

Ethanollic extract of *Tulipa edulis* Bak induces apoptosis in SGC-7901 human gastric carcinoma cells via the mitochondrial signaling pathway

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Abstract. *Tulipa edulis* Bak (TEB) is an active ingredient in various traditional Chinese medicine compounds and is commonly used to treat swelling and redness, remove toxicity and eliminate stagnation, as well as to prevent and treat certain cancer types. However, the underlying molecular mechanism of the anticancer activity of TEB remains unclear. The aim of the current study was to investigate the effect and underlying mechanism of the ethanollic extract of TEB (EETEB) on SGC-7901 human gastric carcinoma cells. An MTT assay was performed to analyze cell viability. In addition, transmission electron microscopy, an Annexin V/fluorescein isothiocyanate assay, a JC-1 assay and laser scanning confocal microscopy with DAPI staining were used to determine the rate of apoptosis. Furthermore, reverse transcription-polymerase chain reaction and western blot analysis were used to detect the expression levels of the apoptosis gene and protein. EETEB was identified to inhibit the growth of SGC-7901 cells in a dose-dependent manner and induce changes in cell morphology. At the molecular level, EETEB induced SGC-7901 cell DNA fragmentation, loss of plasma membrane and asymmetrical collapse of the mitochondrial membrane potential, while it increased the expression of pro-apoptotic B-cell lymphoma-2 (Bcl-2)-associated X protein and reduced expression of anti-apoptotic Bcl-2. Thus, the results of the current study revealed that the application

of EETEB may inhibit the growth of the SGC-7901 cells due to mitochondria-mediated apoptosis.

Introduction

Gastric cancer (GC) is the most commonly-diagnosed cancer with the second highest mortality rate worldwide (1). In Asian countries, the prognosis for early-stage GC is good; however, the five-year survival rate is only 40% (2). Furthermore, although surgery, chemotherapy and radiation therapy are predominantly used for the treatment of GC, the median overall survival is <1 year (3). Due to the lack of currently available effectual treatment strategies for patients with advanced GC patients, the development of novel therapeutic approaches for the treatment of this life-threatening disease is crucial (4). Plants are considered to be one of the most important sources for the development of novel anticancer agents, with plant-derived agents typically exhibiting relatively fewer side-effects. Various plants have been used in clinical practice in China for thousands of years as important alternative treatments for a variety of diseases. *Tulipa edulis* Bak (TEB), a Chinese herb, appears to exhibit a curative effect on swelling and redness, removing toxicity and eliminating stagnation (5). In addition, TEB has been used to treat various diseases, such as furunculosis, as well as liver, gastric and breast cancer (6). However, the precise mechanism of the potential antitumor activity of TEB in GC has yet to be investigated.

Anticancer agents commonly target cancer cells through the induction of cell apoptosis, typically by targeting the mitochondrial signaling pathway (7). The B-cell lymphoma-2 (Bcl-2) family of proteins includes critical apoptosis regulators, such as the apoptotic suppressor Bcl-2, and promoters, such as Bcl-2-associated X protein (Bax) (8-12). Bax and Bcl-2 are typically considered as the molecular hallmarks of apoptosis (13). In mammalian cells, intrinsic apoptotic signaling leads to mitochondrial outer membrane (MOM) permeabilization, the release of apoptogenic factors (such as cytochrome c) and the subsequent activation of caspase protein expression (14). Thus, one possible mechanism through which the proteins of the Bcl-2 family regulate apoptosis is by altering

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the MOM permeability following homo- or hetero-association (15). During the early apoptosis of cancer cells, the MOM is known to change; thus, the anti-apoptotic Bcl-2 protein may bind to active Bax in order to prevent damage to the MOM (16). During the process of apoptosis, cell fate appears to be determined by the ratio of active anti- and pro-apoptotic Bcl-2 family members (17). This ratio is altered by aberrant expression of Bax and Bcl-2 proteins, impairing the normal apoptotic program and contributing to various apoptosis-associated diseases. Therefore, controlling the permeability of mitochondria using Bcl-2 family proteins ultimately results in mitochondrial changes that induce cell apoptosis (18). Previously, it has been demonstrated that treatment with ethanolic extract of TEB (EETEB) resulted in a decreased number of microvilli and shrinkage of the cytoplasmic organoid volume in human gastric carcinoma SGC-7901 cells, indicating that TEB may induce apoptosis at the molecular level (19).

