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Full Length Research Paper

Scutellaria barbata D. Don induces apoptosis of human colon carcinoma cell through activation of the mitochondrion-dependent pathway

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***Scutellaria barbata* D. Don has long been used in China for the clinical treatment of various cancers including colorectal cancer. However, the precise mechanisms of its tumorcidal activity remain unclear. In the present study, we investigated the cellular effects of the ethanol extract of *S. barbata* D. Don (EESB) in the HT-29 human colon carcinoma cell line. We found that EESB inhibited the growth of HT-29 cells as demonstrated EESB-induced cell morphological changes and reduced cell viability in dose- and time-dependent manners. Furthermore, we observed that EESB treatment resulted in DNA fragmentation, loss of plasma membrane asymmetry, collapse of mitochondrial membrane potential, activation of caspase-9 and caspase-3, and increase of the ratio of pro-apoptotic Bax to anti-apoptotic Bcl-2. These results suggest that promotion of cancer cell apoptosis through activation of the mitochondrion-dependent pathway is one of the mechanisms by which *S. barbata* D. Don can be effective in the treatment of cancer.**

Key words: *Scutellaria barbata* D. Don, apoptosis, colorectal cancer, HT-29 cells, herbal medicine, mitochondria.

INTRODUCTION

Colorectal cancer (CRC) is a serious public health concern throughout the world, with nearly one million new cases and over half a million deaths each year (Boyle and Leon, 2002). Currently, chemotherapy plays a major role in the therapeutic approach toward CRC (Gustin and Brenner, 2002). 5-Fluorouracil (5-FU)-based regimens are the standard treatment for patients with advanced CRC. However, due to drug resistance, systemic chemotherapy using 5-FU-based regimens produces objective response rates of less than 40% (Gorlick and Bertino, 1999; Longley et al., 2006). Moreover, many currently used anti-cancer agents have potent cytotoxic

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Abbreviations: EESB, Ethanol extract of *Scutellaria barbata* D. Don; **DMSO,** dimethyl sulfoxide; **MTT,** 3-(4, 5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide.

effects in normal cells (Boose and Stopper, 2000). Both drug resistance and toxicity limit the effectiveness of current CRC chemotherapy, thus there is a need for the development of new therapeutic agents. Natural products have received recent interest as therapeutic agents for CRC as they have relatively few side effects and have been used as alternative remedies for a variety of diseases including cancer (Tang and Eisenbrand, 1992; Huang, 1999; Gordaliza, 2007).

Apoptosis is crucial for tissue homeostasis by eliminating excess or damaged cells. Disturbed regulation of apoptotic death contributes to various diseases including cancer (Adams and Cory, 2007; Cory and Adams, 2002). Mitochondria play an important role in this process (Gross et al., 1999). Apoptosis is highly regulated by Bcl-2 family proteins including both anti-apoptotic members such as Bcl-2 and pro-apoptotic members such as Bax. Following activation, Bax can induce mitochondrial outer membrane permeabilization (MOMP), releasing pro-apoptotic proteins such as cytochrome *c*. This process eventually leads to the activation of caspases, resulting in destruction of the cell (Hsu et al., 1997; Wolter et al., 1997; Kluck et al., 1997; Jürgensmeier et al., 1998; Antonsson et al., 2000). Bcl-2 protects cells from apoptosis by inhibiting Bax-mediated MOMP (Gross et al., 1999; Yang et al., 1997; Antonsson et al., 1997; Thomenius et al., 2003). Therefore, the ratio of anti- and pro-apoptotic Bcl-2 family proteins determines the fate of cells.

Scutellaria barbata D. Don is a medicinal herb that has long been used in China to treat various types of cancer including CRC (Chinese Pharmacopoeia Commission, 2010; Qian, 1987). Extracts of *S. barbata* D. Don have been shown to inhibit the growth of many cancer cells (Lee et al., 2004; Yin et al., 2004; Cha et al., 2004; Suh et al., 2007), however, the precise mechanisms of its tumorcidal activity remain unclear. In this study, we evaluated the cellular effect of the ethanol extract of *S. barbata* D. Don (EESB) in HT-29 human colon carcinoma cells, and investigated the molecular mechanisms mediating its biological effect. We found that EESB inhibited the growth and induced apoptosis of HT-29 cells. EESB-induced apoptosis was accompanied by loss of mitochondrial membrane potential, caspase-9 and caspase-3 activation, and up-regulation of Bax to Bcl-2 ratio. Our findings suggest that promotion of cancer cell apoptosis throuigh activation of the mitochondrion-dependent pathway is one of the mechanisms by which *S. barbata* D. Don can be effective in the treatment of cancer.

MATERIALS AND METHODS

Materials and reagents

Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin, Trypsin-EDTA, TriZol reagent and iBlot western detection stack/iBlot dry blotting system, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethyl-benzimidazol- carbocyanine iodide (JC-1), DNA ladder detection kit, and caspase-3 and -9 colorimetric protease assays were purchased from invitrogen (Carlsbad, CA, USA). Superscript II reverse transcriptase was obtained from promega (Madison, WI, USA). Bcl-2 and Bax antibodies, horseradish peroxidase (HRP)-conjugated secondary antibodies were obtained from cell signaling (Beverly, MA, USA). A fluorescein isothiocyanate (FITC)-conjugated annexin V apoptosis detection kit was obtained from Becton Dickinson (San Jose, CA, USA). All other chemicals, unless otherwise stated, were obtained from sigma chemicals (St. Louis, MO, USA).

