Full Length Research Paper

Spica Prunellae extract promotes mitochondrion-dependent apoptosis in human colon carcinoma cell line

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Spica Prunellae (*Prunella vulgaris* fruiting spikes) has long been used as an important component in formulated prescriptions of Chinese traditional medicine to treat various kinds of cancer. However, the precise mechanism of the anti-cancer activity of Spica Prunellae remains to be elucidated. In this report, we investigated the cellular effects of the ethanol extract of Spica Prunellae (EESP) in the HT-29 human colon carcinoma cell line. We found that EESP inhibited the growth of HT-29 cells as evidenced by EESP-induced cell morphological changes and reduced cell viability in dose- and time-dependent manners. Furthermore, we demonstrated that the HT-29 cell growth inhibitory activity of EESP was due to apoptosis, as EESP treatment resulted in the loss of plasma membrane asymmetry (externalization of phosphatidylserine), collapse of mitochondrial membrane potential, activation of caspase-9 and caspase-3, and increase in the ratio of pro-apoptotic Bax to anti-apoptotic Bcl-2. Taken together, these results suggest that Spica Prunellae inhibits the growth of HT-29 colon cancer cells through mitochondrion-mediated apoptosis, which may, in part, explain its anti-cancer activity.

Key words: Apoptosis, anti-tumor, HT-29 cells, phytotherapy, mitochondria, Spica Prunellae.

INTRODUCTION

Spica Prunellae, the fruit-spikes of the perennial plant Prunella vulgaris L., is a medicinal herb widely distributed in Northeast Asia. As a well-known traditional Chinese folk-medicine, Spica Prunellae is believed to have heat-clearing and detoxifying properties and used to treat poor vision, blood stasis, edema, acute conjunctivitis, lymphatic tuberculosis, scrofula, acute mastitis, mammary gland hyperplasia, thyromegaly, and hypertension (Pharmacopoeia of the People's Republic of China, 2010). Spica Prunellae has also been used as a major component in several TCM formulas for the clinical treatment of several kinds of cancer (Liu et al., 2003; Sun et al., 2003; Wang and Zhang, 2010), since according to the theory of traditional Chinese medicine (TCM), accumulation of toxic dampness and heat is one of the major causative factors in the pathogenesis of cancers and therefore clearing heat and detoxification is a principle of anti-cancer treatment. Although it has been reported that extracts of Spica Prunellae can inhibit the growth of

many cancer cells (Wang et al, 2000; Zhang et al, 2005, 2006; Ma et al, 2006; Du et al, 2009), the precise mechanism of its tumoricidal activity still remains largely unclear.

The biological role of apoptosis is to eliminate redundant or damaged cells and hence is crucial for maintaining tissue homeostasis. Disturbed regulation of this vital physiological process underlies many diseases including cancer (Adams and Cory, 2007; Cory and Adams, 2002; Reed, 2000). Apoptosis can be triggered by either intrinsic stimuli such as cytokine deprivation and DNA damage or by extrinsic stimuli such as death ligand-receptor engagement. Both intrinsic and extrinsic signals eventually lead to the activation of cysteine-dependent aspartate-directed proteases (caspases) and nucleases, resulting in destruction of the cell (Cory and Adams, 2002; Borner, 2004). The best understood intrinsic apoptotic pathway is centered at the mitochondria, which therefore is referred to as mitochondrion-dependent apoptosis. Bcl-2 family proteins are key regulators of mitochondrion-dependent apoptosis (Cory and Adams, 2002; Reed, 2000), functioning as either suppressors such as Bcl-2, or promoters such as Bax. One mechanism by which Bcl-2 family proteins regulate apoptosis is through their effect on the permeability of the mitochondrial outer membrane (MOM) via homo- or hetero-association (Vaux and Korsmeyer, 1999). Activation of either of the pro-apoptotic proteins Bax or Bak is sufficient to induce mitochondrial outer membrane permeabilization (MOMP) (Gross et al., 1999; Hsu et al., 1997; Wolter et al., 1997; Wei et al., 2000). This event leads to the release of pro-apoptotic proteins such as cytochrome c and Diablo/Smac that, in turn, trigger the activation of the caspase cascade (Wei et al., 2000; Antonsson et al., 2000; Jurgensmeier et al., 1998; Kluck et al., 1997; Yang et al., 1997). The anti-apoptotic Bcl-2 protein protects cells from apoptosis by interacting with Bax and inhibiting Bax-mediated MOMP (Groos et al., 1999; Yang et al., 1997; Thomenius et al., 2003; Antonsson et al., 1997). The ratio of active anti- and pro-apoptotic Bcl-2 family proteins determines the fate of cells. Alteration of this ratio by aberrant expression of these proteins impairs the normal cellular apoptotic program and can contribute to various apoptosis-related diseases including several types of cancer (Youle and Strasser, 2008; Yip and Reed, 2008). Therefore, promoting cell apoptosis via regulation of the Bcl-2 family proteins has been a major focus in the development of anti-cancer therapies.

