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ORIGINAL ARTICLE

Naturally occurring davanone exhibits anticancer potential against ovarian cancer cells by inducing programmed cell death, suppression of cell migration and invasion and modulation of PI3K/AKT/MAPK signaling pathway

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Summary

Purpose: *The current study was undertaken to examine the anticancer potential of davanone against human ovarian cancer cells along with evaluating its effects on cell apoptosis, PI3K/AKT/MAPK signaling pathway and cell migration and invasion.*

Methods: *CCK-8 assay was performed for cell viability and clonogenic potential was examined through clonogenic assay. Acridine orange (AO)/Ethidium bromide (EB) dual staining assay was performed to detect apoptosis and quantification of apoptosis was achieved through annexin V-FITC/propidium iodide (PI) staining assay. Mitochondrial membrane potential (MMP) was studied via flow cytometric analysis of ovarian cancer cells. Cell migration and invasion potential of ovarian cancer cells was monitored via transwell assay. Western blotting technique was used to study PI3K/AKT/MAPK pathway.*

Results: *The results indicated that davanone induced dose as well as time dependent inhibition in cell viability of OVACAR-3 cells. Next, AO/EB staining suggested that the antiproliferative effects of davanone are apoptosis-mediated. There was a remarkable increase in apoptotic cell percentage with the molecule dose. Caspase-3, -8 and -9 activity along with Bax activity were observed to be increasing with davanone doses and Bcl-2 activity decreased with increasing molecule concentration. Transwell assay indicated potential inhibition of invasive and migratory ability of OVACAR-3 cells after davanone exposure. Finally, western blotting analysis revealed that davanone resulted in blocking of PI3K/AKT/MAPK signaling pathway in OVACAR-3 cells.*

Conclusions: *The results indicate that davanone is a potential anticancer agent against human ovarian cancer mediated via caspase-dependent apoptosis, loss of MMP, inhibition of cell migration and invasion and targeting PI3K/AKT/MAPK signaling pathway.*

Key words: *davanone, ovarian cancer, apoptosis, cell migration, flow cytometry*

Introduction

Terpenoids are the largest class of naturally occurring chemical entities with nearly 25,000 different chemical structures [1]. This huge structural diversity leads to their significant practical applications including chemical, pharmaceutical, flavor and fragrance industries [2,3]. Due to huge diversity in structure terpenoids are subdivided into various subclasses like tetraterpenoids, triterpenoids, diterpenoids, sesquiterpenoids, and monoterpenoids [4,5]. Traditional Chinese medicine used for thousands of years by the people found different terpenoids that serve their medicinal po-

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tency. As the cancer incidence and anticancer drug resistance increase day by day, the research and development are diverted to find novel potential anticancer drugs. This leads to the identification of various terpenoids with immense potential as anticancer drugs such as limonene, artemisinin, triptolide and celastrol [6-11]. Ovarian cancer (OC) is a gynecological malignancy and a leading cause of cancer mortality among women globally [12,13]. OC's lethality enhances due to late diagnosis, as upon diagnosis the cancer already has spread to the maximum parts of ovary/and beyond [14]. OC has a very low 5-year survival rate (<30%) mainly due to late diagnosis and ineffectiveness of available treatment options. Currently used treatment options for OC include platinum-based chemotherapy and surgery [15,16]. Platinum-based therapy still lacks effectiveness as nearly 20% of the total OC patients do not respond and hence relapse. OC metastasis, side-effects of current chemotherapeutics and drug-resistance are the biggest hurdles in OC treatment [17,18]. Thus, the underlying mechanism of OC development should be understood for the development of novel therapeutic targets. *Artemisia aucheri* Boiss (Asteraceae), also known as "Dermaneye koohi" in Persian, is a frequently growing species found in Iran and Himalayan region of India. Aerial parts of the plant have been reported with the presence of different oxygenated monoterpenoids. Davanone molecule is a sesquiterpene and is found in major quantity in the aerial parts of the herb. This herb has been reported with several medicinal activities including antimicrobial, antifungal, acaricidal, antioxidant and antimalarial [19-21].

