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ORIGINAL ARTICLE

Anticancer activity of ursolic acid on human ovarian cancer cells via ROS and MMP mediated apoptosis, cell cycle arrest and downregulation of PI3K/AKT pathway

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Summary

Purpose: *Ovarian cancer (OC) is perhaps the most difficult problem in gynaecologic oncology; in particular the drug-resistant ovarian cancer remains a challenge for the clinicians. Therefore there is a pressing need for novel and effective chemotherapeutic agents against OC. The main objective of the current research work was to study the anticancer effects of a naturally occurring triterpene acid, ursolic acid, against SKOV-3 OC cells. Its effects on reactive oxygen species (ROS)-mediated apoptosis were also studied along with cell cycle phase distribution and PI3K/AKT signalling pathway.*

Methods: *Cell proliferation was checked by CCK8 cell viability assay. Apoptosis-related studies were examined by fluorescent microscopy using acridine orange (AO)/ethidium bromide (EB) and DAPI staining as well as flow cytometry using annexin V/propidium iodide (PI) assay. Further, western blot assay was used to study effects of ursolic acid on the apoptosis-related protein expressions including Bax, Bcl-2 as well as PI3K/AKT signalling pathway. Effects on cell cycle were examined by flow cytometry while effects on ROS production were evaluated by fluorescent microscopy.*

Results: *Ursolic acid caused significant reduction in the viability of the SKOV-3 ovarian carcinoma cells in a dose-dependent manner, exhibiting an IC_{50} of 35 μM in cancer cells and IC_{50} of 75 μM in normal cell lines (normal ovarian surface epithelial (OSE). Ursolic acid inhibited the viability of cancer cells via induction of apoptotic cell death which was associated with increase in Bax and decrease in Bcl-2 levels. DAPI staining results also confirmed that ursolic acid induced apoptotic cell death. Ursolic acid also induced dose-dependent G2/M phase cell cycle arrest along with causing significant upsurge in ROS production. Western blot analysis revealed that ursolic acid had the potential to inhibit I3K/AKT signalling pathway.*

Conclusion: *The results of this study clearly indicate that ursolic acid has the potential to be developed as a potent drug candidate against OC provided further in vivo and toxicological studies are carried out.*

Key words: *ursolic acid, ovarian cancer, apoptosis, cell cycle, flow cytometry*

Introduction

Ovarian carcinoma (OC) is a destructive malignancy related to women's reproductive system, with high morbidity and mortality [1,2]. There are three main types of OC (epithelial, germ cell and sex-cord stromal), accounting for 90%, 3% and 2% patients respectively [3]. The subtype epithelial OC is further subdivided into serous, endometrioid, mucinous and clear cell tumors (accounting for 52, 10, 6, and 6 % of patients respectively). The progression of epithelial OC development has mystified researchers for long. As the advancements made towards biological understanding regarding OC progressed, epithelial subtypes are gradually categorized as distinctive diseases with unlike risk

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factors, molecular pathways, and treatment [4-7]. Serous tumors are frequent prime serous carcinomas, involving both ovaries, late-stage diagnosis, aggressive behavior and minimum survival. Evidence from various studies suggests that serious tumors basically evolve in the epithelial cells of the fallopian tube as infinitesimal primary lesions that afterwards settle in ovaries and/or peritoneum [8,9]. Other subtypes of ovarian carcinoma usually affect only one ovary. Lethality of OC gets enhanced due to absence of symptoms, results in late diagnosis and hence leads to reduced 5-year survival rate (25 to 30%) [10]. In addition to high relapse rate, chemo-resistance also promotes high mortality and poor prognosis among these patients [11,12]. Therefore, novel and sophisticated investigations of therapeutic strategies and identification of new and effective agents for OC are immediately needed to improve the survival chances. Natural products act as a source of large number of active phytochemicals which have pharmacological importance [13,14]. Ursolic acid (UA) is a naturally occurring molecule belonging to triterpenoids (pentacyclic triterpenoid carboxylic acid), a potent anticancer agent. It has revealed various pharmacological activities including antitumorigenesis, prevention of tumor growth (especially breast carcinoma, colorectal carcinoma and cervical carcinoma) and suppression of angiogenesis [15-17]. Herein, the current study was performed to reveal the anticancer activity of ursolic acid on human OC cells via ROS-mediated apoptosis, cell cycle arrest and downregulation of PI3K/AKT pathway.

