

## ORIGINAL ARTICLE

# Suppression of *in vitro* and *in vivo* human ovarian cancer growth by isoacteoside is mediated via sub-G1 cell cycle arrest, ROS generation, and modulation of AKT/PI3K/m-TOR signalling pathway

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## Summary

**Purpose:** The purpose of the present study was to investigate the anticancer properties of isoacteoside against OVCAR-3 human ovarian cancer cells. Its effects on apoptosis, reactive oxygen species (ROS) generation, cell invasion, cell cycle arrest and its effects on tumor volume and weight were also evaluated in the current study.

**Methods:** MTT assay was used to study the cytotoxic effects of the compound on the cell viability. Effects on apoptosis and cell cycle arrest were evaluated by flow cytometry. In vitro wound healing assay and matrigel assay were carried out to study the effects of isoacteoside on cell migration and cell invasion respectively. Non-cancer ovarian cell line SV-40 served as control.

**Results:** Isoacteoside exerted both dose-dependent as well as time-dependent growth inhibitory effects on ovarian cancer cells with IC<sub>50</sub> values of 15  $\mu$ M at 24h incubation. Isoacteo-

side led to early and late apoptosis induction in these cells. Isoacteoside also led to sub-G1 cell cycle arrest which showed strong dose-dependence. Isoacteoside treatment also led to inhibition of cell migration and cell invasion. The results revealed that OVCAR-3 tumor growth was significantly suppressed by isoacteoside administration, compared with that in the control group. At the end of the 5-week period of isoacteoside treatment, the average tumor growth and volume in the untreated control group were considerably higher than those in the treated groups.

**Conclusion:** In brief, the current study indicates that isoacteoside has a great potential in suppressing both *in vitro* and *in vivo* ovarian cancer cell growth and can be used as a possible anticancer agent.

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

## Introduction

Natural products, especially plants and microbes, have served as important sources of anti-cancer drugs in the past and because of the huge diversity of plant species, it is believed that they can serve as the source of more drugs to combat deadly diseases such as cancer [1]. Among plant metabolites that have shown potential to inhibit the growth of cancers, phenylethanoid glycosides

are considered of utmost importance [2]. Isoacteoside is a phenylethanoid glycoside that has been identified and isolated from a number of plant species [3]. Studies carried out previously have shown that phenylethanoid glycosides, such as isoacteoside, exhibit the capacity to inhibit the proliferation of cancer cells [4]. In the current study the anticancer effects of isoacteoside were examined

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Received: 03/06/2018; Accepted: 20/06/2018

against ovarian cancer cell line OVCAR-3. Cancer is considered as the second main cause of death throughout the world and more than 14 million patients are diagnosed with cancer annually as per the reports of World Health Organization (WHO). In 2012 alone, around 8 million people died of cancer [5]. Of all the gynecologic cancers, ovarian cancer is responsible for significant mortality in women [6]. Ovarian cancers are mostly detected at advanced stages as there are no apparent symptoms during the development of this disease and hence, it is often difficult to treat [7]. Nonetheless, ovarian cancers generally respond well to the first line chemotherapy; however there are frequent relapses which are mostly associated with the development of chemoresistance [8]. Despite recent advancements in the field of cancer research, there is still lack of reliable markers for the diagnosis of ovarian cancers [9]. In addition, the currently used chemotherapeutic agents for the treatment of ovarian cancer are associated with unwanted adverse effects [10]. In this study it was found that isoacteoside inhibits the growth of ovarian cancer cells and exhibits an  $IC_{50}$  of 15  $\mu$ M. Next, the underlying mechanism of the anticancer activity of isoacteoside was investigated and the results showed that isoacteoside could trigger generation of ROS which leads to apoptotic cell death of the OVCAR-3 cancer cells. Further, flow cytometric analysis revealed that isoacteoside could cause arrest of the cells in the sub-G1 phase of the cell cycle. The effects of isoacteoside were also examined on the AKT/PI3K/m-TOR signalling cascade and it was found that this molecule could inhibit this pathway. *In vivo* evaluation of isoacteoside in the xenografted mice models revealed that isoacteoside could suppress tumor growth. In conclusion, these observations indicate that isoacteoside may prove important in the treatment of ovarian cancer.