Preparation of Ethanol Extract from *S. barbata* D. Don

500 g of *S. Barbata* D. Don were extracted with 5000 ml of 85% ethanol using a refluxing method and were filtered. The resulting ethanol solvent was evaporated on a rotary evaporator (Shanghai Yarong, Model RE-2000, China) and concentrated to a relative density of 1.05. The EESB was dissolved in 50% DMSO to a stock concentration of 500 mg/ml and stored at –20°C. The working concentrations of EESP were made by diluting the stock solution in the cell culture medium. The final concentration of DMSO in the medium for all experiments was < 0.5%.

Cell culture

Human colon carcinoma HT-29 cells were obtained from American type culture collection (ATCC, Manassas, VA, USA). The cells were grown in DMEM containing 10% (v/v) FBS, and 100 Units/ml penicillin and 100 µg/ml streptomycin in a 37°C humidified incubator with 5% CO_2. The cells were subcultured at 80 to 90% confluency. Cells used in this study were subjected to no more than 20 cell passages.

Evaluation of cell viability by MTT assay

Cell viability was assessed by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) colorimetric assay. HT-29 cells were seeded into 96-well plates at a density of 1 x 10^4 cells/well in 0.1 ml medium. The cells were treated with various concentrations of EESB for 24 h or with 2.5 mg/ml of EESB for different periods of time. Treatment with 0.5% DMSO was included as vehicle control. At the end of the treatment, 10 µl MTT (5 mg/ml in phosphate buffered saline, PBS) were added to each well, and the samples were incubated for an additional 4 h at 37°C. The purple-blue MTT formazan precipitate was dissolved in 100 µl DMSO. The absorbance was measured at 570 nm using an ELISA reader (BioTek, Model ELX800, USA).

Observation of morphologic changes

HT-29 cells were seeded into 6-well plates at a density of 2 x 10^5 cells/well in 2 ml medium. The cells were treated with various concentrations of EESB for 24 h. Cell morphology was observed using a phase-contrast microscope (Olympus, Japan). The photographs were taken at a magnification of 200 x.

Detection of apoptosis by flow cytometry analysis with annexin V/PI staining

After incubation with various concentrations of EESB, apoptosis of HT-29 cells was determined by flow cytometry analysis using a fluorescence-activated cell sorting (FACS) caliber (Becton Dickinson, CA, USA) and Annexin V-fluorescein isothiocyanate

(FITC)/Propidium iodide (PI) kit. Staining was performed according to the manufacturer's instructions. The percentage of cells in early apoptosis was calculated by Annexin V-positivity and PI-negativity, while the percentage of cells in late apoptosis was calculated by Annexin V-positivity and PI-positivity.

Detection of DNA fragmentation by gel electrophoresis

DNA fragmentation was determined using a DNA ladder detection kit (invitrogen), following the manufacturer's instructions. Briefly, HT-29 cells were detached from the plates by scraping and washed in PBS. 2 x 10^6 cells were resuspended in 70 µl TE lysis buffer. The cells were lysed with the manufacturers provided lysis solutions and the DNA precipitated with ammonium acetate and cold absolute ethanol followed by centrifugation at 16,000 x g for 10 min. The DNA pellet was washed with 70% ethanol and centrifuged at 16,000 x g for 10 min. The precipitated DNA was resuspended in the manufacturers provided DNA suspension buffer and analyzed after separation by gel electrophoresis (1.5% agarose). The DNA bands were examined using a Gel Documentation System (BioRad, Model Gel Doc 2000, USA).

Measurement of mitochondrial membrane potential (ΔΨm) by flow cytometric analysis with JC-1 staining

JC-1 is a cationic dye that exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green to red, which thus can be used as an indicator of mitochondrial potential. In this experiment, 1 x 10^6 treated HT-29 cells were resuspended after trypsinization in 1 ml of medium and incubated with 10 µg/ml of JC-1 at 37°C, 5% CO_2, for 30 min. Both red and green fluorescence emissions were analyzed by flow cytometry after JC-1 staining.

Analysis of caspase activation

The activities of caspase-3 and -9 were determined by a colorimetric assay using the caspase-3 and -9 activation kits, following the manufacturer's instructions. Briefly, after treatment with various concentrations of EESB for 24 h, HT-29 cells were lysed with the manufacturer's provided lysis buffer for 30 min on ice. The lysed cells were centrifuged at 16,000 x g for 10 min. The protein concentration of the clarified supernate was determined and 100 µg of the protein were incubated with 50 µl of the colorimetric tetrapeptides, Asp-Glu-Val-Asp (DEAD)-p-nitroaniline (pNA) (specific substrate of caspase-3) or Leu-Glu-His-Asp (LEHD)-pNA (specific substrate of caspase-9) at 37°C in the dark for 2 h. Samples were read at 405 nm in an ELISA plate reader (BioTek, Model ELX800, USA). The data were normalized to the activity of the caspases in control cells (treated with 0.5% DMSO vehicle) and represented as "fold of control".