Methods

Cell proliferation/viability assay

Cell viability assay CCK-8 (Cell Counting Kit-8) was performed to detect cell viability of SKOV-3 cancer cells and normal ovarian cells (normal ovarian surface epithelial cells (OSE). Briefly, 96-well plates were used for seeding, at a concentration of 2×10^4 cells per well. After culturing at 37°C for 24 h, these cells were subjected to treatment with the test molecule at varying concentrations i.e. 0, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 and 200 µM for 48 h. Ten µL of CCK-8 were added to each well, and the plate was further incubated for 1 h at 37°C, and then absorbance at 450 nm was recorded with a microplate recorder at 630 and 540 nm wavelengths.

Apoptosis investigation by inverted microscopy and flow cytometry (DAPI and Annexin V-FITC/PI assay)

The human OC cell line SKOV-3 was seeded in 6-well plates for the determination of apoptosis. Target cells were incubated for 12 h and then exposed to varying concentrations of ursolic acid i.e. 0, 6, 12 and 24 µM at 37°C for 24 h. Later 20 µl of cell culture were

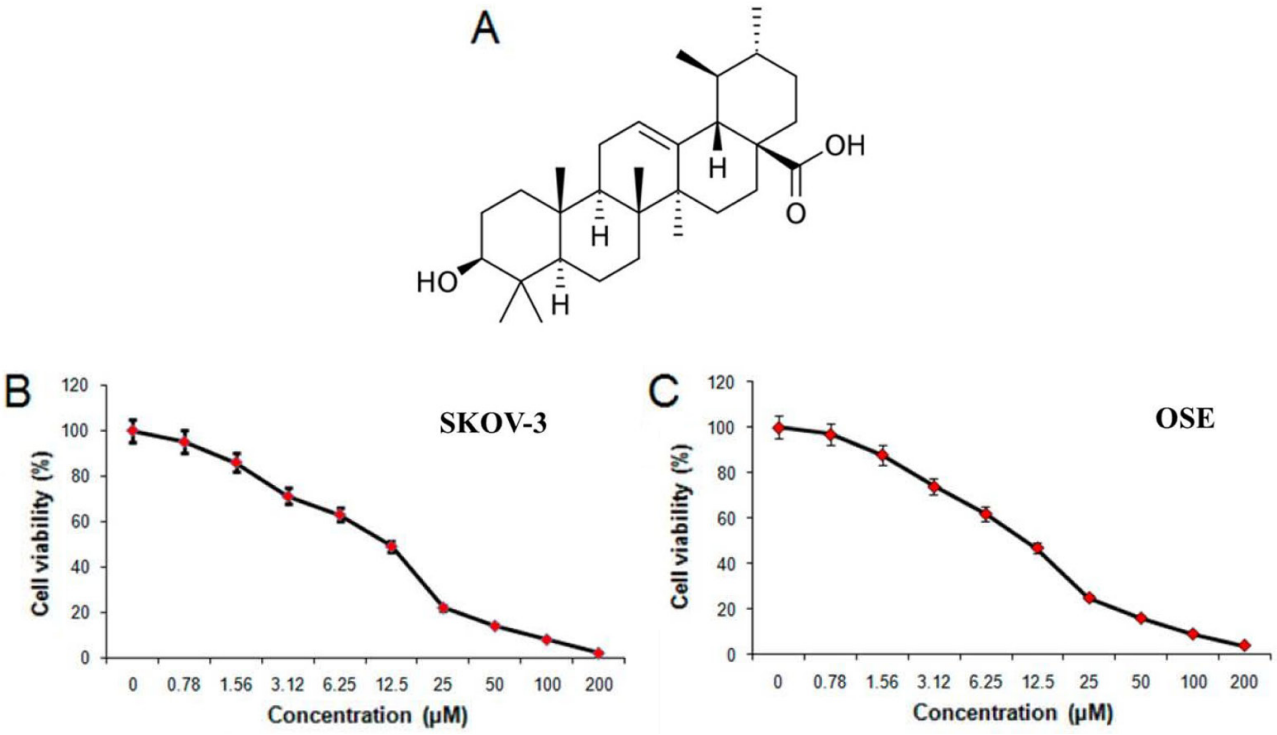

Figure 1. A: Chemical structure of ursolic acid. **B:** Ursolic acid supressed cell viability of SKOV-3 ovarian carcinoma cells as revealed by CCK-8 assay. The cells were subjected to varying concentrations of ursolic acid for 12h and then revealed a dose-dependent and selective inhibition in cell viability of these cancer cells, also showing less toxicity in normal ovarian surface epithelial cells (OSE). The data is the mean±SD of three independent experiments. $p<0.01$. C???

drawn out of the plate at the time cells started to cast-off, and placed on glass slides and staining of ursolic acid-treated cells was performed with DAPI. The slides were then cover-slipped and studied under fluorescent microscope.

Following the manufacturer's protocol (BD PharmingenTM, USA), the mode of cell death was obtained by Annexin V-FITC/PI binding assay. Briefly, treated OC cell line SKOV-3 was washed with phosphate-buffered saline (PBS) and cells were harvested by adding 300 µL of trypsin-EDTA. After harvesting, cells were suspended in 0.1M Hepes, 0.1M NaOH pH 7.4, 1.4M NaCl, 25mM CaCl2 (1x binding buffer) at a concentration of 1×10^4 cells/mL. Subsequently, 5 µL of Annexin V-FITC/PI were added to the suspensions. Later, gently vortexing of the suspensions was performed, and 400 µL of 1x binding buffer cells at room temperature were incubated for 15 min in the dark. Staining was done with AO/EB staining assay, and fluorescence activated cell sorter (FACS) Calibur flow cytometer was used for the determination of the number of apoptotic cells at 488 nm with argon ion laser.

