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Original Article

In vitro evaluation of the pogostone effects on the expression of *PTEN* and *DACT1* tumor suppressor genes, cell cycle, and apoptosis in ovarian cancer cell line

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Abstract

Background and purpose: Ovarian cancer is one of the most dangerous cancers among women. Pogostone has anticancer effects and is rich in polyphenol compounds. In the present study, we investigated the effects of pogostone on ovarian cancer cell lines (OVCAR-3).
Experimental approach: OVCAR-3 cells were treated with pogostone at IC_{50} (90 µg/mL) for 24 and 48 h. Cell viability and apoptotic rate in the cells were measured using MTT assay and flow cytometry. Real-time PCR was used to determine the expression of genes involved in the cell cycle and apoptosis. The expression of caspase-3 (CASP3) protein was evaluated by the CASP3 assay.
Findings/Results: Treatment of OVCAR-3 cells with pogostone increased the expression levels of phosphatase and tensin homologue deleted on chromosome ten (*PTEN*) and Dapper antagonist of catenin-1 (*DACT1*) tumor suppressor genes, as well as the apoptotic genes *CASPs3*, *8*, and *9*. Moreover, the ratio of the expressed *BCL2* associated X (*BAX)/BCl2* genes, as pro- and anti-apoptotic genes, was increased. The expression levels of the genes related to the cell cycle progression including cyclin D1 (*CCND1*) and cyclin-dependent kinase 4 (*CDK4*) were inhibited. The data obtained from flow cytometry indicated that pogostone induced cell apoptosis in 24 and 48 pogostone groups. The CASP3 colorimetric assay revealed that pogostone increased the expression of CASP3 protein in the treated groups.
Conclusion and implication: Pogostone, by inducing the expression of *PTEN* and *DACT1* tumor suppressor genes and regulation of downstream genes may decrease cell proliferation and increase the rate of apoptosis in OVCAR-3.

Keywords: Apoptosis; Cell cycle; *DACT1*; Ovarian cancer; Pogostone; *PTEN*.

INTRODUCTION

Nowadays, many women suffer from ovarian cancer, which is the most common cancer in the world and has a high mortality rate with 14,000 deaths annually (1). Presently, treatments such as surgery, radiotherapy, and chemotherapy are the best approaches to treat ovarian cancer. These treatments are not always effective and have shown severe side effects such as hair loss, nausea, vomiting, and drug resistance (2-4). Hence, achieving new drugs with minimal side effects that overcome cancer cell drug resistance, is one of the purposes of recent studies in order to improve the treatment of this deadly disease (4).

In most types of cancers, excessive activity of oncogenic factors and loss of activity of tumor suppressor genes are observed. Hence, concentrating on the genetic and epigenetic factors, finding a way to increase the expression of these genes, and inducing the apoptosis process could be several new approaches to treat different kinds of cancers including ovarian cancers (5,6).

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Improvement in the knowledge about medicinal plants in the past years has led to the increase in using herbal medicines which prevent various diseases (7). Among various treatment modalities, herbal drugs, with their noticeable antitumor activity, are the preferred choice (8).

Pogostemon cablin, which belongs to the Lamiaceae family, grows in Southeast Asia (9). Many studies have shown the effects of this plant, such as the antimicrobial, antioxidant, analgesic, anti-inflammatory, anti-mutagenic, antithrombotic, antiemetic, and cytotoxic activities (10).

Pogostone is a natural substance isolated from *Pogostemon cablin* and has various pharmacological activities (11). Although the anticancer effects of pogostone have been recognized in some cancers, the exact mechanism of its function in ovarian cancer is not yet known.

The phosphoinositide 3-kinases (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) pathway is an essential signaling pathway in cell processes, such as growth and proliferation, motility, survival, and apoptosis. Increased activity of this cellular signaling pathway leads to the growth and proliferation of cancer cells in many human cancers (12).

The phosphorylated form of phosphatase and tensin homologue deleted on chromosome ten (*PTEN*) acts as a tumor suppressor gene by negatively regulating *PI3K*. PTEN catalyzes the dephosphorylation of phosphatidylinositol (3,4,5)-trisphosphate (PIP3*)*, which leads to PI3K/AKT phosphorylation. *PTEN* is phosphorylated and strongly suppressed with the activation of PI3K/AKT in cancer cell lines (13). Inducing *PTEN* prevented ovarian cancer cell growth and prolonged the survival time of mice with peritoneal disseminated tumors (14).

It was shown that pogostone induced autophagy and apoptosis through the PI3K/AKT/mTOR axis pathway in colorectal tumors (15). Co-existing with human ovarian cancer, the activated PI3K/AKT/mTOR pathway is a potential predictor of invasiveness for ovarian tumor cells (16).

