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ORIGINAL ARTICLE

Arctigenin induces apoptosis in colon cancer cells through ROS/p38MAPK pathway

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Summary

Purpose: *In the current study the antiproliferative effect of arctigenin, plant lignin, was evaluated on human colon cancer cell line HT-29. Furthermore, attempts were made to explore the signaling mechanism which may be responsible for its effect.*

Methods: *Cell growth inhibition was assessed by MTT and LDH assays. Flow cytometric analysis was performed to determine cell arrest in the cell cycle phase and apoptosis. Furthermore, to confirm the apoptotic activity of arctigenin, caspase-9 and -3 activities analysis was performed. The levels of reactive oxygen species (ROS) and p38 mitogen activated protein kinase (MAPK) were investigated to determine their role in inducing apoptosis in arctigenin-treated HT-29 colon cancer cell line.*

Results: *MTT and LDH results demonstrated significant cell growth inhibitory effect of arctigenin on HT-29 cells in a dose-dependent manner. Furthermore, increase in cell number arrested at G2/M phase was observed in flow cytometric analysis upon arctigenin treatment. In addition, arctigenin increased the apoptotic ratio in a dose-dependent manner. The involvement of intrinsic apoptotic pathway was indicated by the activation of caspase-9 and -3. Moreover, increased ROS production, activation of p38 MAPK and changes in mitochondrial membrane potential (ΔΨm) also revealed the role of intrinsic apoptotic signaling pathway in cell growth inhibition after arctigenin exposure.*

Conclusion: *Arctigenin induces apoptosis in HT-29 colon cancer cells by regulating ROS and p38 MAPK pathways.*

Key words: *antiproliferative, apoptosis, arctigenin, MAPK, ROS*

Introduction

Despite advancements in systemic therapies of colon cancer, this disease still remains one of the leading therapeutic challenges due to a rather high mortality rate [1]. Colorectal cancer ranks third in mortality globally, with around 1.36 million cases [2]. The existing chemotherapeutic drugs cause significant side effects [3]. In addition, the moderate response rates of the existing chemotherapeutic agents calls for discovery of safe and novel natural products with antineoplastic activities [4]. Previous studies have reported the use of natural compounds as anticancer agents against various colon cancer cell lines [5,6].

Traditional chinese herbs are rich in various natural compounds possessing medicinal properties. Arctigenin, a phenyl propanoid dibenzyl butyrolactone lignin (Figure 1), is among such well known natural compounds found in many herbs of Asteracea family such as Arctium lappa and *Saussurea medusa* [7]. Arctigenin displays several pharmacological properties including antiinflammatory, antioxidant and anticancer activities [7]. Recent studies demonstrate that arctigenin induces cancer cell growth inhibition via apoptosis [8] or cell cycle arrest [9]. The ability of arctigenin in inducing apoptosis through wnt/β-catenin

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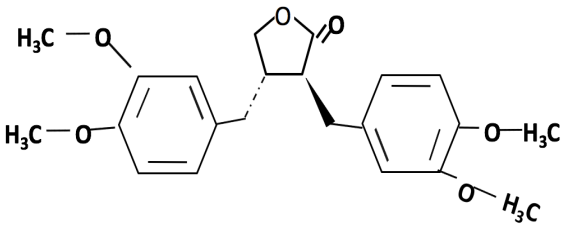

Figure 1. Structure of arctigenin.

signaling pathway in colon cancer was recently studied [10]. Apoptosis has been identified as the major molecular phenomenon via which natural products perform their anticancer activity [11]. Apoptosis, being an essential process for maintaining cellular balance, when disturbed leads to uncontrolled cell growth. This makes apoptosis as a targeting therapeutic strategy for combating cancer. Various stimuli can induce apoptosis in cells through different pathways. The generation of excess of ROS leads to oxidative stress which may regulate/activate MAPK pathway leading to cell apoptosis [12].

However, the role of arctigenin in inducing ROS-mediated p38 MAPK involved apoptosis in colon cancer cells is still unknown. Therefore, the present study was designed to uncover the antiproliferative effect of arctigenin on colon cancer cell line HT-29, by inducing apoptosis via ROS-mediated p38 MAPK pathway.

