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Original Research

Betulin terpenoid targets OVCAR-3 human ovarian carcinoma cells by inducing mitochondrial mediated apoptosis, G2/M phase cell cycle arrest, inhibition of cell migration and invasion and modulating mTOR/PI3K/AKT signalling pathway

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Abstract: The main purpose of the current research work was to study in vitro anticancer effects of betulin in OVCAR-3 human ovarian carcinoma cells along with examining its effects on cellular apoptosis, cell cycle phase distribution, cell migration and invasion and mTOR/PI3K/AKT signalling pathway. The cell proliferation of OVCAR-3 cells at various doses of the drug was studied by CCK8 cell viability assay. Effects on cell apoptosis were studied by fluorescence microscopy and western blot. Effects on cell cycle were evaluated by flow cytometry and western blot. Transwell assays were used to study effects on cell migration and invasion. The results indicated that betulin led to significant reduction of OVCAR-3 cell viability in a dose-dependent as well as time dependent manner. Betulin also led to reduction in cell colonies. The anticancer effects of betulin were due to the induction of apoptosis which was seen by increased apoptotic cells with yellow and orange fluorescence. Betulin prompted mitochondrial apoptosis which was also associated with alteration in the apoptosis-related protein expression (Bax, Bad and Bcl-2 and Bcl-xL). The molecule also led to G2/M phase cell cycle arrest on OVACR-3 ovarian carcinoma cells. It was also observed that betulin could inhibit the migration and invasion of the ovarian cancer cells in a concentration-dependent manner. Betulin molecule also resulted in blocking of mTOR/PI3K/AKT signalling pathway. In conclusion, this study clearly indicates the anticancer effects of betulin natural product in OVCAR-3 human ovarian cancer cells are mediated via apoptosis induction, G2/M phase cell cycle arrest, cell migration and invasion inhibition and targeting of mTOR/PI3K/AKT signalling pathway.

Key words: Ovarian cancer; Betulin; Flow cytometry; Apoptosis, Cell migration.

Introduction

Terpenoids are a diverse and huge class of phytochemicals found in medicinal plants, vegetables and fruits (1). Terpenoids show structural resemblance with hormones found in human beings. These are classified on the basis of isoprene units present in their structure like hemiterpenoids (1 unit), monoterpenoids (2 unit), sesquiterpenoids (3 units), diterpenoids (4 units), triterpenoids (6 units), etc. Terpenoids have been extensively studied and have been reported of multiple pharmacological properties including anticancer, anti-inflammation and antioxidant (2,3). Several *in vitro* and *in vivo* studies have reported that terpenoids inhibit tumor growth and proliferation in different human cancers (4). A diet rich in terpenoids content have been inversely related to the development of chronic diseases like cancer. Several terpenoids have entered clinical trials against different human cancers including diterpenoid paclitaxel and docetaxels (semi-synthetic analogue of paclitaxel) (5). Nowadays numerous studies are reporting the effectiveness of natural products as potential anti-tumor agents (6). Betulin belongs to a pentacyclic class of triterpenoids mainly present in the bark of birch species (*Betulaceae*). Betulin has been reported of several medicinal and pharmacological effects including anti-osteoclastogenic, anti-amnesic and anti-inflammatory effects (7,8). Moreover, betulin exhibits protective effects against chronic obstructive pulmonary disease, kidney injury and hepatitis (9). Betulin induces remarkable anticancer effects against a number of human cancers including prostate cancer, breast cancer, lung cancer, gastric cancer, melanoma and leukemia (10-12). Ovarian cancer is a disastrous female malignancy associated with their reproductive system. It ranks 3rd in prevalence after cervical and uterine cancers (13). Ovarian cancer has a very high mortality rate and worst prognosis among all the cancers in women (14). It is thrice as lethal as breast cancer although being less prevalent than breast cancer. The huge mortality rate associated with ovarian cancer has been attributed to secret and asymptotic tumor growth, delayed symptoms and improper screening resulting in late diagnosis (15). Therefore, this lethal malignancy has been given the name “silent killer” (16). The ovarian cancer incidences vary globally depending on the life style and different risk factors contributing to the development of cancer. Keeping in view the lethality of ovarian cancer, there is a need for novel therapeutic and screening agents that could benefit in ovarian cancer detection and treatment. Betulin has been shown to

exhibit remarkable anticancer activities against several human cancers. Therefore, this study was designed to estimate the anti-tumor effects of betulin against human OVACAR-3 ovarian cancer cells. its effects on cellular apoptosis, cell cycle, cell migration, cell invasion and mTOR/PI3K/AKT signalling, were also examined.