Overexpression of Dapper antagonist of catenin-1 (*DACT1*) inhibits cell growth and plays a crucial role in tumor suppression. It was suggested that *DACT1* expression was associated with decreased nuclear β-catenin and positive regulation of Wnt/β-catenin signaling (17). Moreover, it was reported that β-catenin controls G2/M transition and apoptosis in epidermal keratinocytes (18). β-catenin signaling targets cyclin D1 (*CCND1*) and *C-MYC* and mediates the cell cycle progression and cell proliferation (19). CCND1 is a protein required for progression through the G1 phase of the cell cycle. *C-MYC* is a proto-oncogene and a cell master regulator (20).

Cao *et al.* reported that pogostone has anti-colorectal tumor effects by inducing autophagy and apoptosis through the PI3K/AKT/mTOR axis (15).

There are two pathways of apoptosis including the extrinsic and the intrinsic pathways. In the intrinsic or mitochondrial pathway of apoptosis, which is regulated by pre-apoptotic factors such as *BAX* genes, the caspase (*CASP) 9* gene is finally expressed as the *CASP* initiator. Activated *CASP9* then activates further downstream caspases including *CASP8*. Activation of *CASP8* eventually leads to the activation of terminal caspases (*CASP3*) and poly (ADP-ribose) polymerase (*PARP*). On the other hand, the intrinsic pathway is activated by several different stimuli, leading to a dramatic decrease in transmembrane mitochondrial potential and consequently the release of cytochrome C and pro-apoptotic effectors across the mitochondrial membrane (21). It has been shown that pogostone induces apoptosis through the activation of *CASP3* pathways (15). Tsai *et al.* indicated that the survival and proliferation of Ishikawa cells decreased with a higher dosage of pogostone. They revealed that pogostone significantly elevates apoptosis in endometrial cancer cells, and may delay cancer cell growth by apoptosis *via* the upregulation of the expression of BCL-associated athanogene3 (*BAG3*), *CASP4*, and *CASP5* genes (8). Another study also found that pogostone treatment increased the activity of *CASP9* and *3* (22).

The OVCAR-3 cell line is highly metastatic and resistant to drugs, therefore it is a suitable model to study the effects and mechanisms of

various anticancer substances (23). Extensive studies on 39 ovarian cancer cell lines including OVCAR-3 have shown that *PTEN* and *DACT1* tumor suppressor genes were the wild-type form (24).

The present study aimed to investigate the effects of pogostone on the expression of *PTEN* and *DACT1* tumor suppressor genes, cell cycle arrest, and apoptosis in the ovarian cancer cell line OVCAR-3.

MATERIALS AND METHODS

Compounds and reagents

Pogostone was purchased from Sigma (St. Louis, MO, USA). It was dissolved in dimethyl sulfoxide (DMSO, 10 mg/mL) and diluted by culture medium (Dulbecco's modified eagle medium, DMEM/F12). The mixture was heated for 30 min at 70 °C, centrifuged for 10 min at 1800 rpm, and sterilized using a 0.22-µm syringe filter (Millipore, USA). It was stored at -20 °C until use. The final concentration of DMSO in the test solutions was less than 0.1%.

Cell culture media, DMEM/F-12, fetal bovine serum (FBS), and streptomycin-penicillin were procured from Bioscience Ltd (Wokingham, UK). Phosphate-buffered saline (PBS), ethanol (95%), and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) were obtained from Santa Cruz Biotechnology (CA, USA). Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) apoptosis detection kit were purchased from Beyotime (Shanghai, China).

Cell lines and cell culture

The ovarian cancer cell line OVCAR-3 was purchased from the Pasteur Institute of Iran (Tehran, Iran). All the cells were seeded into 25-cm^2 flasks with DMEM/F12 medium (Falcon, Grand Island, NY, USA) containing 10% FBS (Bioscience, UK) supplemented with 1% penicillin and streptomycin (Sigma, USA) and incubated at 37 °C, 95% humidity, and 5% CO_2. At 80% confluency, the cells were trypsinized and incubated for the downstream experiments. OVCAR-3 cells were passaged every 2-3 days.

Experimental groups

OVCAR-3 cells were treated with 90 µg/mL pogostone which was equal to the IC_{50} concentration (pogostone group), DMSO as the solvent of pogostone (DMSO group), or the medium as the control group for 24 and 48 h.

The IC_{50}, which is the concentration of pogostone that inhibits half-maximal proliferation of OVCAR-3 cells, was determined as follows: 10,000 cells per well were seeded in 96-well plates and incubated overnight. Then, the cells were treated with 200 µL of serial dilution of pogostone (10 to 250 µg/mL) for 24 h. The assays included blank wells containing only the medium, untreated control cells, and test cells treated with pogostone in serial dilutions. Afterward, the MTT assay was performed on the cells to determine the cell viability rate. Then, the IC_{50} curve was plotted.