Methods

Chemicals and cell culture

Arctigenin (purity ≥98%) was purchased from Sigma–Aldrich, St. Louis, MO, USA. Stock solution of arctigenin (50 mM) was prepared using dimethyl sulfoxide (DMSO; Sigma). Human colon carcinoma cell line HT-29 was cultured in 10% fetal bovine serum (FBS; Sigma), 100 U/mL penicillin and 100 µg/mL streptomycin (Lonza, Basel, Switzerland) and L-glutamine (2 mM) supplemented RPMI 1640 (Invitrogen, Carlsbad, CA) in a humidified chamber at 37 °C with 95% air and 5 % CO_2. The HT-29 cells were maintained at 80-90% confluence level.

MTT assay

MTT assay was performed to analyze the antiproliferative effect of arctigenin on HT-29 colon carcinoma cell line. In brief, $2x10^4$ cells/well were plated in 96-well plate for 24 hrs at 37 °C for cell viability assay. Then, cells were exposed to different doses of arctigenin (0 µM, 0.5 µM, 1 µM, 2.5 µM, 5 µM and 10µM) for 0, 24 and 48 hrs. After the incubating period, 20 µl of 3-(4,5-dimethlthiazol-2-yl)-2,5-diphenyl-tetrazoliumbromide (MTT; Sigma) solution were added to the treated cells and were left for incubation at 37 °C for 3 hrs. Cell viability was recorded by mitochondrial succinate mediated MTT formazan production. Afterwards, the supernatant was removed and the formed MTT formazan was dissolved by adding of DMSO. The optical density of the samples at 570 nm wavelength was recorded to calculate the cell viability. For accuracy and reproducibility all the experimental sets were performed in triplicate and expressed as mean ± SD.

LDH assay

Cytotoxicity detection kit (Takara Bio Inc., Shiga, Japan) was used according to manufacturer's instructions to determine cell cytotoxicity by evaluating lactate dehydrogenase (LDH) in the damaged cells [13]. For LDH assay 2x104 HT-29 cells per well were first seeded in 96-well plate. The plates were then incubated at 37 °C for 24 hrs, after which cells were treated with 0 µM, 0.5 µM, 1 µM, 2.5 µM, 5 µM and 10µM of arctigenin for 0, 24 and 48 hrs. Culture medium from wells, PBS and LDH (1:4:5) were further mixed and incubated in the dark at 25 °C, which was followed by addition of stop solution. Cell cytotoxicity was calculated after measuring the optical density of samples at 490 nm.

Flow cytometric study for apoptosis evaluation

To determine the dose-dependent apoptotic effect of arctigenin HT-29 cells were treated with arctigenin at varying doses (0 µM, 0.5 µM, 1 µM, 2.5 µM, 5 µM and 10µM). After 24 hrs of treatment, samples were centrifuged at 1000 rpm for 5 min and cells were collected as pellet. Afterwards cells were fixed, washed and treated with 1% v/v Triton X-100. After incubation of cells at 37 °C for 10 min, propidium iodide solution (50 µg/ml; Sigma) was mixed at 4 °C in the dark to stain the DNA. FACScan flow cytometer (BD Biosciences, San Jose, CA, USA) was then used to determine the cell cycle phase distribution of cells and apoptosis rate.

Caspase-3 and caspase-9 activity assay

The activity of caspase-3 and caspase-9 was analyzed to confirm the apoptotic activity of arctigenin. Caspase-3 and Caspase-9 colorimetric assay kit (Biovision, Inc, Milpitas, CA, USA) was used to this purpose, according to the manufacturer's protocol. Briefly, HT-29 cells were treated with 0 µM, 0.5 µM, 1 µM, 2.5 µM, 5 µM and 10µM doses of arctigenin for 24 hrs and were further collected as pellet (12,000g for 10 min). After resuspension of the pellet in ice-cold lysis buffer for 10 min, the supernatant was collected (10,000g for 1 min) and quantified for total protein. Equal amount of proteins were then mixed with respective substrates at 37 °C for 30 min and p-nitroaniline was quantified in a

microtiter plate reader at 400 nm.

Measurements of intracellular reactive oxygen species

To determine the ROS level, HT-29 cells (2x105 cells/well) were cultured in 12-well plate and incubated with 0 μM, 1 μM, 2.5 μM, 5 μM and 10μM of arctigenin for 24 hrs. After incubation cells were harvested as pellet and washed by PBS (Sigma). The cells were further resuspended in fluorescent probes 2',7'-dichlorofluorescin diacetate (H2DCF-DA; Life Technologies, Carlsbad, CA, USA) for 20 min in the dark at 37 °C. Afterwards, the stained cells were analyzed by FACScan flow cytometer at excitation wavelength of 480 nm and emission wavelength of 525, as previously described [14].