Materials and Methods

Estimation of cell proliferation

OVACAR-3 cells were harvested at logarithmic phase of growth and inoculated at a density of 1×10^4 cells/well within 96-well plates. These cells were then exposed to varying concentrations of betulin viz 0, 15, 30, 60 and 120 µM for 12 h and 24 h, respectively. Post betulin treatment, cells were added with 100 µl of CCK-8 solution (Sigma) diluted in RMPI-1640 medium followed by 2 h of incubation in dark at 37°C. Finally, optical density was taken at 450 nm by using a microplate reader (Bio-Rad laboratories, Inc.). Each betulin concentration was experimented three repeats.

Apoptosis analysis

OVACAR-3 cells were cultured in 24-well plates with a density of 5×10^3 cells/well. Cells were harvested by trypsinzing post reaching 20-80% of growth confluence. Afterwards, cells were exposed to changing betulin concentrations viz 0, 15, 60 and 120 µM, at 37°C for 24 h in 5% CO_2. 0.1% of dimethyl sulphoxide (DMSO) was used in case of controls. Post betulin treatment, cells were fixed in formaldehyde (4%) followed by staining with 10 μL of Acridine Orange/Ethidium Bromide (AO/EB) for 5 min. Finally, betulin treated and AO/EB stained OVACAR-3 cells were investigated under a fluorescence microscope (Olympus Co., Tokyo, BX51TRF, Japan).

Flow cytometry

Human OVACAR-3 cells were cultured within 6-well plates at a density of 1×10^5 cells/well. Afterwards, each well was supplemented with altering concentrations of betulin viz 0, 15, 60 and 120 µM for 24 h. Post betulin treatment, cells were washed and centrifuged for 10 min at 2000 rpm. Cell pellets were resuspended and fixed in ethanol (70%) for 2 h at 4°C. Post fixation, cells were washed using phosphate buffered saline (PBS) followed by centrifugation again for 10 min at 2000 rpm. Cell pellets were disintegrated through vortexing and then resuspended in PBS (250 µl) maintaining 20 µg/ml of PI, 20 µg/ml of RNase and 0.1% of Triton X-100. Cells were then incubated in dark for 30 min followed by flow cytometric analysis (BD FACSCalibur, BD Biosciences, United States) for determination of different cell cycle phases.

Transwell chambers assay

Transwell chambers assay was used to study the effects of betulin on cell migration and cell invasion of OVACAR-3 cells. Briefly, cells were precultured for 24 h in 24-well plates with a density of 1×10^5 cells/well. These cells were then transfected with betulin at varying concentrations viz 0, 15, 60 and 120 µM for 48 h. Post betulin treatment, cells were detached with trypsin followed by transference of the 100 µl of cell suspension to upper transwell chambers maintaining 500 µl of RMPI-1640 cultural medium with fetal bovine serum (10%). Lower transwell chambers were supplied with medium alone. These transwell chambers were placed in basolateral chamber and removed after 12 h of incubation. The non-migrated cells were rubbed off with a cotton swab and migrated cells were fixed with ethanol (95%) for 15 min. Thereafter, cells were stained using crystal violet staining dye (0.1%) for 12 min. finally, individual chambers were inverted over glass slides and photographed under a fluorescence microscope (Olympus Co., Tokyo, BX51TRF, Japan). Five random fields were selected from each filter membrane to number the transmembrane cells. Similar methodology was adopted for invasion detection except transwell chambers were coated with Matrigel.