Thus, the aims of the present study were to investigate the effect of EETEB on the apoptosis of SGC-7901 human GC cells and determine the possible molecular mechanisms underlying this effect.

Materials and methods

TEB alcohol extraction. A preparation of dry TEB extract powder (Tongrentang, Fuzhou, China) was weighed (58.7 g) and added to a 1,000-ml flask with 500 ml 95% (v/v) ethanol. The mixture was refluxed at 95°C for 3 h. A second sample was mixed with an additional 500 ml 95% (v/v) ethanol and a second reflux was performed. The two samples were mixed and concentrated on a rotary evaporator at a temperature of 50°C for 48 h. Subsequently, the sample was successively washed two times with distilled water and three times with petroleum ether to obtain the final extract (EETEB), weighing 1.71 g.

Cell culture and cytotoxicity analysis. SGC-7901 human GC cells, from the Tumor Hospital of Fujian (Fujian, China) were grown and maintained in RPMI 1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum, 80 U/ml penicillin and 80 U/ml streptomycin (all from Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere of 5% CO₂. The SGC-7901 cells were inoculated into 96-well plates at a concentration of 100 μ l/well and cultured for 24 h. Subsequently, the cells were treated with various concentrations of EETEB (0.75, 1.0 and 1.5 mg crude EETEB/ml); the control cells were treated with PBS. Following incubation for 24 h, 50 μ l MTT solution (1.1 mg/ml) was added to each well and incubated for an additional 4 h. Finally, the medium was replaced with 1.5 mg/ml MTT and incubated at 37°C for ~4 h. The crystallization was resolved using dimethyl sulfoxide and the absorbance was measured using a microplate spectrophotometer (SpectraMax 190; Molecular Devices, LLC, Sunnyvale, CA, USA) at a wavelength of 580 nm.

Apoptosis detection. SGC-7901 cells were seeded into 6-well plates in 2 ml medium and treated with various concentrations of EETEB (0.75, 1.0 and 1.5 mg/ml) for 24 h. Flow cytometric analysis with an Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) kit (BD Biosciences,

Franklin Lakes, NJ, USA) was used to detect the apoptosis of SGC-7901 cells. The staining procedure was performed according to the manufacturer's instructions. In the apoptosis assay, an Annexin V/PI double-negative population [labeled as LL in the fluorescence-activated cell sorting (FACS) diagram] indicated the viable cells; by contrast, an Annexin V-positive/PI-negative or Annexin V/PI double-positive population represented cells undergoing early or late apoptosis, respectively.

Apoptosis analysis using DAPI staining. DAPI staining followed by laser scanning confocal microscopy (LSCM; LSM 710; Carl Zeiss AG, Oberkochen, Germany) was used to detect the apoptosis of the SGC-7901 cell nuclei. A minimum of 5x10⁴ cells treated with various concentrations of EETEB (0.75, 1.0 and 1.5 mg/ml) were evaluated per chamber. A 488-nm, 10-MW argon laser beam was used for excitation of blue DAPI fluorescence. Subsequently, LSCM was performed to observe the nuclei of the SGC-7901 cells and detect apoptosis. The collected data were analyzed using Microsoft Excel 2000 software (Microsoft Research, Redmond, WA, USA).

