Original Article
Osthenol inhibits the viability of HCT116 cells through suppressing PI3K/AKT signaling pathway

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Abstract: Colon cancer is one of the leading causes of cancer related deaths. The natural products show a great potential for anti-cancer use. The objective of the present study to explore the anti-tumor ability of osthenol in human colon cancer (HCT116) cells. The MTT assay revealed the antiproliferative activity of osthenol in a concentration dependent manner. And it also can promote HCT116 cells apoptosis in a concentration dependent manner. Moreover the expression of caspase-3, caspase-8 and caspase-9 in HCT116 were significant enhanced after treated with osthenol. However the results also showed the osthenol was able to decrease HCT116 cells migration and arrest cell cycle in G1 phase. Furthermore the signal mechanism study demonstrated that the anti-tumor effect of osthenol was associated with PI3K/AKT signaling pathway. The current study strongly reveals that osthenol inhibits colon cancer cells proliferation by inducing apoptosis mediated through cell cycle arrest and activation of caspases. These findings may provide a promising method to treat the colon cancer.

Keywords: Colon cancer, apoptosis, osthenol, cell cycle, PI3K/AKT

Introduction

Colon cancer is the most common cancer in the world with an estimated 750,000 new cases diagnosed annually, and the severe cause of cancer death worldwide with an estimated 598,000 death annually [1]. More than 80% of colon cancer deaths occur in developing countries. In China, colon cancer is the fourth most common cause of all cancer deaths and accounts for most of colon cancer deaths of the world [2]. Therefore, colon cancer represents a significant public health issues in developing countries.

Surgery is the most common treatment approach for early stage colon cancer and have high rate of cure for patients with early stage colon cancer [3]. Unfortunately, more than 80% colon cancer cases are diagnosed in the intermediate or late stages with or unresectable advanced disease. The current life expectancy for patients with late stage colon cancer is only about a few months [4]. These late stage unresectable or metastatic colon cancers often carry a poor prognosis, and systemic therapy with cytotoxic agents provides marginal benefit. Because of the poor response of late stage colon cancer to systemic therapy, there has been a sense of nihilism for this disease for decades. The search for effective reagents with minimal adverse effects for the treatment of colon cancer remains the top priority of cancer research [3].

In recent decades, a number of studies have drawn attention to natural products extracted from Chinese medicinal herbs as anticancer agents in colon cancer therapy [5, 6]. Over 60% of the current anticancer drugs have their origin in one way or another from natural sources. Nature continues to be the most prolific source of biologically active and diverse chemotypes. Among natural products, natural coumarin represent a structurally diverse group of organic compounds with potent bioactivity including antitumor effects [7]. However the antitumor mechanisms of these natural products are still not clear.

Phosphatase and tension homolog deleted on chromosome 10 (PTEN)/phosphatidylinositol
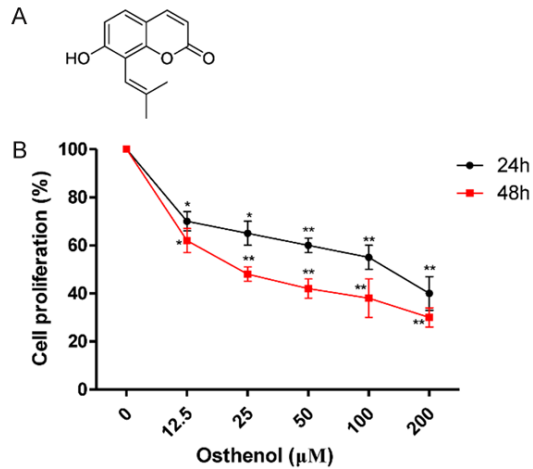
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Figure 1. Osthenol inhibits HCT16 cells proliferation. A. The chemical structure of osthenol. B. The proliferation of HCT116 cells treated with osthenol for 24 and 48 respectively.