MTT assay and determination of cell viability assays

OVCAR-3 cells were seeded in 96-well plates (10,000 cells per well) and incubated overnight. Next, the cells were treated with pogostone and DMSO for 24 and 48 h. For the controls, the cells were incubated with only the medium. Each group was repeated in six wells. Then, 50 µL of the MTT solution (5 mg/mL, 0.4%; Thermo Fisher Scientific) was incubated at 37 °C, with 95% humidity, and 5% CO_2 for 4 h. The medium was removed and 200 µL of DMSO was added to each well to dissolve the formazan. The wells were covered and agitated in an orbital shaker for 15 min. The absorbance in each well was measured at 570 nm in a microtiter plate reader. The reference wavelength was higher than 650 nm. The blanks were given values close to zero (±0.1).

Analysis of apoptosis by flow cytometry

OVCAR-3 cells were seeded into 6-well culture plates and treated for 24 and 48 h. Annexin V and PI staining was performed followed by flow cytometry. The cells were trypsinized and washed with PBS. After adding the binding buffer, the cells were treated with 5 µL of Annexin V-FITC. The cells were incubated at room temperature for 15 min and then washed with washing buffer. Finally,

200 µL of buffer and 5 µL of PI were added to the cells and the apoptotic OVCAR-3 cells were counted by flow cytometry (Becton Dickinson, USA).

The CASP3 activity assay

The CASP3/CPP32 colorimetric assay kit (BioVision, Catalog, and K105-25) was utilized to evaluate the activity of CASP3. A critical executioner of apoptosis, CASP3 is responsible for the proteolytic cleavage of many key proteins (23). At the end of the treatment, the cells were trypsinized and washed with PBS. The cell pellet was suspended in 50 µL of chilled cell lysis buffer. Then, 50 µL of 2X reaction buffer, containing 10 mM dithiothreitol (DTT), was added to each sample. Next, the cells were incubated on ice for 10 min. Subsequently, 50 µL of 2X reaction buffer, 1 µL of DTT (1 M), and 5 µL of N-acetyl Asp-Glu-Val-Asp 7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC, 1 mM) were added to the cell lysates. The reactions were incubated for 2 h at 37 °C, 5% CO2, and 95% humidity. Finally, 50 µL of the cell lysates were transferred into a 96-well plate and the absorbance was determined using a spectrophotometer with 400 excitation and 505-nm emission filters.

Isolation of total RNA and real-time polymerase chain reaction

The expression level of the target genes in this study was determined by real-time polymerase chain reaction (RT-PCR). Total RNA from the cells of different treatment groups was extracted using the YTA total RNA purification mini kit (Yekta Tajhiz Azma, Iran) and according to the manufacturer's protocol. After treatment with DNase I to remove genomic DNA, cDNA was reverse transcribed using RevertAid™ first-strand cDNA synthesis kit (Fermentas, USA). Maxima SYBR Green ROX qPCR master mix kit (Fermentas) was used according to the manufacturer's protocol in an ABI StepOnePlus™ RT-PCR system (Applied Biosystems, USA). The cycling parameters were as follows: 10 min at 95 °C for the initial denaturation followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension for 1 min at 60 °C. β-Actin was used as a reference gene for internal control. The data were analyzed using the comparative Ct (ΔΔCt) method (25). The experiments were carried out in triplicate and were independently repeated at least three times. Gene-specific primer sequences are presented in Table 1.

Statistical analysis

All data were statistically analyzed by SPSS 23 software and are expressed as mean ± SD. Comparison between groups was done using a one-way ANOVA test followed by Post hoc Tukey test and P values < 0.05 were considered as significant.

Table. 1. Sequences of the used primers

Genes	Forward primer sequences	Reverse primer sequences
PTEN	ACCAGTGGCACTGTTGTTTC	TCCTCTGGTCCTGGTATGAAG
DACT1	CCCCAAATCTGCAGATGTG	TGACGGCATCTAGCTCAGATC
AKT	TCTTTGCCGGTATCGTGT	TGTCATCTTGGTCAGGTGGT
MTOR	TCCGAGAGATGAGTCAAGAGG	TCCCACCTTCCACTCCTATG
GSK3β	TGTCAAGTTGTATATGTATCAGC	AATACAGCAGTATCAGGATCC
C-MYC	GCGACTCTGAGGAGGAACAAG	TCCAGACTCTGACCTTTTGCC
CDK4	TCTTTGACCTGATTGGGCTG	CCATCTCAGGTACCACCGAC
CCND1	ACAAACAGATCATCCGCAAACAC	TGTTGGGGCTCCTCAGGTTC
BAX	GGAGCTGCAGAGGATGATTG	GTCCAATGTCCAGCCCATG
BCL2	AAAATACAACATCACAGAGGAAG	CTTGATTCTGGTGTTTCCC
CASP3	AGCACTGGAATGACATCTCG	ACATCACGCATCAATTCCAC
CASP8	ACTGGATGATGACATGAACCTG	GCTGAATTCTTCATAGTCGTTG
CASP9	CCTTTGTTCATCTCCTGCTTAG	CCTCAAACTCTCAAGAGCACC
β-actin	TTCGAGCAAGAGATGGCCA	CACAGGACTCCATGCCCAG

