

Stevioside induced cytotoxicity in colon cancer cells via reactive oxygen species and mitogen-activated protein kinase signaling pathways-mediated apoptosis

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Abstract. The role of mitogen-activated protein kinase (MAPK) signaling pathways in cell growth and differentiation has been well established. The present study aimed to investigate the anti-proliferative effect of stevioside on human colon cancer HT-29 cells. Additionally, the effect of stevioside on cell cycle arrest and MAPK signaling pathways in HT-29 cells was explored. Stevioside was observed to significantly inhibit cancer cell growth at a dose of 5 μ M at 48 and 72 h. A dose-dependent increase in the apoptosis rate was observed with cell cycle arrest at G2/M phase. In addition, caspase-9 and caspase-3 activity also increased. An increase in reactive oxygen species (ROS) production and a decrease in the mitochondrial membrane potential indicated that the mitochondrial-mediated intrinsic pathway is responsible for apoptotic activity. These results were additionally verified by the elevated expression level of phosphorylated p38 and extracellular signal-regulated kinase mitogen-activated protein kinases (MAPKs). Additionally, by inhibiting ROS production and MAPK activation, the antiproliferative effect of stevioside was suppressed, confirming the hypothesis that ROS and MAPK proteins induce apoptosis in human colon cancer HT-29 cells.

Introduction

Colon cancer ranks third among all type of cancers with respect to global mortality (1). Due to poor responsiveness against currently available chemotherapeutic drugs and side effects, the treatment of colon cancer requires the use of natural therapeutic agents (2,3). Several natural compounds

have been previously investigated against colon cancer cell lines such as nobiletin (4) and arctigenin (5). Herbs are well known for their medicinal properties due to the presence of natural components within them. Stevioside has been investigated among such natural medicinal agents (6).

Stevioside, a diterpenoid glycoside, comprises of steviol, an aglycone, and glucose molecules, and is isolated from *Stevia rebaudiana* (Fig. 1). This small shrub is a member of the genus *Stevia* of the Asteraceae family and is commonly known as sugar leaf. Although this shrub is native to Brazil and Paraguay, at present it is cultivated in various countries of the world including East Asian countries, the United States of America, Canada and Europe (7-9). As stevioside is ~300 times sweeter than sucrose and contains numerous medicinal properties against diabetes mellitus, hypertension and obesity, it is used in the food, cosmetics and pharmaceutical industries (10-12). Natural products have been well known to exhibit anti-cancer activity through apoptosis as a major molecular phenomenon (13). Apoptosis is programmed cell death that maintains the cellular balance. If this balance is disturbed this leads to uncontrolled cell growth. Thus, the discovery of novel anticancer therapeutics largely focuses on apoptosis-stimulating agents in the cell via various pathways. One of these well-recognized molecular pathways leading to apoptosis is the production of excessive reactive oxygen species (ROS) (14).

Normal cells tend to produce ROS constantly, which is counteracted by the available antioxidant protein in the cells (15). However, if ROS production exceeds the limit of counteraction it causes a state of oxidative stress. Such states of oxidative stress can be a consequence of various diseases including cancer (16). ROS regulates several signaling pathways, which are involved in the normal functioning of the cell. Therefore, altered ROS levels can lead to the impaired functioning of the cellular processes. One of the most well-studied signaling pathways regulated by ROS is the mitogen-activated protein kinase pathway (MAPK) (17). Extracellular signal-related kinases (ERK), c-Jun amino-terminal kinases (JNK) and phosphorylated P-38 (p-p38) are 3 well-known subgroups of the MAPK family that are involved in cell growth and cell death, and are activated by a cascade of phosphorylation (18).

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A previous study demonstrated that stevioside induces ROS-mediated apoptosis in breast cancer cell lines (19). However, the role of stevioside in inducing ROS-mediated apoptosis in colon cancer cells remains unknown. Therefore, the present study aimed to reveal the ROS-mediated apoptotic phenomenon of the stevioside-induced antiproliferative effect on human colon cancer HT-29/HCT-15 cells. Also, the involvement of ERK and P-38 MAPK in inducing antiproliferative effect was assessed.