Measurements of mitochondrial membrane potential ($\Delta\Psi m$)

HT-29 cells in exponential growth phase were cultured in 12-well plate ($2x10^5$ cells/well) and treated with varying doses of arctigenin. Following cell harvesting, the cells were stained with 3,3'-dihexy¬loxacarbocyanine iodide (DiOC6(3); Life Technologies, Carlsbad, CA, USA) in the dark for 20 min at 37 °C and analyzed by flow cytometer as previously reported [15]. This dye is voltage-dependent and mitochondria-specific.

Western blot analysis

After the treatment of HT-29 cells with arctigenin at varying doses, total cellular protein was collected following cell treatment with lysis buffer. Protein samples were prepared in accordance to a previously reported study with slight modifications [16]. Thereafter, the supernatant was collected from the samples after vortex and centrifuged at 12,000 rpm for 30 min. The retrieved supernatant was then used to determine protein amount using protein assay reagent (Bio-Rad) to adjust the same protein level in the samples using PBS. Protein samples were separated by sodium deodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) purchased from Sigma. Separated proteins were then transferred to nitrocellulose membrane electrophoretically and blocked with tween 20-trisbuffered saline (TBST) and nonfat dry milk (5%) at room temperature for 2 hrs. Further. Next, the membrane was incubated with primary antibodies (p-p38 MAPK, p38 MAPK and β-actin; Santa Cruz Biotech, CA, USA) overnight at 4 °C. Following triple washing with tris-buffered saline and tween solution (TBST), consisting of 10mmol/L tris-HCl (Sigma), pH 7.14, 150 mmol/L NaCl and 0.05% tween-20, the membrane was incubated with horseradish peroxidase labeled rabbit anti-mouse secondary antibody (Santa Cruz Biotech, CA, USA) at room temperature for 1 h. Again, the membrane was washed thrice with TBST and the bands were detected using enhanced chemiluminescence detection system (FUJFILM Las-3000 mini, Tokyo, Japan).

Inhibition of MAPK signaling pathway

The effect of ROS scavenger N-acetyl-L-cysteine (NAC; Sigma), and MAPK inhibitor, hydrochloride4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) 1 H-imidazole (SB203580; Sigma), on arctigenin-induced apoptosis and antiproliferative effect in HT-29 cell was also studied. Briefly, HT-29 were plated ($3.5 X 10^4$ cells/well) in a 48-well dish were treated with arctigenin for 24 hrs. The cells were pre-incubated with or without 5 mM of NAC or 20 μM of p38 specific inhibitor, SB203580, for 2 hrs. Cell viability of HT-29 cells was then analyzed using MTT assay.

Statistics

Statistical analysis was evaluated using graphpad Prism 5.0 (San Diego, CA) and data was assessed using two-tailed Student's t test and two-way ANOVA test. A p value <0.05 was considered statistically significant.

Results

Antiproliferative effect of arctigenin on HT-29 cells

The inhibitory effect of various doses of arctigenin on HT-29 colon carcinoma cell line was assessed by MTT assay following 24 and 48 hrs of treatment. The antiproliferative effect of arctigenin on HT-29 cells was dose-dependent at all time points (Figure 2A). Approximately 45 and 70% decrease in the viability of HT-29 cells were observed after 24 hrs of arctigenin treatment at a dose of 5 μM and 10 μM, respectively. Significant growth inhibitory effect was also observed by arctigenin at 1 and 2.5 μM doses after 48 hrs of treatment. The observed results of LDH assay were also in concordance with MTT assay results, demonstrating dose-dependent cell cytotoxicity of arctigenin on HT-29 cells (Figure 2B).

Apoptotic effect and cell cycle arrest induced by arctigenin on HT-29 cells

To determine whether the observed antiproliferative effect of arctigenin on HT-29 cells was due to apoptosis, flow cytometry was used after 24 hrs of treatment. The results showed dose-dependent increase in the apoptotic rate of HT-29 cells upon treatment with arctigenin. The highest apoptotic rate observed was 32.22±2.15% at the dose of 10 μM (Figure 3). Furthermore, it was also observed that arctigenin promoted cell cycle arrest at G2/M phase in HT-29 cells in a dose-dependent manner (Table 1).