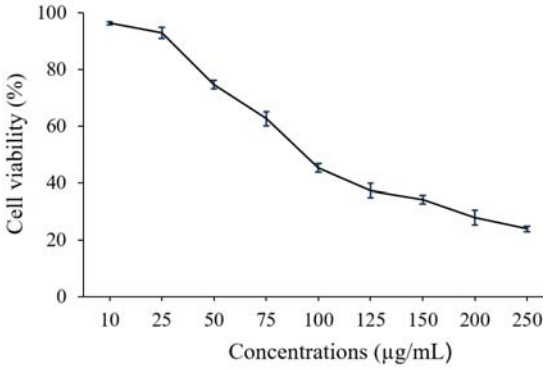

Fig. 1. Effect of pogostone on the viability of OVCAR-3 cells. The cells were incubated with different concentrations (10-250 ug/mL) of pogostone for 24 h. Cell viability was measured with an MTT assay. Based on the results, IC_{50} of pogostone was in the range of 90 µg/mL.

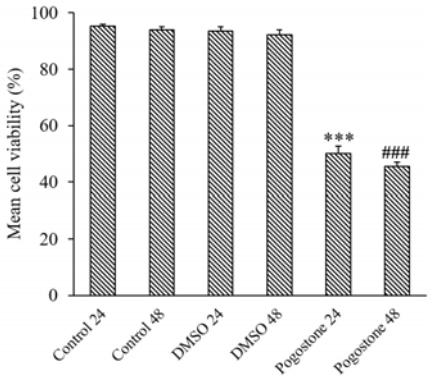

Fig. 2. Comparison of mean cell viability between groups. $^{***}P < 0.001$ Indicates significant differences compared to DMSO 24 and $^{###}P < 0.001$ versus DMSO 48.

RESULTS

Cytotoxicity assay of pogostone

The IC_{50} is a measure of the effectiveness of a compound in inhibiting biological or biochemical function. The OVCAR-3 ovarian cancer cells were treated with serial dilutions of pogostone (10-250 µg/mL). Then, the MTT assay was performed on the cells and the cell viability against pogostone concentrations was constructed.

According to the depicted curve, the concentration of 90 µg/mL corresponded to 50% cell viability of the OVCAR-3 cells following treatment with pogostone, and further investigations cells were exposed to this concentration. (Fig. 1).

Inhibition of OVCAR-3 cell growth by pogostone

OVCAR-3 cells were treated with 90 µg/mL pogostone (the IC_{50} concentration) for 24 and 48 h. The cells cultured in pogostone-free media were used as the treatment control and the cells treated with DMSO were used as the vehicle control.

The viability of the cells incubated with pogostone decreased significantly after 24 h (50 ± 2.64%) and 48 h (45.66% ± 1.52%) treatment. The results of MTT assay showed that the antiproliferative effects of pogostone increased time-dependently (Fig. 2).

Annexin V assay and flow cytometry

To quantify the apoptotic OVCAR-3 cells, annexin V and PI were used for staining. Annexin V^+ and PI^- cells were designated as apoptotic. As shown in Figs. 3 and 4, compared with the DMSO-treated cells, in which almost no apoptotic cells were detected, the apoptotic rate in the cells after 24 h of treatment with 90 µg/mL pogostone was 31.2 ± 2.8% and for 48 h was 34.9 ± 1.96%.

RT-PCR

RT-PCR was used to evaluate the effects of pogostone on the expression levels of genes involved in the cell cycle and the apoptosis process.

The results showed that treatment with the IC_{50} of pogostone increased the expression levels of *PTEN* and *DACT1* tumor suppressor genes and consequently affected the expression levels of the downstream genes such as *AKT*, *MTOR*, *GSK3B*, and *C-MYC* which were involved in cell growth and proliferation (Fig. 5). Cell cycle-related genes including *CCND1* and cyclin-dependent kinase 4 (*CDK4*), were significantly decreased following pogostone treatment in OVCAR-3 cells compared with their respective control cells (Fig. 5).

The results indicated that pogostone up-regulated the expression of *BAX*, a pro-apoptotic gene, and down-regulated the expression of *BCL2*, an anti-apoptotic gene (Fig. 5).