Determination of ROS and MMP

For the estimation of ROS and mitochondrial membrane potential (MMP), human OC cell line SKOV-3 was cultured at 37oC for 24 h. Consequently, the target cells were exposed to the test molecule at varying concentrations i.e. 0, 6, 12, and 24 µM for 24 h. After that Dulbecco's modified Eagle's medium (DMEM) was entirely drawn off and sampling of the cultured cells was done at thousand cells per sample. For ROS estimation, treated cells were subjected to 5 µM DCH-DA treatment and data was obtained by flow cytometry. MMP was estimated by addition of a fluorescent dye rhodamine 123 (Rh123) to each sample and then MMP was established by flow cytometry.

Cell cycle analysis

For cell cycle analysis human OC cell line SKOV-3 was cultured at a density of 1×10^5 cells/ml in DMEM medium. These target cells were treated with ursolic acid with various concentrations i.e. 0, 6, 12, and 24 µM, and then harvested. After harvesting, these cells were fixed with 70% ethanol and later exposed to 15 µg/ml of RNase A. Further, target cells were washed with 20 µl of PBS, followed by staining with PI solution at a concentration of 20 µg/mL. FACS Calibur flow cytometry (FACS Calibur; BD Biosciences) was utilized to observe the different phases of the cell cycle of SKOV-3 cell line.

Western blot analysis

RIPA lysis buffer comprising protease inhibitor was used for lysing the SKOV-3 cells. About 30 µg of proteins from each sample were separated through electrophoresis via SDS-PAGE gels, followed by transference to polyvinylidene difluoride membrane H bond-C supermembrane (Amersham Pharmacia Biotech, Piscataway, NJ). Further, blocking at 25°C for 1 h was completed by using fat-free milk, followed by primary antibody exposure overnight at 4°C. Consequently, these cancer cells were laid for incubation with secondary antibodies. Finally, signal detection was obtained by Odyssey Infrared Imaging System and normalization was done by Actin.

Statistics

The experiments were performed in triplicate; data was shown as the mean ± SD (standard deviation) and percentages (%). Student's *t*-test and chi-square test were used to determine major differences among the groups. A p value <0.05 was taken as statistically significant.