## Methods

### *Cell lines and culture conditions*

Ovarian cancer cell line OVCAR-3 and non-cancer ovarian cell line SV40 were procured from American Type Culture Collection. Both cell lines were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS), antibiotics (100 units/mL penicillin and 100  $\mu$ g/mL streptomycin), and 2 mM glutamine. The cells were cultured in incubator (Thermo Scientific, Waltham, Massachusetts, USA) at 37°C with 98% humidity and 5% CO<sub>2</sub>.

### *MTT assay*

The proliferation of the ovarian cancer and normal cells was assessed by MTT assay. In brief, the cells were cultured in 96-well flat-bottom microtiter culture plates

at a density of  $5 \times 10^4$  cells per well and subjected to treatment with isoacteoside at varied concentrations for 24 hrs. Afterwards, the addition of fresh MTT solution followed, and then the cells were incubated for another 4 hrs at 37°C. This was followed by addition of DMSO to dissolve the formazan crystals. Finally, the absorbance was taken at 570 nm using a microplate reader.

### *DAPI, annexin V/PI staining and ROS estimation*

The nuclear morphology of the OVCAR-3 cancer cells was assessed by fluorescence microscopy after subjecting the cells to DAPI staining. Ten fields with 100 cells/field were randomly selected for estimation of the cells with condensed nuclei. Annexin V/PI double staining was used for the determination of apoptotic ovarian cancer cells as described previously [11]. The estimation of ROS was carried out as described earlier [12].

### *Wound heal assay*

The migration of OVCAR-3 cells was determined with wound healing assay. The cells were grown till 70% confluence and a scratch was made with a scratching device. The cells were then incubated again for 48 hrs and the wound healing capacity was evaluated by comparing the widths of the wounds.

### *Matrigel invasion assay*

Invasion was evaluated with the help of Matrigel®-coated invasion chambers. The isoacteoside-treated and untreated OVCAR-3 cells that reached the lower surface of the membrane were subjected to staining with crystal violet (CV), and images of CV-stained cells were taken. The crystal violet complexes formed were dissolved in 10% acetic acid and the cell invasion was determined by measuring the absorbances of the resultant solutions at 600 nm in a spectrophotometer.

### *In vivo study*

The experiments were performed in accordance with National Institutes of Health standards for the care and use of laboratory animals and approved by the Institutional Animal Care and Use Committee of the Institute. In this study 4 week-old female mice were procured from the Shanghai SLAC Laboratory Animal Co. Ltd. Mice were injected with  $5 \times 10^6$  OVCAR-3 cells subcutaneously at the left flank. The mice were then randomly divided into two groups. As the tumors became apparent, one group of mice (n=5) was injected intraperitoneally with DMSO (0.1%) dissolved isoacteoside and diluted with 100  $\mu$ L normal saline at 30 mg/kg body weight and this time point was taken as the first day of the experiment. Isoacteoside was administered to the mice thrice a week, while the control mice were administered DMSO (0.1%) in normal saline only. At the end of 5 weeks, the mice were sacrificed and tumors were harvested for assessment of tumor growth and other investigations.

### *Western blotting analysis*

The OVCAR-3 ovarian cancer cells were collected and treated with lysis buffer. The extracts were boiled for 10 min in the presence of loading buffer, followed by

separation of cell extracts using 15% SDS-PAGE gel. The samples were then put onto polyvinylidene fluoride membranes and subjected to blocking with 5% skim milk. Membrane incubation with primary antibodies was performed overnight at 4°C and subsequently incubated with horseradish peroxidase-conjugated antimouse secondary antibody at 1:1,000 dilution for one hr. The membranes were then subjected to washing with phosphate buffered saline (PBS) and the immune-reactive bands were visualized using ECL-PLUS kit (Thermo Scientific Waltham, Massachusetts, USA). Development of the immune complexes was carried out using an ECL detection kit according to the manufacturer's protocol. The bands were analyzed with GelDoc 2000 imaging system.

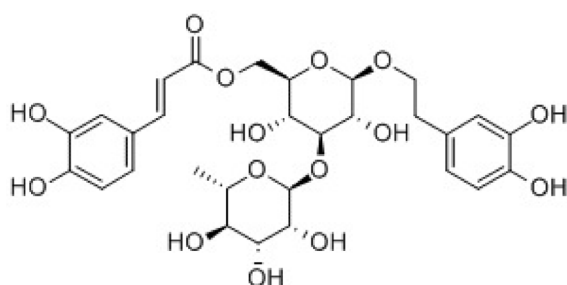
#### Statistics

Each test was performed thrice and the values are presented as mean  $\pm$  SD. Student's *t*-test was employed for statistical analysis using GraphPad prim 6 software. Statistical significance was set at  $p < 0.05$ .