In this study, we investigated the cellular effect of the ethanol extract of Spica Prunellae (EESP) and the molecular mechanism of EESP-induced apoptosis in HT-29 human colon carcinoma cells. We found that EESP inhibited the growth and induced apoptosis of HT-29 cells. EESP-induced apoptosis was accompanied by loss of the mitochondrial membrane potential, activation of caspase-9 and caspase-3, and increase in the Bax to Bcl-2 ratio.

Our findings demonstrate that Spica Prunellae promotes apoptosis of cancer cells via activation of the mitochondrion-dependent pathway, which likely is one of the mechanisms underlying the anti-cancer effect of Spica Prunellae.

MATERIALS AND METHODS

Materials and reagents

Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin, Trypsin-EDTA, TriZol reagent, 5,5',6,6'-tetrachloro-1,1',3,3 -tetraethyl-benzimidazol -carbocyanine iodide (JC- 1), iBlot western detection stack/Iblot dry blotting system, caspase-3 and -9 colorimetric protease assay kits were purchased from Invitrogen (Carlsbad, CA, 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 *Spica Prunellae*

The fruiting spikes of *P. vulgaris (Spica Prunellae)* were collected in Hubei province, China, in July, 2008. 500 g of *Spica Prunellae* were extracted with 5000 ml of 85% ethanol using a refluxing method and filtered. The ethanol solvent was evaporated on a rotary evaporator (Shanghai Yarong, Model RE-2000, China) and concentrated to a relative density of 1.05. A dried powder of ethanol extract of *Spica Prunellae* (named EESP) was obtained by spray desiccation using a spray dryer (Buchi, Model B-290, Swiss). The EESP was dissolved in DMSO to a stock concentration of 200 mg/ml and stored at –20˚C. The working concentrations of EESP were made by diluting the stock solution in the culture medium. The final concentration of DMSO in the medium for all experiments was < 0.5%.

PLC analysis

EESP was analyzed on a Shimadzu LC20-AT HPLC system (Kyoto, CA, Japan) using a C- 18 column. The absorbance was measured at 210 nm (Figure 1). The mobile phase consisted of methanol:H_2O:acetic acid = 86:14:0.1 at a flow rate of 1 ml/min with an injection volume of 20 l. Ursolic acid and oleanolic acid were used as controls.