Cell ultrastructure. The cell lysates were collected by centrifugation at 15,000 x g for 10 min at 25°C, the cells treated with various concentrations of EETEB (0.75, 1.0 and 1.5 mg/ml) were fixed in 1.5% paraformaldehyde and 4% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) buffer (pH 7.2-7.4) for 12 h at 4°C. Next, the cells were washed with PBS buffer and post-fixed with 1% OsO₄ in 0.1 M PBS buffer for 2 h. The cells were then dehydrated in a graded ethanol alcohol series and embedded in Epoxy resin 618 (E-51, Ganxi Chemical Co. Ltd., Jiangxi, China). Ultrathin sections (100 nm) were cut using an ultramicrotome (EM UC6; Leica Microsystems GmbH, Wetzlar, Germany). The sections were stained in 2.0% uranyl acetate for 20 min and lead citrate for 15 min. Finally, transmission electron microscopy (TEM; H-7650; Hitachi, Ltd., Tokyo, Japan) was used to examine and capture images of the cell sections.

Measurement of mitochondrial membrane potential. The mitochondrial membrane potential was detected using a JC-1 fluorescent probe (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China), which is a cationic dye that exhibits potential-dependent accumulation in mitochondria. A change in JC-1 fluorescence emission from red to green indicates depolarization of the mitochondrial membrane; thus, this dye can be used as an indicator of changes in mitochondrial membrane potential. In the current experiment, following trypsin digestion (Thermo Fisher Scientific Inc.), SGC-7901 cells (1x10⁶) treated with various concentrations of EETEB (0.75, 1.0 and 1.5 mg/ml) were resuspended in 1 ml medium and incubated with 10 μ g/ml JC-1 at 37°C in an atmosphere of 5% CO₂ for 30 min. JC-1 fluorescence was recorded using a FACSCalibur flow cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) at emission wavelengths of 525 and 590 nm for green and red fluorescence, respectively.

Reverse transcription-polymerase chain reaction (RT-PCR). SGC-7901 cells (1x10⁶ cells/well) were seeded into 6-well plates with 2 ml medium and treated with various concentrations of

EETEB (0.75, 1.0 and 1.5 mg/ml) for 24 h. Total RNA was extracted from the SGC-7901 cells using a Beyzol reagent kit (Shanghai Biyuntian Bio-Technology Co., Ltd., Shanghai, China). Subsequently, oligo(dT)-primed RNA (1 μ g) was reverse-transcribed using SuperScript II[®] Reverse Transcriptase (Promega Corporation, Madison, WI, USA), according to the manufacturer's instructions. PCR was performed to determine the quantity of Bcl-2 and Bax mRNA in the obtained cDNA samples. GAPDH was used as the internal control and the DNA bands were examined using a Gel Documentation system (Gel Doc 2000; Bio-Rad Laboratories, Hercules, CA, USA).

Western blotting. SGC-7901 cells (1×10^6) were seeded in 6-well plates in 2 ml medium and treated with various concentrations of EETEB (0.75, 1.0 and 1.5 mg/ml) for 24 h. The cells were washed twice with cold PBS and then lysed in radioimmunoprecipitation assay buffer containing 1 mM phenylmethyl sulfonyl fluoride (Roche Diagnostics GmbH, Mannheim, Germany). Next, the lysates were centrifuged at $12,000 \times g$ for 10 min at 4°C to acquire the total Bax and Bcl-2 proteins from the mitochondria. The cytosolic and mitochondrial fraction proteins were collected. A single aliquot of the supernatant (50 mg protein) was subjected to electrophoretic separation using SDS-PAGE and transferred to a polyvinylidene fluoride (PVDF) membrane (EMD Millipore, Billerica, MA, USA). The membranes were incubated in blocking buffer (non-fat milk) and then incubated overnight at 4°C with rabbit polyclonal antibodies against Bax (1:1,000, 20 kDa, Cell Signaling Technology, Inc., Danvers, MA, USA), Bcl-2 (1:1,000, 26 kDa, Cell Signaling Technology, Inc.) or β -actin (1:4,000, 43 kDa, Beyotime Institute of Biotechnology, Haimen, China). The membranes were stringently washed and incubated with HRP-conjugated secondary antibodies (Proteintech Group, Chicago, IL, USA), for 1 h at room temperature. Images were captured using a Kodak Image Station 400R (Eastman Kodak Co., Rochester, NY, USA).