Western blotting

Western blotting assay was performed for assessment of protein expressions allied with apoptosis, cell cycle and mTOR/PI3K/AKT signalling pathway. OVACAR-3 cells were cultured at a density of 1×10^4 cells/well of 24-well plates and harvested at >90% of growth confluence. Cells were the exposed to different betulin concentrations viz 0, 15, 60 and 120 µM, for 24 h. Afterwards, betulin treated OVACAR-3 cells were lysed using RIPA lysis buffer (Sigma) followed by quantification of protein content with bicinchoninic acid assay. 35 µg of proteins from each lysate was subjected to separation with SDS-PAGE followed by electrophoretic transference to PVDF membranes (Merck Millipore, Billerica, MA, U.S.A.). PVDF membranes were then blocked for one hour using skimmed milk (5%) followed by primary antibodies treatment at 4°C in dark for 12 h. Antibodies with 1:1000 dilution were used against Bax, Bad, Bcl-2, Bcl-xl, Cycli-B1, Cyclin-D1, Cyclin-E1, m-TOR, PI3K and AKT (Bioss Biotechnology, Beijing, China). Post primary antibodies treatment; membranes were subjected to horseradish peroxidase-labeled goat anti-rabbit IgG (1:2000, Cusabio Biotech Co., Ltd., Wuhan, China) for 1 h in dark. Finally, electro-chemical luminescence kit (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) was used to detect the protein signals.

Statistical analysis

Statistical analyses were performed by one-way ANOVA and Student's t-test using SPSS software version 23.0 (IBM Corporation, Chicago, IL, United States). Each individual drug concentration was experimented in triplicates and data was expressed as mean ± SD. The statistically significant values of p were taken as $*p < 0.05$ and $**p < 0.01$.

Results

Betulin induced cytotoxicity in OVACAR-3 cells

The cytotoxic effects of betulin (Figure 1A) against OVACAR-3 cells were examined by CCK-8 kit. OVACAR-3 cells were treated with betulin for 12 h and 24 h at variant concentrations ranging from 0-120 µM. Results revealed that betulin significantly reduced the proliferation of OVACAR-3 cells in a concentration and time-dependent manner (Figure 1B). When compared

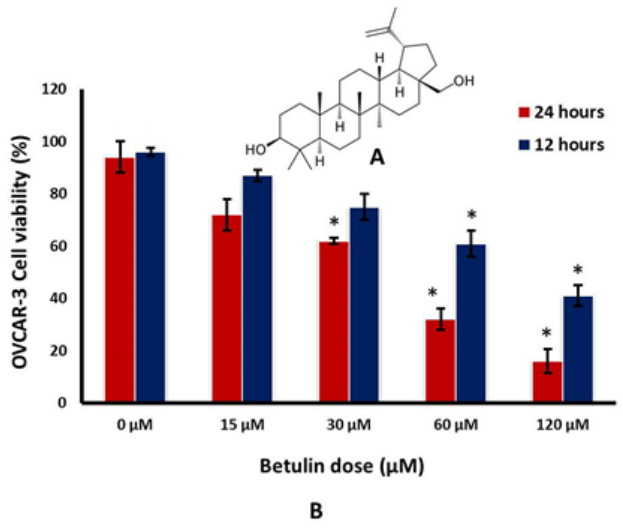

Figure 1. (A) Chemical structure of betulin molecule. (B) The proliferation of OVACAR-3 cells post betulin treatment for 12 h and 24 h at indicated concentrations. Results showed that betulin inhibited the proliferation in dose- and time-dependent fashion. All individual betulin concentrations were experimented in triplicates and data was represented as mean ± SD (*$p < 0.05$).