The current study was planned to unveil the anticancer effects of one of the major constituents of the *Artemisia aucheri,* that is davanone molecule, against human OC, along with studying Caspase-dependent apoptosis, cell migration and invasion, MMP and PI3K/AKT/MAPK signaling pathway.

Methods

Cell cytotoxicity of davanone

OC OVACAR-3 cell line viability was estimated by performing CCK-8 assay. Briefly, cancer cells with a density of 1×10^3 cells/well were seeded in 96-well plates and harvested at 70% confluence. After that, cells were exposed to changing davanone concentrations (0, 10, 20, 40, and 80 µM) for 24h and 72h. Drug exposure was followed by phosphate buffered saline (PBS) washing and reculturing for 72h. Ten µl of CCK-8 solution with a final concentration of 0.5 mg/ml (Sigma-Aldrich, St.Louis, MO, USA) were supplied to each well with incubation for 1h. Thereafter, a microplate auto reader (BioTek instruments, Inc., Winooski, United States) was used to measure absorbance at 490nm for optical density (OD) calculations.

Clonogenic potential estimation assay

The ability of OC OVACAR-3 cells to form colonies was checked by performing clonogenic assay. After the ovarian cancer cells were pre-treated with davanone molecule at changing concentrations (0, 10, 40, and 80 µM), for 24 h, 2,000 cells/well were plated in 6-well plates. Plates were incubated at 37°C in a humidified CO_2 (5%) incubator for 14 days. RPPI-1640 culture medium was used which was replaced every 4 days. Plates containing davanone-treated cells were washed with ice-cold PBS and then fixed by using paraformaldehyde (4%). Afterwards, cell colonies were stained with crystal violet for about 15 min at 25°C and finally washed with distilled water. Each well plate was dehydrated in air and the number of OC cell colonies were counted under a light microscope.

Acridine orange/ethidium bromide (AO/EB) dual staining assay

The OVACAR-3 OC cells were cultured in 96-well plates followed by davanone treatment at different concentrations (0, 10, 40, and 80 µM). Afterwards, trypsinisation of cells was performed with 20 µl of trypsin and when the cells start to slough off, about 25 µl of suspensions were transferred to glass slides. Each cell suspension was supplemented with 100 µg/ml AO and 100 µg/ml EB (dual fluorescent staining solution) and then covered with coverslips. Finally, using a fluorescent microscope (Olympus, Japan) 500 cells were counted within 20 min and cellular morphology was examined.

Apoptosis and mitochondrial membrane potential ($\Delta\Psi m$) assessment

Following davanone treatment at varying doses (0, 10, 40, and 80 µM), OC cells were harvested after 24h. Harvested OC OVACAR-3 cells were then washed with PBS. Annexin V-FITC/PI staining assay was performed with apoptosis detection kit (Invitrogen, Carlsbad, CA, United States) comprising of annexin V/FITC and PI following the manufacturer's instructions. The MMP was estimated through staining with JC-1 staining. All the data was recorded with flow cytometric analysis through FACSCalibur flow cytometer (Becton-Dickinson, San Jose, USA). Individual experiments were repeated thrice.

Caspase-3, -8 and -9 expression evaluation

OVACAR-3 OC cells were treated with varying davanone doses (0, 10, 40, and 80 µM) for 24h followed by isolation of cellular extracts. Human caspase-3, -8 and -9 active ELISA kit (Invitrogen, Grand Island, NY, USA) was used to determine the caspase-3 and -9 levels strictly obeying the manufacturer's protocol. Microplate Autoreader with 450nm wavelength was used for OD calculations.

