

ORIGINAL ARTICLE

Cytotoxic and Pro-Apoptotic Effects of Honey Bee Venom and Chrysin on Human Ovarian Cancer Cells

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Abstract

Background: The anti-cancer effects of honey bee venom (BV) and chrysin might open a new window for treatment of chemo-resistant cancers. This study was designed to evaluate cytotoxic and pro-apoptotic effects of BV and chrysin on A2780cp cisplatin- resistant human ovarian cancer cells.

Methods: As per the study objectives, A2780cp cells were categorized to 4 groups: 3 experiment groups (treated either with BV or chrysin or BV + chrysin) and 1 control group (untreated cells). Experiment group cells were cultured and treated by different concentrations of BV and chrysin for 24 hours. Then, experiment and control cells were studied with MTT assay, Annexin V-FITC, DAPI and Acridine Orange / Propidium Iodide staining, flow cytometry, caspase-3 and -9 assay, measurement of intracellular level of reactive oxygen species (ROS) and RT-PCR.

Results: MTT assay showed that 8 µg/mL BV, 40 µg/ml chrysin and 6 + 15 µg/mL BV + chrysin co-treatment induced 50% cell death on A2780cp cells compared with controls ($P < 0.001$). Morphological observations by inverted and fluorescent microscopy revealed ROS generation and apoptotic cell death under exposure to BV or chrysin or BV + chrysin co-treatment. Caspase-3 and -9 assay demonstrated that BV and chrysin triggered apoptosis through intrinsic pathway and RT-PCR demonstrated down-regulation of Bcl-2.

Conclusion: Honey bee venom and chrysin are effective for destroying chemoresistant ovarian cancer cells through activation of intrinsic apoptosis, which propose them as potential candidates to be used in development of improved chemotherapeutic agents in the future.

Keywords: Apoptosis Inducing Factor; Bee Venoms; Chrysin, Cytotoxicity; Ovarian Neoplasms

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INTRODUCTION

Ovarian cancer has been considered as one of the most fatal diseases in women. It causes more deaths than any other cancer of the female reproductive system, but it is responsible for only about 3% of all cancers in women (1,2). Aggressive surgical treatment followed by paclitaxel or platinum-based therapy can enhance the survival rate of ovarian cancer patients (3). However, use of this chemotherapeutic agents is accompanied by serious side effects including neutropenia, kidney dysfunction and peripheral neuropathy (4). Moreover, such conventional treatments are less likely to completely eradicate neoplastic tissue because of irregular tumor microenvironment and drug/radiation resistance (5). Hence, women are still at high risk of recurrence, while, treatment modalities against metastatic and recurrent ovarian cancers are not adequately effective (3,6).

Apoptosis has an essential role in biological processes and homeostasis. In ovarian cancer, apoptosis resistance reduces the chance of treatment success. In this respect, apoptosis induction has been considered as a promising technique to

overcome the drug resistance and increase the treatment outcome in ovarian cancer (7-9). Previous studies have proven the effectiveness of natural products in treatment of various cancers through the mechanism of apoptosis induction (10,11). The use of natural metabolites and toxins has greatly helped in development of enhanced therapeutics for overwhelming chemo-resistant cancer cells (10,12). Honey bee venom (BV) contains a variety of active components including melittin, phospholipase A2, apamin and adolapin with various biological activities such as anti-microbial, antioxidant, anti-inflammatory and anti-cancer effects (13,14). Moreover, bee-related natural products such as honey and propolis have therapeutic applications (15). Propolis is a bee product derived from plant resins that is composed of many polyphenolic, fatty acid, flavones and flavonones compounds (16). Chrysin (5,7-dihydroxyflavone) is one of the natural flavonoids derived from honey and propolis (17), and known as having antioxidant, anti-inflammatory, anti-aging and anti-cancer properties (18,19). The anti-cancer effects of BV and chrysin might open a new window for treatment of chemo-resistant cancers. Therefore,

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this study was designed to evaluate cytotoxic and pro-apoptotic effects of BV and chrysin on A2780cp human ovarian cancer cells.

METHODS

Cell culture

A2780cp human ovarian cancer cell line, known for being cisplatin resistant, was provided from Iranian National Cell Bank and was cultured in RPMI 1640 medium supplemented with 10% Gibco™ Fetal Bovine Serum (Thermo Fisher Scientific, Massachusetts, USA) and 1% antibiotic in a humidified incubator with 5% CO₂. The cells were maintained sub-confluent and all experiments were repeated at least three times. As per the study objectives, A2780cp human ovarian cancer cells were categorized to 4 groups: 3 experiment groups (treated either with BV or chrysin or BV + chrysin) and 1 control group (untreated cells).