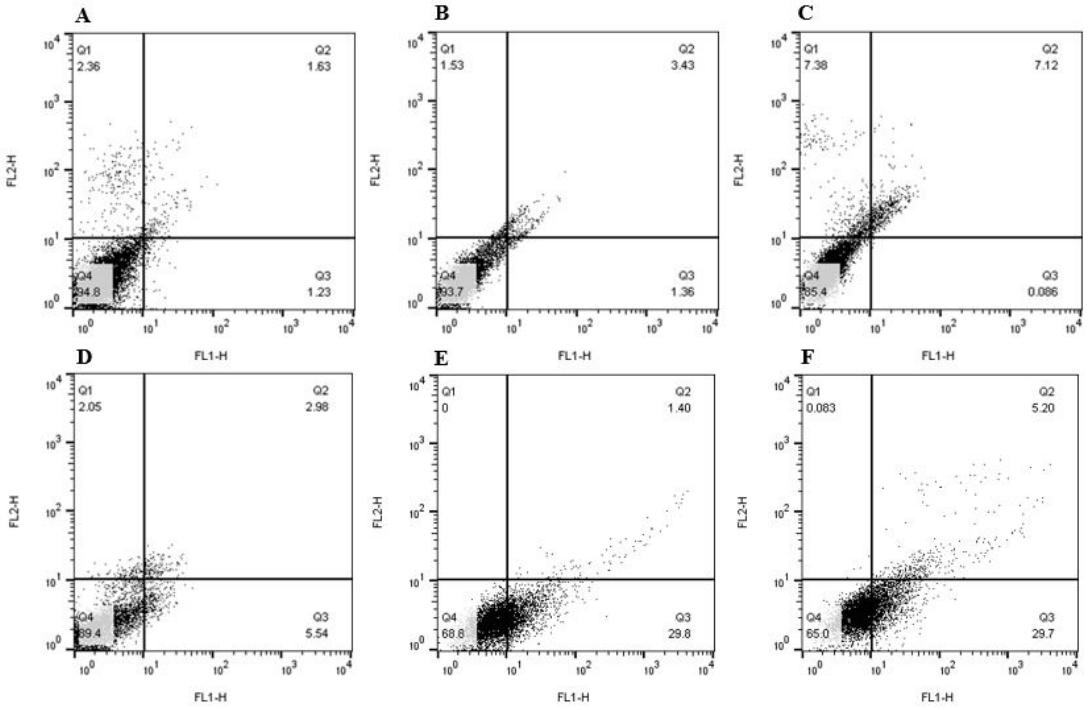

Fig. 3. The effects of pogostone on cell apoptosis which was determined by flow cytometry. Treatment with 90 µg/mL pogostone for 24 and 48 h, significantly induced apoptosis in OVCAR-3 cells compared with the DMSO treated cells. (A, B) Cells without treatment with any substance in 24 and 48 h, respectively; (C, D) cells treated with DMSO, as the solvent of pogostone, for 24 and 48, respectively; (E, F) cells treated with 90 µg/mL pogostone for 24 and 48 h, respectively.

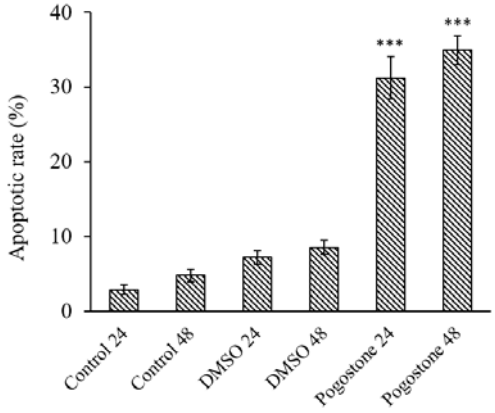

Fig. 4. The effect of pogostone on the apoptotic cell in different groups evaluated by flow cytometry. ***$P < 0.001$ Indicates significant differences compared to DMSO 24 and $^{###}P < 0.001$ versus DMSO 48.

The results also showed that treatment with pogostone led to increasing the expression of *CASP* genes (*3*, *8*, and *9*), which are involved in intrinsic and extrinsic apoptotic pathways (Fig. 5).

CASP3 activity assay

The protein expression of CASP3 in the OVCAR-3 cells were measured to ensure cell death *via* apoptosis. The CASP colorimetric assay indicated that the protein expression levels of CASP3 in the pogostone-treated groups increased significantly compared with the DMSO-treated group. However, the difference between the pogostone-treated groups was not significant at different times (Fig. 6).