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
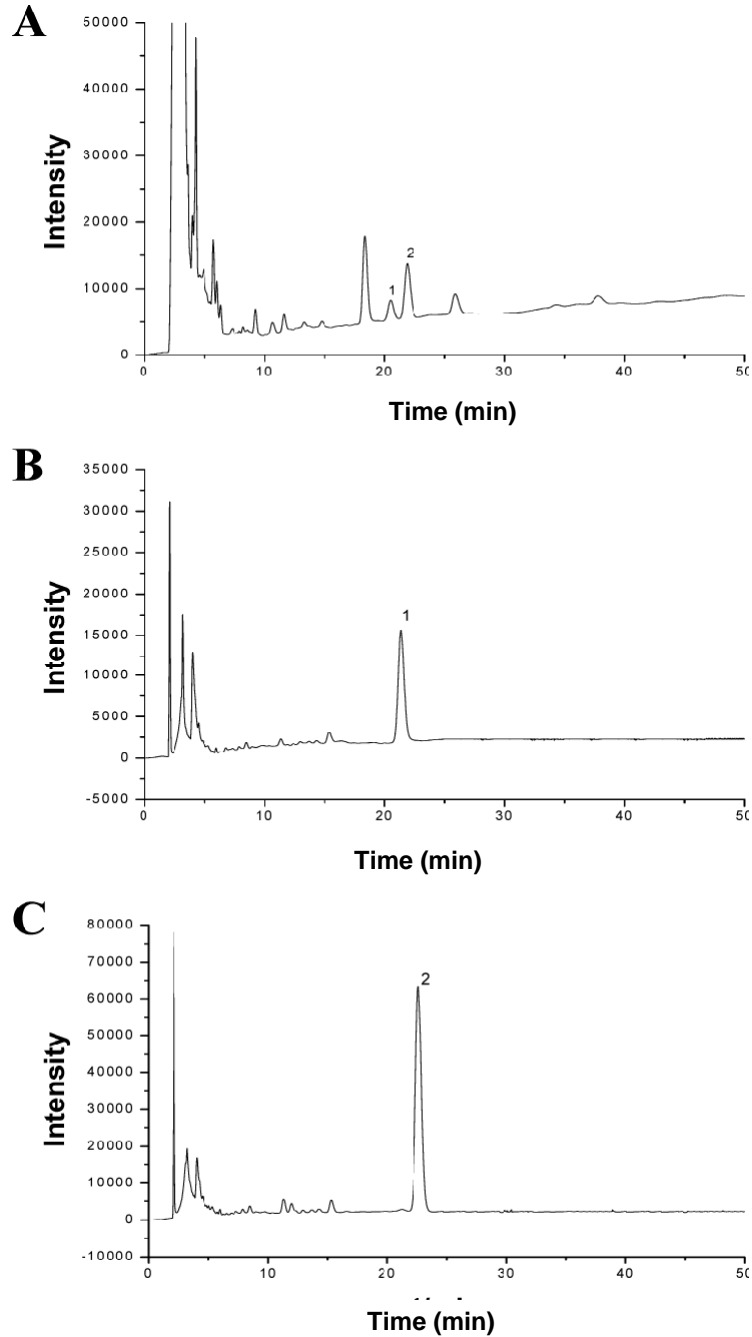
Figure 1. HPLC profiles of EESP (A), oleanolic acid (B) and ursolic acid (C). The mobile phase consisted of methanol : H_2O : acetic acid = 86:14:0.1 at a flow rate of 1 ml/min with an injection volume of 20 l. Peak 1: Oleanolic acid; peak 2: Ursolic acid.

were seeded into 96-well plates at a density of 1×10^4 cells/well in 0.1 ml medium. The cells were treated with various concentrations of EESP for 24 h or with 1.5 mg/ml of EESP for different periods of time. Treatment with 0.5% DMSO was included as the vehicle control. At the end of the treatment, 10 l 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 l DMSO. The absorbance was measured at 570 nm using an ELISA plate reader

(BioTek, Model EXL800, USA).

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

After incubation with various concentrations of EESP, apoptosis of HT-29 cells were 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.

Measurement of mitochondrial membrane potential (m) by flow cytometry 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 can be used as an indicator of mitochondrial potential. In this experiment, 1×10^{6} treated HT-29 cells were resuspended after trypsinization in 1 ml of medium and incubated with 10 g/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 EESP 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×g for 10 min. The protein concentration of the clarified supernate was determined and 100 g of the protein were incubated with 50 l 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 EXL800, 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

2×10^{5} HT-29 cells were seeded into 6-well plates in 2 ml medium and treated with various concentrations of EESP for 24 h. Total RNA from the HT-29 cells was isolated with TriZol reagent. Oligo(dT)-primed RNA (1 μg) was reverse-transcribed with SuperScript II reverse transcriptase (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. The obtained cDNA was used to determine the amount of Bcl-2 or Bax mRNA by PCR. GAPDH was used as an internal control. 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'. The DNA bands were examined using a Gel Documentation System (BioRad, Model Gel Doc 2000, USA).