Materials and methods

Chemicals. Stevioside hydrate (purity $\geq 98\%$), fetal bovine serum (FBS), MTT, propidium iodide and SDS were purchased from Sigma-Aldrich; Merck Millipore, Darmstadt, Germany. The medium for the cell culture RPMI 1640 and Dulbecco's modified Eagle's medium (DMEM) medium were purchased from Invitrogen; Thermo Fisher Scientific, Inc. Waltham, MA, USA. The supplements for the cell culture media such as penicillin, streptomycin and L-glutamine were obtained from Lonza Group AG, Basel, Switzerland. The fluorescent dyes 2',7'-dichlorofluorescein diacetate (H_2DCF -DA) and 3,3'-dihexyloxacarbocyanine iodide ($DiOC_6(3)$) were purchased from Thermo Fisher Scientific, Inc. To determine the signaling pathways involved, the ROS scavenger N-acetyl-L-cysteine (NAC) and the MAPK inhibitor hydrochloride 4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl) 1 H-imidazole (SB203580) were purchased from Sigma-Aldrich; Merck Millipore, while the ERK inhibitor 2'-amino-3'-methoxyflavone (PD98059) was obtained from Calbiochem; Merck Millipore. The primary antibodies directed against p-p38 (cat. no. sc-166182), pERK (cat. no. sc-7383) and β -actin (cat. no. sc-130656), and the horseradish peroxidase (HRP) labelled rabbit anti-mouse secondary antibody (cat. no. sc-2357) were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Primary antibodies were used at dilution 1:1,000 and secondary antibodies were used at dilution 1:10,000.

Cell culture. Stock solution of 50 mM stevioside hydrate was prepared using dimethyl sulfoxide (DMSO). HT-29 was used as the human colon carcinoma cell line for the present study, which was cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, 100 μ g/ml streptomycin and 2 mM L-glutamine. The HT-29 cells were cultured at 37°C in a humidified chamber air and CO_2 (95 and 5%, respectively) and were maintained at ~90% confluence level.

Effect of stevioside on HT-29 cell viability. The antiproliferative activity of stevioside on colon cancer HT-29 cell line was studied using an MTT assay. Initially, in a 96-well plate, 2×10^4 cells/well were cultured for 24 h at 37°C and subsequently treated with different doses, 0.5, 1, 2.5 and 5 μ M, of stevioside for 24, 48 and 72 h. Subsequent to the respective incubation times, the treated cells were added to MTT solution for 3 h at 37°C. Subsequent to the completion of the reaction time, medium along with MTT reagent was discarded and cells were lysed in DMSO to record the optical density at 570 nm for the production of mitochondrial succinate-mediated MTT formazan, a cell viability indicator. The experiments were

conducted in triplicate for accuracy and results are expressed as the mean $\pm$ standard deviation.

Effect of stevioside on HT-29 cell cytotoxicity. To determine the cell cytotoxicity of stevioside, the production of lactate dehydrogenase (LDH) within damaged cells was used as marker using a cytotoxicity detection kit (Takara Bio Inc., Otsu, Japan). A total of 2×10^4 HT-29 cells per well were seeded in a 96-well plate and incubated at 37°C for 24 h. Subsequent to the incubation time the seeded cells were treated with stevioside at different concentrations, 0.5, 1, 2.5 and 5 μ M, for varying times; 24, 48 and 72 h. PBS (40 μ l) and the reaction mixture provided in the cytotoxicity detection kit (50 μ l) were added to the cell free culture medium samples (10 μ l) in 96-well plates and incubated in the dark at 25°C. Subsequent to the reaction time (45 min), stop solution (50 μ l) was added and cell cytotoxicity was calculated by measuring the optical density at 490 nm wavelength.

Effect of stevioside on apoptosis and cell cycle distribution. The apoptotic effect of stevioside on the HT-29 cells at different concentrations was determined via a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA). A total of 50,000 cells/well were seeded in a 6-well culture plate and incubated for 24 h at 37°C in 10% FBS-supplemented DMEM medium. The adherent cells were treated with 0.5, 1, 2.5 and 5 μ M stevioside for 48 h. Following treatment, the samples were centrifuged (45 x g for 5 min at 4°C) and the pellets were collected, fixed and washed. Pelleted cells were then treated with 1% volume/volume Triton X-100 and incubated at 37°C for 10 min. To stain the DNA, the cells were mixed with 50 μ g/ml propidium iodide solution at 4°C. Apoptotic rate and cell cycle phase distribution were further analyzed using a FACScan flow cytometer.

