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ORIGINAL ARTICLE

Boswellic acid exerts potent anticancer effects in HCT-116 human colon cancer cells mediated via induction of apoptosis, cell cycle arrest, cell migration inhibition and inhibition of PI3K/AKT signalling pathway

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Summary

Purpose: *Boswellic acid is an important plant-derived natural product with tremendous pharmacological potential and has been reported to inhibit the growth of several types of cancer cells. In this study we report the anticancer activity of boswellic acid against human colon cancer cells via induction of apoptosis, cell cycle arrest and inhibition of cell migration and PI3K/AKT signalling pathway.*

Methods: *The antiproliferative effects of boswellic acid were assessed by MTT assay using different doses of the drug. The apoptotic effects were studied by DAPI and annexin V/PI staining assays, and cell cycle distribution was studied by flow cytometry. The effects of the drug on PI3K/AKT protein expression were studied using western blot analysis.*

Results: *The results of this study showed that boswellic acid suppresses the growth of HCT-116 colon cancer cells. The anticancer effects were found to be dose-dependent and the IC_{50} value was 15 μM against the HCT-116 colon cancer cells. The inhibition of growth of these cancer cells was mainly due to apoptosis and G2/M cell cycle arrest. Besides, boswellic acid altered the Bax/Bcl-2 ratio in the HCT-116 cancer cells and inhibited their migration as indicated by the cell migration assay. It was observed that boswellic acid decreased the expression of p-PI3K and p-AKT in a concentration-dependent manner. However, the expression of PI3K and AKT remained almost unaltered.*

Conclusion: *In conclusion, these results clearly suggest that boswellic acid could be employed in the treatment of colon cancer provided further in vivo and other in depth experiments are done.*

Key words: *apoptosis, boswellic acid, cell cycle arrest, colon cancer, PI3K/AKT*

Introduction

Plants contain a number of secondary metabolites which have been utilized as medicines since the very past [1]. Amongst these metabolites, tritepenoids have been reported to be of immense pharmacological potential [2]. Boswellic acid is a triterpenoid mainly isolated from the plants of *Boswellia serrata* [3]. Boswellic acid has been shown to exhibit a number of properties that include anticancer and antimicrobial activities and has been reported to eliminate different types of cancer cells via a number of mechanisms that include apoptosis and cell cycle arrest [4]. Over the years there have been drastic alterations in human lifestyles, accompanied with increased incidence of cancer

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around the globe. Colon cancer is one of the leading causes of cancer-related mortality worldwide with the majority of patients diagnosed with advanced cancer [5]. Currently, the treatment of colon cancer involves surgery followed by chemotherapy. However, the prognosis of this disease is rather poor and the mortality rate is high [6]. Therefore there is an urgent need to develop novel treatment strategies or explore novel targets for the treatment of this disease.

In this study we report on the anticancer activity of boswellic acid against human colon cancer cells via induction of apoptosis, cell cycle arrest and inhibition of cell migration and PI3K/AKT signalling pathway. To the best of our knowledge, this is the first such report on this type of cancer using such bioassays.

Methods

Chemicals, reagents and culture conditions

The chemicals and the reagents that were used in this study were procured from Sigma-Aldrich Co, St. Louis, USA. The antibodies were purchased from Santa Cruz Biotechnology Inc., St. Louis, USA. Human colon cancer HCT-116 cell lines were purchased from American Type Cell Collection. The cells were grown in RPMI-1640 medium containing 10% fetal bovine serum (FBS), streptomycin and penicillin (100 U/mL each) and maintained in air containing 5% CO_2.

Cell viability assay

For assessment of the cell viability, the HCT-116 cells were cultured in 96-well plates at a density of 5×10^3 cells/well. The cells were incubated overnight and then the RPMI-1640 medium was removed and replaced with a fresh RPMI-1640 medium with boswellic acid separately at different concentrations (0-100 µM) for 24 hrs. Thereafter, MTT solution of 0.5 mg/ml was added for 4 hrs and finally the absorbance was measured at 570 nm.