3-kinase (PI3K)/AKT represent a critical pathway regulating the signaling of multiple biological processes such as apoptosis, metabolism, cell proliferation and cell growth [8]. PTEN acts as a tumor suppressor factor in AKT-mediated survival pathway via binding phosphatidyl-inositol, 3, 4, 5 triphosphate (PIP3), the product of PI3K. Increase in PIP3 recruits AKT to the membrane, where it is activated by other kinases. Thus in the present study, we explored the anticancer effects of osthenol, isolated from *Angelica dahurica*, in human colon cancer (HCT116) cells and its possible mechanism.

Materials and methods

Materials

HPLC grade authentic standard of osthenol was purchased from Chengdu Congon Bio-tech Co., Ltd. (Sichuan, China), and dissolved in DMSO. Phospho-PTEN, PI3K, AKT, phospho-AKT, p38, phospho-p38, c-Jun, c-Fos, Bim, Bid, Puma, Bax, Bcl, Mcl-1, caspase-9, cleaved caspase-9, caspase-8, caspase-3, and cleaved caspase-3 anti-rabbit antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). Loading control β-actin antibody was purchased from CST. All other chemicals and solvents used were of the highest purity grade.

Cell culture

Human colon cancer HCT116 cell line was purchased from American Type Culture Collection

(ATCC, Rockville, MD). The normal colonic epithelial cells were obtained from Hongshun Biotechnology Company (Shanghai, China). The two cell lines were routinely cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; GIBCO/BRL, NY), 100 mg/l penicillin G and 100 U/ml streptomycin sulfate at 37°C in 5% CO_2.

Cell viability assay

Cells were seeded at a 1×10^4 density per well in 96-well plates and exposed to various concentrations of osthenol (0-200 μM) at different time points within 48 h. 10 μl 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (5 mg/ml) (Sigma-Aldrich) were added to each well and cells were kept at 37°C for 4 h. The resulting formazan crystals were solubilized in 200 ml dimethyl sulfoxide (DMSO). The density of the solubilized formazan was read at 570 nm spectrophotometrically (bio-RAD, Hercules, CA, USA).

Invasion assays

Invasion assays were performed in 24-well Transwell chambers (Corning, Acton, MA, USA) containing polycarbonate filters with 8-μm pores coated with Matrigel (BD Biosciences, Bedford, MA, USA). Briefly, HCT116 cells were allowed to grow to subconfluency and were serum-starved for 48 h. After detachment with trypsin, the cells were washed with PBS, resuspended in serum-free medium and 2×10^4 cells were added to the upper chamber. Complete medium was added to the bottom chamber as a chemoattractant. Then, different concentrations of osthenol (0, 25, 50, 100 μM) was added to the inside of each insert and incubated for 24 h in a cell culture incubator. Uninvaded cells on the upper surface of the filter were mechanically removed with a cotton swab, and the invasive cells on the lower membrane surface were fixed with methanol and stained with 0.1% crystal dark blue. The invading cells were counted and photographed under a microscope at ×100 magnification. Five fields were counted per filter in each group and the experiment was conducted in triplicate.