Effect of stevioside on caspase activity. A caspase-3 and caspase-9 colorimetric assay kit (BioVision, Inc., Milpitas, CA, USA) was used to confirm the apoptotic effect of stevioside on the HT-29 cells, according to protocol of the manufacturer. Following stevioside treatment at varying concentrations, the HT-29 cells were incubated for 48 h and centrifuged at 12,000 x g for 10 min followed by cell collection as pellets. The cells were additionally re-suspended in ice-cold lysis buffer for 10 min and centrifuged at 10,000 x g for 1 min to collect supernatant. Equal amounts of protein, 200 μ g, were then added to the respective substrates in reaction buffer and incubated at 37°C for 30 min. Finally, an ELISA microplate reader was used to measure the released p-nitroaniline quantity at 400 nm.

Effect of stevioside on ROS production. A total of 2×10^5 HT-29 cells were cultured per well in a 12-well plate. Subject to reaching confluency, the cells were treated with predetermined concentrations of stevioside and incubated for 48 h at 37°C. Following incubation, the cells were collected subsequent to centrifugation (45 x g for 10 min at 4°C) as a pellet and washed thoroughly using PBS. Furthermore, the cells were added to a fluorescent probe, H_2DCF -DA, at room temperature in the dark for 20 min. A FACScan flow cytometer at 480 nm and 525 nm (excitation and emission

wavelengths, respectively) was used to analyze the stained cells (20).

Effect of stevioside on mitochondrial membrane potential ($\Delta\Psi_m$) level. The HT-29 cells were prepared and treated with stevioside in a similar manner as aforementioned. Subsequent to collecting the cells as pellets, the cells were stained with DiOC₆ (3) and analyzed by flow cytometry as discussed previously (21).

Western blot analysis. Cellular protein was collected from the HT-29 human colon cancer cells subsequent to treatment with stevioside at various concentrations for 48 h. The cells were then treated with lysis buffer followed by centrifugation at 6,400 x g for 30 min at 4°C. A protein assay reagent kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) was then used to estimate the total amount of protein in the collected supernatant. Furthermore, the expression levels of p-p38 and pERK were estimated by first separating proteins by western blot analysis using SDS-PAGE, according to a protocol of a previous study, with slight modifications (22). The gel was then transferred electrophoretically to a nitrocellulose membrane, followed by membrane treatment with the primary antibodies of p-p38, pERK and β -actin. Following overnight incubation at 4°C, the membranes were then treated with HRP conjugated secondary antibodies specific to primary antibodies for 1 h at room temperature. The protein bands were then visualized using an enhanced chemiluminescence detection system (FUJIFILM LAS-3000 mini, Tokyo, Japan).

Inhibition of MAPK signaling pathway. THE HT-29 cells were plated in 48-well culture dish at a density of 3.5×10^4 cells/well and pre-incubated at 37°C with 10 μ M of the MAPK inhibitor, SB203580, the ERK inhibitor, PD98059, or the ROS scavenger, NAC, for 2 h, followed by treatment with 5 μ M stevioside to analyze the effect of the inhibitors on stevioside-induced antiproliferation. Furthermore, HT-29 cell viability was studied using MTT assay, as will be described subsequently.

Statistical analysis. An unpaired two-tailed Student's *t* test and Prism software (version 5; GraphPad Software, Inc., La Jolla, CA, USA), were used to obtain and evaluate the statistical data. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Stevioside inhibited proliferation of HT-29 cells. In the present study, to investigate the growth inhibitory effect of stevioside on the colon carcinoma HT-29 cell line, an MTT assay was performed subsequent to 24, 48 and 72 h of treatment at various doses. Fig. 2A presents the dose-dependent cell growth inhibition by stevioside on the HT-29 cells at all the aforementioned time points studied. With respect to cell viability, ~60-70% inhibition of HT-29 cells was observed between 48 and 72 h of stevioside treatment at a dose of 5 μ M, which was considered significant ($P < 0.05$). In addition, similar results were obtained by LDH assay (Fig. 2B), showing dose-dependent cytotoxicity on HT-29 cells by

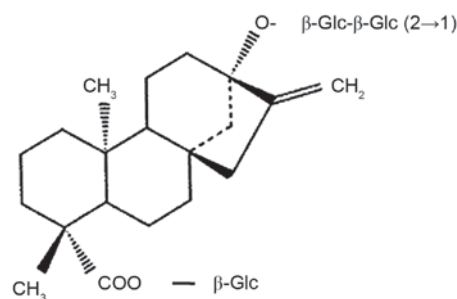


Figure 1. Structure of stevioside.