Statistical analysis. All the data are presented as the mean of three experiments and were analyzed using SPSS software (version 16.0; SPSS, Inc., Chicago, IL, USA). Statistically significant differences between the control and treatment groups were obtained by performing one-way analysis of variance. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

EETEB inhibits the proliferation of SGC-7901 cells. The effect of EETEB on the proliferation of SGC-7901 cells was determined by performing an MTT assay. As demonstrated in Fig. 1, treatment with 0.75, 1.0 and 1.5 mg/ml EETEB resulted in cell viability of 71.99 ± 7.26 , 54.28 ± 5.28 and $34.48 \pm 5.84\%$, respectively. These values were significantly higher compared with the untreated control cells ($P < 0.05$), indicating that EETEB inhibited SGC-7901 cell proliferation in a dose-dependent manner.

EETEB induces the apoptosis of SGC-7901 cells. To determine whether the cell-growth suppressive effect of EETEB

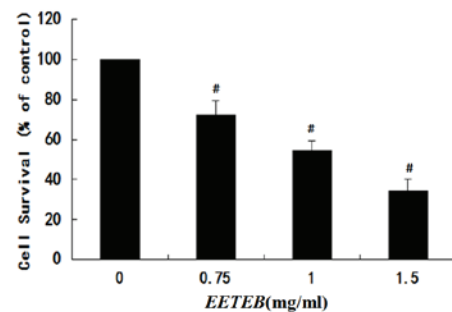


Figure 1. Effect of EETEB on the viability of SGC-7901 gastric cancer cells. Cells were grown and treated with increasing concentrations of EETEB (up to 1.5 mg/ml) for 24 h. Cell viability was analyzed using an MTT assay and data are presented as the mean \pm standard error of the mean of three independent experiments ($n=3$). # $P < 0.05$ vs. control cells. EETEB, ethanolic extract of *Tulipa edulis* Bak.