DAPI staining

HCT116 cells were treated with osthenol at the final concentrations of 0, 25, 50 and 100 μM for 48 h, and then washed once in phosphate
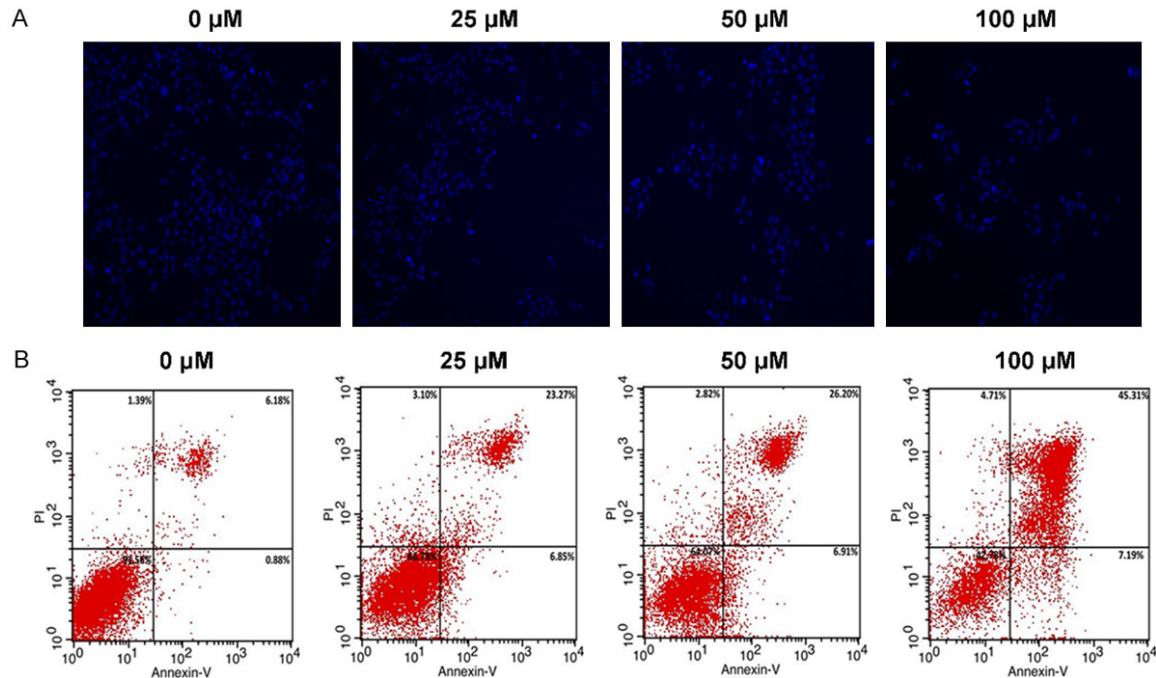
Figure 2. Osthenol promotes HCT116 cells apoptosis. A. DAPI staining showed the nucleus of HCT16 cells treated with 0, 25, 50, 100 μM osthenol. B. The apoptosis of HCT116 cells treated with 0, 25, 50, 100 μM osthenol.

buffer saline (PBS) followed by fixation in cold methanol: acetone (1:1) for 5 min. After washing twice in PBS for 5 min, these cells were stained with 4 μg/ml DAPI for 10 min at room temperature and subsequently examined by fluorescence microscopy (Eclipse E-800; Nikon, Tokyo, Japan). Apoptotic cells were identified by chromatin condensation and nuclear fragmentation.

Apoptosis assay

Annexin V-FITC/propidium iodide (PI) double-staining was per-formed with an Annexin V-FITC Kit (BDBioscience, USA). Human colon cancer (HCT116) cancer cells were treated with different concentrations (0, 25, 50 and 100 μM) of osthenol for 48 h. The cells were trypsinized, rinsed twice with PBS, and resuspended in 1×binding buffer. The cells were labeled with 10 μl of FITC-conjugated annexin V and 10 μl of propidium iodide. The cells were incubated for 20 min in dark at 37°C and then 450 μl of binding buffer was added and the samples were immediately analyzed with a flow cytometer (Becton Dickinson, San Jose, CA). The annexin V-FITC-/PI- cell population was considered as normal, while the annexin V-FITC+/PI- and Annexin V-FITC+/PI+ cell populations were con-

sidered as indicators of early and late apoptotic cells, respectively.

Activity assay of caspases

Cells were treated with various concentrations of osthenol (0-100 μM) for 48 h. For the activity assay, the cell lysate was added into Protease Assay Buffer in 96-well plate. Reaction mixtures with lysis buffer were used as negative controls. Cells treated with DMSO (0.1%) were treated as vehicle control. The reaction mixtures were incubated for 1 h at 37°C. The AMC liberated from the substrates was measured using spectrofluorometer of Victor 2 plate reader (Perkin Elmer, Massachusetts, USA) with an excitation wave-length of 380 nm and an emission wavelength of 430 nm.

