associated with swerchirin-triggered cell apoptosis in SKOV3 cells.

Swerchirin targets Raf/MEK/ERK signalling pathway

The protein expressions of Raf/MEK/ERK signalling pathway were evaluated using Western blot assay. The findings are shown in Figure 7 and indicate an interesting outcome. Compared to the untreated control cells, swerchirin-treated cells showed a concentration-dependent downregulation phospho-MEK and phospho-ERK in a dose-dependent manner. Thus it may be concluded that swerchirin induced anticancer partially through via Raf/MEK/ERK signalling pathway.

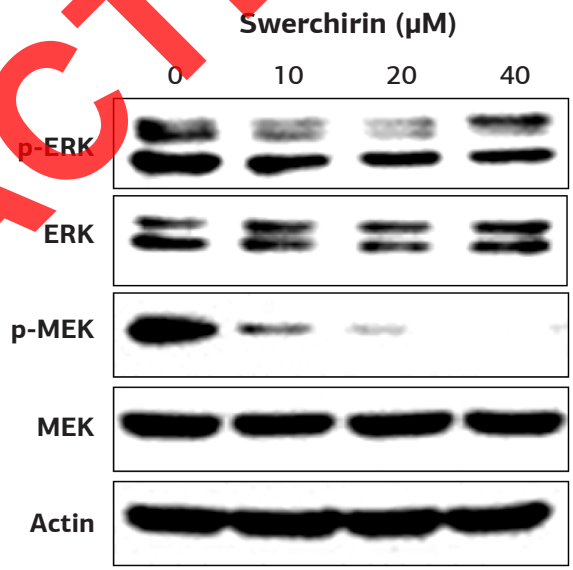

Figure 7. Effect of indicated doses of swerchirin on protein expression of MEK/ERK signaling pathway determined by western blotting. β-actin was used as a control. The images are representatives of three biological experiments. The results of this Figure indicate that swerchirin decreases the expression of p-ERK and p-MEK in a concentration-dependent manner.

Discussion

Ovarian cancer is one among the main reasons of gynecological cancer-related deaths around the globe. Despite initial responses to chemotherapy, the disease consistently relapses. Xanthones, like swerchirin, have recently received attention for their antitumorigenic activity [5,6]. In the present study swerchirin showed potential growth inhibiting activity against SKOV3 cells as evidenced

from the MTT cell viability assay. As previously reported, many drugs exhibit antiproliferative effect through apoptotic cell death. For example several chemotherapeutic drugs, such as cisplatin [12,13], have been reported to alter apoptotic pathways. Additionally, drug resistance is partially explained by the ability of cancer cells to bypass apoptosis [14]. One of the reasons for apoptosis might be the observed capacity of swerchirin to cause cell cycle arrest as it induced the G2/M phase increase of SKVO3 cancer cells in a dose–dependent manner. Cell cycle and apoptosis are known as the main controlling mechanisms for cell growth and proliferation. Apoptotic cell death is triggered when explicit checkpoints are arrested during cell cycle [12]. Consistent with this, several anticancer agents lead to cell cycle arrest and have been found to be clinically effective for cancer treatment [15]. Further, drugs with apoptosis-inducing properties may potentially minimize drug resistance. Our results indicated that cells treated with swerchirin induced apoptosis *in vitro* in a dose-dependent manner as evidenced by annexin V/PI staining. Although apoptosis is triggered though different routes, the mitochondrial pathway is a crucial signalling pathway in the induction of apoptosis. It is well established that Bcl-2 family proteins are frequently main players in the mitochondrial apoptotic pathway. The anti-apoptotic and pro-apoptotic protein members of Bcl-2 protein family control apoptosis by regulating the mitochondrial membrane permeability [15]. While Bcl-2 is a strong antiapoptotic protein, Bax is inducer of apoptosis. Bax is present in the outer membrane of the mitochondria, facilitating the discharge of cytochrome c and stimulating caspase 9. Caspase 3 is activated by proteolytic cleavage of caspase 9 and is a key apoptotic executive caspase. Stimulation of the caspase signalling and concomitant PARP cleavage is considered as the main feature of the apoptotic cascade. In the present study involvement of the mitochondrial apoptotic pathway in swerchirin-induced cell death were first detected by the observed reduction in the MMP. This was further strengthened by the changes observed in the Bcl-2 and Bax expression levels since mitochondrial malfunction is often due to MMP loss and discharge of cytochrome c release into the cytosol. We observed swerchirin lessened the MMP, leading to cytochrome c release from the mitochondria into the cytoplasm. Protein expression analysis revealed that swerchirin caused considerable downregulation of Bcl-2 expression and upregulation of Bax protein, therefore ultimately favoring apoptosis. Additionally, swerchirin elevated caspase 3 and caspase 9 as well as cleaved PARP expression in a concentration-dependent manner. It was also observed that swerchirin induced intracellular ROS alterations in SKVO3 ovarian cells in a dose–dependent manner. Flow cytometry using PI as a probe was used to study the effects of swerchirin on cell cycle progression. Further, it was shown that swerchirin could inhibit SKOV3 cancer cells in a concentration-dependent manner. These findings are promising since it is well established that ovarian cancer is one of the most lethal malignancies and swerchirin could inhibit this process. The induction of the Raf/MEK/ERK signalling pathway is one of the main Ras-controlled cascades and has been reported to be linked to cancer cell proliferation and survival. It is believed that that inhibition of the Raf/MEK/ERK pathway may prove a handy target for inhibition of tumor cell growth [16]. Therefore, targeting Raf/MEK/ERK pathway is a favorable strategy for cancer treatment. Our experiments indicated that SKOV3 ovarian cancer cells were sensitive to swerchirin *in vitro*. Swerchirin inhibited the Raf/MEK/ERK pathway through suppression of p-MEK and p-ERK in a dose-dependent manner which is consistent with studies carried out previously.