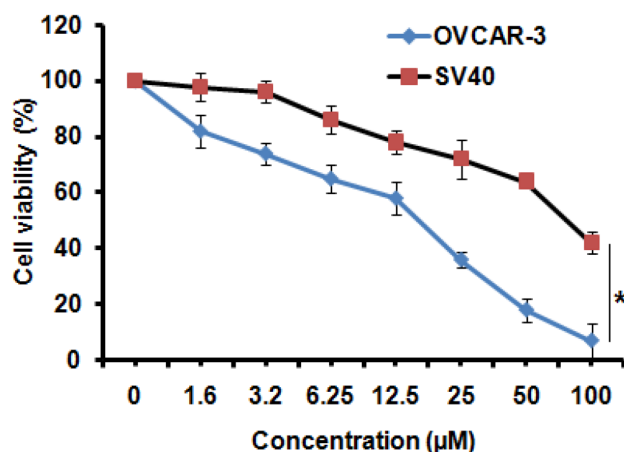
## Results

### *Isoacteoside exerts antiproliferative effects on ovarian cancer*

The antiproliferative effects of isoacteoside (Figure 1) was examined against the OVCAR-3 cell

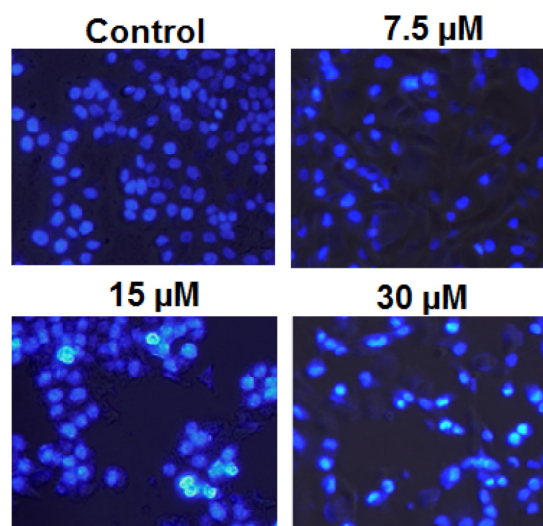


**Figure 1.** Chemical structure of isoacteoside.

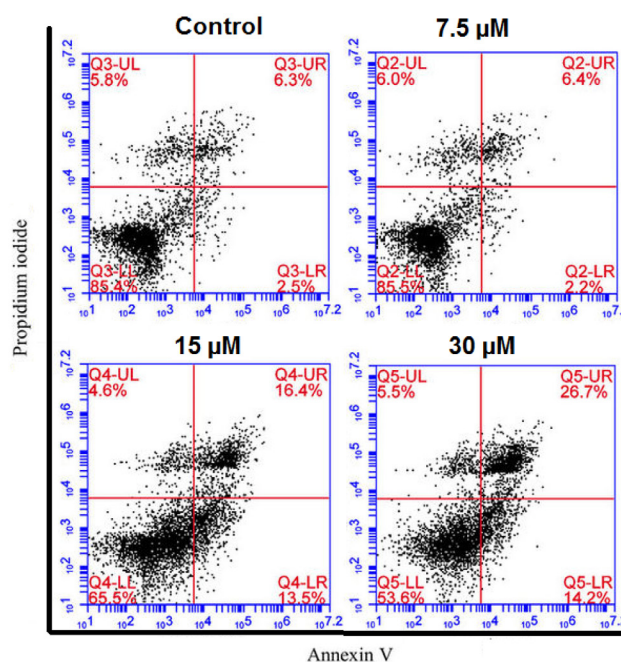


**Figure 2.** Effect of isoacteoside on the viability of OVCAR-3 and SV40 cells by MTT assay. The experiments were performed in triplicate and shown as mean  $\pm$  SD ( $p < 0.05$ ).

line and normal SV40 cell line by MTT assay. The results of the proliferation assay showed that isoacteoside exerts antiproliferative effects on the ovarian cancer cell line with  $IC_{50}$  of 15  $\mu$ M. However, the  $IC_{50}$  of isoacteoside was found to be much higher against the normal ovarian cells ( $IC_{50}$  80  $\mu$ M). In addition, it was observed that the anticancer effects of isoacteoside on the ovarian cancer cells were concentration-dependent (Figure 2).



**Figure 3.** Isoacteoside induced apoptosis in OVCAR-3 cells at the doses shown as depicted by DAPI staining. The experiments were performed in triplicate.