Western blot analysis

2×10^{5} HT-29 cells were seeded into 6-well plates in 2 ml medium and treated with various concentrations of EESP 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 resolved in 12%

SDS-PAGE gels and electroblotted using the iBlot Western Detection Stack/iBlot Dry Blotting System. The PVDF membranes were blocked with SuperBlock T20 (TBS) blocking buffer (Thermo Scientific, Rockford, IL) for 30 min and washed in TBS with 0.25% tween-20 (TBST), followed by incubation overnight at 4°C with primary antibody. After washing with TBST, the membranes were incubated with secondary antibody for 1 h. The membranes 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 and the data were analyzed using the SPSS package for Windows (Version 11.5). Statistical analysis of the data was performed with the student's t-test and ANOVA. Differences with $P < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Effect of EESP on the growth of HT-29 cells

The effect of EESP on the viability of HT -29 cells was determined by MTT assay to compare the relative number of cells in EESP treated monolayers to untreated controls. As shown in Figure 2A, treatment with 0.5 to 3 mg/ml of EESP for 24 h reduced cell viability by 16 to 84% compared to untreated control cells ($P<0.01$) in a dose-dependent manner, with an estimated half maximal inhibitory concentration (IC $_{50}$) value of 1.5 mg/ml. The cell viability was decreased to 16% at the highest concentration of EESP (3 mg/ml) used in this study. We also evaluated the effect of 1.5 mg/ml of EESP (IC$_{50}$ value) on cell viability with incubation for different periods of time. As shown in Figure 2B, treatment with 1.5 mg/ml of EESP led to a gradual decrease in cell viability with the increase of exposure time. Taken together, these data demonstrate that EESP inhibits the growth of HT-29 cells.

Effect of EESP on apoptosis of HT-29 cells

Loss of plasma membrane asymmetry is one of the characteristic features of the apoptotic program. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a phospholipid-binding protein that has a high affinity for PS, and binds to cells with exposed PS. Therefore, to determine whether the cell-growth inhibitory effect of EESP is due to apoptosis, we examined EESP's pro-apoptotic activity in HT- 29 cells via Annexin-V/PI staining followed by FACS analysis. In this assay, Annexin V/PI double-negative population (labeled as LL in the FACS diagram) identifies viable cells; Annexin V-positive/PI-negative and Annexin V-positive/PI-positive population (labeled as LR or UR in the FACS diagram)

A

B
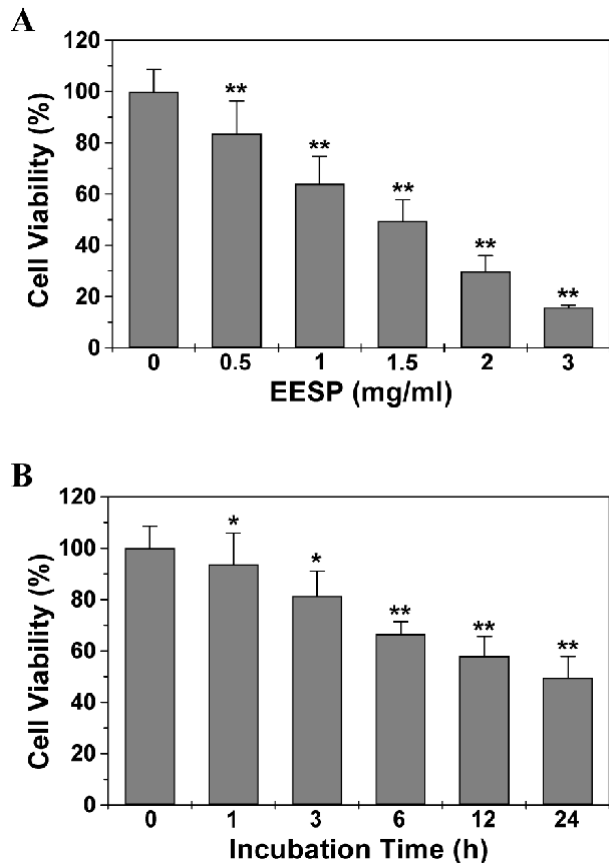
Figure 2. Effect of EESP on cell viability of HT-29 cells. (A) HT-29 cells were treated with the indicated concentrations of EESP for 24 h. (B) Cells were treated with 1.5 mg/ml of EESP for the indicated time periods. Cell viability was determined by the MTT assay. The data were normalized to the viability 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< 0.05, **P< 0.01, versus control cells.