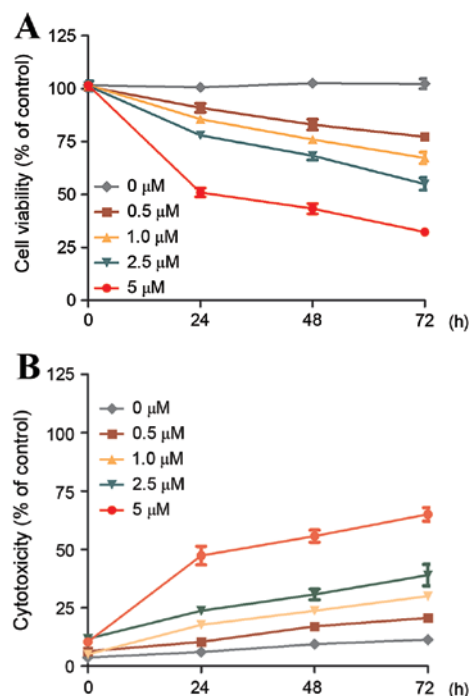


Figure 2. Cell growth inhibition of stevioside on HT-29 colon cancer cells (A) MTT assay, (B) LDH assay. LDH, lactate dehydrogenase.

stevioside. Therefore, for additional experiments 48 h was selected as a significant time point for showing anti-proliferative effect.

Stevioside induced apoptotic effects and cell cycle arrest in HT-29 cells. The present study then assessed the involvement of apoptosis in the antiproliferative effect of stevioside on HT-29 cells using flow cytometry subsequent to 48 h of treatment. Stevioside treatment was observed to increase the apoptotic rate of HT-29 cells in a dose-dependent manner, with a maximum observed rate of $24.15 \pm 1.56\%$ at a 5 μ M dose (Fig. 3A). As presented in Table I, a significant increase ($P < 0.001$) in G2/M phase at a 5 μ M dose for 48 h, $24.65 \pm 0.70\%$ cell population compared with $8.13 \pm 0.95\%$ of control value, and simultaneously a significant decrease ($P < 0.001$) in cell population from $62.40 \pm 1.65\%$ of control value to $46.92 \pm 1.02\%$ in cells in G0/G1 phase at the same dose was observed. Furthermore, to validate the phenomenon of apoptosis, the involvement of apoptotic pathways was investigated in stevioside-treated HT-29 cells.

Table I. Cell cycle and apoptosis ratios with various concentrations of stevioside on HT-29 cells.

Group	Concentration (μ M)	G0/G1 (%)	S (%)	G2/M (%)	Apoptosis ratio (%)
Stevioside	0	62.40 $\pm$ 1.65	29.47 $\pm$ 1.72	8.13 $\pm$ 0.95	2.02 $\pm$ 0.06
	0.5	59.23 $\pm$ 2.84	30.25 $\pm$ 1.62	10.52 $\pm$ 0.80 ^a	3.06 $\pm$ 0.20 ^a
	1.0	56.80 $\pm$ 2.10 ^a	31.37 $\pm$ 1.12	11.83 $\pm$ 0.81 ^b	8.28 $\pm$ 0.08 ^b
	2.5	52.68 $\pm$ 2.35 ^b	29.43 $\pm$ 3.54	17.89 $\pm$ 1.26 ^c	14.54 $\pm$ 1.86 ^b
	5.0	46.92 $\pm$ 1.02 ^c	28.43 $\pm$ 1.26	24.65 $\pm$ 0.70 ^c	24.15 $\pm$ 1.56 ^c

^aP<0.05, ^bP<0.01, ^cP<0.001 vs. the untreated cells.