Transwell assay for cell migration and invasion

OC OVACAR-3 cells were transfected with davanone at changing doses (0, 10, 40, and 80 µM) for 48h and then transfected cells were collected by using RPMI-1640

culture medium deprived of serum. Upper and lower chambers of transwell were filled differently. The upper chambers were filled with 150 µl OC cell suspension containing 2.5×10^5 cells and the lower chambers were filled with 10% FBS in 600 µl of culture media (Corning Incorporated, Corning, NY, USA). Membranes (Corning) were then incubated for about 12h and then fixation was performed in methanol at 4°C for 10 min. Next, migratory cells were stained with crystal violet for 5 min at room temperature. Finally, the results were observed and photographed under an inverted light microscope with a magnification power of 200× (TS100; Nikon Corporation, Tokyo, Japan).

For cell invasion potential determination of ovarian cancer cells the same procedure was followed except transwell chambers were pre-coated with Matrigel for 6h.

Western blotting assay for monitoring of PI3K/AKT/MAPK signaling pathway

OC cells were harvested at 85% confluence after treatment with varying davanone doses (0, 10, 40, and 80 µM), for 24h. Complete cellular extracts were organized with RIPA lysis buffer comprising of protease inhibitors. Bicinchoninic acid (BCA) assay was performed to isolate proteins from each lysate and about 40 µg protein from each sample were subjected to separation via SDS-PAGE (12%) followed by transferal to nitrocellulose membranes. Trisbuffered saline (TBS) containing skimmed milk (5%) was used for blocking of membranes at room temperature for 1h. Blocking of the membranes was followed by primary antibody treatment (those antibodies which recognize Bax, Bcl2, PI3K, AKT and MAPK) overnight at 4°C. Next, secondary antibody (coupled with horseradish peroxidase) treatment was given to the membranes at room temperature for 1h. Electron chemiluminescence (ECL) detection system (Amersham, Piscataway, NJ, USA) was used to observe protein signals.

Statistics

All the experimental data were analyzed through SPSS 13.0 software for windows. The data was presented as mean±SD and the differences in mean were analyzed using one-way ANOVA. Experiments performed were repeated thrice. Statistical significance was set at $p<0.05$.

Results

Effect on cell proliferation rate of OVACAR-3 cells by davanone treatment

The effects of davanone (Figure 1) on cell proliferation rate of OVACAR-3 cells were estimated via CCK-8 proliferation assay. The results from CCK-8 assay indicated effective time- as well as concentration-dependent suppression in cell proliferation rate. Untreated control cells were taken as 100% viable cells. The viable cell percentage at 10, 20, 40, and 80 µM was recorded as nearly 90%, 70%, 45% and 15%, respectively after 24h of drug treatment. After 72h of exposure, under the same concentrations, the OVACAR-3 cell percent viability was 70%, 50%, 20% and 5% (Figure 2).

Inhibition of OVACAR-3 cell colonies by davanone

Clonogenic analysis was performed for evaluation of the effects of davanone on OVACAR-3 cell colonies. The number of colonies in controls were nearly 275. On drug exposure the number of cell colonies reduced dramatically. At concentrations of 10, 40, and 80 µM the number of cell colonies

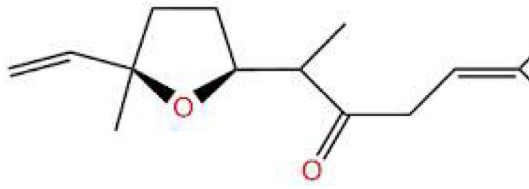

Figure 1. Chemical structure of davanone molecule.

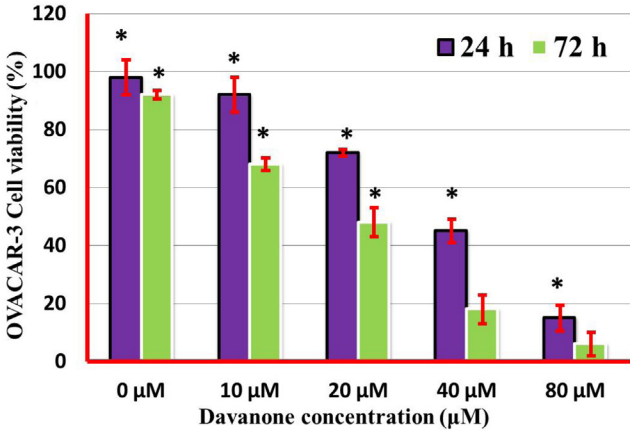

Figure 2. Results from CCK-8 assay presenting cell viability percentage after davanone treatment of OVACAR-3 cells at indicated doses and times. The experiments performed were repeated thrice (*p<0.05).