RNA extraction and RT-PCR analysis

2 x 10^5 HT-29 cells were seeded into 6-well plates in 2 ml medium and treated with various concentrations of EESB for 24 h. Total RNA was isolated with TriZol reagent. Oligo (dT)-primed RNA (1 µg) was reverse-transcribed with superscript II reverse transcriptase (Promega) according to the manufacturer's instructions. The obtained cDNA was used to determine the mRNA amount of Bcl-2 or Bax by PCR. GAPDH was used as an internal control. The sequences of the primers used for amplification of Bcl-2, Bax, and GAPDH transcripts are as follows: Bcl-2 forward 5'-CAG CTG CAC CTG ACG CCC TT-3 and reverse 5'-GCC TCC GTT ATC CTG GAT CC-3'; Bax forward 5'-TGC TTC AGG GTT TCA TCC AGG-3' and reverse 5'-TGG CAA AGT AGA AAA GGG CGA-3'; GAPDH forward 5'-GT CAT CCA TGA CAA CTT TGG-3' and reverse 5'-GA GCT TGA CAA AGT GGT CGT-3'.

Western blot analysis

2 x 10^5 HT-29 cells were seeded into 6-well plates in 2 ml medium and treated with various concentrations of EESB for 24 h. The treated cells were lysed with mammalian cell lysis buffer (M-PER; Thermo Scientific, Rockford, IL, USA) containing protease and phosphatase inhibitor cocktails (EMD Biosciences and Sigma Chemical, USA, respectively), and the lysates were separated by NuSep 12% long life Tris Glycine iGels (NuSep Ltd, Australia) under a reducing condition using 200 V for 1 h. The proteins were then electrophoretically transferred onto nitrocellulose membranes using the iBlot western detection stack/iBlot dry blotting system (invitrogen). Membranes were blocked for 30 min with agitation at RT in superblock T20 (TBS) blocking buffer (Thermo Scientific, Rockford, IL). Membranes were washed in TBS with 0.25% tween-20 (TBST) and exposed to primary antibodies against Bcl-2 or Bax (1:1000) overnight at 4°C with rocking. β-actin (1:1000) was measured as an internal control for protein loading. After the membranes were washed in TBST, secondary horseradish peroxidase (HRP)-conjugated antibodies (anti-rabbit) were added at 1:2500 dilutions for 1 h at room temperature and the membranes were washed again in TBST. Blots were developed using Super Signal Pico Substrate (Thermo Scientific, Rockford, IL, USA), and images were taken using a Kodak image station 400R (Kodak, Rochester, NY, USA).

Statistical analysis

All data are the means of three determinations. The data was analyzed using the SPSS package for Windows (Version 11.5). Statistical analysis of the data was performed with Student's t-test and ANOVA. Differences with $P < 0.05$ were considered statistically significant.

RESULTS

EESB inhibited the growth of HT-29 cells

The effect of EESB on the viability of HT-29 cells was determined by MTT assay to compare the relative number of cells in EESB-treated monolayer's to untreated controls. As shown in Figure 1A, treatment with 0.5 to 4 mg/ml of EESB for 24 h dose-dependently reduced cell viability by 14 to 73% compared to untreated control cells ($P < 0.01$), with an estimated half maximal inhibitory concentration (IC_{50}) value of 2.2 mg/ml. The cell viability was decreased to 27% at the highest concentration of EESB (4 mg/ml) used in this study. We also evaluated the effect of 2.5 mg/ml of EESB on cell viability with incubation for different periods of time. As shown in Figure 1B, treatment with 2.5 mg/ml of EESB led to a gradual decrease in cell viability with the increase of exposure time. These results suggest that EESB inhibits HT-29 cell growth or viability in both dose- and time-dependent manners. To further verify these results, we evaluated the effect of EESB on HT-29 cell morphology