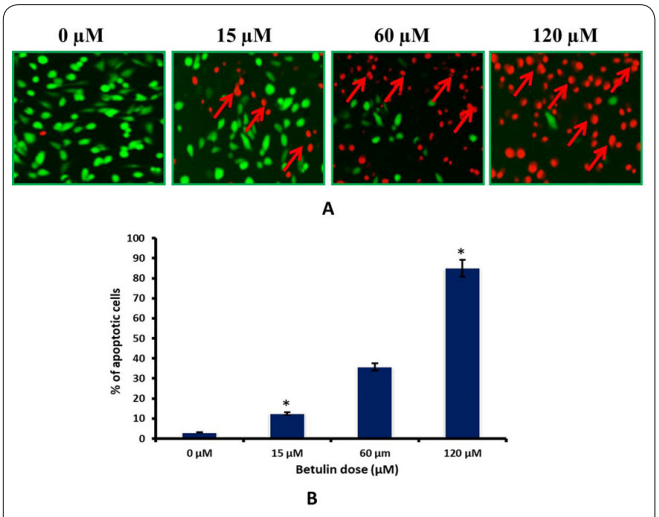

Figure 2. (A) Apoptosis analysis of betulin treated OVACAR-3 cells through fluorescence microscopy. Green fluorescence represents normal cells and red fluorescence indicates apoptotic cells. Results indicated that the number of apoptotic cells increased on increasing betulin concentrations. (B) The graph represents percentage apoptotic OVACAR-3 cells after betulin exposure. All individual betulin concentrations were experimented in triplicates and data was represented as mean ± SD (*$p < 0.05$).

to control group, betulin reduced the number of viable cells from 100 % to almost 40% and 15% after 12 h and 24 h of exposure, respectively.

Betulin induced apoptosis in OVACAR-3 cells

The apoptotic effects of betulin in OVACAR-3 cells were examined via AO/EB staining and western blotting assay. Results revealed that betulin increased the population of apoptotic cells in a concentration-dependent manner (Figure 2A). The number of non-apoptotic cells (green fluorescence) reduced significantly and the number of apoptotic cells (red fluorescence) increased substantially after betulin exposure. Betulin treatment to OVACAR-3 cells increased the apoptotic cell percentage from almost 1% to 80% at 120 µM (Figure 2B). Moreover, western blotting showed that betulin increased the levels of expressions of proapoptotic Bax and Bad proteins and reduced the expression levels of antiapoptotic Bcl-2 and Bcl-xl proteins in OVACAR-3 cells (Figure 3). When compared to control groups Bax and Bad levels were found to be significantly elevated in OVACAR-3 cells. These results indicated that betulin exhibit proapoptotic effects against human ovarian OVACAR-3 cancer cells.

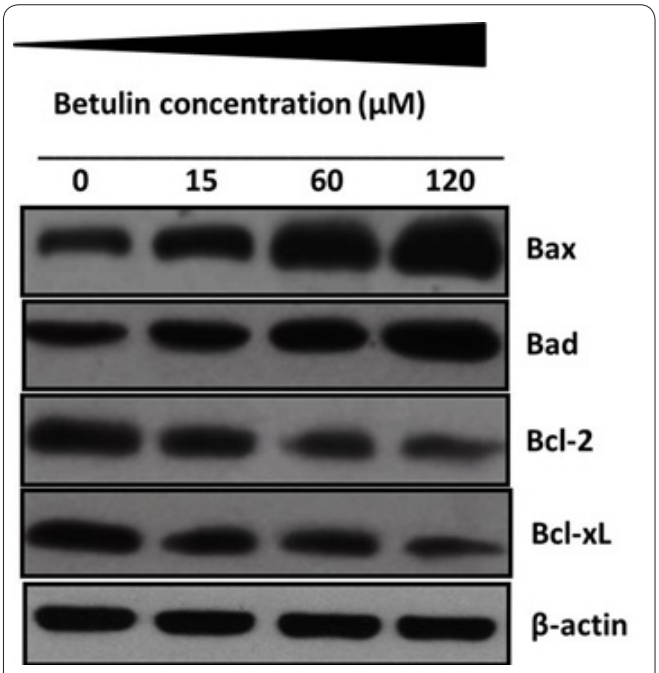

Figure 3. Western blotting assay was performed to monitor the activity of apoptosis allied proteins. Results revealed that the expressions of Bax and Bad (proapoptotic) increased while that of Bcl-2 and Bcl-xl (antiapoptotic) decreased in betulin treated group as compared to control group. β-Actin was used as normalization control.