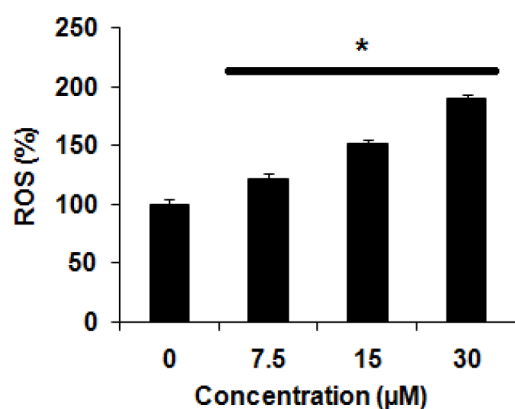


**Figure 4.** Estimation of apoptotic cell populations of OVCAR-3 cells determined by annexin V/PI staining. The Figure shows that isoacteoside increases the percentage of OVCAR-3 apoptotic cells in a concentration-dependent manner. The experiments were performed in triplicate.

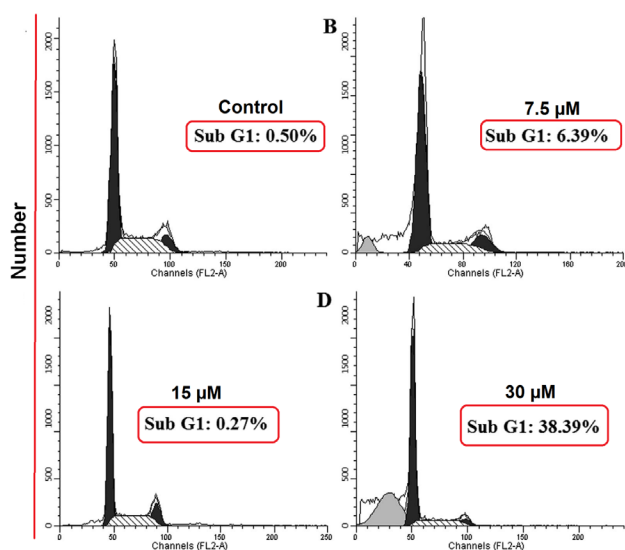


### *Isoacteoside triggers apoptotic cell death of OVCAR-3 cells*

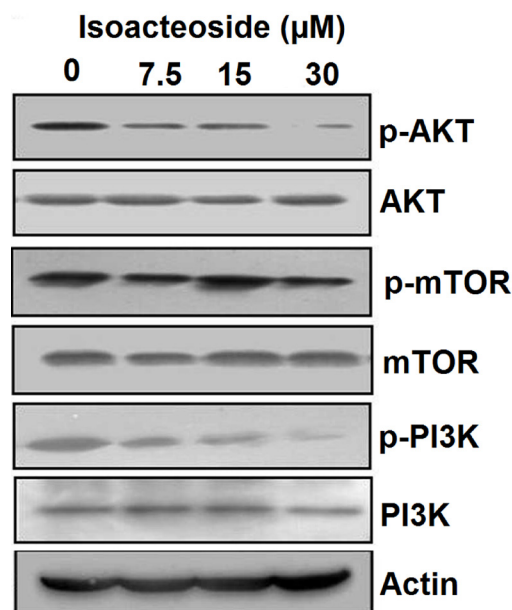
The influence of isoacteoside on the apoptotic pathway in OVCAR-3 cells was examined by DAPI and annexin V/PI staining. The results showed that isoacteoside caused considerable changes in the nuclear morphology of the OVCAR-3 cancer cells (Figure 3). These changes were characteristic of apoptosis and therefore annexin V/PI staining was performed to determine the percentage of the apoptotic cells (Figure 4). Furthermore, we evaluated the ROS levels of the isoacteoside-treated cells and found that the ROS levels increased up to 190% at 30  $\mu$ M concentration (Figure 5).