identifies cells undergoing early and late apoptosis, respectively. As shown in Figures 3A and B, the percentage of cells undergoing apoptosis following treatment with 0, 1, 1.5 and 2 mg/ml of EESP (including early and late apoptotic cells) was 0.7 ± 0.21, 10.5 ± 1.49, 14.2 ± 1.91 and $27.3 \pm 1.28\%$, respectively ($P<0.01$, versus untreated control cells). This demonstrates that EESP treatment induces HT-29 cell apoptosis in a dose-dependent manner.

Effect of EESP on the loss of mitochondrial potential (m)

The mitochondrion-dependent pathway is the most common apoptotic pathway in vertebrate animal cells. MOMP, accompanied by the collapse of electrochemical gradient across the mitochondrial membrane, is a key

commitment step in the induction of mitochondrion-dependent apoptosis, as 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 (Mantymaa et al., 2000; Korper et al., 2003). To investigate the mechanism of how EESP induces HT-29 cell apoptosis, we used FACS analysis with JC-1 staining to examine the change in mitochondrial membrane potential after EESP 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/red- dim signal in cells treated with EESP. The percentage of cells with reduced JC -1 red fluorescence following treatment with 0, 1, 1.5 and 2 mg/ml of EESP was 13 ± 2.0, 14.5 ± 3.5, 33 ± 5.8 and $73.2 \pm 4.9\%$, respectively ($P<0.01$, versus untreated control cells), indicating that EESP dose-dependently induces loss of mitochondrial membrane potential in HT-29 cells.

**Effect of EESP on the activation of caspase-9 and caspase-3**

Caspases, represented by a family of cysteine proteases, are the key proteins that modulate the apoptotic response. Caspase-3, a key executioner of apoptosis, is activated by an initiator caspase such as caspase-9 during mitochondrion- mediated apoptosis. To identify the downstream effectors in the apoptotic signaling pathway, 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, EESP treatment significantly and dose-dependently induced activation of both caspase-9 and caspase- 3 in HT -29 cells ($P<0.01$, versus untreated control cells). These data suggest that EESP promotes HT-29 cell apoptosis via the mitochondrion-dependent pathway.