Cell cycle analysis

Briefly, human colon cancer cells (1×10^6) were seeded into each well of 6-well plates and incubated for 24 h for cell attachment and recovery. The cells were treated with different concentrations (0, 25, 50 and 100 μM) of osthenol. Untreated cells (control) were also incorporated. After incubation for 24 h, the cells were harvested and fixed with ice-cold 70% ethanol (5 mL) at -20°C for 2 h. Prior to analysis, the cells
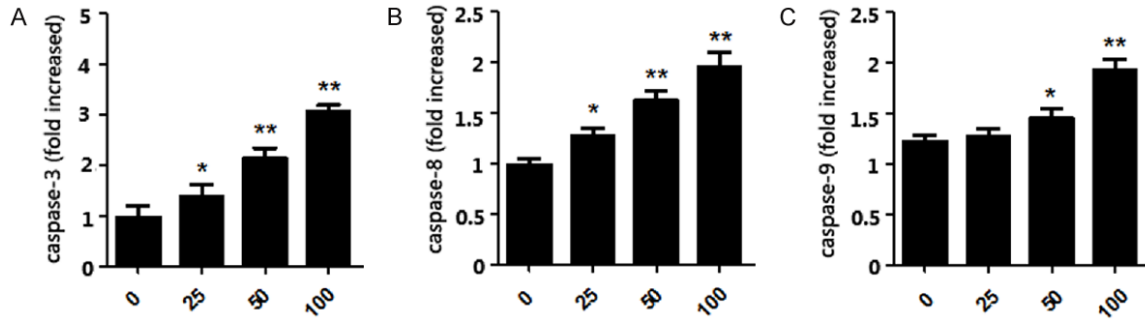
Figure 3. The expression of caspase-3, caspase-8 and caspase-9 in HCT116 cells treated with 0, 25, 50, 100 μM osthenol respectively. *P<0.05, **P<0.01.

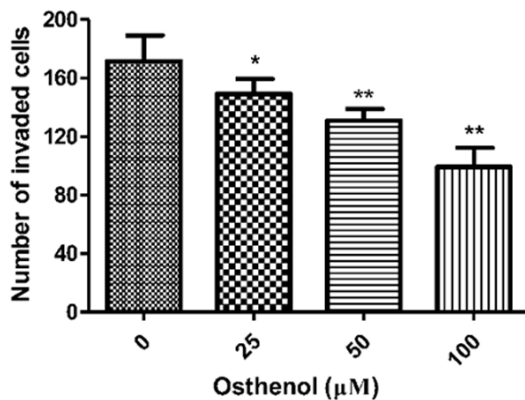**Figure 4.** The number of invaded HCT116 cells treated with 0, 25, 50, 100 μM osthenol for 48 h respectively. *P<0.05, **P<0.01.

were washed with cold PBS and re-suspended in 400 μl of PBS,20 μl PIand 20 μl RNase A. The DNA contents were recorded by a flow cytometer (Becton Dickinson, San Jose, CA) equipped with Cell Quest software.

Western blot analysis

Western blot assay was done as previously reported with slight modifications [9]. Bradford assay (Bio-Rad) was used to determine the protein content. After electrophoresing a total of 20-40 Ag of protein on 15% SDS-PAGE gels, it was transferred to nitrocellulose membranes. Membranes were blocked, incubated with primary Abs at the suitable dose, and consequently incubated with primary antibody, washed and incubated with horseradish peroxidase conjugated secondary antibody (1:2500 dilution; Bio-Rad). Detection was performed using a chemiluminescent western detection kit (Cell Signaling Technology, Inc., Danvers, MA, USA).