Results

Ursolic acid induced antiproliferative effects in human OC cell line SKOV-3

For the determination of SKOV-3 cell viability treated with ursolic acid (Figure 1A), we performed CCK-8 assay. The results showed that the test molecule induced inhibition of cell proliferation of these cancer cells in a dose-dependent manner. As the dose of the tested molecule increased from 0, 0.78, 1.56, 3.12, 6.25, 12.5, 25, 50, 100 and 200 µM (Figure 1B), the inhibitory effect on OC cells increased too. No significant cytotoxic effects on normal ovarian cell line (OSE) were seen to be induced by ursolic acid (Figure 1C). Thus it was concluded that ursolic acid exhibits antiproliferative effect in a dose-dependent manner with an IC_{50} of 35 µM in cancer cells and IC_{50} of 75 µM in the normal cell line.

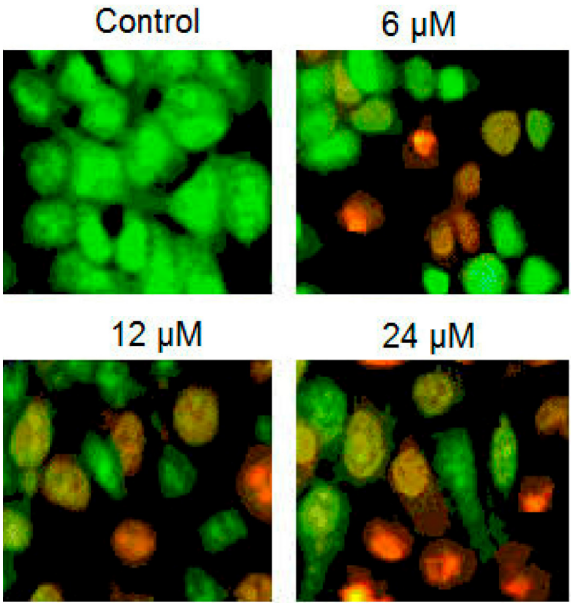

Figure 2. Acridine orange/ethidium bromide (AO/EB) staining of treated SKOV-3 OC cells showing chromatin condensation and nuclear fragmentation which increased with increasing dose of ursolic acid. The experiment was performed in triplicate. Apoptotic cells emit yellow and orange fluorescence which can be easily seen with increasing drug doses.

Induction of apoptosis by ursolic acid on human OC SKOV-3 cells

Earlier studies have revealed that ursolic acid is a potent anticancer agent against different human cancer cell lines. Herein, we investigated apoptosis by inverted microscopy and flow cytometry (DAPI and Annexin V-FITC/PI assay). The results suggested that the test molecule exposure of SKOV-3 cells induced cell death via apoptosis. Treated cells when observed under fluorescent microscope indicated condensed, contracted, and disjointed nuclei (Figure 2). It was completely contrary in the case of untreated cells, which demonstrated a normal, smooth, flattened, nuclei and low fluorescence as well as the chromatin was uniformly distributed (Figure 3). Further, to quantify the effect of apoptosis induction by ursolic acid, we performed flow cytometry through Annexin V-FITC/PI assay (Figure 4). Ursolic acid-treated cells revealed that apoptosis induction was dose-dependent. The percentage of apoptotic cells at varying concentrations of ursolic acid i.e., 0, 6, 12, and 24 μM was 0.91, 7.88, 12.47 and 19.10 u% respectively, for one complete day at 37°C (Figure 4). This data indicated that following ursolic acid treatment, the cell death predominantly occurred via apoptosis. Further investigation regarding apoptosis induction by ursolic acid was