Cell viability assay

The effects of BV and chrysin on A2780cp cells were evaluated by MTT cell viability assay according to methodology described in the study by Sun et al in 2011 (20). Briefly, A2780cp cells were cultivated at a concentration of 10⁴ cells/well in 96-well plates at 37°C, overnight. Then the cells were treated with the venom of honey bee (*Apis mellifera*) and/or chrysin (C80105, Sigma, St. Louis, USA) at the following concentrations: 0, 2, 4, 8, 16, and 32 µg/mL of BV; 0, 20, 40, 60, 80, 100 µg/mL of chrysin, and 4 + 10, 6 + 15, 2 + 20 µg/mL of BV + chrysin for 48 hours. Subsequently, 20 µL of MTT ((3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium) solution was added and followed by addition of 150 µL dimethylsulfoxide. Optical density was then determined with a micro-plate spectrophotometer at 570 nm (BioTech Epoch, Winooski, USA).

Apoptosis detection by flow cytometry

After exposure of cells to BV and/or chrysin, detached cells were washed in phosphate buffer saline (PBS), resuspended in binding buffer and subjected to Annexin V-FITC-PI staining or hypotonic buffer of propidium iodide for detection of sub-G1 peak. Briefly, after treatment, A2780cp cells were trypsinized and resuspended in 500 µL of 1X binding buffer. Then, 5 µL of Annexin-V-FITC and 5 µL of propidium iodide were added and maintained in the dark. For detection of sub-G1 peak, the rinsed cells were resuspended in propidium iodide solution containing 0.1% sodium citrate plus 0.1% Triton X-100 and incubated at 37°C for 30 minutes. Finally, the cells were analyzed using flow cytometry (FACSCalibur BD Bioscience, San Jose, USA) (21).

Apoptosis assessment by AO/PI and DAPI staining

The untreated cells (control group) and experiment groups cells were harvested, rinsed and stained with 10 µL of a dye mixture composed of Acridine Orange (AO) and Propidium Iodide (PI) in concentration of 100 µg/mL. Moreover, A2780cp cells were fixed with methanol for 5 minutes, rewashed and stained with 10 µg/mL 4',6-diamidino-2-phenylindole (DAPI) at 37°C for 15 minutes. Finally, morphological alterations were visualized under a fluorescence microscope (Olympus, Tokyo, Japan).

Measurement of ROS generation

Reactive oxygen species (ROS) generation was assessed

by fluorescence microscopy with 2',7'-dihydrofluorescein-diacetate (DCF-DA) fluorescence probe. Briefly, untreated and treated A2780cp cells were treated with 10 µM DCF-DA in PBS at 37°C for 30 minutes. Trapping the probe inside cells occurred through intracellular esterase action with removal of acetate groups on DCF-DA. Using fluorescence microscopy, ROS generation was assessed through observation of DCF oxidation (8).

Caspase-3 and -9 activity assay

After treatment with inhibitory concentrations of BV, chrysin or BV + chrysin, cells were harvested with lysis buffer. Then, 2X reaction buffer and DTT were mixed with cytosolic protein mixture and caspase substrate, and the resultant mixture was placed in incubator for 120 minutes. Finally, the activity of caspase-3 and -9 was measured via spectrophotometry by recording the absorbance at 405 nm (Epoch, Winooski, USA).

Evaluation of gene expression by RT-PCR

The expression of B-cell lymphoma-2 (Bcl-2), an anti-apoptotic protein, at the mRNA level was assessed by reverse transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated from untreated and treated cells using the high pure RNA isolation kit (Roche, Mannheim, Germany). Complementary DNA (cDNA) was synthesized using the easy cDNA synthesize kit (Pars Tous, Tehran, Iran). The reaction PCR was performed in a final volume of 20 µL containing 10 µL Taq premix, 2 µL cDNA, 2 µL primer (forward and reverse) and water. RT-PCR was started with an initial cycle of reverse transcription at 95°C/4 min followed by 35 cycles of denaturation at 94°C/30s, annealing (58°C/30s), and extension (72°C/30s). The sequences of primers used were as follow: β-2-microglobulin (B2M) forward 5' TGGTGCTTGGCTCACTGACC 3' and reverse 5' TATGTCGGCTTCCCATTCT 3' (which was used as the housekeeping gene). The forward primer and reverse primer Bcl-2 were designed as 5'CATGTGTGGAGAGCGTCAAC3' and 5'CAGATAGGCACCCAGGGTGTAT3' 223 base pair. Following amplification, the PCR products were electrophoresed in a 2% agarose gel.