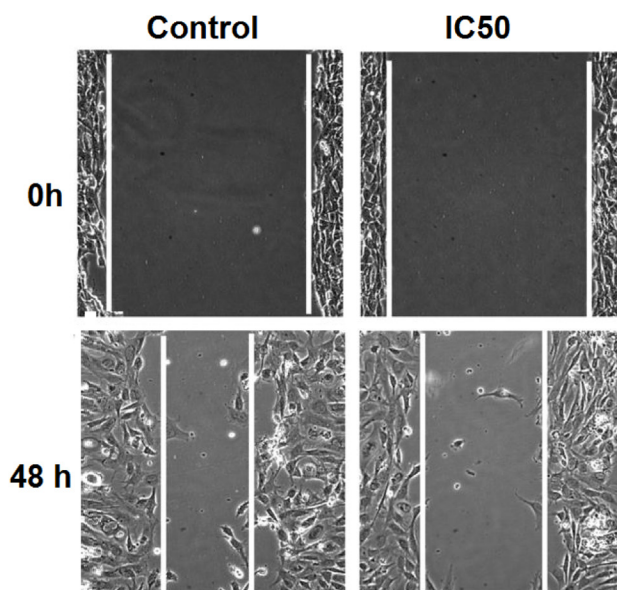
**Figure 5.** Isoacteoside caused generation of ROS in OVCAR-3 cells. The Figure shows that isoacteoside increases the ROS levels concentration-dependently. The experiments were performed in triplicate and shown as mean  $\pm$  SD (\* $p$ <0.05).



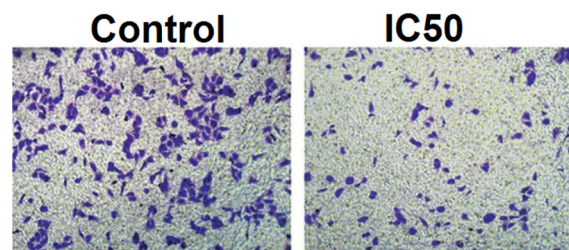
**Figure 6.** Isoacteoside effects cell cycle phase distribution of OVCAR-3 cells. The experiments were performed in triplicate and show that the percentage of the G2/M phase cells increases upon isoacteoside treatment, leading to G2/M cell cycle arrest.



**Figure 7.** Isoacteoside inhibited the AKT/PI3K/mTOR pathway as shown by western blot analysis. The experiments were performed in triplicate.



**Figure 8.** Isoacteoside inhibited the migration of OVCAR-3 cells as shown by wound healing assay. The experiments were performed in triplicate.



**Figure 9.** Isoacteoside inhibited the invasion of OVCAR-3 cells as shown by transwell assay. The experiments were performed in triplicate.

### *Isoacteoside induces Sub-G1 cell cycle arrest in OVCAR-3 cells*

To assess whether isoacteoside causes cell cycle arrest of cancer cells, the OVCAR3 cells were subjected to treatment with isoacteoside followed by flow cytometry. The results revealed that this molecule caused remarkable increase in the percentage of the OVCAR-3 cells in the Sub-G1 phase of the cell cycle. These effects of isoacteoside were found to be concentration-dependent (Figure 6).

### *Isoacteoside targets AKT/PI3K/mTOR pathway in ovarian cancer cells*

The effects of isoacteoside were also investigated on the AKT/PI3K/mTOR signalling pathways in OVCAR-3 cells and the results showed that this

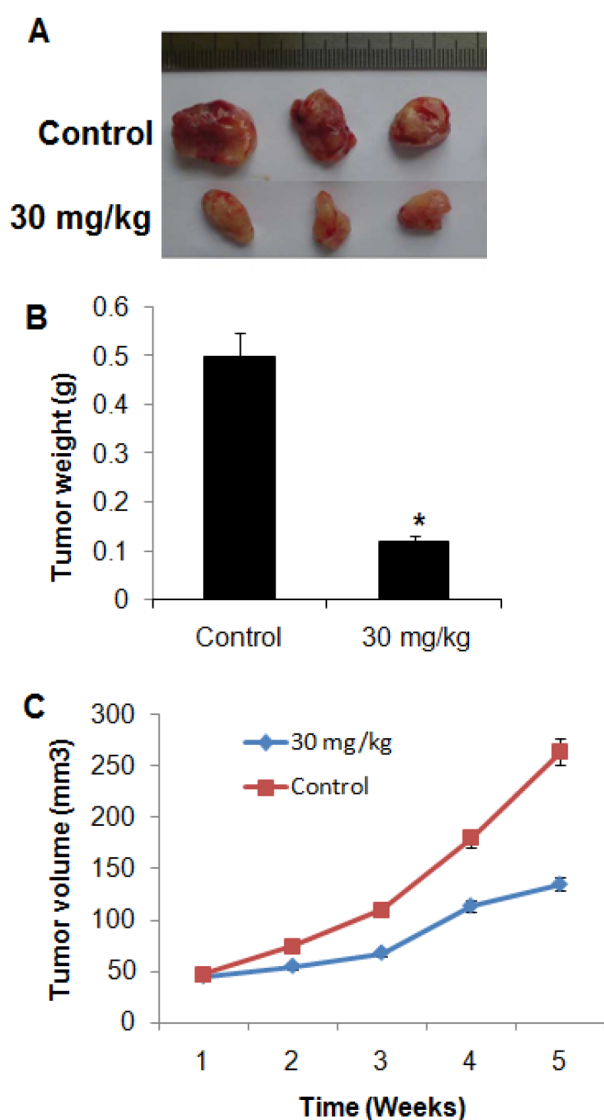
molecule caused downregulation of the p-AKT, p-mTOR, p-PI3K protein expression. However, the expression of the AKT, PI3K and mTOR showed no apparent change upon treatment with isoacteoside (Figure 7).