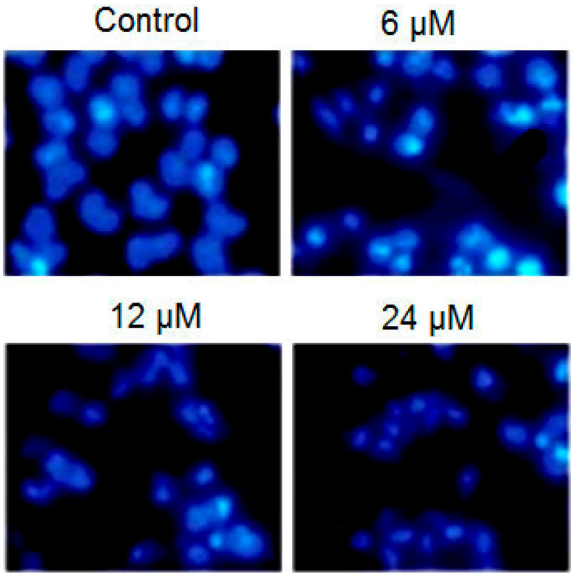

Figure 3. DAPI staining of treated SKOV-3 OC cells showing membrane blebbing and formation of apoptotic bodies with the doses given. The experiments were performed in triplicate using fluorescence microscopy.

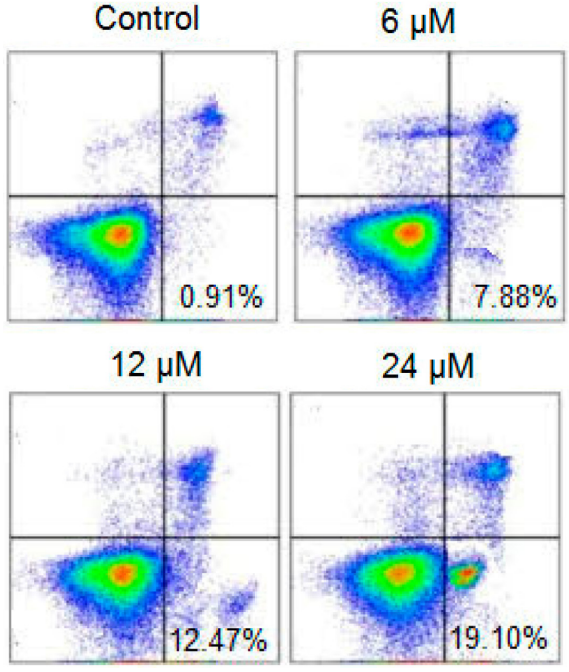

Figure 4. Flow cytometric analysis of ursolic acid-treated SKOV-3 human OC cells using annexin V/PI staining. Both early and late apoptotic cell percentage increased with increasing dose of ursolic acid. The experiments were performed in triplicate.

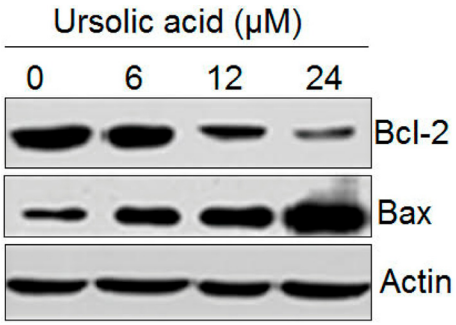

Figure 5. Western blot analysis of ursolic acid-treated SKOV-3 human OC cells on the expression of apoptosis-related proteins Bax and Bcl-2. Ursolic acid led to dose-dependent increase in Bax and decrease in Bcl-2 expressions. The experiments were performed in triplicate.

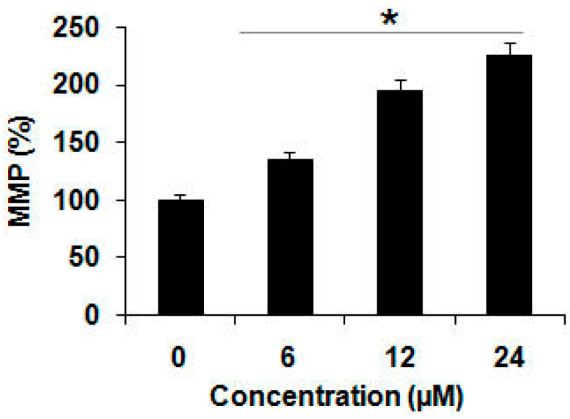

Figure 6. Effect of various doses of ursolic acid on the mitochondrial membrane potential of SKOV-3 human OC cells as indicated by flow cytometry. Ursolic acid led to increase in MMP. The data is the mean ± SD of three independent experiments. *p<0.01.

done by western blotting technique which showed that ursolic acid resulted in retarding of Bcl-2 and enhancing of Bax expression in a concentration-dependent manner, i.e. 0, 6, 12, and 24 µM (Figure 5).