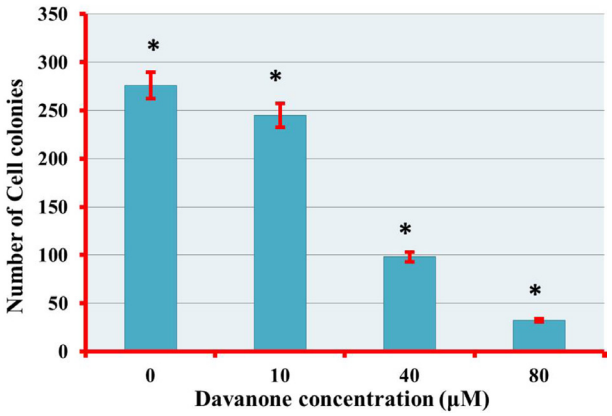

Figure 3. Graphical presentation of the number of cell colonies after davanone treatment of OVACAR-3 cells. The experiments performed were repeated thrice (*p<0.05).

calculated were 250, 100 and 30 (Figure 3). Thus the results from clonogenic assay revealed dose-dependent inhibition in colony formation potential of OVACAR-3 cells.

Davanone induced apoptosis in OVACAR-3 cells

Different assays were performed to check the apoptosis inducing potential of davanone in OVACAR-3 OC cells. AO/EB staining assay was performed to check the status of apoptosis in target cells. The results showed that the negative control cells showed no sign of apoptosis. But on drug exposure early apoptotic cells could be seen with crescent-shaped yellow-green AO fluorescence, late apoptotic cells with disproportionately situated orange EB fluorescence and necrotic cells with irregular orange-red fluorescence situated at their periphery (Figure 4). Next, as evidenced from the AO/EB staining, the anti-proliferative effects might be due to apoptosis induction, therefore it was quantified by annexin V-FITC/PI staining assay. The apoptotic cell percentage was observed as annexin V+/PI- and annexin V+/PI+. Minimum number of apoptotic cells were observed in controls. After drug exposure apoptotic cell percentage in case of annexin V+/PI- started to increase from about 12% to 22% on higher drug doses. As indicated by annexin V+/PI+ apoptotic cell percentage also increased from 6% to 25% with increasing drug doses (Figure 5).

Davanone led to loss of mitochondrial membrane potential (MMP) in OVACAR-3 cells

Flow cytometry was performed for MMP assessment of ovarian cancer cells after davanone exposure. The results revealed tremendous dose-dependent loss in MMP after drug exposure of target cells. The MMP observed at 0, 10, 40, and 80 µM was nearly 100%, 80%, 40% and 20% (Figure 6), suggesting dose-dependent MMP loss in OVACAR-3 cells by davanone exposure.

Davanone induced caspase-dependent apoptosis in OVACAR-3 cells

Caspase activity plays a pivotal role in apoptosis. Davanone treatment of OVACAR-3 cells resulted in enhancing the activity of caspase-3, -8 and -9 in a dose-dependent manner. Along with that, the expressions of Bax protein was increased and Bcl-2 protein decreased (Figure 7), clearly evidencing that davanone induced apoptotic cell death in OVACAR-3 OC cells.