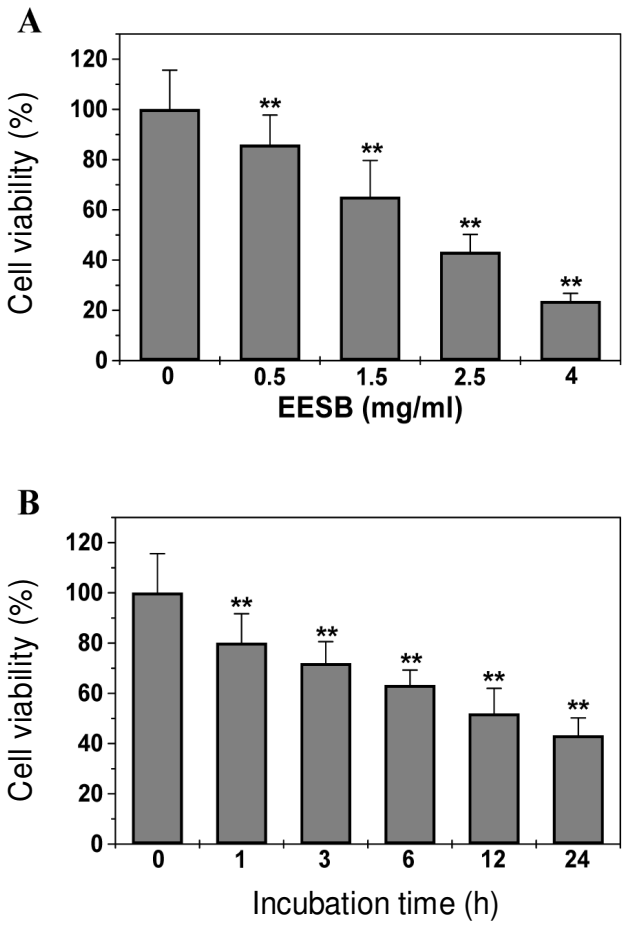

Figure 1. Effect of EESB on cell viability of HT-29 cells. (A) HT-29 cells were treated with the indicated concentrations of EESB for 24 h. (B) Cells were treated with 2.5 mg/ml of EESB for the indicated time periods. Cell viability was determined by the MTT assay. The data were normalized to the viability of control cells (100%, treated with 0.5% DMSO vehicle). Data are averages with S.D. (error bars) from at least three independent experiments. **P<0.01, versus control cells.

through phase-contrast microscopy, since cell morphology in culture is an indicator of the status of the cell's health. As shown in Figure 2, untreated HT-29 cells appeared as densely packed and disorganized multilayers. In contrast, after incubation for 24 h with various concentrations of EESB many of the cells were rounded, shrunken, and detached from adjacent cells adhering to the plate or floating in the medium. In addition, with 24 h exposure to EESB, the treated monolayer's were less confluent than the untreated control. Taken together, these data demonstrate that EESB inhibits the growth of HT-29 cells.

EESB induced apoptosis of HT-29 cells

To determine whether the cell-growth suppressive effect of EESB is due to apoptosis, we examined EESB's pro-apoptotic activity in HT-29 cells through Annexin-V/PI staining followed by FACS analysis. In this assay, Annexin V/PI double-negative population (labeled as LL in the FACS diagram) indicates viable cells; Annexin V-positive/PI-negative or Annexin V/PI double-positive population (labeled as LR or UR in the FACS diagram) represents cells undergoing early or late apoptosis, respectively. As shown in Figures 3A and B, the percentage of cells undergoing apoptosis following treatment with 0, 0.5, 1.5 and 2.5 mg/ml of EESB (including early and late apoptotic cells) was 12.7 ± 0.6%, 17.5 ± 2.4%, 29.5 ± 2.9%, and 46.1 ± 3.3%, respectively (mean ± S.D.; $P < 0.05$, versus untreated control cells). This indicates that EESB treatment induces HT-29 cell apoptosis in a dose-dependent manner. To confirm the pro-apoptotic function of EESB, we investigated the effect of EESB on cellular DNA fragmentation, a typical feature of apoptosis. As shown in Figure 3C, EESB treatment (4 mg/ml) resulted in a characteristic ladder pattern of discontinuous DNA fragments in HT-29 cells.

EESB induced the loss of mitochondrial potential (ΔΨm)

The mitochondrion-dependent pathway is the most common apoptotic pathway in vertebrate animal cells. MOMP, accompanied by collapse of the electrochemical gradient across the mitochondrial membrane, is a key commitment step in the induction of mitochondrion-dependent apoptosis (Mantymaa et al., 2000; Korper et al., 2003). To investigate the mechanism of how EESB induces HT-29 cell apoptosis, we used FACS analysis with JC-1 staining to examine the change in mitochondrial membrane potential after EESB treatment. JC-1 is a lipophilic, cationic dye that selectively enters mitochondria. In healthy cells with high mitochondrial potential, JC-1 forms J-aggregates with intense red fluorescence (590 nm), whereas under apoptotic condition, the mitochondrial membrane potential collapses, so that JC-1 does not accumulate within the mitochondria but remains in the cytoplasm in monomeric form displaying green fluorescence (525 nm). These fluorescence differences can be detected by FACS analysis using JC-1 green and red channels. As shown in Figure 4, JC-1 fluorescence was shifted from a JC-1-green-bright/JC-1-red-bright signal in untreated HT-29 cells to a JC-1-green-bright/JC-1-red-dim signal in cells treated with EESB in a dose-dependent fashion, indicating EESB-induced loss of mitochondrial membrane potential in HT-29 cells.