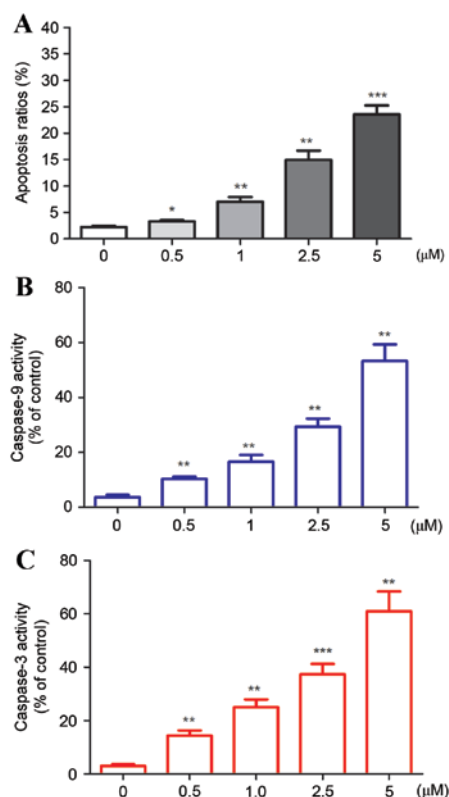


Figure 3. (A) Apoptotic effect of stevioside on HT-29 colon cancer cells, (B) effect of stevioside on caspase-9 activity, (C) Effect of stevioside on caspase-3 activity. Data is shown as the mean $\pm$ standard deviation. *P<0.05, **P<0.01, ***P<0.001.

Stevioside increased caspase-9 and -3 activity in HT-29 cells. Furthermore, to confirm the apoptotic effect of stevioside on HT-29 cells, caspase-9 and caspase-3 activities were assessed as indicators of apoptosis (23). Significant increases in caspase-9 (P<0.01; Fig. 3B) and caspase-3 (P<0.01; Fig. 3C) activities were observed in stevioside-treated HT-29 cells in a dose-dependent manner subsequent to 48 h of treatment. A significant increase in the activities of caspase-9 and caspase-3, to ~53 (P<0.01) and 61% (P<0.01), respectively, were observed subsequent to treatment with 5 μ M stevioside, confirming their involvement in inducing cell apoptosis.

Stevioside increased ROS level and decreased $\Delta\Psi$ m level in HT-29 cells. Fig. 4A and B shows the intracellular ROS and $\Delta\Psi$ m levels in HT-29 cells subsequent to treatment with

stevioside at 48 h. ROS has been reported to be an apoptosis inducer and the level of ROS was measured using H₂DCF-DA, a fluorescent dye (24). Furthermore, altered $\Delta\Psi$ m has been hypothesized to be a significant event of apoptosis (25) and the level of this event was assessed using DiOC₆ (3), a voltage-dependent and mitochondria-specific fluorescent dye, in stevioside-treated HT-29 cells. As shown in Fig. 4A and B, intracellular levels of ROS increased and $\Delta\Psi$ m levels decreased dose-dependently in HT-29 cells subsequent to stevioside treatment. The maximum increase in ROS level was ~58% and maximum decrease in $\Delta\Psi$ m level was ~29% in HT-29 cells treated with 5 μ M stevioside. The involvement of ROS in inducing apoptosis in stevioside-treated HT-29 cells was therefore confirmed, and the contribution of underlying mitochondrial signaling pathways in the induction of apoptosis was indicated.

Stevioside increased the expression levels of ERK and p38 MAPK protein. ERK and p38 MAPK are involved in cell proliferation by regulating cell growth, oxidative stress and differentiation. The activation of MAPKs has been reported in a previous study (26). To verify the involvement of p38 MAPK and ERK in the apoptotic activity of stevioside in HT-29 cells, dose-dependent phosphorylation of p38 MAPK and ERK was monitored by western blotting. Fig. 5 demonstrates the increases in pERK and p-p38 MAPK levels at 5 μ M stevioside for 48 h. Thus, the involvement of ERK and the p38 MAPK pathway in stevioside-induced apoptosis is indicated.

Stevioside-induced apoptosis in HT-29 cells was associated with ROS production and MAPK proteins. The HT-29 cells were treated with an ROS scavenger, NAC, to verify whether ROS is essential for stevioside-induced cell apoptosis. The results obtained showed an increase in HT-29 cell viability following NAC pretreatment, which is a clear indication of the role of ROS in apoptosis induction (Fig. 6A). Furthermore, to determine whether p38 MAPK and ERK are essential in inducing apoptosis in stevioside-treated cells, the cells were pretreated with an ERK inhibitor, PD98059, and a p38 MAPK inhibitor, SB203580, for 2 h. Pretreatment with PD98059 and SB203580 in stevioside-treated cells exhibited a significant increase in the cell viability (P<0.05 and P<0.01, respectively, vs. treatment with stevioside alone), confirming that ERK and p38 MAPK are involved in ROS-mediated apoptosis in HT-29 cells treated with stevioside (Fig. 6B and C, respectively).