Statistical analysis

Data were analyzed using SPSS software for windows (SPSS Inc., Chicago, USA) and Microsoft Excel (Microsoft, Redmond, USA). Results are expressed as mean ± standard deviation. The difference between each experiment group and the control group was analyzed with Student's t-test. Probability values of less than 0.05 were considered statistically significant.

RESULTS

Cytotoxicity of chrysin and honey bee venom on ovarian cancer cells

To evaluate the cytotoxicity, A2780cp cells were treated with BV and chrysin in different concentrations for 24 hours and then the cells viability was measured by MTT assay. As shown in figure 1, the viability of A2780cp cells was significantly suppressed in a dose-dependent manner; showing that compared with control cells (100%), the viability of experiment cells was 44.7 ± 3.0 % and 30.5 ± 2.9 % after treatment with 8 and 16 µg/mL BV, respectively

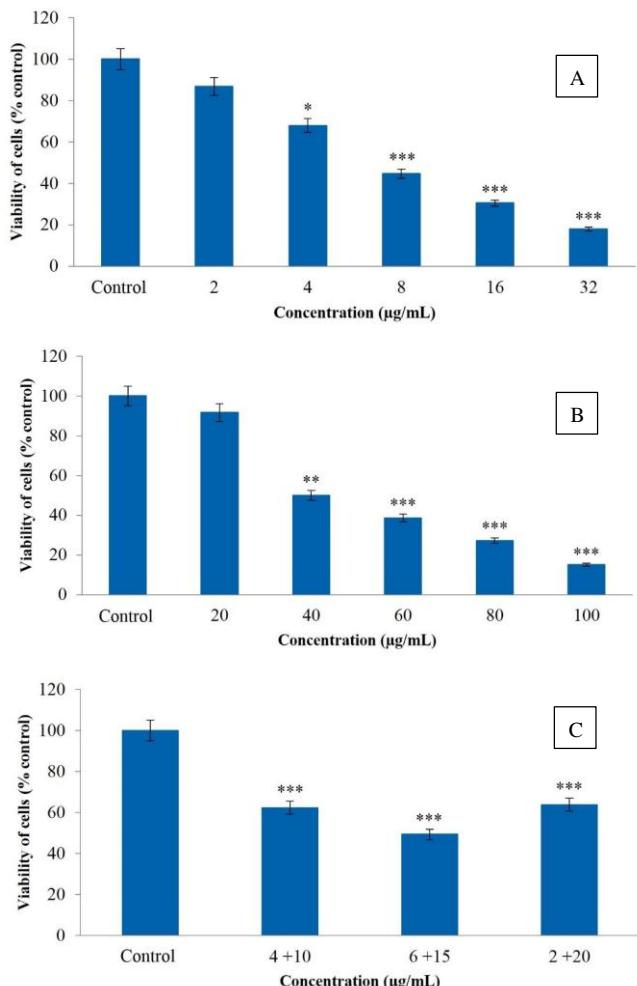


Figure 1. Effect of treatment with bee venom (A), chrysin (B) and co-treatment of bee venom and chrysin (C) on viability of human A2780cp cells after 24 hours. Graphs show mean (histograms) and standard errors (whiskers) of ten independent experiments in each concentration
*P < 0.05, **P < 0.01 and ***P < 0.001 show the significance of difference between mean viability of untreated (control) and treated (experimental) cells

(P < 0.001 for both). In addition, compared with control cells, the viability of experiment cells was $49.9 \pm 7.2\%$ and $38.6 \pm 6.1\%$ after treatment with 40 and 60 $\mu\text{g/mL}$ chrysin, respectively (P, <0.01 and < 0.001). By co-treatment with BV and chrysin at doses of 4 + 10, 6 + 15, 2 + 20 $\mu\text{g/mL}$, viability was $62.3 \pm 5.2\%$, $49.3 \pm 3.8\%$, and $63.8 \pm 7.1\%$, respectively (P < 0.001 for all analyses). Hence, the half maximal inhibitory concentration (IC_{50}) values for A2780cp cells were considered to be approximately 8 $\mu\text{g/mL}$ BV, 40 $\mu\text{g/mL}$ chrysin and 6 + 15 $\mu\text{g/mL}$ BV + chrysin.