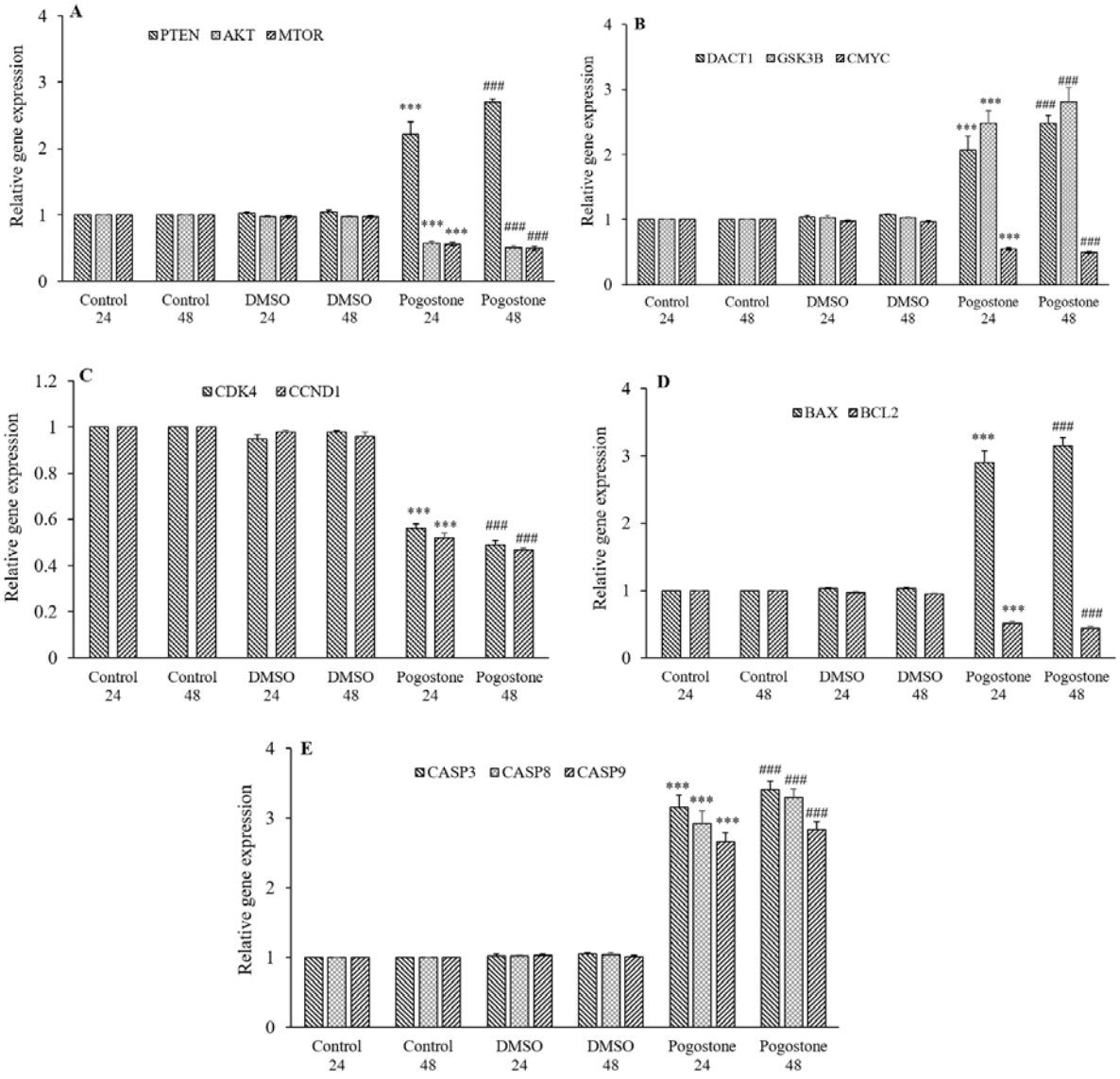

Fig. 5. The effects of pogostone on the expression level of genes involved in the signaling pathway, cell cycle, and apoptosis process evaluated by real-time polymerase chain reaction. (A) The expression level of genes involved in phosphoinositide 3-kinases (PI3K)/AKT/mTOR signaling pathway (PTEN, AKT, and mTOR) in different groups; (B) the expression level of genes involved in Wnt/β-catenin signaling pathway (DACT1, GSK3B, and C-MYC) in different groups; (C) the expression level of genes involved in the cell cycle (CDK4 and CCND1) in different groups; (D) the expression level of pro- and anti-apoptotic genes (BAX and BCL2) in different groups; (E) the expression level of genes involved in the apoptotic process (CASP3, CASP8, and CASP9) in different groups. ***$P < 0.001$ Indicates significant differences compared to DMSO 24 group and $^{###}P < 0.001$ versus DMSO 48 group. PTEN, phosphatase, and tensin homologue deleted on chromosome ten; AKT, protein kinase B; mTOR, mammalian target of rapamycin; DACT1, Dapper antagonist of catenin-1; CDK4, cyclin-dependent kinase 4; CCND1, cyclin D1; CASP, caspase.

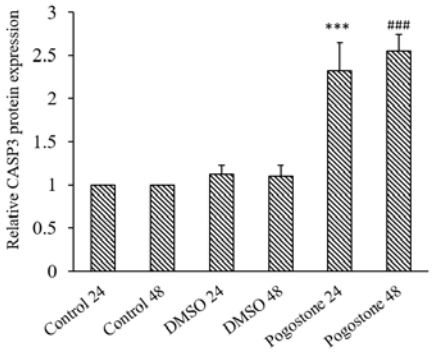

Fig. 6. Protein expression of caspase3 in different groups evaluated by caspase colorimetric. ***$P < 0.001$ Indicates significant differences compared to DMSO 24 and $^{###}P < 0.001$ versus DMSO 48.