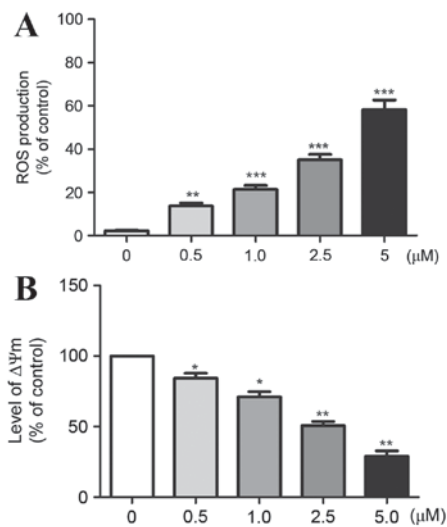


Figure 4. (A) Effect of stevioside on ROS production, (B) effect of stevioside on $\Delta\Psi_m$ level. Data are shown as the mean $\pm$ standard deviation. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. ROS, reactive oxygen species; $\Delta\Psi_m$, mitochondrial membrane potential.

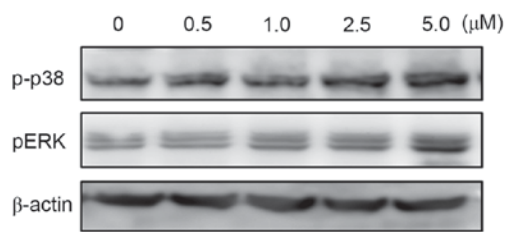


Figure 5. Regulation of p-p38 MAPK, pERK and β -actin expression in HT-29 cells upon stevioside treatment. p-p38 MAPK, phosphorylated P-38 mitogen-activated protein kinase pathway; pERK, phosphorylated extracellular signal-regulated kinases.

Discussion

The ability of natural products to reverse or suppress cancer development is well known (27). However, the exact mode of action of natural products remains unknown. In the search for novel anticancer molecules, the use of dietary phytochemicals as natural chemotherapeutic agents has been discussed previously (28). Stevioside is a type of dietary phytochemicals, which is known to possess anticancer properties (29). Nakamura *et al* (30) showed the antitumor activity of stevioside on 12-O-tetradecanoylphorbol-13-acetate-induced skin cancer. Furthermore, isosteviol, a hydrolysis product of stevioside, inhibited DNA polymerases and DNA topoisomerase II, which are potential cellular targets for oncotherapy (31). In accordance with previous studies, the treatment of stevioside at various doses, 0.5, 1.0, 2.5 and 5.0 μM for 24, 48 and 72 h on colon cancer HT-29 cells resulted in decreased cell viability and increased cell cytotoxicity via MTT and LDH assay, respectively (Fig. 2).

Cell cycle and cell growth are well-regulated phenomena that are mediated by the checkpoints G0/G1 and G2/M between growth phases, and apoptosis or cell cycle arrest is initiated if DNA damage occurs or if DNA replication is not completed in the previous phase (32,33). As reported previously, the anti-proliferative effect of various natural