Targeting MMP and ROS by ursolic acid in SKOV-3 cells

The effect of ursolic acid on MMP and ROS of human OC cell line SKOV-3 was evaluated by flow cytometry. The results indicated that MMP percentage in ursolic acid-treated cells increased significantly (from 100% to around 240%) with increasing concentrations i.e. 0, 6,12 and 24 of the molecule (Figure 6). Afterwards, analysis for ROS was performed and showed significant rise in ROS percentage nearly reaching 200 (Figure 7A). Further, the time interval of exposure of target cells with the test molecule was altered to confirm time dependency of ROS, which revealed that the ursolic acid caused an increase in intracellular ROS concentrations in both time- and dose-dependent manner (Figure 7B).

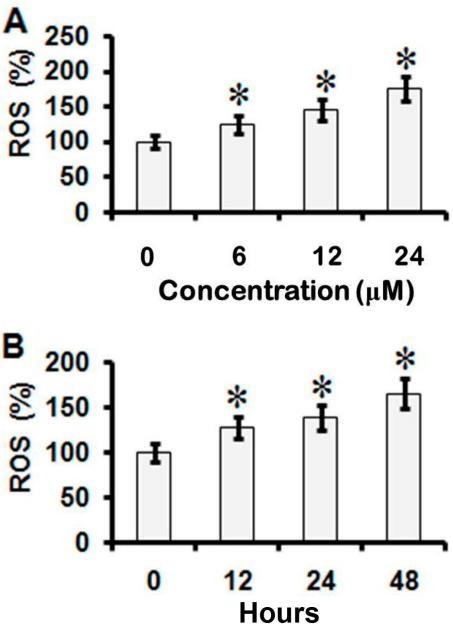

Figure 7. Dose-dependent **(A)** as well as time-dependent **(B)** increase in reactive oxygen species (ROS) production in SKOV-3 cells induced by ursolic acid treatment and analyzed by flow cytometry. The data is the mean ± SD of three independent experiments. *p<0.01.

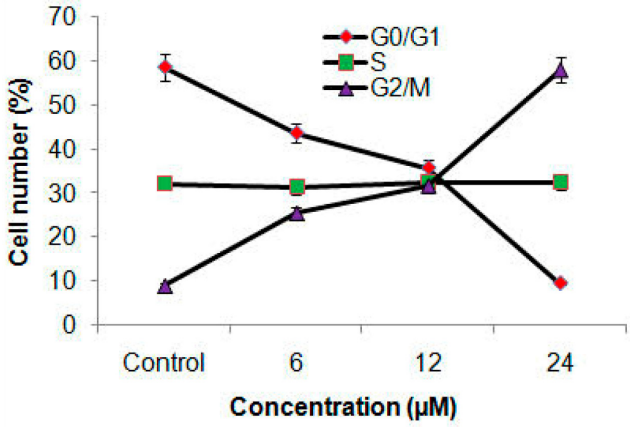

Figure 8. Ursolic acid treatment in SKOV-3 human OC cells led to dose-dependent G2/M phase cell cycle arrest as indicated by flow cytometry. The data is the mean ± SD of three independent experiments. p<0.01.

Induction of G2/M cell cycle arrest by the test molecule

The SKOV-3 cell line was treated with varied doses of ursolic acid and the distribution of SKOV-3 cells at each phase of cell cycle was studied with flow cytometry. On exposure to test molecule there was an increase in G2/M phase cells. The percentage of G2/M SKOV-3 cells was 10, 20, 30 and nearly 60 % at control, and 6, 12 and 24 µM concentrations of ursolic acid, respectively. Further, it was observed that the number of S0 cells remained unchanged and G0/G1 phase cell number declined significantly (Figure 8).