Betulin induced cell cycle arrest in OVACAR-3 cells

Different cell cycle phases in betulin treated OVACAR-3 cells and control groups were determined by performing flow cytometric analysis. Results suggested that the number of G2/M-phase cells increased significantly on betulin exposure to OVACAR-3 cells while the S- and G0/G1-phase cells progressed to next levels (Figure 4A). The augmented quantity of G2/M-phase cells indicated that betulin inhibited the progression of cell cycle in OVACAR-3 cells at this phase of cell cycle. Moreover, western blotting showed that the expressions of cell cycle allied proteins Cyclin-B1 and Cyclin-E1 increased remarkably with increasing concentrations of betulin drug (Figure 4B). The levels of Cyclin-D1 were observed to reduce with increasing betulin concentrations. Therefore, the antiproliferative effects of betulin could be mediated via G2/M-phase cell cycle arrest.

Betulin inhibited migration and invasion of OVACAR-3 cells

The effects of betulin on cell migration and invasion of OVACAR-3 cells were examined via transwell chambers assay. Results revealed that betulin treated group showed reduced number of migrated cells in comparison to that of control group (Figure 5A). The

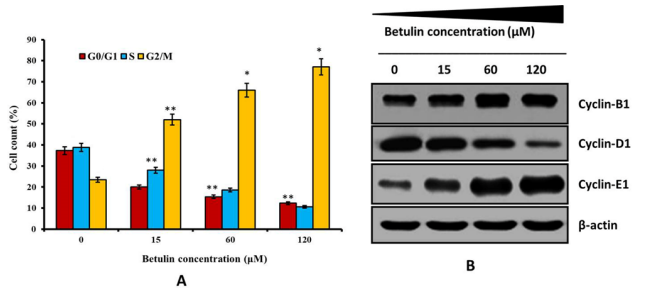

Figure 4. Flow cytometry was used to estimate the effects of betulin on cell cycle phase distribution of OVACAR-3 cells. Results indicated that S and G0/G1-phase cells normally passed to higher phases while the number of G2/M-phase cells increased. This indicated cell cycle arrest at G2/M-phase of cell cycle. All individual betulin concentrations were experimented in triplicates and data was represented as mean ± SD (*$p < 0.05$ and **$p < 0.01$). (B) Western blotting was used to analyse the expressions of key cell cycle allied proteins. Results showed increased levels of Cylin-B1 and Cyclin-E1 and decreased levels of Cyclin-D1. β-Actin was used as normalization control.

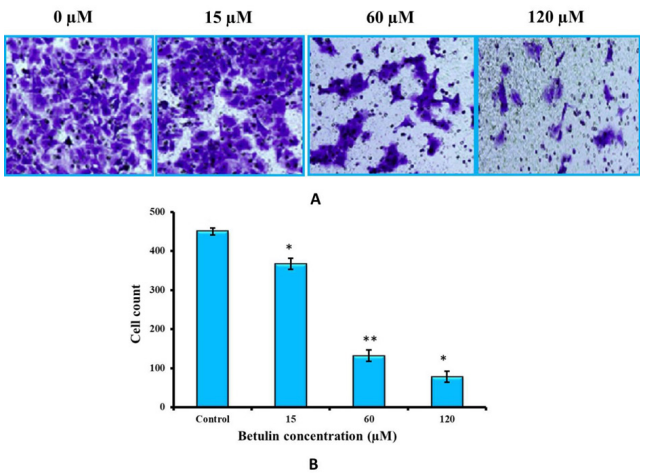

Figure 5. (A) Transwell chambers assay was executed to check the effects of betulin on cell migration of OVACAR-3 cells. Results indicated that betulin supressed the migration tendency of OVACAR-3 cells as indicated. (B). The graph represents the number of migrated cells post betulin treatment to OVACAR-3 cells. The number of migrated cells decreased significantly with increasing betulin concentrations. All individual betulin concentrations were experimented in triplicates and data was represented as mean ± SD (*$p < 0.05$ and **$p < 0.01$).

number of migrated cells in control group was found to be almost 450 while the number was almost limited to 70 in betulin treated group at 120 μM (Figure 5B). Furthermore, betulin inhibited the invasive ability of OVACAR-3 cells by reducing the number of invasive cells in a concentration dependent fashion (Figure 6A, B). Therefore, betulin exhibited significant inhibition on cell invasion and cell migration of OVACAR-3 cells.