EESB induced the activation of caspase-9 and caspase-3

To identify the downstream effectors in the apoptotic

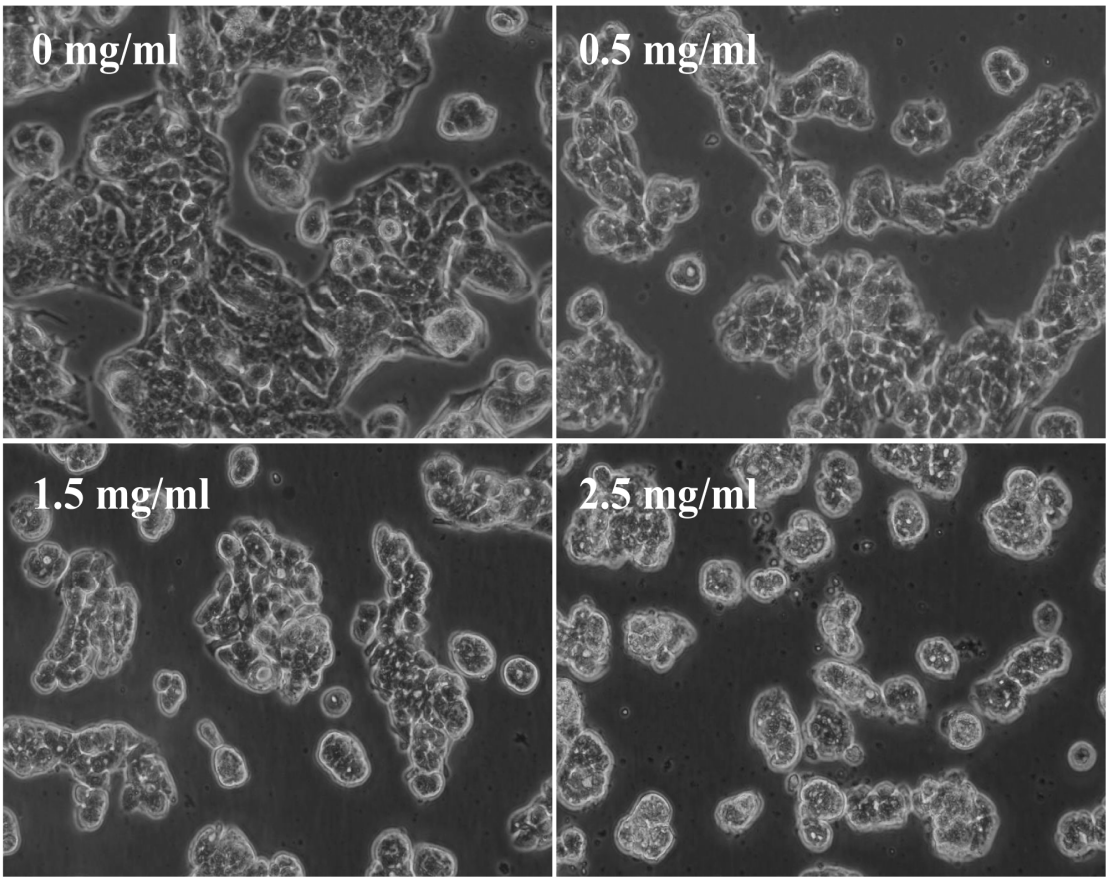

Figure 2. Effect of EESB on the morphology of HT-29 cells. HT-29 cells were treated with the indicated concentrations of EESB for 24 h and morphological changes were observed using phase-contrast microscopy. The photographs were taken at a magnification of 200 ×. Images are representative of three independent experiments.

signaling pathway induced by EESB, the activation of caspase-9 and caspase-3 was examined by a colorimetric assay using specific chromophores, DEVD-pNA (specific substrate of caspase-3) and LEHD-pNA (specific substrate of caspase-9). As showed in Figures 5A and B, EESB treatment significantly and dose-dependently induced activation of both caspase-9 and caspase-3 in HT-29 cells ($P < 0.05$, versus untreated control cells). These data suggest that EESB promotes HT-29 cell apoptosis through the mitochondrion-dependent pathway.

EESB up-regulated the ratio of pro-apoptotic Bax to anti-apoptotic Bcl-2

Bcl-2 family proteins are key regulators of mitochondrion-mediated apoptosis, including anti-apoptotic members such as Bcl-2 and pro-apoptotic members such as Bax. Tissue homeostasis is maintained by controlling the ratio of active anti- and pro-apoptotic Bcl-2 family proteins. Higher Bcl-2-to-Bax ratio by aberrant expression of the proteins is found commonly in various cancers (Kitada et al., 2002). To further study the mechanism of EESB's anti-cancer activity, we performed Reverse Transcription polymerase chain reaction (RT-PCR) and western blot analysis to examine the mRNA and protein expression of Bcl-2 and Bax in EESB-treated HT-29 cells. The results of the RT-PCR assay showed that EESB treatment profoundly increased Bax mRNA expression and reduced Bcl-2 mRNA expression in HT-29 cells (Figure 6A). Data from western blot analysis showed that the pattern of protein expression of Bax and Bcl-2 was similar to that observed at the mRNA level (Figure 6B), suggesting that EESB induces mitochondrion-dependent apoptosis in HT-29 cells through the regulation of expression of Bcl-2 family proteins.