Induction of apoptosis by honey bee venom and chrysin on A2780cp cells

Morphological changes such as loss of adhesion and the formation of apoptotic bodies under exposure to BV and

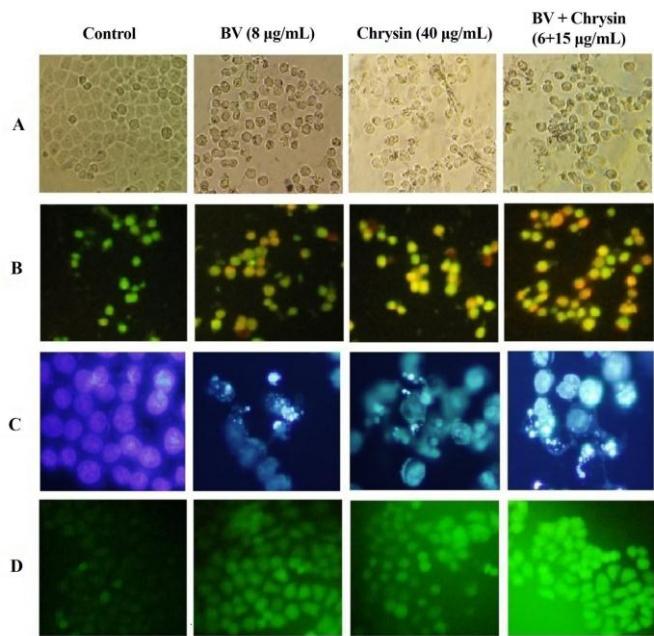


Figure 2. A) Microscopic images of A2780cp cells treated with bee venom (BV), chrysin and BV + chrysin after 24 hours ($\times 200$ magnification), B) Detection of apoptosis by AO/PI staining, bright green and yellow color are indicators of apoptosis induction, C) Fluorescence profiles of treated and untreated A2780cp cells using DAPI staining indicating DNA fragmentation in treated cells ($\times 400$ magnification) D) Effect of bee venom and chrysin on intracellular ROS formation in A2780cp cells

chrysin indicated typical morphological changes of apoptosis (Figure 2A). In addition, AO/PI and DAPI staining showed that incubation of ovarian cancer cells with inhibitory concentrations of BV and chrysin produces bright green or orange color in AO/PI staining and DNA fragmentation in DAPI staining which are clearly suggestive of apoptosis. Furthermore, appearance of red color in co-treatment group suggests that the suppression of A2780cp cell growth were caused by apoptosis and necrosis (Figure 2B, 2C).

Apoptosis induction via ROS-dependent mechanism

Free radicals generation by chemotherapeutic agents can activate pro-apoptotic signals. As shown in figure 2D, treatment of A2780cp cells with IC_{50} concentrations of BV, chrysin and the combination of both for 24 hours increases ROS generation. This indicates that BV and chrysin can sensitize A2780cp cells to apoptosis via ROS-dependent mechanism.

Detection of apoptosis using flow cytometry

For confirming fluorescent microscopic findings, we performed flow cytometry based on annexin V-FITC/PI. As shown in figure 3, the IC_{50} concentrations of BV and chrysin induced apoptosis in A2780cp cells, so that the proportion of apoptotic cell death (annexin V-positive / PI-negative) increased from 1.6% in control cells to 12%, 15% and 23.2% in 8 $\mu\text{g/mL}$ BV, 40 $\mu\text{g/mL}$ chrysin, and 6 + 15 $\mu\text{g/mL}$ BV + chrysin treatment groups, respectively. Moreover, necrotic cell death was at the highest rate in co-treatment group. These results revealed that chrysin and BV