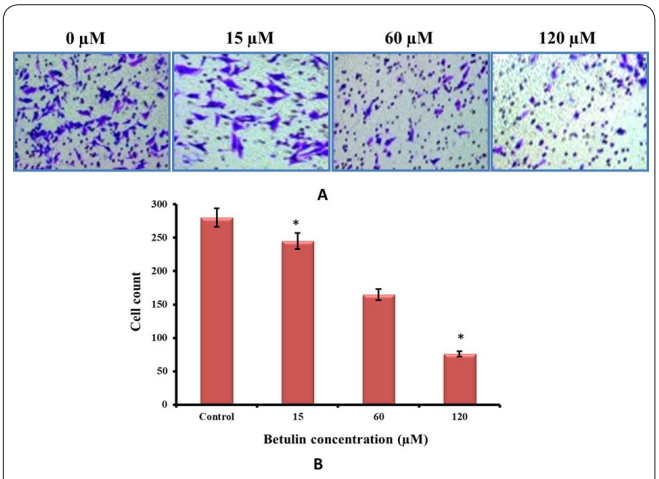

Figure 6. (A) Transwell chambers assay was executed to check the effects of betulin on cell invasion of OVACAR-3 cells. Results indicated that betulin supressed the invasion tendency of OVACAR-3 cells as indicated. (B). The graph represents the number of invasive cells post betulin treatment to OVACAR-3 cells. The number of invasive cells decreased significantly with increasing betulin concentrations. All individual betulin concentrations were experimented in triplicates and data was represented as mean ± SD (*$p < 0.05$ and **$p < 0.01$).

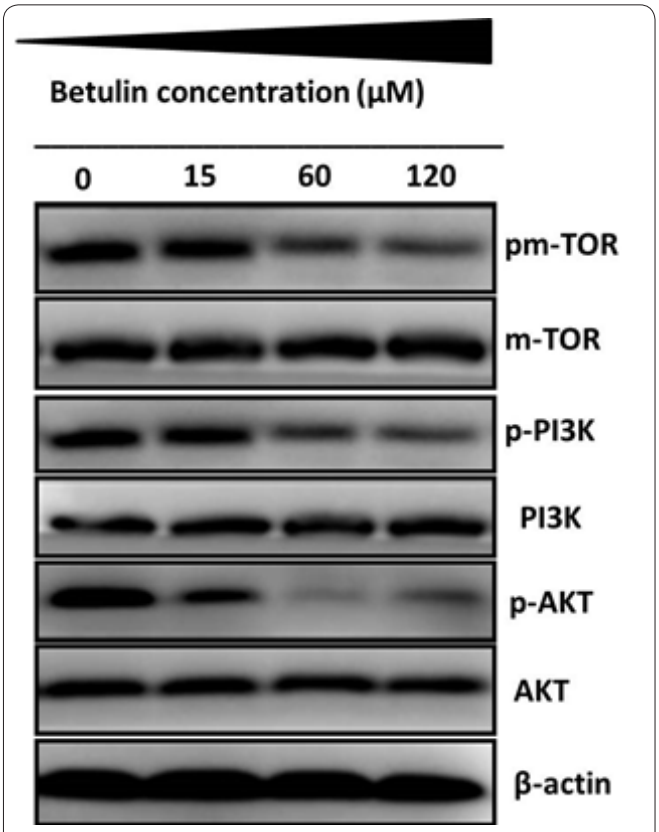

Figure 7. Western blotting assay was used to demonstrate the effects of betulin on mTOR/PI3K/AKT signalling pathway in OVACAR-3 cells. Results revealed that betulin reduced the levels of p-mTOR, p-AKT and p-PI3K indicating inactivation of mTOR/PI3K/AKT signalling. β-Actin was used as normalization control.