Caspase-9 and -3activity enhancement by arctigenin

A significant dose-dependent increase in

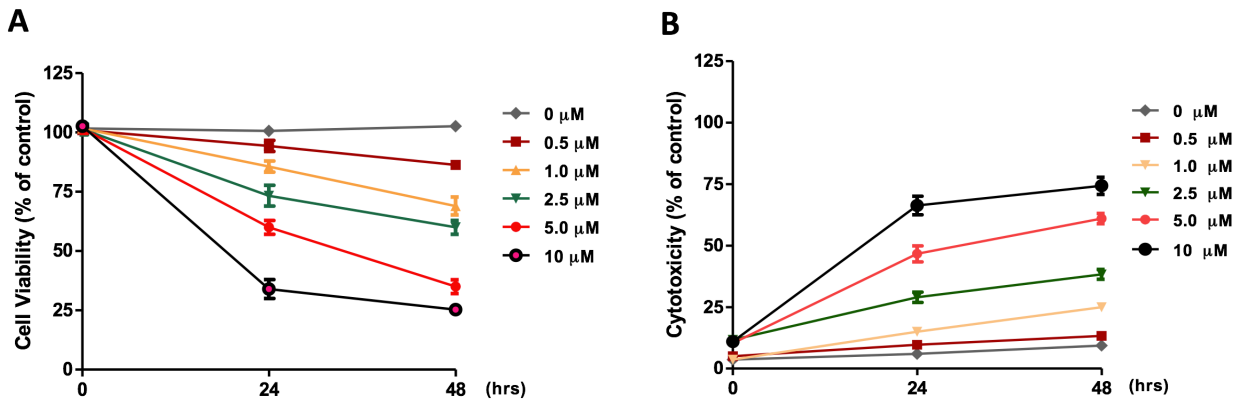

Figure 2. Growth inhibitory effect of different doses of arctigenin on HT-29 cells. **A:** MTT assay. **B:** LDH assay. Data are shown as mean±SD. ***p<0.001.

Table 1. The cell cycle and apoptosis ratios with treated concentrations of arctigenin on HT-29 cells

Concentration (μM)	*G_0/G_1(%) mean±SD*	*S (%) mean±SD*	*G_2/M (%) mean±SD*	*Apoptosis ratio (%) mean±SD*
0	60.47±1.75	32.68±2.60	6.85±0.70	1.50±0.08
0.5	57.22±1.80	30.48±2.66	12.30±0.74	2.40±0.25
1.0	52.85±1.30	28.32±2.50	18.83±0.65	6.81±0.09
2.5	48.56±1.20	27.38±2.64	24.06±1.32	12.06±1.51
5.0	45.95±1.16	27.40±2.95	26.65±1.73	24.77±1.48
10	42.31±2.35	25.20±2.23	32.49±1.05	32.22±2.15

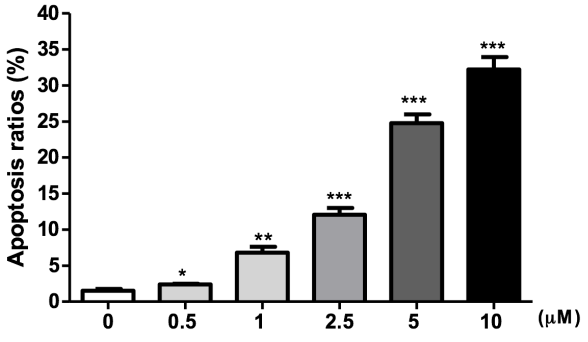

Figure 3. Apoptotic effect of different doses of artigenin on HT-29 cells. Data are shown as mean±SD. *p<0.05, **p<0.01, ***p<0.001.

caspase-9 and -3 activities was observed in HT-29 cells after treatment with arctigenin for 24 hrs (Figure 4A and 4B). Highest caspase-9 and -3 activities were increased from a range of approximately 4% to 64% in response of 0 μM to 10 μM of arctigenin. A rise in the activity of both caspases indicated that caspase-9 and -3 dependent signaling pathways were involved in stimulating cell apoptosis by arctigenin.