DAPI and annexin V/PI staining

Colon cancer HCT-116 cells were cultured (2×10^5 cells/well) in 6-well plates. The cells were then exposed to 0, 5, 10 and 20 µM of boswellic acid and incubated for 24 hrs. DAPI was carried out by treating the cells in 6-well plates. The cells were then washed with PBS and then fixed with 10% formaldehyde. The DAPI-stained cells were then subjected to fluorescence microscopy. For estimation of the apoptotic cell populations, a similar procedure was carried out except for the cells stained with annexin V/PI and analyzed by flow cytometry.

Cell cycle analysis

In order to estimate the number of cells in each phase of the cell cycle, the boswellic acid-treated HCT-116 colon cancer cells were harvested and washed with PBS. Thereafter the cells were fixed with 70% ethanol for about an hr and then washed again with PBS. The cells were finally resuspended in PI solution (50 µl/ml) and RNase1 (250 µg/ml). This was followed by incubation for 30 min at room temperature and the final investigation was performed under a fluorescence-activated cell sorting cater-plus cytometer using 10, 000 cells/group.

Wound healing assay for cell migration

The cell migration potential of boswellic acid-treated colon cancer HCT-116 cells was investigated by wound healing assay. Briefly, 5×10^4 cells/well were seeded in 96-well plates. Afterwards the plates were incubated overnight at 37°C to allow the cells to adhere. Then a wound was scratched using a sterile pipette tip after the cells reached 95% confluence. The cells were then washed with PBS to clear the detached cells, were monitored after a 20-h interval and photographed.

Determination of protein expression by western blotting

Total protein from untreated and boswellic acid-treated HCT-116 colon cancer cells was extracted in RIPA lysis buffer. Equal concentrations of the protein samples were run on SDS PAGE. This was followed by transfering to a polyvinylidene fluoride membrane. Afterwards, blocking was done with 5% non-fat milk, followed by incubation at room temperature for 1 hr. The membranes were then subjected to treatment with specific primary antibody at 4°C for 20 hrs. Thereafter, washing in washing buffer was carried out and then the membranes were incubated with secondary antibody for 1 hr. The protein bands were then visualised by an ECL Advanced Western Blot Detection Kit.

Results

Boswellic acid decreased the viability of HCT-116 colon cancer cells

The effects of boswellic acid (Figure 1) on cell viability were evaluated by MTT assay. The

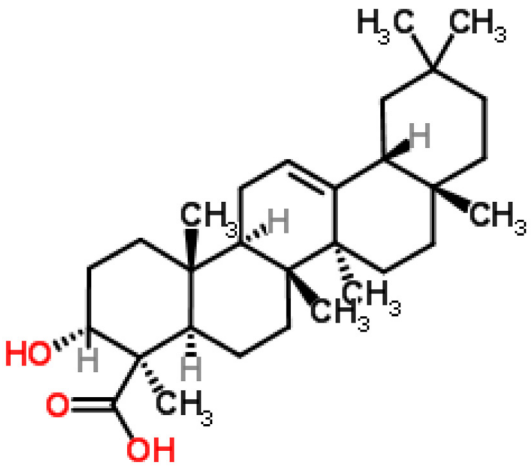

Figure 1. Structure of boswellic acid.

HCT-116 cells were treated with boswellic acid at varied concentrations (0, 5, 10 and 20 μM). The results of MTT assay revealed that boswellic acid exhibited considerable antiproliferative effects on the HCT-116 cells and the antiproliferative effects were found to be concentration-dependent (Figure 2). It was observed that the IC_{50} of boswellic acid against HCT-116 colon cancer cells was 15 μM. Moreover, boswellic acid also induced changes in the morphology of the HCT-116 cancer cells (Figure 3). These results clearly show that boswellic acid exerts anticancer effects on colon cancer cells.