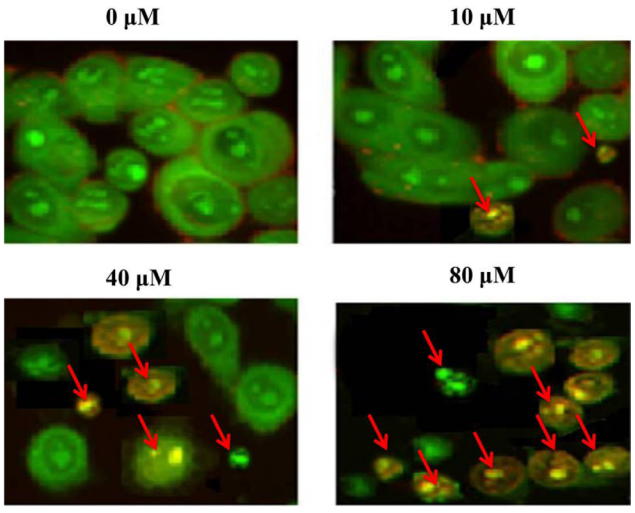

Figure 4. AO/EB staining assay results showing early, late apoptotic and necrotic cells on davanone exposure of OVACAR-3 cells. The arrows show nuclear damage, indicating apoptotic cell death in OVCAR-3 cell post-davanone exposure. The experiments performed were repeated thrice.

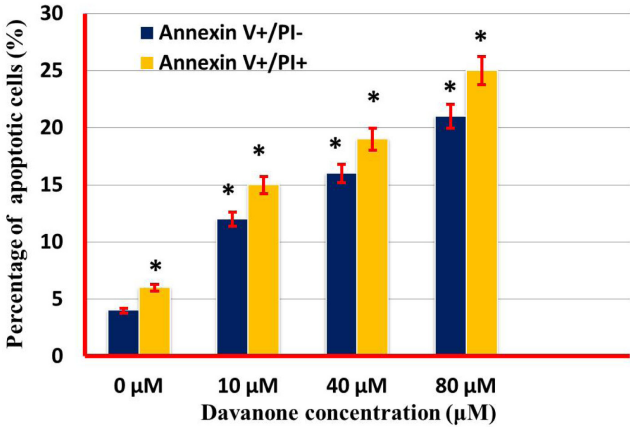

Figure 5. Results presenting early and late apoptotic cells after annexin V-FITC/PI staining. The experiments were repeated thrice (*p<0.05).

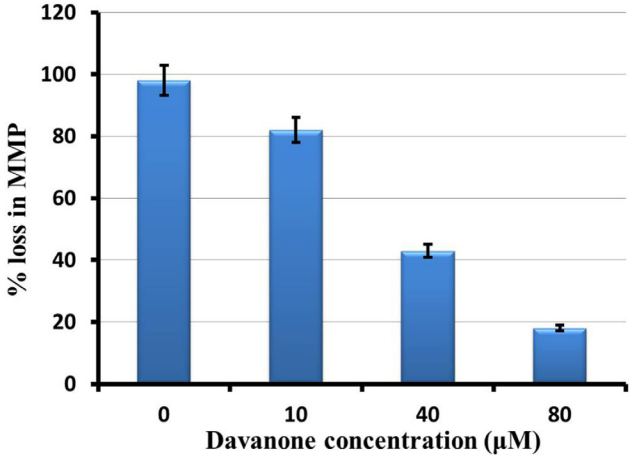

Figure 6. Flow cytometric analysis of OVACAR-3 cells after davanone exposure at indicated doses depicting loss in MMP. The experiments performed were repeated thrice (p<0.05).

Davanone suppressed migration and invasion of OVACAR-3 cells

Transwell assay was carried out to monitor the migration and invasion ability of OVACAR-3 cells. The results revealed that the migratory potential was suppressed in a dose-dependently manner (Figure 8). It was also observed that the number of invasive cells decreased with increasing drug doses (Figure 9). Thus, the results from transwell assay indicate that both migration and invasion of OVACAR-3 cells was reduced on test drug exposure.