Statistical analysis

Data are expressed as mean ± SD of three independent experiments. SPSS.13.0 software was used to perform statistical analysis. Differences were analyzed using one-way analysis of variance (ANOVA) or two-way ANOVA. P<0.05 was considered statistically significant.

Results

Antiproliferative activity of osthenol in human colon cancer cells

To examine the inhibitory effect of osthenol on the proliferation of human colon cancer cells, MTT assay was conducted. HCT116 cells were treated with different concentrations (0, 12.5, 25, 50, 100 and 200 μM) of osthenol dissolved in DMSO and the same volume of DMSO was used as a control. The results showed that osthenol exerted potent and dose-dependent as well as time dependent antiproliferative effects on HCT116 cancer cells after treatment for 24 and 48 h (**Figure 1**).

Effects of osthenol on the apoptosis of HCT116 cells

As shown in **Figure 2A**, DAPI staining showed the nucleus of HCT116 cells gradually reduced with the increased concentration of osthenol. To further confirm the pro-apoptotic effect of osthenol, fluorescent Annexin V-FITC/PI double staining was performed. HCT116 cells were treated with different concentration (0, 25, 50 and 100 μM) of osthenol for 48 h. Osthenol induced both early and late apoptosis in a concentration-dependent manner as compared to the untreated control cells (**Figure 2B**). When the cells were treated with 25, 50 and 100 μM
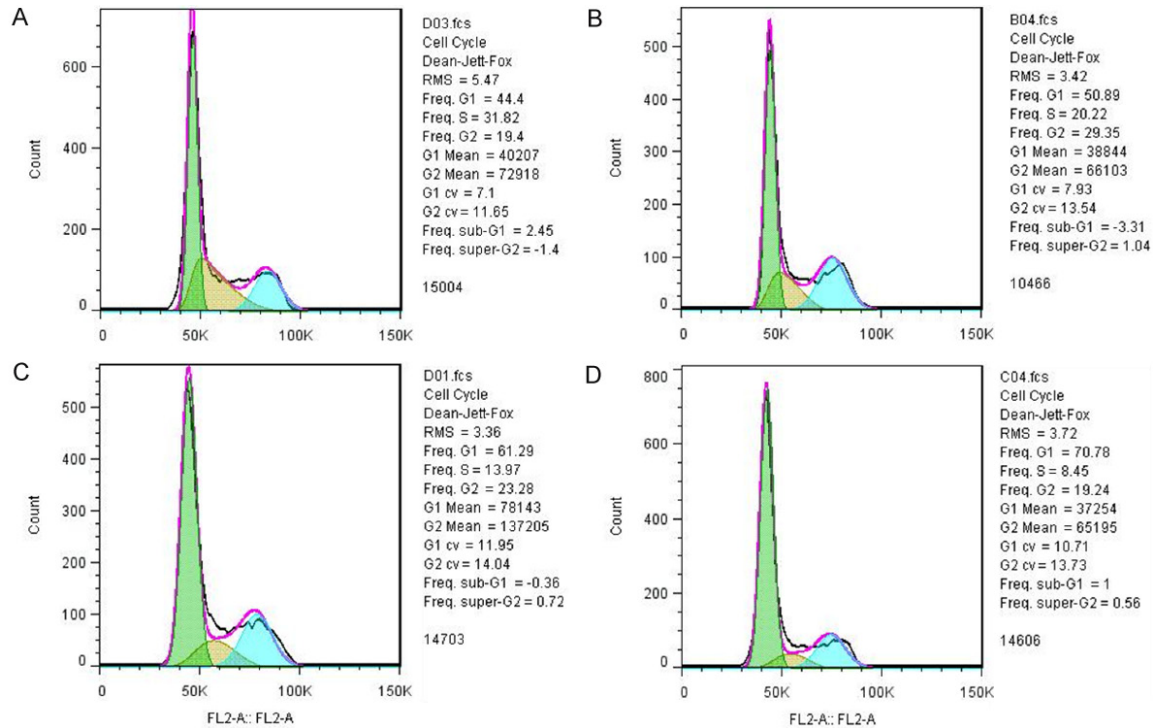
Figure 5. Effect of osthenol on the cell cycle arrest in human colon cancer (HCT116) cells. Cells were treated with (A) 0 µM, (B) 25 µM, (C) 50 µM and (D) 100 µM of osthenol for 48 h.