Increased ROS level and decreased ΔΨm level in HT-29 cells by arctigenin

In regard with ROS, the results obtained showed a concentration-dependent increase in intracellular ROS level in HT-29 cells upon arctigenin treatment (Figure 5A). Furthermore, as changes in ΔΨm have been hypothesized to be an important phenomenon of apoptosis, we determined the ΔΨm level in arctigenin-treated HT-29 cells. In regard with ΔΨm, the results showed significant and dose-dependent decrease in ΔΨm level in arctigenin-exposed HT-29 cells (Figure 5B). These results signify that arctigenin is involved in the production of ROS and stimulates intrinsic signaling pathways leading to apoptosis in HT-29 cells.

Increased expression level of p38 MAPK protein

The level of p38 MAPK protein was analyzed to authenticate its involvement in arctigenin

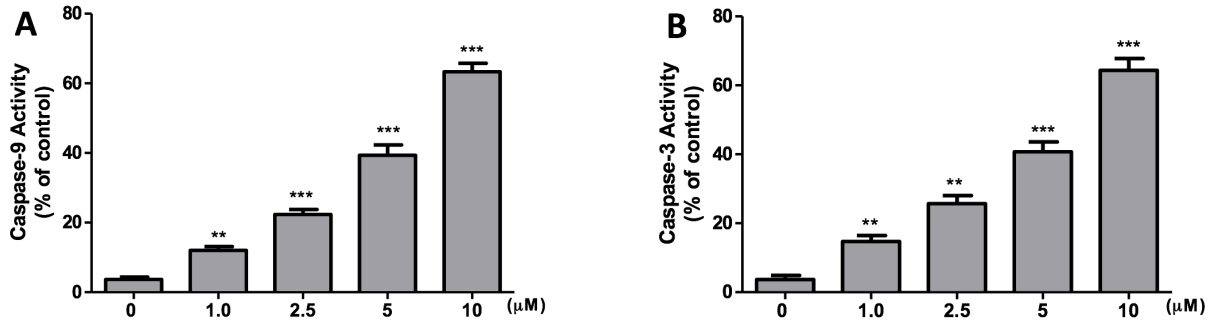

Figure 4. Effect of different doses of arctigenin on (**A**) caspase-9 and (**B**) caspase-3 activities. Data are shown as mean±SD. **p<0.01, ***p<0.001

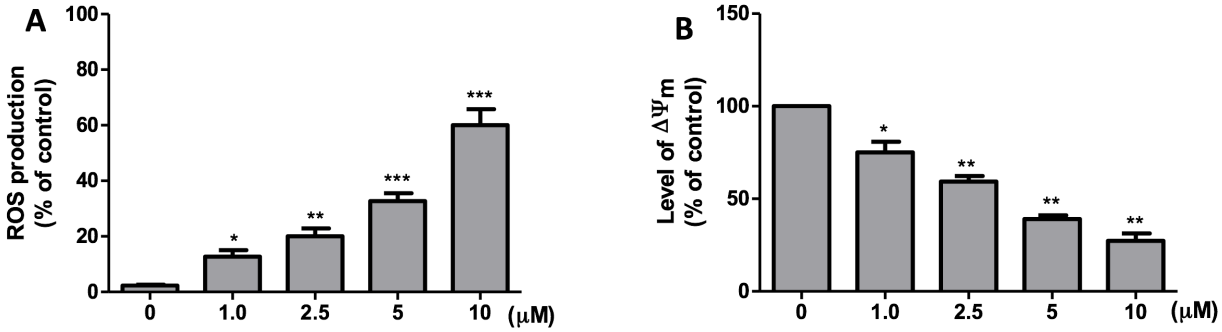

Figure 5. Arctigenin-induced ROS production (**A**) and decrease in ΔΨm (**B**).Data are shown as mean± SD. *p<0.05, **p<0.01, ***p<0.001.

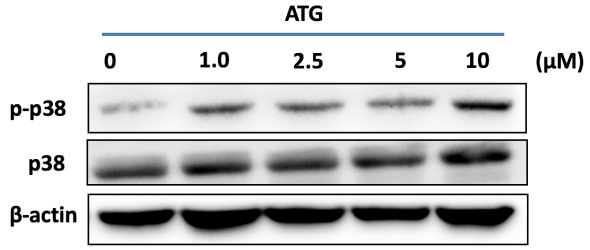

Figure 6. Regulation of p-p38 MAPK, p38 MAPK and β-actin expression in HT-29 cells upon arctigenin (ATG) treatment.

stimulated apoptosis in HT-29 cells. Western blotting results showed significantly dose-dependent increased phosphorylation of p38 (p-p38), implicating the activation of p38 signaling after the 24 hrs treatment with arctigenin (Figure 6). The result depicts the possible involvement of p38 MAPK pathway in the response of HT-29 cells to arctigenin.