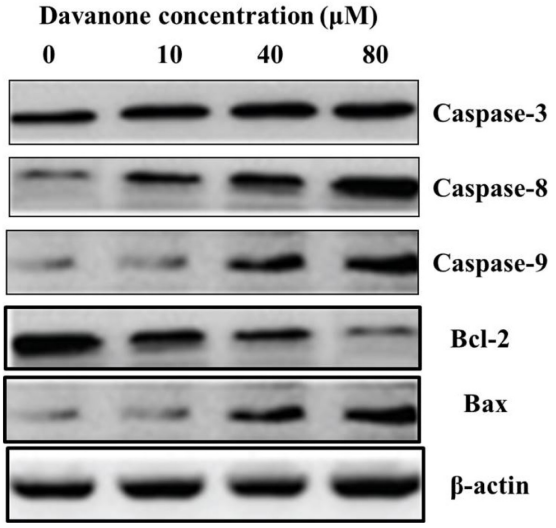

Figure 7. Results presenting activity of proapoptotic and antiapoptotic proteins at presented doses. The Figure shows increased expressions of proapoptotic caspases and Bax proteins and a sharp decrease in antiapoptotic Bcl-2 expression on davanone exposure. The experiments were repeated thrice.

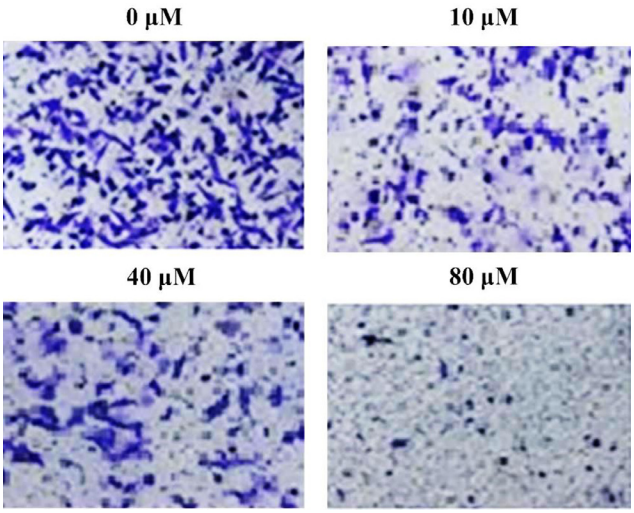

Figure 8. Transwell assay results showing suppressed cell migration in OVACAR-3 cells after increasing davanone dosage. The experiments repeated thrice.

Davanone targeted PI3K/AKT/MAPK signaling pathway

The impact of davanone treatment on PI3K/AKT/MAPK signaling pathway in OVACAR-3 OC cells was checked by western blotting assay. The results revealed that the levels of PI3k, AKT and MAPK remained almost the same on lower as well

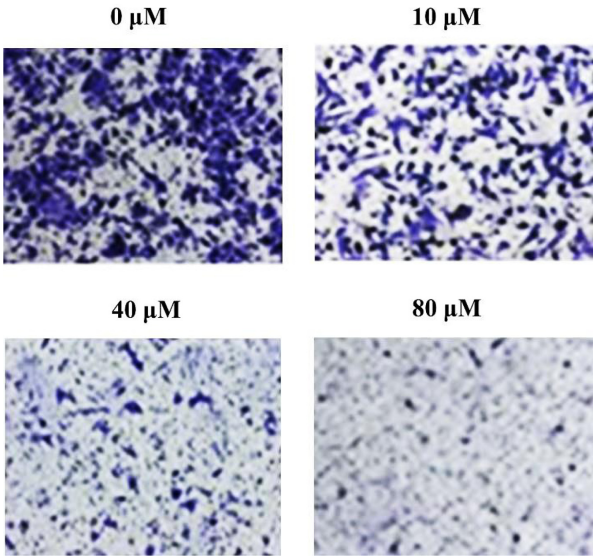

Figure 9. Transwell assay results showing suppressed cell invasion in OVACAR-3 cells with increasing davanone exposure. The experiments were performed in triplicate.