for 48 h, the average proportion of Annexin V-staining positive cells (total apoptotic cells) significantly increased from 7.06% in control to 30.12%, 33.11% and 52.5% respectively.

Osthenol induced caspase apoptosis in colon cancer cells

Osthenol induced a concentration-dependent activation of caspase-3, caspase-9, and caspase-8. As shown in **Figure 3**, the expression of caspase-3, caspase-9, and caspase-8 was increased with the increased concentration of osthenol from 0 to 100 µM. And the expression of caspase-3, caspase-9, and caspase-8 in HCT 116 cells treated with 25, 50, 100 µM osthenol were higher than HCT cells without osthenol treatments (P<0.05).

Osthenol inhibits invasion of HCT116

The effects of osthenol on HCT116 cells invasion were evaluated using Matrigel-coated transwell invasion assays. The results clearly revealed that osthenol significantly inhibited HCT116 cells invasion in a dose-dependent manner (**Figure 4**). The invaded HCT116 cells in 25 µM osthenol treated group were reduced

than HCT116 cells without osthenol treatment (P<0.05). Moreover the invaded HCT116 cells in 50, 100 µM osthenol treated group were significantly reduced than HCT116 cells without osthenol treatment (P<0.01).

Osthenol induced G1 cell cycle arrest in HCT116 colon cancer cells

To determine the distribution of osthenol-treated HCT116 cells in different phases of the cell cycle, DNA content in cells was detected by propidium iodide (PI) staining and flow cytometry. The results showed that treatment with different concentrations of osthenol for 48 h led to an increase in the population of cells in the G1 phase (apoptotic population) (P<0.01) (**Figure 5**). As compared to the control (**Figure 5A**), where 44.4% of the cells were in G1 phase, 25, 50 and 100 µM osthenol-treated cells showed 50.9% (**Figure 5B**), 60.3% (**Figure 5C**) and 70.8% (**Figure 5D**) of the cells in the G1 phase (apoptotic phase) of the cell cycle.

Osthenol altered PI3K/AKT activation in HCT116 cells

To understand the effect of osthenol on PI3K/AKT signaling cascade, we first investigated the
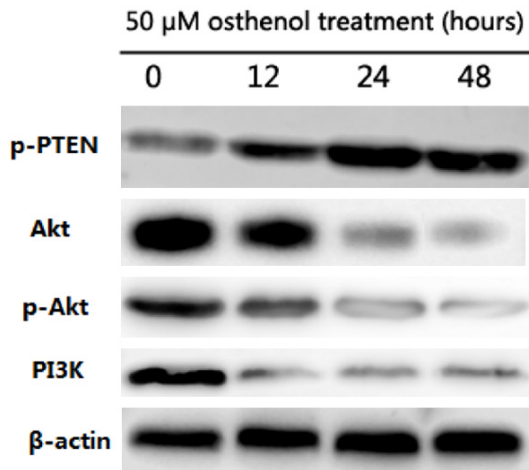
Figure 6. The expression of p-PTEN, AKT, p-AKT, PI3K in HCT116 cells treated with 50 μM osthenol respectively. β-actin was used as a loading control.

expression level of phospho-PTEN, the phosphorylated form of major negative regulator of the PI3K/Akt signaling pathway. Our results indicated that PTEN phosphorylation increased time-dependently following osthenol treatment in HCT116 cells (**Figure 6**). We also detected that PI3K and AKT expression profiles were both downregulated within 48 h after osthenol treatment in HCT116 cells. In addition, the phosphorylated form of AKT, which is required for its activation, was also found dephosphorylated in HCT116 cells.