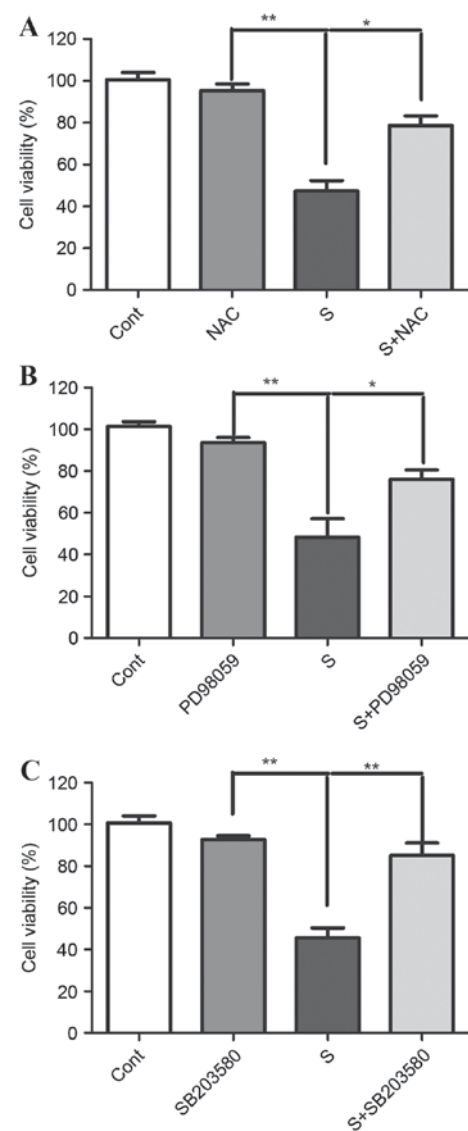


Figure 6. Growth inhibitory effect of stevioside on HT-29 cells subsequent to treatment with (A) NAC, an ROS scavenger, (B) the ERK inhibitor PD98059 and (C) the MAPK inhibitor SB203580. Data are shown as the mean $\pm$ standard deviation. * $P<0.05$, ** $P<0.01$. S, stevioside; NAC, N-acetyl-L-cysteine; ROS, reactive oxygen species; ERK, extracellular signal-regulated kinases; MAPK, mitogen-activated protein kinase pathway.

phytochemicals was found to be associated with apoptosis in different cellular systems (34,35). Therefore, to additionally identify whether the stevioside-induced antiproliferative effect on HT-29 cells was accompanied by apoptosis and cell cycle arrest, flow cytometry was performed subsequent to stevioside treatment at various doses for 48 h, based on significant MTT and LDH results. The highest number of apoptotic cells was observed at a 5.0 μM dose of stevioside (Fig. 3A). Furthermore, a significant reduction in cell numbers was observed in the G0/G1 phase in treated cells as compared to the control. An increase in the cell population was observed in the G2/M phase compared with the control value indicating cell arrest in this phase (Table I).

The effect of stevioside on the human breast cancer MCF-7 cell line through various pathways including cell viability suppression, DNA synthesis inhibition and ROS-mediated apoptotic cell death has also been previously demonstrated (19). ROS

is mainly produced by mitochondria and acts as a secondary messenger for various cellular processes, such as apoptosis and cell proliferation. Though the actual role of ROS remains unknown, it can be assumed that the constant production and removal of ROS is necessary to maintain and regulate the physiological balance of the cell (36,37). In addition, elevated ROS have been revealed to be associated with the lowering of the $\Delta\Psi_m$ (38). Therefore, the present study aimed to correlate the induction of apoptosis with ROS production in stevioside-treated HT-29 cells. As shown in Fig. 4, ROS production increased and $\Delta\Psi_m$ decreased dose-dependently subsequent to stevioside treatment, suggesting that apoptosis was induced by an elevated ROS level. In agreement with this result, cells pretreated with NAC, an ROS scavenger, showed increased cell viability, confirming ROS-dependent apoptosis in HT-29 cells subsequent to stevioside treatment (Fig. 6). Additionally, the decrease in $\Delta\Psi_m$ suggests the involvement of mitochondrial apoptotic factors in the present study. Changes in $\Delta\Psi_m$ may initiate cytochrome *c* release from mitochondria, which may additionally activate caspase-9 and caspase-3 (39). Thus, in the present study the enhanced level of caspase-9 and caspase-3 following stevioside treatment also verified the involvement of a mitochondrial-mediated intrinsic apoptotic pathway (Fig. 3).

Since ROS and caspase levels increased in stevioside-treated cells, the present study also assessed the expression of MAPK family members such as p38 and ERK. A wide range of extracellular signals may trigger MAPK pathways, which activates a cascade of signaling mechanisms. Extracellular stress is mainly responsible for p38 activation while mitogenic stimuli are responsible for ERK activation, which is involved in cell differentiation and proliferation (40,41). The present study observed that in HT-29 cells the levels of phosphorylated p38 and ERK increased subsequent to incubation with stevioside for 48 h in a dose-dependent manner (Fig. 5). However, stevioside did not induce antiproliferative effects subsequent to pre-treatment with the inhibitors of p38 and ERK signaling pathways, suggesting that their pathways affect the cellular response to stevioside (Fig. 6).

In conclusion, the results of the present study showed that in stevioside-treated colon cancer HT-29 cells, ROS and MAPK serve critical roles in inducing apoptosis. Therefore, in the search for novel anticancer compounds, stevioside may be an interesting molecule to be additionally explored. Being natural in origin, stevioside may exhibit decreased toxicity compared with artificial chemotherapeutic drugs. However, the exact potential and mode of action of stevioside as anticancer drug require investigation.

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