**Effect of EESP on the expression of anti-apoptotic Bcl-2 and pro-apoptotic Bax**

Mitochondrion-dependent apoptosis is primarily regulated by Bcl- 2 family proteins. MOMP is thought to occur through the formation of pores in the mitochondria by
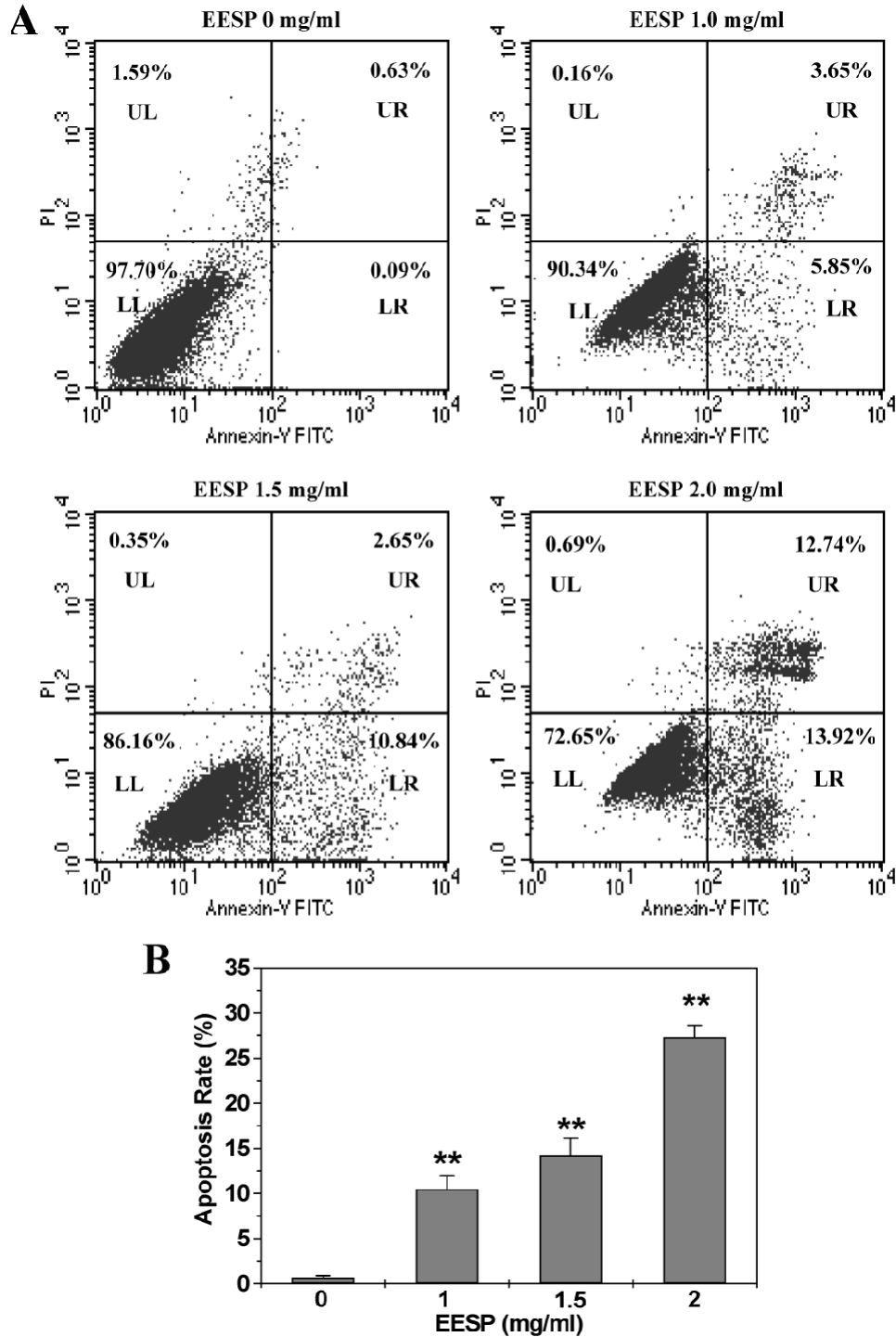
Figure 3. Effect of EESP on apoptosis of HT-29 cells. (A) HT-29 cells were treated with the indicated concentrations of EESP for 24 h, stained with Annexin V/PI, and analyzed by FACS. Representative FACS analysis scatter-grams of Annexin V/PI staining displays four different cell populations labeled 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. $**P < 0.01$, versus control cells.
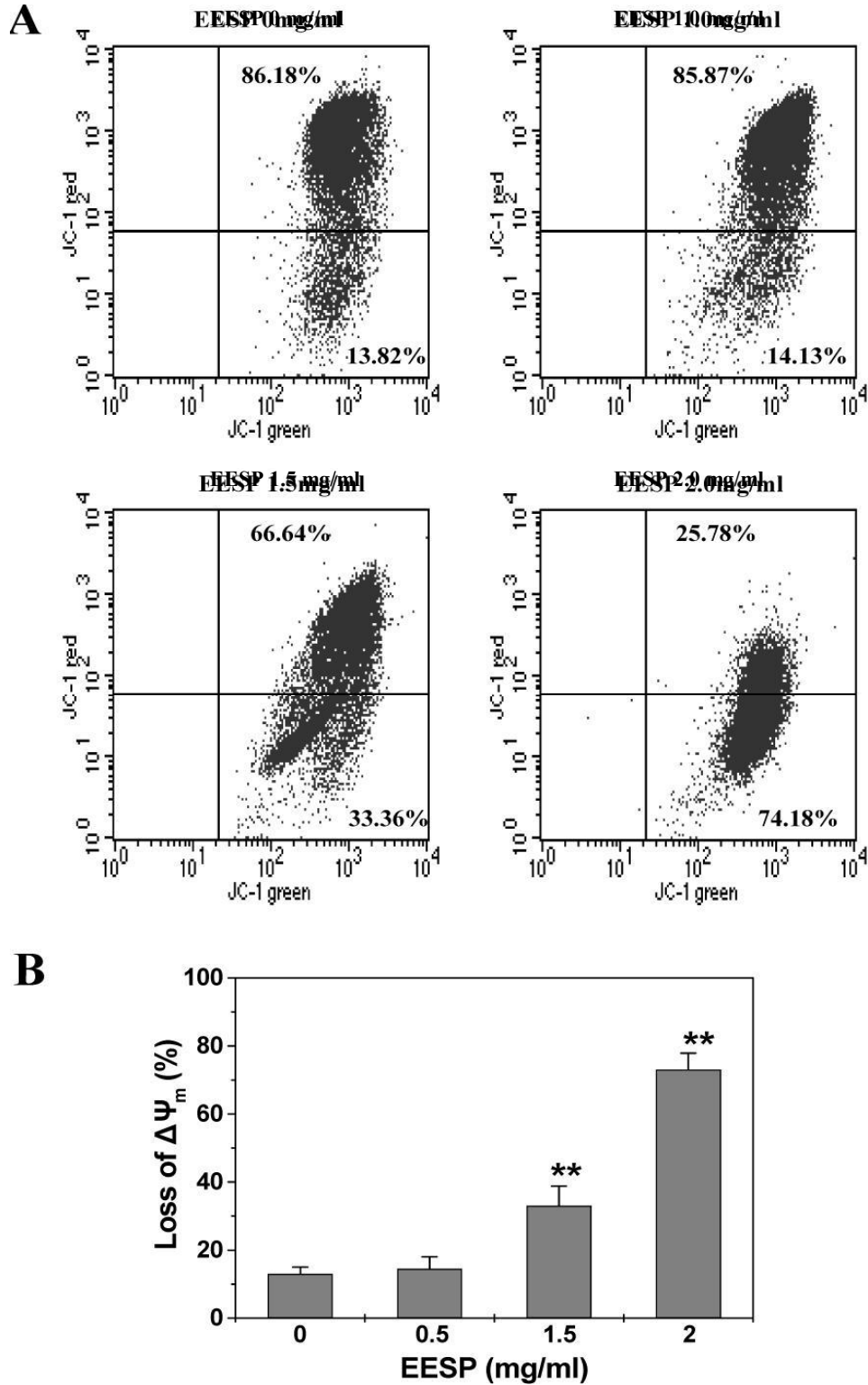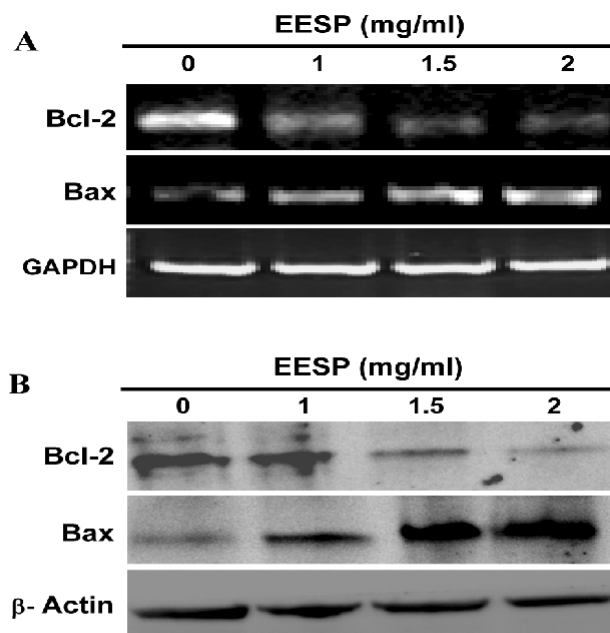
Figure 4. Effect of EESP on the loss of mitochondrial membrane potential in HT-29 cells. (A) HT-29 cells were treated with the indicated concentrations of EESP for 24 h and stained with JC-1. The mean JC-1 fluorescence intensity was detected using FACS analysis. Data shown are