### *Isoacteoside inhibits cell migration and invasion*

The effect of isoacteoside was also assessed on the cell migration and invasion ability of the ovarian cancer cells at  $IC_{50}$  of isoacteoside (15  $\mu$ M). The results revealed that isoacteoside could inhibit the migration of the OVCAR-3 cancer cells (Figure 8). Similar effects were also observed on the cell invasion of the OVCAR-3 cancer cells (Figure 9).

### *Isoacteoside inhibits the tumor growth in vivo*

The effects of isoacteoside were also evaluated *in vivo* on the xenografted tumors and it was observed that this molecule significantly inhibited the tumor growth (Figure 10A). At 30 mg/kg of the test compound the tumor weight was considerably reduced (Figure 10B). Furthermore, the tumor volume showed a time-dependent decrease upon treatment with isoacteoside (Figure 10C).



**Figure 10.** (A) Isoacteoside inhibited tumor growth, (B) tumor weight and (C) tumor volume at 30 mg/kg in xenografted mice models. The experiments were performed in triplicate and shown as mean  $\pm$  SD (\* $p < 0.05$ ).

## Discussion

Ovarian cancer is probably the most deadly malignancy among all the gynecological cancers and is responsible for a large number of deaths in women [13]. The prevalence of ovarian cancer is increasing and the current treatments are far from satisfactory [14]. Moreover, the chemotherapeutic drugs currently used for the treatment of ovarian cancer have a number of adverse effects [4]. In the present study the anticancer effects of isoacteoside were evaluated against the OVCAR-3 cell line and normal ovarian SV40 cells. What it was observed was that isoacteoside exerted anticancer effects specifically on ovarian cancer cells and exhibited an  $IC_{50}$  of 15  $\mu$ M. However, the effects were minimal on the normal SV40 cells with an  $IC_{50}$  of 80  $\mu$ M. The results of the present investigation are complemented by previous investigations wherein isoacteoside has been reported to inhibit the growth of cancer cells [2]. The underlying mechanism by which isoacteoside exerts its anticancer effects was also investigated. Firstly, DAPI staining was carried out which indicated that isoacteoside induced apoptotic cell death of the OVCAR-3 cells and this was further confirmed by the annexin V/PI staining. Estimation of ROS levels in the isoacteoside-treated OVCAR-3 cells revealed that this molecule might be inducing apoptosis via production of ROS. This is also supported by previ-

ous studies wherein several natural products have been reported to induce ROS-mediated apoptosis in cancer cells [15]. In addition to apoptosis, cell cycle arrest is also an impressive mechanism to curb the growth of the cancer cells and in this study we observed that isoacteoside induced Sub-G1 cell cycle arrest in the OVCAR-3 cancer cells, thus inhibiting their growth. The fact that this compound inhibits the migration and invasion of the OVCAR-3 cells indicated that isoacteoside may prove to be an important antimetastatic agent *in vivo*. The capacity of cancer cells to metastasize to other parts of the body is an important factor for the prognosis of malignancies [17]. The anticancer effects of isoacteoside were also evaluated *in vivo* in the xenografted mice models and it was observed that this molecule could regress the tumor weight and

volume, indicating the potential of isoacteoside usage as a prospective anticancer agent.

## Conclusion

The results of the present study clearly indicated that isoacteoside inhibits the growth of ovarian cancer cells specifically by triggering apoptosis and cell cycle arrest. Besides, it also suppresses the migration and invasion of cancer cells and inhibits tumor growth *in vivo*. Taken together, we conclude that isoacteoside may prove beneficial in the clinical management of ovarian cancer.

## Conflict of interests

The authors declare no conflict of interests.

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