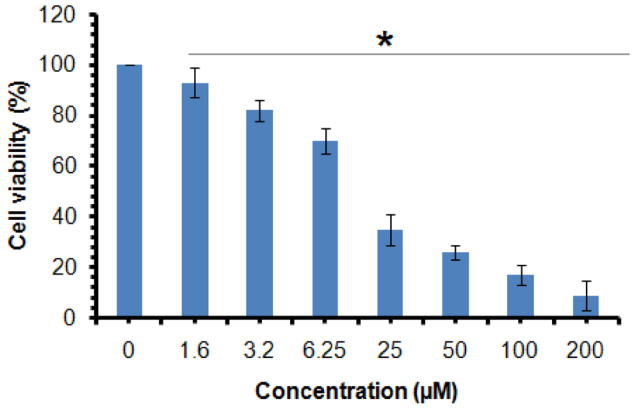

Figure 2. MTT assay showing the effect of boswellic acid on cell viability of HCT-116 cells. The values are mean±SD of three experiments (*p<0.05).

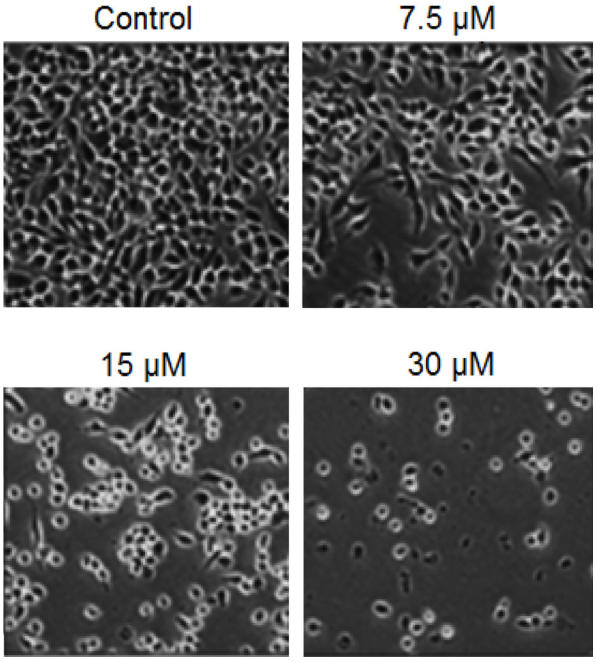

Figure 3. Effect of boswellic acid on cell morphology using phase contrast microscopy. Experiments were carried out in triplicate. Boswellic acid induced changes in the morphology of HCT-116 cancer cells, indicating that the drug exerts cytotoxic effects in these cells.

Boswellic acid induced apoptosis in HCT-116 colon cancer cells

The HCT-116 colon cancer cells were first treated with boswellic acid at different concentrations, subjected to DAPI staining, and finally observed under fluorescence microscope. Boswellic acid induced apoptosis in HCT-116 colon cancer

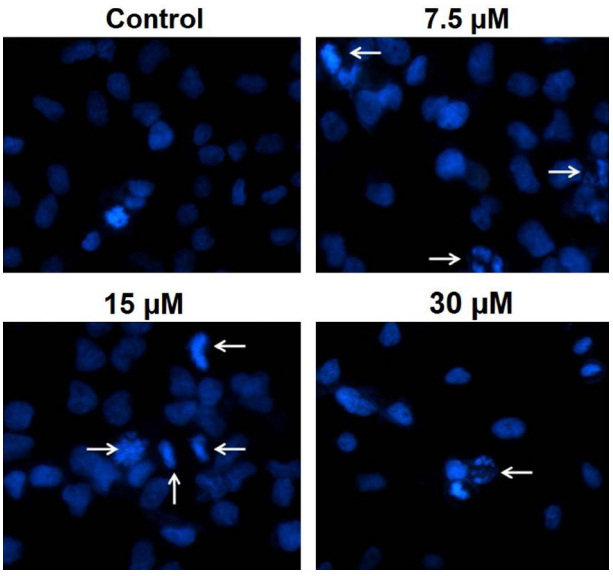

Figure 4. Effect of boswellic acid on the apoptosis of HCT-116 cells using fluorescence microscopy and DAPI. Experiments were carried out in triplicate. Boswellic acid induced apoptosis in HTC-116 colon cancer cells as evidenced from the increased number of cells with bright color nuclei. Arrows show the apoptotic cells.