DISCUSSION

Since most cancers are characterized by the overexpression of oncogenes and/or the inactivation of tumor suppressor genes, implementing genetic and epigenetic modifications through introducing new ways to suppress/express the driver genes alongside the induction of apoptosis could be considered as a novel strategy to treat several types of cancer, including ovarian malignancies (5,6).

The current study evaluated the antiproliferative and anti-apoptotic activities of pogostone on the drug-resistant ovarian cancer cell line OVCAR-3. For this purpose, the effects of pogostone on the expression of *PTEN* and *DACT* tumor suppressor genes and the association of their expression with the degree of activation of intracellular signaling pathways, cell cycle progression, cell proliferation, and cell apoptosis were investigated. One of the intracellular signaling pathways that play an important role in the regulation of cell survival, growth, and proliferation is the PI3K/AKT/mTOR pathway (12). Dysregulation of this pathway has been reported previously in human disorders, especially in ovarian cancer and related malignancies (26).

Pogostone is a natural substance isolated from *Pogostemon cablin*, which possesses various pharmacological properties such as antibacterial, antifungal, anti-inflammatory, and immunosuppressive activities (15). *PTEN* is considered as one of the tumor suppressor elements in the human genome and the loss of function of this gene occurs in some cancers (13,27). In the present study, it was shown that pogostone decreased the functionality of the PI3k/AKT pathway *via* increasing the phosphorylation processes through the higher expression of *PTEN*, which resulted in the decrease of cell growth and the increase of pre-apoptotic factors.

Takei *et al.* suggested that the overexpression of *PTEN* in ovarian cancer cells suppressed the growth of the tumor and also prolonged the survival time in mice with peritoneal disseminated tumors (14). Russo *et al.* demonstrated that PTEN loss in the fallopian tube induced hyperplasia and ovarian tumor tissue formation (28). Saito *et al.* showed that PTEN played a principal role in the development of ovarian tumors (29).

The results of the present work showed that pogostone led to the induction of *PTEN* expression as well as the reduction of the levels of *AKT* and *MTOR* genes, which are the components of the PI3K/AKT/mTOR signaling pathway in ovarian cancer cells. The decrease in cell growth and proliferation can be attributed to the overexpression of *PTEN* and its inhibitory effect on the expression of *AKT* and *MTOR* genes. This may explain the significant reduction of cell proliferation observed after treating cells with pogostone, as proved by the MTT assay.

The *AKT* controls the regulation of various cellular functions as well as apoptosis, cell cycle, cell growth, metabolism, and transcription. Following activation, *AKT* directly triggers *mTORC1*. mTOR is a serine-threonine protein kinase that can influence the cell cycle through the passage from G1 to the S phase (30,31).

DACT1 is another tumor suppressor gene that has an essential role in the apoptosis and proliferation of ovarian cancer cells. *DACT1* regulates the cell cycle and inhibits cancer cell growth by decreasing the nuclear β-catenin levels. The molecule also affects the Wnt signaling pathway. It has been shown that aberrant activation of the Wnt/β-catenin pathway in ovarian cancer leads to the hyperactivity of β-catenin (32).

Li *et al*. suggested that *DACT1* inhibited Wnt signaling and active autophagy in type one epithelial ovarian cancer (EOC) (5).

C-MYC and *CCND1* are the main downstream target genes of the Wnt/β-catenin signaling pathway and may impact the biological behaviors of cancer cells, such as the cell cycle, proliferation, and apoptosis. It has been suggested for a long time that *C-MYC* amplification is a common finding in the advanced stages of ovarian cancer (33,34). Recently, it has also been proven that the *MYC* status is a determinant of the synergetic drug response in ovarian cancer (35).

The results of the present study revealed that treatment of OVCAR-3 ovarian cancer cells with pogostone led to the upregulation of *DACT1* and *GSK3B* and the downregulation of the *C-MYC* genes. This may reflect the inductive effects of pogostone on the Wnt/β-catenin signaling pathway and its subsequent influences on *C-MYC* expression. The *CDK4* gene is also an important factor for a successful cell cycle. Activation of its upstream mitogenic pathways, including PI3K/AKT/mTOR can enhance the *CDK4* activity. Studies have reported that *CCND1-CDK4/6* is a prerequisite factor for maintaining the tumorigenic potential of breast cancer cells (35).

Treatment of two cell lines of colorectal cancer with pogostone led to the increase of *P21* expression and inhibition of *CCND1* and *CDK4* expressions, resulting in an increased level of apoptosis and decreased cell growth and proliferation. Most likely, the slowdown in the growth and proliferation of cancer cells is due to the inhibition of the *C-MYC* expression and the apoptotic activity resulting from a decrease in HDAC2 (histone deacetylase 2). The results of the present study showed that 24 and 48 h after treatment with pogostone, the expression levels of *CCND1* as well as *CDK4* in ovarian cancer cells decreased.