ROS and p38 MAPK induced apoptosis

To further validate the role of ROS in arctigenin-induced apoptosis in HT-29 cells, the cells were treated with the ROS scavenger NAC along with arctigenin. Cell viability of HT-29 cells was increased from around 51.3% of arctigenin-treated cells to 77.3% of NAC pretreated cells, suggesting ROS mediated apoptotic activity (Figure 7A). As it has been previously reported that ROS induces MAPK pathway, we further elucidated p38 MAPK mediated apoptosis by adding the p38 MAPK inhibitor SB203580 to the arctigenin-treated cells and testing the cell viability. Figure 7B shows significant increase in arctigenin-treated cells upon addition of the p38 MAPK inhibitor. Thus, in agreement to previous studies, our results also depicted the activation of p38 MAPK pathway by ROS in arctigenin-treated HT-29 cells.

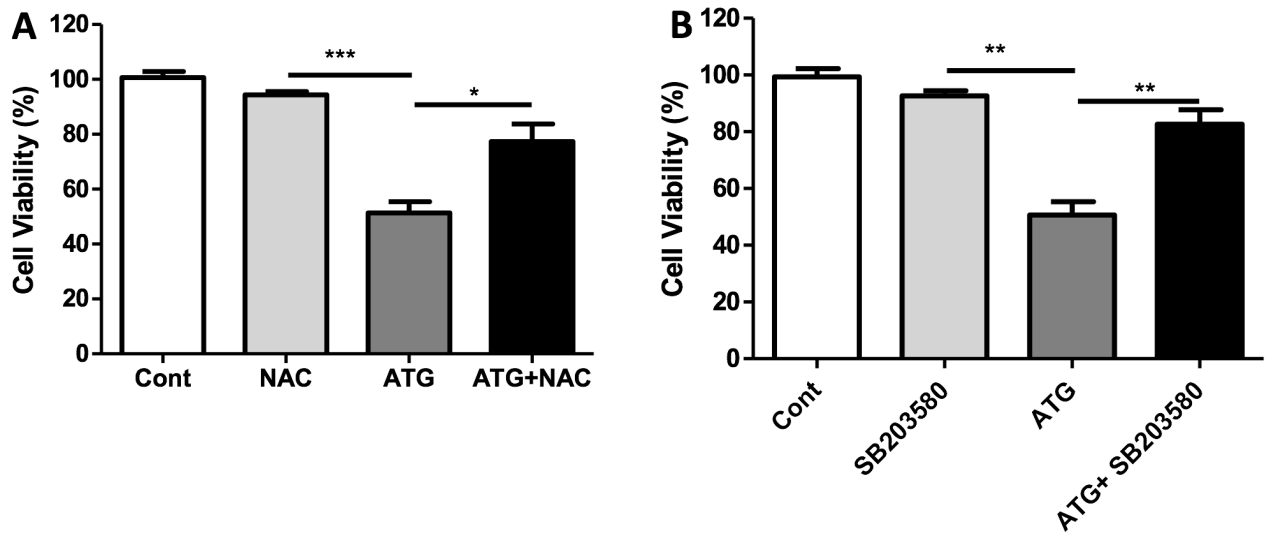

Figure 7. Growth inhibitory effect of arctigenin on HT-29 cells after treatment with (**A**) NAC (ROS scavenger) and (**B**) MAPK inhibitor (SB203580). Data are shown as mean± SD. *p<0.05, **p<0.01, ***p<0.001.

Discussion

In biological systems ROS (such as hydrogen peroxide (H_2O_2) and hydroxyl radical (¬-OH)) are constantly produced and removed from the cells to maintain the physiological balance and to precede necessary regulatory pathways [19]. Evidence supports that during oxidative stress ROS causes activation of MAPK family members such as p38 MAPK, further regulating downstream processes and leading to cell death or apoptosis [20]. Therefore, the search for novel molecules, able to exert potential oxidative stress on cells, has been an area of great interest.