DISCCUSSION

Cancer cells are characterized by an unregulated increase in cell proliferation and/or a reduction in cell apoptosis (Adams and Cory, 2007). In addition, disrupted

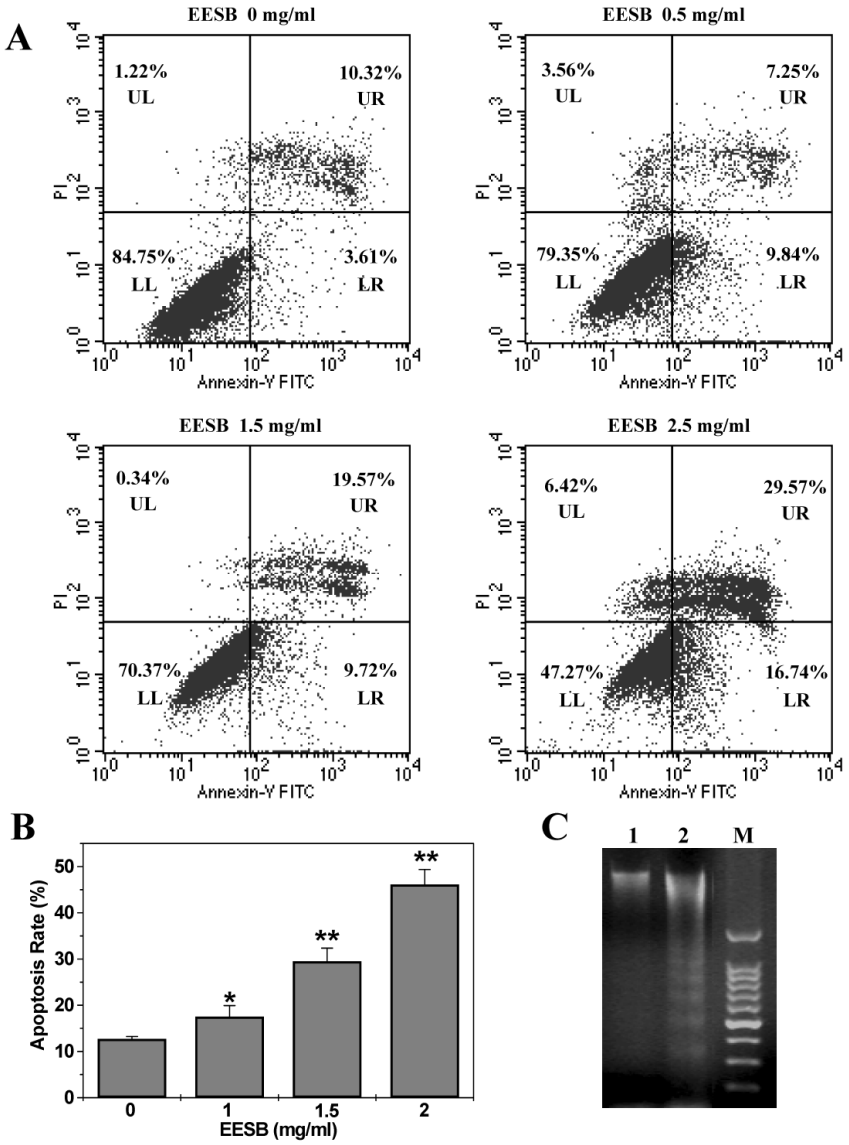

Figure 3. Effect of EESB on apoptosis of HT-29 cells. (A) HT-29 cells were treated with the indicated concentrations of EESB for 24 h, stained with Annexin V/PI, and analyzed by FACS. Representative FACS analysis scatter-grams of Annexin V/PI staining displays four different cell populations labeled as: double-negative stained cells (LL, lower left) indicating live cell population; Annexin V-positive/PI-negative stained cells (LR, lower right) and Annexin V/PI double-positive stained cells (UR, upper right) representing early apoptosis and late apoptosis, respectively; Annexin V-negative and PI-positive stained cells (UL, upper left) showing dead cells. Data shown are representative of three independent experiments. (B) Quantification of FACS analysis. Data shown are averages with S.D. (error bars) from three independent experiments. *P <0.05, **P < 0.01, versus control cells. (C) EESB-induced DNA fragmentation. HT-29 cells were treated with 2.5 mg/ml of EESB for 24 h. DNA was extracted and analyzed by 1.5% agarose gel electrophoresis. M: 100 bp DNA marker, lane 1: control, lane 2: 2.5 mg/ml EESB.

apoptosis contributes to drug resistance of tumor cells, which has become a significant obstacle for the successful management of patients with malignant tumors including colorectal cancer (CRC) (Gorlick and Bertino, 1999; Longley et al., 2006). Moreover, many currently used anti-cancer agents contain potent intrinsic cytotoxicity to normal cells. This limits their long-term use and thereby their therapeutic effectiveness (Boose and