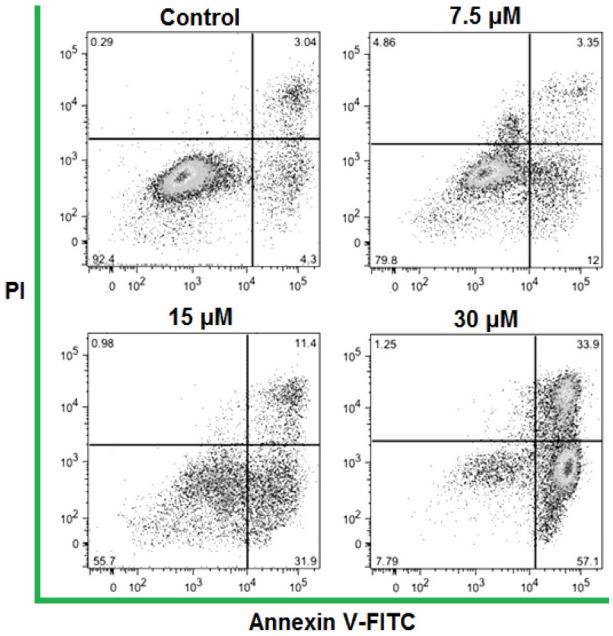

Figure 5. Estimation of apoptotic cell populations by annexin V/PI staining of HCT-116 cells using flow cytometry. Experiments were carried in triplicate. The results revealed that the apoptotic cell populations increased from 3.04% in the controls to 33.9% at 30μM concentration.

cells as evidenced from the increased number of cells with white color nuclei (Figure 4). The results of annexin V/PI further revealed that the apoptotic cell populations increased from 3.04% in the controls to 33.9% at 30 μM concentration (Figure 5). To further confirm the apoptosis at molecular level, we determined the expression of Bax and Bcl-2 proteins. The results showed that boswellic acid treatment increased the expression of Bax and decreased the expression of Bcl-2 in a concentration-dependent manner (Figure 6).

Boswellic acid caused G2/M cell cycle arrest in HCT-116 colon cancer cells

The distribution of HCT-116 colon cancer cells in the different cell cycle phases after treatment with boswellic acid at varied concentrations was determined by flow cytometry. The results showed that boswellic acid led to accumulation of HCT-116 colon cancer cells in G2/M phase of the cell cycle, ultimately prompting G2/M cell cycle arrest (Figure 7).

Boswellic acid inhibited cell migration of HCT-116 colon cancer cells

The effects of boswellic acid at IC_{50} were determined on the migration of HCT-116 colon cancer cells by the wound healing assay. The results showed that boswellic acid significantly inhibited

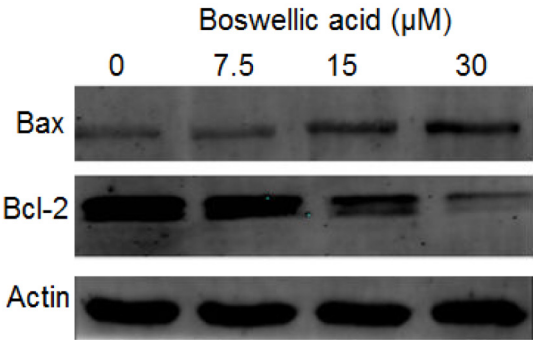

Figure 6. Effect of boswellic acid on the expression of Bax and Bcl-2 as indicated by western blotting. Experiments were carried out in triplicate. Boswellic acid treatment increased the expression of Bax and decreased the expression of Bcl-2 in a concentration-dependent manner.