Betulin inhibited mTOR/PI3K/AKT signalling pathway

The effects of betulin on mTOR/PI3K/AKT signalling pathway were analysed using western blotting assay. OVACAR-3 cells were treated with variant concentrations of betulin ranging from 0-120 μM for 24 h. Results showed that betulin reduced the expression levels of p-mTOR, p-PI3K and p-AKT in OVACAR-3 cells in comparison to that of control groups (Figure 7). The levels of mTOR, PI3K and AKT remained almost constant. Therefore, it is evident that betulin supressed the phosphorylation of mTOR/PI3K/AKT signalling allied protein thereby blocking its expressions.

Discussion

Apoptosis has been a leading target in cancer chemotherapy from past few decades. It is often termed as type-I programmed cell death (17). It is a natural maintenance process operating in multicellular organisms. Delayed apoptosis or its inhibition leads to several chronic disorders in human beings like cancer (18). Cancer has been a great cause of concern globally as millions of people fall to this malignancy each year (19). Ovarian cancer is one of the top three cancers prevalent in females worldwide. These diseases not always but sometimes result in female infertility (20). The disease mostly remains silent until later stages leading to poor prognosis and high mortality. Therefore there is an immediate need for potential agents that can supress ovarian cancer, improve prognosis and overall survival rate. This study was undertaken to study the effects of betulin- a potential anticancer agent, against OVACAR-3 ovarian cancer cells. Betulin has been previously reported of inducing antiproliferative effects against different human cancer lines including lung, breast and gastric cancers (21). Herein, betulin was reported to inhibit the proliferation of OVACAR-3 cells in a concentration and time-dependent manner. Further, efforts were taken to check the underlying mechanism of action of antiproliferative effects of betulin in OVACAR-3 cells. Several studies have reported that betulin exhibits proapoptotic effects against different human cancer cells including gastric and colon cancer (22, 23). It has been reported of remarkable potential of stimulating apoptosis via upregulation of Caspases, PARP and Bax proteins and downregulation of Bcl-2 and Bcl-xl proteins. Herein, betulin exhibited proapoptotic effects in OVACAR-3 cells in a dose-dependent manner. The levels of Bax and Bad increased while betulin decreased the levels of Bcl-2 and Bcl-xl in OVACAR-3 cells. Further, betulin has been previously reported of inducing cell cycle arrest in colorectal CT26 and HCT116 cancer cells. The G0/G1-phase cell cycle arrest in CT26 and HCT116 cells was linked to decreased Cycli-D1/CDK4 complex expressions (23). Herein, betulin induced G2/M-phase cell cycle arrest in OVACAR-3 cells may be due to enhanced expressions of Cylin-B1 and Cyclin-E1 or reduced expressions of Cyclin-D1. Additionally, we found that betulin could obstruct OVACAR-3 cell migration as well as invasion. The mTOR/PI3K/AKT signalling is one of the important signalling transduction taking part in cell survival, development and differentiation (24). Betulin has been previously reported of inactivating mTOR/PI3K/AKT signalling pathway thereby inducing autophagy and promoting apoptosis in colorectal cancer cells (23). Herein, betulin blocked the mTOR/PI3K/AKT signalling through in activation of key regulatory proteins and promoted apoptosis in OVACAR-3 human ovarian cancer cells.

Taken together, the results of current study pointed out that betulin molecule could significantly inhibit the proliferation of ovarian OVACAR-3 cancer cells. The anticancer effects were found to intercede via apoptosis initiation, cell cycle arrest and downregulation of mTOR/PI3K/AKT signalling. Moreover, betulin reduced the cell migratory and invasive capability OVACAR-3 cells.

Conflict of interest

The authors declare that there is no conflict of interest.

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