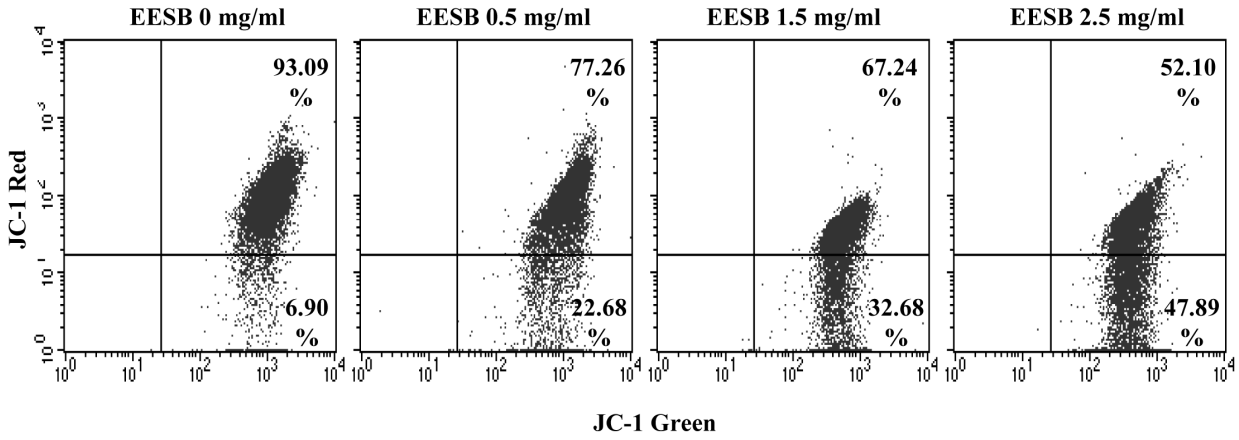

Figure 4. Effect of EESB on the loss of mitochondrial membrane potential in HT-29 cells. HT-29 cells were treated with the indicated concentrations of EESB for 24 h and stained with JC-1. The mean JC-1 fluorescence intensity was detected using FACS analysis. Data are representative of three independent experiments.

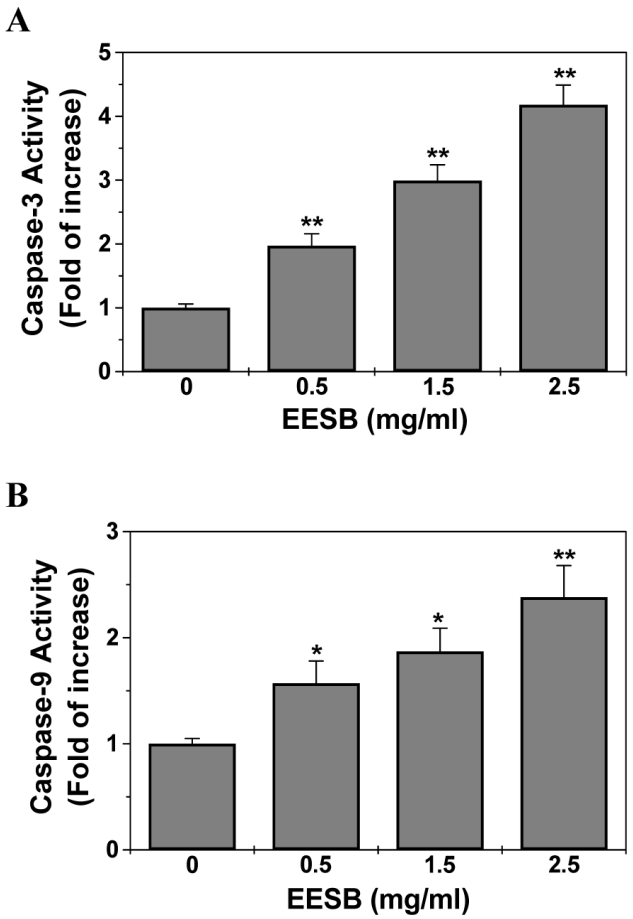

Figure 5. Effect of EESB on the activity of caspases in HT-29 cells. The cells were treated with the indicated concentrations of EESB for 24 h. Caspase-3 (A) and -9 (B) activities were determined by a colorimetric assay. The data were normalized to the caspase activities within control cells (treated with 0.5% DMSO vehicle) and represented as "fold of control". Data are averages with S.D. (error bars) from at least three independent experiments. *P< 0.05, **P< 0.01, versus control cells.

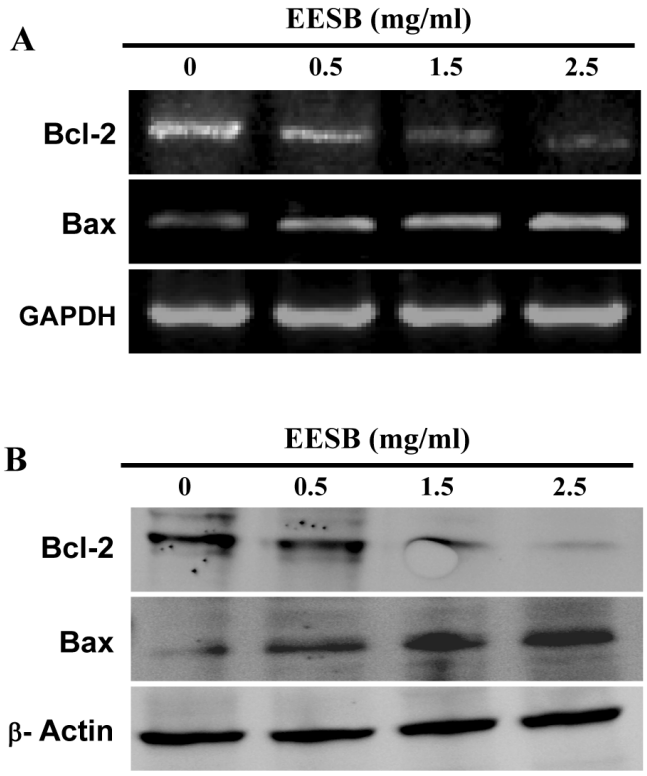

Figure 6. Effect of EESB on the expression of Bax and Bcl-2 in HT-29 cells. Cells were treated with the indicated concentrations of EESB for 24 h. (A) The mRNA levels of Bax and Bcl-2 were determined by RT-PCR. (B) The protein expression levels of Bax and Bcl-2 were determined by western blot analysis. GAPDH and β-actin were used as the internal controls for the RT-PCR or western blot assays, respectively. Data are representative of three independent experiments.