Inactivation of *PTEN* and *DACT1* tumor suppressor genes due to the genetic and epigenetic changes is very common in the progression of some cancers, especially ovarian cancer. Thus, eliminating these negative changes and motivating the expression of these genes can inhibit the growth of ovarian cancer cells, stop their cell cycle in the G1 phase, and induce the apoptotic process (5,6).

Apoptosis induction is now considered as an effective way for cancer chemotherapy and can also be a good indicator for cancer treatment and prevention (15). Various natural compounds have been shown to suppress the growth of tumor cells by inducing apoptosis (36).

Safarzadeh *et al*. reported that herbal extract, through the induction of apoptosis, killed the cancerous cell population with minimal side effects on normal cells (4).

However, the present findings suggested that pogostone as a natural compound shows antiproliferative and anti-apoptotic effects, which are indicated by the annexin V assay and flow cytometry. The apoptotic rate after 24 and 48 h of treatment with pogostone increased in OVCAR-3 cells.

Another study reported that gallic acid, a natural phenolic compound isolated from fruits and vegetables, had a more potent growth-inhibitory effect on two ovarian cancer cell lines OVCAR-3 and A2780/CP70 (37). Cao *et al.* also demonstrated that pogostone has anti-colorectal tumor effects by inducing autophagy and apoptosis *via* the PI3K/AKT/mTOR pathway (15).

In the present study, we investigated the expression of *CASP8* and *CASP9*, which are required for the initiation of apoptosis through the extrinsic/intrinsic apoptotic pathways and *CASP3* that is activated in both extrinsic and intrinsic (mitochondrial) pathways.

Tsai *et al*. reported that pogostone induced apoptosis through the intrinsic pathway, which is related to mitochondrial dysfunction. In addition, this occurs through the activation of caspases. The results of their study indicated that pogostone may delay cancer cell growth by inducing apoptosis *via* the upregulation of the expression of BCL-associated athanogene 3 (*BAG3*), *CASP4*, and *CASP5* genes. Moreover, their findings indicated that pogostone significantly reduced the mitochondrial membrane potential in Ishikawa cells. In addition, they reported the increased activation of *CASP3* in Ishikawa cells following pogostone treatment for 24 h (8). Another study found that pogostone activated the mitochondrial apoptotic pathway by increasing *CASP9* and *CASP3* expression (22). As shown in this study, pogostone induced apoptosis

through the significant overexpression of *CASP8, 9,* and *3* genes, which was observed 24 and 48 h after the treatment with pogostone, compared with the control group. In addition, significant increases in the CASP3 protein showed that pogostone certainly induced apoptosis.

It has been proven that pogostone by activating *CASP3*, *CASP8*, and *CASP9* increased the apoptosis of cancer cells. The antioxidation and antimutagenesis properties of pogostone were also reported (8,15). It can be concluded that pogostone exerts its apoptotic effect on OVCAR-3 cells by activating both intrinsic and extrinsic pathways. The *BCL2* gene encodes a 26-kDa protein that prevents programmed cell death without affecting cellular proliferation, but the BAX protein, which is a member of the BCL2 family, promotes apoptosis (38).

Niu *et al*. found that enhancement of the BAX/BCL2 ratio led to the initiation of the mitochondrial pathway of apoptosis (35). The findings of the current study showed that pogostone treatment significantly increased the expression of *BAX* and decreased the level of *BCL2*, so the ratio of *BAX/BCL2* was increased, which indicated the following apoptosis process.

In line with the present results, in another study pogostone inhibited the expression of *BCL2*, but had little effect on the level of *BAX*, which led to a decreased ratio of *BCL2/B*AX in human lung cancer A549 (22). Wang *et al.* showed that the small-molecule inhibitor of Bcl-2 (TW-37) suppressed growth and enhanced cisplatin-induced apoptosis in ovarian cancer cells (39).

CONCLUSION

The present results indicated that treatment of OVCAR-3 with pogostone depressed cell growth and proliferation and induced apoptosis in the cancer cells. The antiproliferation effect of pogostone may be due to provoking the augmentation of the *PTEN* gene expression and its subsequent effects on inhibition of PI3K/AKT/mTOR pathway and increasing the expression of *DACT1* and its subsequent effects on the Wnt/β-catenin signaling pathway. Moreover, pogostone may apply its apoptotic effects on ovarian cancer cells by promoting both intrinsic and extrinsic apoptotic pathways.

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Conflict of interest statement

The authors declared no conflict of interest in this study.

Authors' contribution

M. Homayoun contributed to doing experiments, data analysis, and article writing; N. Sajedi contributed to the analysis of the resulted data; R. Ghasemnezhad contributed revising the article; M. Soleimani contributed to the conception and revision of the article. The final version of the manuscript was approved by all authors.

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