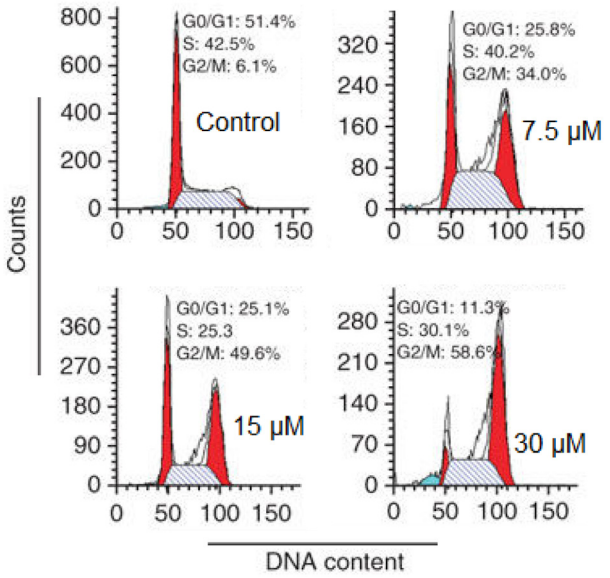

Figure 7. Effect of boswellic acid on cell cycle distribution of HCT-116 cells using flow cytometry. Experiments were carried out in triplicate. Boswellic acid led to accumulation of HCT-116 colon cancer cells in G2/M phase of the cell cycle, ultimately prompting G2/M cell cycle arrest.

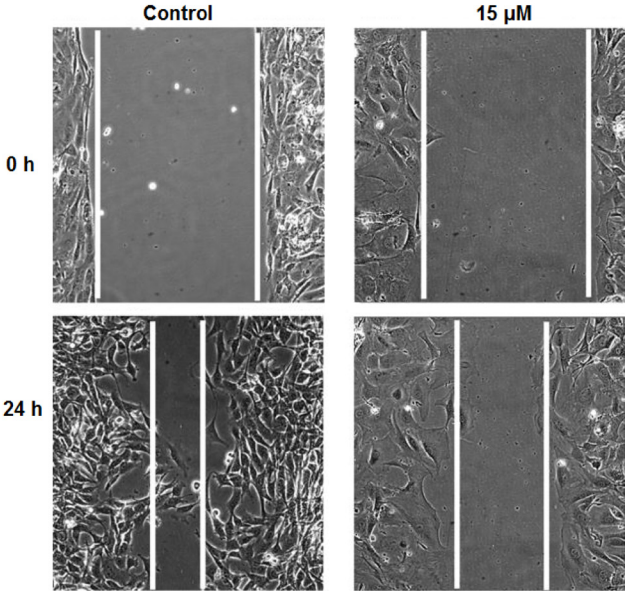

Figure 8. Effect of boswellic acid on migration of HCT-116 cells as indicated by wound healing assay. Experiments were carried out in triplicate. Boswellic acid significantly inhibited the cell migration in a dose-dependent manner.

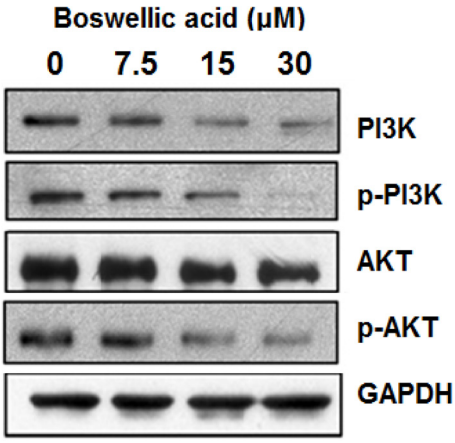

Figure 9. Effect of boswellic acid on protein expression of PI3K/AKT pathway in HCT-116 cells as indicated by western blotting. Experiments were carried out in triplicate. Boswellic acid decreased the expression of p-PI3K and p-AKT in a concentration-dependent manner. However, the expressions of PI3K and AKT remained almost unaltered.

the migration of the HCT-116 colon cancer cells (Figure 8).