Stopper, 2000). These problems highlight the urgent need for the development of novel cancer chemotherapies. Since natural products, such as traditional Chinese herbal medicines (TCM), have relatively fewer side effects as compared to modern chemotherapeutics and have long been used clinically to treat various classes of diseases including cancer (Tang and Eisenbrand, 1992; Huang, 1999; Gordaliza, 2007). The discovery of naturally occurring agents with pro-apoptotic activities is a promising approach to the development of novel anti-cancer treatments.

S. barbata D. Don, a traditional "heat-clearing and detoxifying" Chinese herb used in TCM formulations, has many reported pharmacological applications. *S. barbata* D. Don is reported to be clinically effective in treating various cancers including CRC (Chinese Pharmacopoeia Commission, 2010; Qian, 1987). However, the mode of action for its anti-tumor effect is largely unknown. Therefore, before *S. barbata* D. Don can be further developed as an anti-cancer agent, its anti-tumor activity and underlying molecular mechanism should first be elucidated.

Here we report for the first time that the ethanol extract of *S. Barbata* D. Don (EESB) reduces the viability and inhibits growth of human colon carcinoma HT-29 cells in a dose and time dependent fashion. Furthermore, we demonstrated that these effects on HT-29 cells result from the induction of apoptosis by EESB.

Apoptosis is activated through two major pathways. For the intrinsic pathway, death signals are integrated at the level of the mitochondria (this pathway, also, is referred to as mitochondrion-dependent). For the extrinsic pathway, death signals are mediated through cell surface receptors. Both pathways eventually lead to the activation of caspases and nucleases, resulting in the destruction of the cell (Adams and Cory, 2002, 2007). MOMP is a key commitment step in the induction of cellular apoptosis. It is the point of convergence for a large variety of intracellular apoptotic signaling pathways leading to the release of many apoptogenic proteins from the mitochondrial intermembrane space. During the process of MOMP, the electrochemical gradient across the mitochondrial membrane collapses. Therefore, the loss of mitochondrial membrane potential is a hallmark for apoptosis. Our data clearly showed that treatment with EESB leads to a collapse of mitochondrial membrane potential.

Caspases, represented by a family of cysteine proteases, are the key proteins that modulate the apoptotic response. Caspase-3 is a key executioner of apoptosis, which is activated by an initiator caspase such as caspase-9 during mitochondrion-mediated apoptosis. In this study, we found that EESB induces the activation of both caspase- 9 and caspase- 3 in HT-29 cells in a dose-dependent manner. Thus, EESB-induced HT-29 cell death is accompanied by an increase in the activities of caspases -9 and 3 that then stimulate the molecular cascade for apoptosis.

Mitochondrion-dependent apoptosis is mainly regulated by Bcl-2 family proteins. MOMP is thought to occur through the formation of pores in the mitochondria by pro-apoptotic Bax-like proteins, which can be inhibited by anti-apoptotic Bcl-2-like members. Therefore, the ratio of Bax to Bcl-2 is critical for determining the fate of cells. Alteration of the ratio by aberrant expression of these proteins impairs the normal apoptotic program contributing to various apoptosis-related diseases including cancer (Yip and Reed, 2008; Youle and Strasser, 2008). For instance, higher Bcl-2 to Bax ratios due to the overexpression of Bcl-2 or down-regulation of Bax expression are commonly found in various cancers (Kitada et al., 2002). This not only confers a survival advantage to the cancer cells but also causes resistance to chemo- and radiotherapies. Therefore, induction of apoptosis in tumor cells through the regulation of Bcl-2 family proteins has been suggested as a promising strategy for the development of anti-cancer drugs. In this study, we demonstrated that EESB treatment

dose-dependently enhances Bax and reduces Bcl-2 mRNA expression in HT-29 cells. This indicates that EESB induces apoptosis by affecting the ratio of Bax/Bcl-2 at a transcriptional level. We further studied the role of EESB on the expression of proteins involved in the mitochondrial pathway. The results showed that EESB treatment up-regulates Bax protein expression and down-regulates Bcl-2 protein expression, which is in accordance with the pattern of their mRNA expression after EESB treatment.

In conclusion, our data demonstrate that EESB inhibits the growth of and induces HT-29 cell apoptosis through the mitochondrion-dependent pathway. These results suggest that *S. barbata* D. Don may be a potential novel therapeutic agent for the treatment of colorectal and other cancers.

ACKNOWLEDGEMENTS

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