Conclusion

Taken together, we conclude that swerchirin may prove a potential candidate for the treatment of ovarian cancer by controlling Raf/MEK/ERK signalling pathway. With limited drug options available and limited toxicity associated with naturally occurring plant-derived molecules, swerchirin seems a strong option and deserves further research endeavors.

Acknowledgement

This study was supported by a grant from the project of science and technology bureau of Jining (No: 56-2-4).

Conflict of interests

The authors declare no conflict of interests.

References

1. Lukanova A, Kaaks R. Endogenous hormones and ovarian cancer: epidemiology and current hypotheses. Cancer Epidemiol Prev Biomarkers 2005;14:98-107.
2. Altomare DA, Testa JR. Perturbations of the AKT signaling pathway in human cancer. Oncogene 2005; 24:7455-64.
3. Engelman JA. Targeting PI3K signalling in cancer: opportunities, challenges and limitations. Nat Rev Cancer 2009;9:550-62.
4. Khetwal KS, Joshi B, Bisht RS. Tri- and tetraoxygenated xanthones from Swertia petiolata. Phytochemistry 1990;29:1265-67.
5. Mahendran G, Thamotharan G, Sengottuvelu S, Bai VN. Anti-diabetic activity of Swertia corymbosa (Griseb.) Wight ex CB Clarke aerial parts extract in streptozotocin induced diabetic rats. J Ethnopharmacol 2014; 151:1175-83.
6. Hajimehdipoor H, Esmaeili S, Shekarchi M, Emrarian T, Naghibi E. Investigation of some biologic activities of Swertia longifolia. Res Pharm Sci 2013;8:263-269.
7. Sun SY, Hail N Jr, Lotan R. Apoptosis as a novel target for cancer chemoprevention. J Natl Cancer Inst 2004;96:662-672.
8. Chiang JH, Yang JS, Ma CY et al. Danthron, an Anthraquinone Derivative, Induces DNA Damage and Caspase Cascades-Mediated Apoptosis in SNU-1 Human Gastric Cancer Cells through Mitochondrial Permeability Transition Pores and Bax-Triggered Pathways. Chem Res Toxicol 2011; 24: 20-29.
9. Chiang LC, Ng LT, Lin IC, Kuo PL, Lin CC. Anti-proliferative effect of apigenin and its apoptotic induction in human Hep G2 cells. Cancer Lett 2006; 237: 207-14.
10. Wu M, Zhang H, Hu J, Weng Z, Li C. Isoalantolactone inhibits UM-SCC-10A cell growth via cell cycle arrest and apoptosis induction. PLos One 2013;8:e76000.
11. Azuma M, Tamatani T, Ashida Y, Takashima R, Harada K, Sato M. Cisplatin induces apoptosis in oral squamous carcinoma cells by the mitochondria-mediated but not the NF-kappaB-suppressed pathway. Oral Oncol 2003; 39: 282-9.
12. Yoneda K, Yamamoto T, Osaki T. p53- and p21-independent apoptosis of squamous cell carcinoma cells induced by 5-fluorouracil and radiation. Oral Oncol 1998;34:529-37.
13. Chipuk JE, Bouchier-Hayes L, Green DR. Mitochondrial outer membrane permeabilization during apoptosis: the innocent bystander scenario. Cell Death Diff 2006;13:1396-1402.
14. Simon HU, Haj-Yehia A, Levi-Schaffer F. Role of reactive oxygen species (ROS) in apoptosis induction. Apoptosis 2000;5:415-8.
15. Lo HW. Targeting Ras-RAF-ERK and its interactive pathways as a novel therapy for malignant gliomas. Curr Cancer Drug Targets 2010;10:840-8.
16. Zhang L, Ji Q, Liu X et al. Norcantharidin inhibits tumor angiogenesis via blocking VEGFR2/MEK/ERK signaling pathways. Cancer Sci 2013;104:604-10.