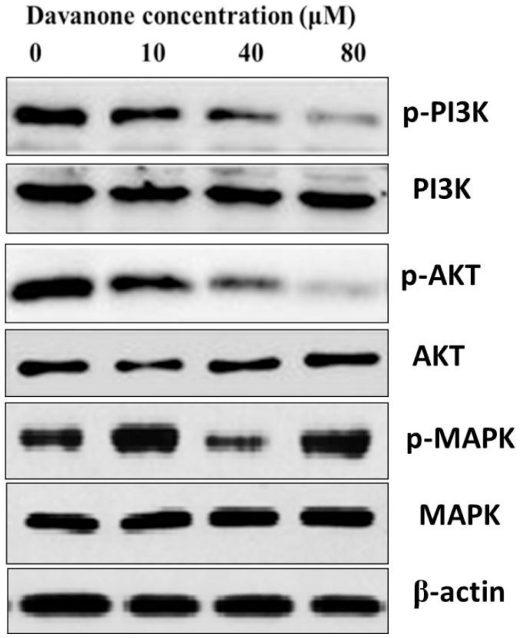

Figure 10. Western blotting analysis presenting the expressions of PI3K/AKT/MAPK signaling pathway-related proteins in davanone-treated OVACAR-3 cells. The Figure shows decreased expression of p-PI3K, p-AKT and p-MAPK, while overall expression of non-phosphorylated PI3K, AKT and MAPK remained constant. The experiments were performed in triplicate.

as higher molecule doses. The expressions of phosphorylated- PI3k, and -AKT significantly reduced and p–MAPK increased only by a small margin, suggesting that davanone treatment blocked PI3K/AKT/MAPK signaling pathway in OVACAR-3 OC cells.

Discussion

Cell differentiation and proliferation, cytoskeleton rearrangement and apoptosis are activated through various growth factors (proteins) which bind to cell surface and control several cell processes. Different growth factor signaling molecules have been found associated with carcinogenesis, including FGF, platelet-derived growth factor, endothelial growth factor, transforming growth factor, colony-stimulating factor and insulin-like growth factor [22]. Various downstream intracellular pathway signals get activated as a consequence of activation of growth factor receptors like Ras-MAPK and PI3K-Akt. These intracellular signalling pathways play a crucial role and are accountable for tumor progression, poor prognosis and serving targets for various chemotherapeutic and chemopreventive agents. In the current study, the effect of naturally occurring davanone sesquiterpene was examined over PI3K/AKT/MAPK signaling pathway in OC cells. Along with apoptosis, MMP, cell migration and invasion were also examined. Cell proliferation was examined by CCK-8 assay and the results revealed tremendous concentration as well as time-dependent inhibition of the viability of OVACAR-3 OC cells. Davanone also exhibited anti-cell colony effects on OVACAR-3 OC cells in a dose-dependent manner. Furthermore, the anti-cell proliferation effects were checked for their apoptosis mediation through AO/EB staining assay and the results showed the formation of necrotic, early and late apoptotic cells with increasing numbers as the molecule doses increased. Apoptosis was quantified by annexin V-FITC/PI staining assay in OVACAR-3 cells after drug treatment which also indicated remarkable rise in the number of early and late apoptotic cells with increasing drug doses. Next, MMP was calculated by flow cytometric analysis and showed dose-dependent loss in MMP of OVACAR-3 cells after davanone treatment. Caspase activity along with Bax and Bcl-2 activity were monitored and the results suggested that proapoptotic protein levels enhanced and antiapoptotic proteins reduced remarkably with increasing davanone doses, strengthening the fact that davanone induced apoptosis in OVACAR-3 cells. Transwell assay for migration and invasion revealed that both were also suppressed by davanone in a dose-dependent-manner in OVACAR-3 cells. Finally, the impact of davanone on PI3K/AKT/MAPK signaling pathway of OVACAR-3 cells was assessed through western blotting assay which showed dose-dependent blocking of PI3K/AKT/MAPK signaling pathway by the molecule.

Conclusion

The above results indicate that davanone terpenoid inhibited strongly cisplatin resistant OC cell growth via induction of caspase-dependent apoptosis, loss of MMP, inhibition of cell migration and invasion, and targeting the PI3K/AKT/MAPK signaling pathway. Hence, davanone is a potent anticancer molecule against human OC cells and can be introduced to the therapy of this disease provided further investigations are performed.

Conflict of interests

The authors declare no conflict of interests.

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