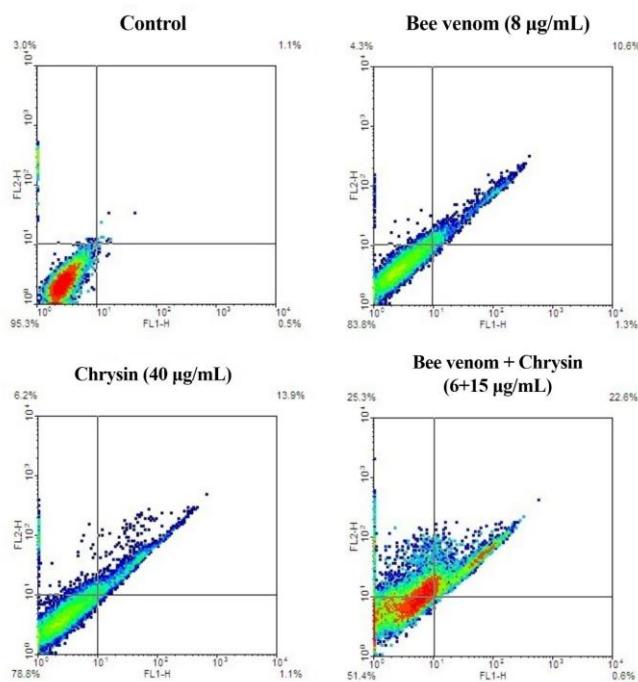


Figure 3. The 50 % inhibitory concentrations of chrysanthemum and honey bee venom separately and combined together induced apoptosis in A2780cp cells after 24 hours (Annexin V-positive and Annexin V-PI double-positive cells were identified as apoptotic cells, whereas PI single-positive cells were identified as necrotic cells)

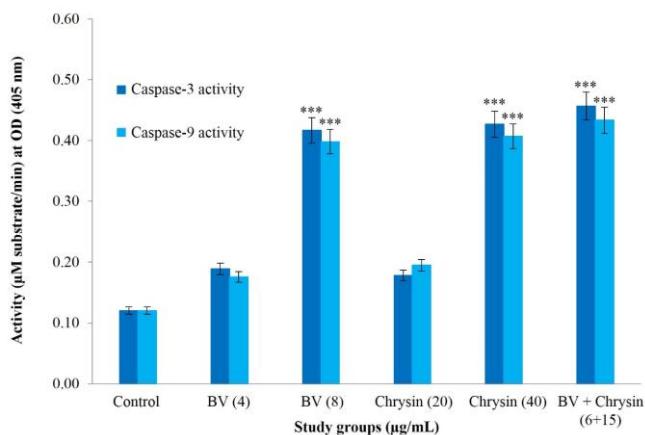


Figure 4. Level of caspase-3 and caspase-9 activation in A2780cp cells treated with bee venom (BV), chrysanthemum and BV + chrysanthemum after 24-hour treatment. Graphs show mean (histograms) and standard errors (whiskers) of three independent experiments in each concentration

***P < 0.001 show the significance of difference between mean caspase activity of untreated (control) and treated (experimental) cells

can separately trigger apoptosis and combined together can induce late apoptosis and necrosis in ovarian cancer cells (Figure 3).

Effects of chrysanthemum and honey bee venom on caspase-3 and 9 activities

As shown in figure 4, caspase-3 activity and caspase-9 activity (μM substrate/min) were significantly higher in 8 $\mu\text{g}/\text{mL}$ BV-treated cells (0.42 ± 0.05 , 0.40 ± 0.04 , respectively), 40 $\mu\text{g}/\text{mL}$ chrysanthemum-treated cells (0.43 ± 0.04 , 0.41 ± 0.05 , respectively) and 6 + 15 $\mu\text{g}/\text{mL}$ BV+chrysanthemum-treated cells (0.46 ± 0.02 , 0.43 ± 0.01 , respectively) compared with control cells (0.12 ± 0.2 , 0.12 ± 0.1 , respectively) ($P < 0.001$ for all analyses). Hence, it can be said that chrysanthemum and BV can also trigger apoptosis via caspase-dependent mechanism in A2780cp cells.

Effects of chrysanthemum and honey bee venom on Bcl-2 mRNA expression

Expression of Bcl-2 and B2M (as internal control) on study groups was assessed using RT-PCR. Figure 5 shows that A2780cp cells under exposure with inhibitory concentrations of BV, chrysanthemum and co-treatment of BV and chrysanthemum had no changes in the expression of B2M. However, chrysanthemum and BV inhibited the expression of Bcl-2 at 40 $\mu\text{g}/\text{mL}$ and 8 $\mu\text{g}/\text{mL}$ doses, respectively, while co-treatment of chrysanthemum and BV less affected Bcl-2 mRNA levels.