Recent studies have focused on the discovery of novel drug formulations targeting colon cancer by arresting cell cycle and inducing apoptosis. Dietary phytochemicals, such as arctigenin (Figure 1), are present in abundance in fruits and vegetables and found to possess therapeutic properties, including anticancer activities [21-23]. The biological activities of arctigenin, such as antiinflammatory [24], antiarrhythmic [25], antimelanogenesis [26], antiviral [27], anticancer [28], etc., have been described in various studies. The antiproliferative effect of arctigenin on various cell lines has been previously discussed e.g. in hepatic cancer, prostate cancer, breast cancer, etc [28,29]. Additionally, it has been observed that arctigenin may exert cytotoxic effect through different mechanisms on different cancer cell types [30-34]. Nevertheless, to the best of our knowledge, the cytotoxic effect of arctigenin on human colon cancer cell line, HT-29, and its mechanism of action has not been studied yet.

Here, to study the antiproliferative effect of arctigenin on HT-29 cells, MTT and LDH assay were conducted, which showed dose-dependent cytotoxic effect when HT-29 cells were treated with arctigenin for 24 and 72 hrs at 0.5, 1.0, 2.5, 5.0 and 10 μM doses (Figure 2). Based on the antiproliferative effect of arctigenin, the cell cycle arrest and its apoptotic effect were further analyzed. The conversion of normal cells into tumor cells depends on the dysregulation of G1/S and G2/M checkpoints in the cell cycle. These checkpoints ensure the completion of upstream processes, e.g. (i) DNA replication and that the replicated DNA is not damaged, (ii) attachment of chromosomes to spindle apparatus, etc., before the initiation of downstream processes. Cell perform apoptosis if any of the upstream functions is not properly conducted [35,36]. Previous reports showed the apoptotic activity of arctigenin in various cell lines [34,37,38]. Being an important phenomenon, we also performed flow cytometry to analyze the induction of apoptosis in HT-29 cells via arctigenin. The obtained results showed increased apoptosis ratio (Figure 3) with increased number of cell arrest in G2/M phase, similar to an earlier reported study on SW480 human colon cancer cell line [39].

Various apoptotic programs are involved in programmed cell death which falls in either of two categories, extrinsic or intrinsic [40]. Intrinsic apoptosis is often called as mitochondrial apoptosis because of the involvement of mitochondrial factors. Caspase-9, being the initiator caspase, rests as inactive monomer in the cell until activation by cytochrome C released from mitochondria upon stress signals. Cytochrome C binds to apop-

totic protease-activating factor 1 (Apaf-1), finally activating caspase-9. This complex is known as apoptosome and it further activates caspase-3. To determine the role of arctigenin in inducing intrinsic apoptosis in HT-29 cells, caspase-9 and -3 activities were measured. Increased caspase-9 and -3 activities indicated the participation of intrinsic pathways in stimulating HT-29 cell apoptosis (Figure 4).

It has been well known that cancer cells are susceptible to therapeutic agents that are able to increase ROS within them [41]. Here, enhanced ROS production in HT-29 cells, upon arctigenin treatment, indicates ROS-dependent induction of apoptosis in the cells. In accordance to this result it was observed that pretreatment of cells with NAC increased the cell viability which also signifies the ROS-dependent apoptosis in arctigenin treated HT-29 cells. Moreover, correlation of mitochondrial ROS production and $\Delta\Psi m$ destabilization is well known [42]. Therefore, decrease in $\Delta\Psi m$ shows the involvement of intrinsic apoptosis in this study (Figure 5). ROS has been observed as upstream regulator of p38 MAPK pathway which further activates apoptotic signals [43]. In the present study, the increase in phosphorylation of p38 MAPK indicates that arctigenin acts as an activator of p38 MAPK (Figure 6). Moreover, as p38 MAPK is associated with apoptotic ability, an increase in phosphorylation of p38 MAPK by arctigenin may be responsible for the increased apoptosis as observed in the case of HT-29 cells (Figure 7). These results were also confirmed by inhibiting p38 MAPK signaling pathway. Taken together, arctigenin provides its potential of anticancer activity by regulating the activity of ROS and p38 MAPK in colon cancer cells, suggesting itself as a potential therapeutic agent for colon cancer.

Authors' contributions

GRH designed the experiments. QUL and YL performed the experiments. YT analyzed and compiled the results.

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