Discussion

Angelica dahurica, belonging to the family Apiaceae, is a perennial plant widely distributed in China, Korea, Japan, and Russia. As a well-known herbal medicine, the roots of *A. dahurica* are commonly used for the treatment of headache, toothache, abscess, furunculosis, and acne. Up to date, more than 100 coumarins have been obtained from *A. dahurica*, exhibiting notable and diverse pharmaceutical properties, such as anti-tumor and, anti-inflammatory, anti-oxidative, and acetylcholinesterase inhibitory activities [10].

Apoptosis is a cellular suicide program that exterminates unwanted, faulty and potentially dangerous cells during the development and maintenance of cell homeostasis. Inducing apoptosis is a key tactic to eliminate cancer cells without stimulating an inflammatory reaction. Regulation of apoptotic signaling path-

ways encompasses a complicated system consisting of several elements. Several conventional drugs are currently used in anticancer chemotherapy which are believed to induce cell apoptosis via activation of these elements [11, 12]. Therefore, the ability of cancer cells to induce the apoptotic program has been recognized as one of the major mechanisms which might serve for the development of novel approaches to treat cancer. Caspases are the vital machineries in the implementation of apoptosis [13, 14]. Usually, caspases associated with apoptosis can be divided into the initiator caspases and the executioner caspases. Caspase-8 and -9 are the initiator caspases in the death receptor and the mitochondrial pathways, respectively. Caspase-3, is the crucial executioner caspase in apoptosis pathway [15, 16]. Our results showed that osthenol induces apoptosis which is mediated through the activation of caspase-3, -8 and -9. Procaspase-9, procaspase-8 and procaspase-3 decreased with the increased dose of osthenol, while as their cleaved form increased especially at higher doses.

Dysregulation in the cell division and apoptosis are connected to the development of most cancers. Many anticancer drugs function primarily to induce apoptosis in cancer cells and prevent tumor development [17, 18]. The morphological changes of apoptosis observed in most cell types initially start with a reduction in cell volume and condensation of the nucleus [19]. In many cases extensive DNA damage age leads to activation of cell cycle check points and results in cell cycle arrest and apoptosis [20]. In the present study, we found that osthenol induced apoptosis in HCT116 colon cancer cells as revealed by fluorescence microscopy as well as Annexin V-FITC assay. When the cells were treated with 25, 50 and 100 μM for 48 h, the average proportion of Annexin V-staining positive cells (total apoptotic cells) significantly increased from 3.2% in control to 34.3%, 46.8% and 65.9% respectively. Further, we evaluated the effect of osthenol on cell cycle phase distribution using flow cytometry. It was observed that osthenol induced cell cycle arrest in the G1 phase.

Various natural products have been reported to induce cell cycle arrest including the triterpenes, coumarins, lignans etc. Coumarins are highly multifunctional compounds and as a

result have promise as agents in the treatment of cancer because of their ability to block the NF-κB activation, induce apoptosis, and prevent proliferation, invasion, metastasis and angiogenesis [21]. As far as osthenol is concerned, we could not find reports of its anticancer action or its effects on the cell cycle arrest. To the best of our knowledge, the current research work on this molecule has not been reported earlier and as such constitutes the first such report.

In conclusion, we can summarize that osthenol exhibits anti-proliferative effects in HCT116 cancer cells by inducing apoptosis which is mediated by activation of caspase-3, caspase-8 and caspase-9. Consequently, the effect of the osthenol on survival and stress-related pathway was described for the first time in the literature and concluded that the alterations in the PI3K/AKT could lead to the apoptosis and the alterations in these pathways might also cause by effects such as the induction of polyamine catabolism and related toxicity.

Disclosure of conflict of interest

None.

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