Boswellic acid inhibited PI3K/AKT signalling pathway

In the present study, the effect of boswellic acid on PI3K/AKT signalling pathway was also investigated. It was observed that boswellic acid decreased the expression of p-PI3K and p-AKT in a concentration-dependent manner. However, the expression of PI3K and AKT remained almost unaltered (Figure 9).

Discussion

Colon cancer is a very frequent malignancy, causing a significant number of deaths worldwide [8]. The systemic treatment options used for colon cancer are very limited and with lot of side effects [9]. Therefore there is an urgent need to look for strong and novel therapeutic targets to curb the growing incidence of the colon cancer. Over the years plant derived secondary metabolites have attained considerable attention as bioactive molecules. They have been shown to exert anticancer activity against a range of cancer types [10]. In this context, the present study was carried out to investigate the anticancer effects of boswellic acid against HCT-116 colon cancer cells. The results showed that boswellic acid exhibits considerable anticancer activity with an IC_{50} of 15 µM against HCT-116 colon cancer cells. To further unveil the reasons behind its anticancer effects we carried out DAPI staining and observed that boswellic acid exerted anticancer effects via induction of apoptosis. Moreover, the apoptotic effects of boswellic acid were concentration-dependent and the apoptotic cell populations increased with increase in the concentration of the boswellic acid as evidenced from the annexin V/PI staining. Apoptosis is a biological event by which programmed series of actions lead to cell death without releasing any harmful chemicals. It is an important mechanism by which several of the chemotherapeutic drugs exert their anti-proliferative activities [11]. The results of the present study are well supported by previous studies wherein boswellic acid has been reported to trigger apoptosis in cancer cells [6,12]. Next, to understand if the boswellic acid -induced apoptosis follows the mitochondrial pathway, we studied and estimated the expression of Bax and Bcl-2 proteins. The results of western blotting revealed that the expression of Bax was increased and that of Bcl-2 was suppressed in response to the boswellic acid treatment. Another important mechanism that has been reported to contribute to the anticancer effects of many well known drugs is cell cycle arrest [13]. Some anticancer drugs halt the progression of the cells from one phase of the cell cycle to the other by targeting specific proteins, leading to accumulation of cancer cells at a particular phase. Arrest of the cell cycle prevents the cancer cell to develop into a tumor and to spread to other parts of the body [15]. Consistent with this, we observed that boswellic acid caused G2/M cell cycle arrest of HCT-116 colon cancer cells in a concentration-dependent manner. Anticancer agents that inhibit the migration of cancer cells have been reported to efficiently inhibit the cancer cells' metastasis [16]. In the present study we also observed that boswellic acid could efficiently inhibit the migration of HCT-116 colon cancer cells. Earlier it has been reported that many anticancer molecules target PI3K/AKT signalling pathway in cancer cells [14-16]. Therefore, we investigated the effect of boswellic acid on the expression of p-AKT, AKT, p-PI3K and PI3K and observed that boswellic acid decreased the expression of p-PI3K and p-AKT in a concentration-dependent manner, indicating that its anticancer effects may in part be due to inhibition of PI3K/AKT signalling pathway.

Conclusion

From the results of this study we conclude that boswellic acid is an important natural product that could be utilized in the treatment of colon cancer. It exerts its anticancer effects through apoptosis and cell cycle arrest. However, this molecule would require further evaluation both *in vitro* and *in vivo*.

Conflict of interests

The authors declare no conflict of interests.

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