DISCUSSION

Chemo-resistance is one of the main factors for poor prognosis and advancement of ovarian cancer in human; hence, scientists have been engaged in designing newer potent therapeutics to overcome this obstacle. It has been ascertained that defective apoptosis is one of the underlying mechanism of chemo-resistance in ovarian cancer (22). Several scientists have focused on apoptosis induction as a potential solution to increase the curability and decrease the chemo-resistance of ovarian cancer. Yin et al investigated the effect of paeonol, a phenolic compound found in peony flower, against ovarian cancer and found that this phenol has significant apoptosis inducing effects on ovarian cancer cells through activation of caspase-3 (23). In another study, Yu et al showed that the extract of *Rauvolfia vomitoria*, a toxic tree native to tropical Africa, can cause apoptosis in ovarian cancer cells in a time and dose-dependent manner (21).

In the same way, in the present study, we sought to investigate the apoptosis inducing capacity of natural derived products of honey bee, its venom and chrysanthemum, on ovarian cancer cells. We found that chrysanthemum and honey bee venom have potent cytotoxic effects on ovarian cancer cells. As we observed, the cytotoxic and pro-apoptotic effects of chrysanthemum and BV are due to three underlying mechanisms: ROS accumulation, caspase activation via mitochondrial pathway and inhibition of Bcl-2 expression in A2780cp cells. In addition, chrysanthemum synergistically multiplied the cytotoxic effects of honey bee venom on ovarian cancer cells. Furthermore, the elevation of caspase -3, -9 and down-regulation of Bcl-2 indicated that bee venom and chrysanthemum exerted their anti-cancer effects through intrinsic apoptotic pathway. Alizadehnohi et al similarly proved the cytotoxic and anti-tumor effects of honey bee venom on A2780cp human ovarian cancer cell line through inhibition of Bcl-2 expression (24). Park et al likewise demonstrated cytotoxic

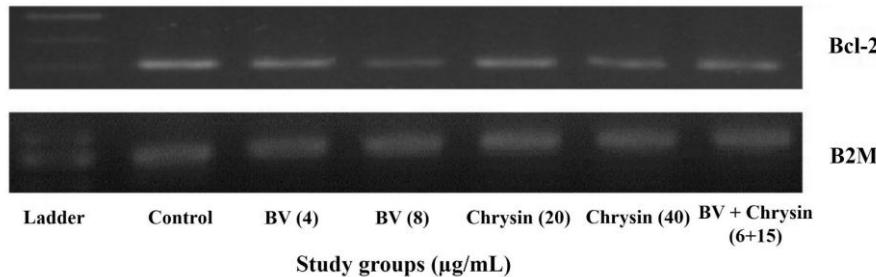


Figure 5. Inhibitory effects of bee venom and chrysin separately and in combination on B2M and Bcl-2 mRNA levels in A2780cp cells after 24-hour treatment

activities of melittin peptide, the main active component of bee venom, against human ovarian cancer cell line (25). The active constituents of honey bee venom, especially melittin, are recognized for their chemotherapeutic effects as they are good cytotoxic agents and they can suppress tumor growth by blocking growth factor receptors, inhibition of cell proliferation, induction of death receptors, inhibition of JAK2/STAT3 pathway and NF- κ B/caspase signal mediated induction of apoptotic cell death (26-28). Jo et al correspondingly ascertained death receptor expression and JAK2/STAT3 pathway inhibition in human ovarian cancer cells by BV and melittin (12).

Despite no evidence on human ovarian cancer cells, chrysin is known as having inhibitory effects on proliferation and triggering effect on apoptosis via activation of caspases and inactivation of Akt signaling in the cells (29). In this respect, Shao et al found growth inhibitory and pro-apoptotic effects of chrysin on lung cancer A549 cells (30). Samarghandian et al similarly showed the cytotoxic and pro-apoptotic effects of honey and chrysin on prostate cancer cells (31). Brechbuhl et al ascertained that chrysin can sensitize the multi-drug resistant non-small cell lung cancer cells to chemotherapeutic agents via enhancement of glutathione efflux and subsequent intracellular glutathione (32). Our investigation was the first proof of anti-cancer effects of chrysin on resistant ovarian cancer cells.

CONCLUSION

Honey bee venom and chrysin are effective for destroying chemoresistant ovarian cancer cells through activation of intrinsic apoptosis, which propose them as potential candidates to be used in development of improved chemotherapeutic agents in the future.

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