representative of three independent experiments. (B) Quantification of FACS analysis. Data shown are averages with S.D. (error bars) from three independent experiments. *$*P <$ 0.01, versus control cells.
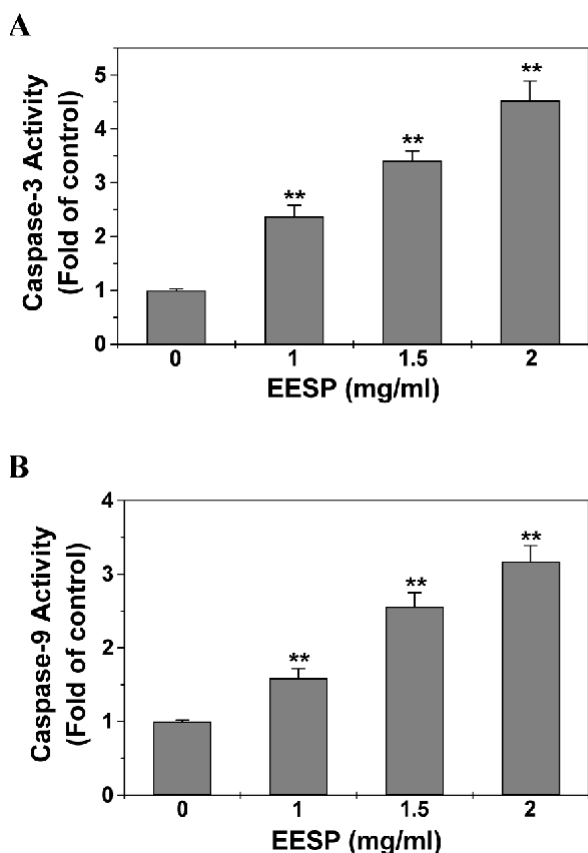
Figure 5. Effect of EESP on the activity of caspases in HT-29 cells. Cells were treated with the indicated concentrations of EESP for 24 h. Caspase-3 (A) and -9 (B) activities were determined by a colorimetric assay. The data were normalized to the caspase activities within control cells (treated with 0.5% DMSO vehicle) and represented as "fold of control". Data are averages with S.D. (error bars) from at least three independent experiments. $**P < 0.01$, versus control cells.

Figure 6. Effect of EESP on the expression of Bax and Bcl-2 in HT-29 cells. Cells were treated with the indicated concentrations of EESP 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 -actin were used as the internal controls for the RT-PCR or western blot assays, respectively. Data are representative of three independent experiments.

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. 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 radio-therapies. To further study the mechanism of EESP's anti-cancer activity, we performed RT-PCR and western blot analysis to examine the mRNA and protein expression of Bcl-2 and Bax in EESP-treated HT-29 cells. The results of the RT-PCR assay showed that EESP 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 their respective mRNA levels (Figure 6B), suggesting that EESP induces mitochondrion-dependent

apoptosis in HT-29 cells through the regulation of expression of Bcl-2 family proteins.

In conclusion, our data for the first time demonstrate that EESP inhibits the growth and induces apoptosis via the mitochondrion- dependent pathway in the colorectal cancer-derived cell line HT-29. These results suggest that *Spica Prunellae* may be a potential novel therapeutic agent for the treatment of colorectal and other cancers.

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