Downregulation of PI3K/AKT pathway by the target molecule on ovarian cancer cells

Further, we performed western blotting analysis for the determination of downregulation of PI3K/AKT pathway-mediated apoptosis. The observations suggested that ursolic acid decreased the expression of PI3K/AKT pathway related proteins in a dose-dependent manner. P-PI3K, PI3k, p-AKT, and AKT proteins showed significantly decreased expression on increasing doses suggesting its dose dependency too (Figure 9).

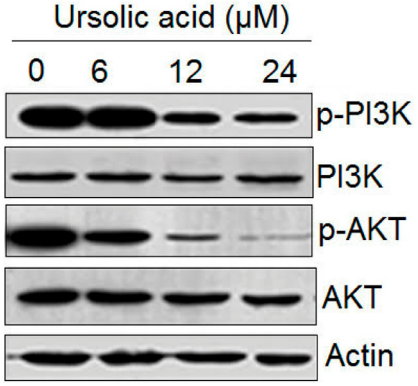

Figure 9. Dose-dependent downregulation of PI3K/AKT signalling pathway by ursolic acid treatment in SKOV-3 OC cells as indicated by western blot analysis. The Figure indicates that ursolic acid decreased the expression of PI3K/AKT pathway related proteins in a dose-dependent manner. P-PI3K, PI3K, P-AKT and AKT proteins showed significantly decreased expression with increasing doses. The experiment was performed in triplicate.

Discussion

OC is a lethal malignancy related to female reproductive system. Usually primary OC does not have evident symptoms, leading to its poor prognosis. Though some studies specify that women experience nonspecific, persisting symptoms, such as back pain, pelvic or abdominal pain, bloating, feeling full quickly and urinary urgency, do exist prior to diagnosis [18]. Currently, there is no competent and effective treatment available to tackle OC. Ursolic acid, a naturally occurring triterpenoid, has shown various biological and pharmacological applications, including anticancer activity. It exhibits anticancer effects against different human cancers i.e. breast, cervical, and colorectal cancers. In nude mice, ursolic acid showed significant sensitization of cancer cells to chemotherapeutic agents to inhibit the cell growth and induce apoptosis in DU145 cells [19-23]. In the current study we examined ursolic acid for its anticancer effects against SKOV-3 OC cells, and for that purpose we performed an array of assays. We performed CCK-8 assay to find out the effect of the target molecule on the viability of SKOV-3 OC cells which revealed significant results by retarding the proliferation of these cells. It was seen that the viability was retarded much more in case of cancer cells with IC_{50} value of 35 μM while the viability of normal ovarian cells showed significantly less toxicity with IC_{50} of 75 μM, revealing its selective antiproliferative effect. Furthermore, the apoptotic potential of ursolic acid was assessed through fluorescence microscopy by DAPI staining, Annexin V-FITC/PI assay and flow cytometry. The results revealed that cells exposed to the target molecule exhibited a significant suppression of BCL-2 and amplified the expression of BAX in a concentration-dependent manner. Next, we performed MMP and ROS estimations through flow cytometry, and the results revealed that the test molecule caused significant increase in both MMP as well as ROS percentage, hence inducing apoptosis through mediation of MMP and ROS with dose as well as time dependency. Afterwards, cell cycle analysis revealed that the ursolic acid caused a substantial increase of G2/M cells, decline of G0/G1 cells, while the effect on S0 phase cells remained negligible. Thereafter, we performed western blotting analysis in order to determine the effect of ursolic acid on PI3K/AKT pathway. P-PI3K, PI3k, p-AKT, and AKT proteins showed significantly decreased expression on increasing doses, suggesting its dose dependency too. Thus it was concluded that the test molecule is a potent anticancer agent against SKOV-3 OC cells and further investigations are required to explore its full potential as anticancer drug.

Conclusion

Ursolic acid showed significant anticancer activities against human OC cell line SKOV-3. The antiproliferative effect of ursolic acid on human OC cells was mediated via apoptosis, ROS and MMP upregulation, cell cycle arrest and downregulation of PI3K/AKT pathway, thus revealing ursolic acid's potential as therapeutic agent against human OC.

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Conflict of interests

The authors declare no conflict of interests.

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