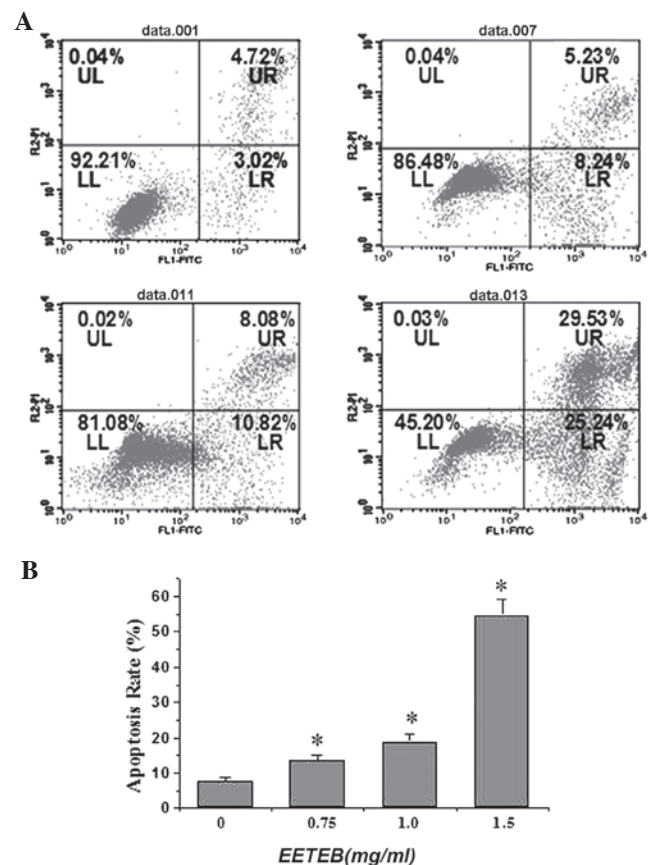


Figure 2. Effect of EETEB on SGC-7901 cell apoptosis. Cells were grown and treated with increasing concentrations of EETEB for 24 h, collected and stained with Annexin V/PI. (A) Representative fluorescence-activated cell sorting (FACS) analysis scattergrams displaying four different cell populations, labeled as: LL, double-negative stained cells = live cell population; LR, Annexin V-positive/PI-negative stained cells - early apoptosis; UR, Annexin V/PI double-positive stained cells - late apoptosis; and UL, Annexin V-negative/PI-positive stained cells - dead cells. Data are representative of three independent experiments. (B) Quantification of FACS analysis. Data are presented as the mean \pm standard error of the mean of three independent experiments ($n=3$). * $P < 0.05$, vs. control cells. UP, upper left; UR, upper right; LL, lower left; LR, lower right; PI, propidium iodide; FITC, fluorescein isothiocyanate; EETEB, ethanolic extract of *Tulipa edulis* Bak.

was due to apoptosis, the pro-apoptotic activity of EETEB on SGC-7901 cells was determined using Annexin-V/PI staining

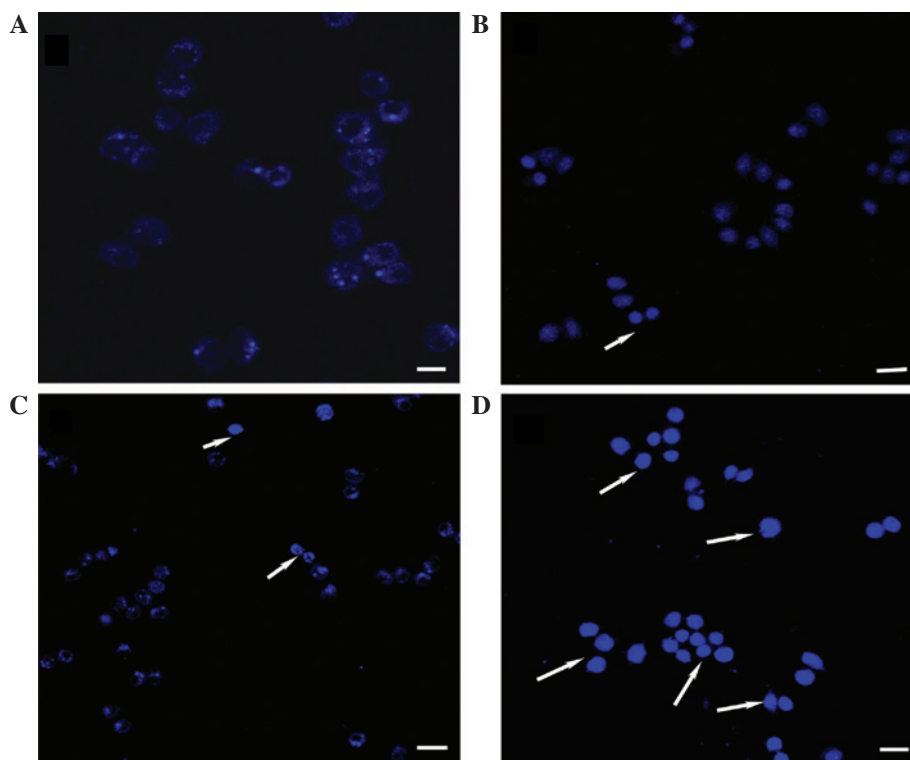


Figure 3. Effect of EETEB on the apoptosis of SGC-7901 cells using DAPI staining. Cells were grown and treated with increasing concentrations of EETEB (up to 1.5 mg/ml) for 24 h, followed by laser scanning confocal microscopy. The cells treated with (A) 0 (control group), (B) 0.75, (C) 1.0 and (D) 1.5 mg/ml EETEB. Arrows indicate apoptotic cells. Scale bars, 20 μ M. EETEB, ethanolic extract of *Tulipa edulis* Bak.

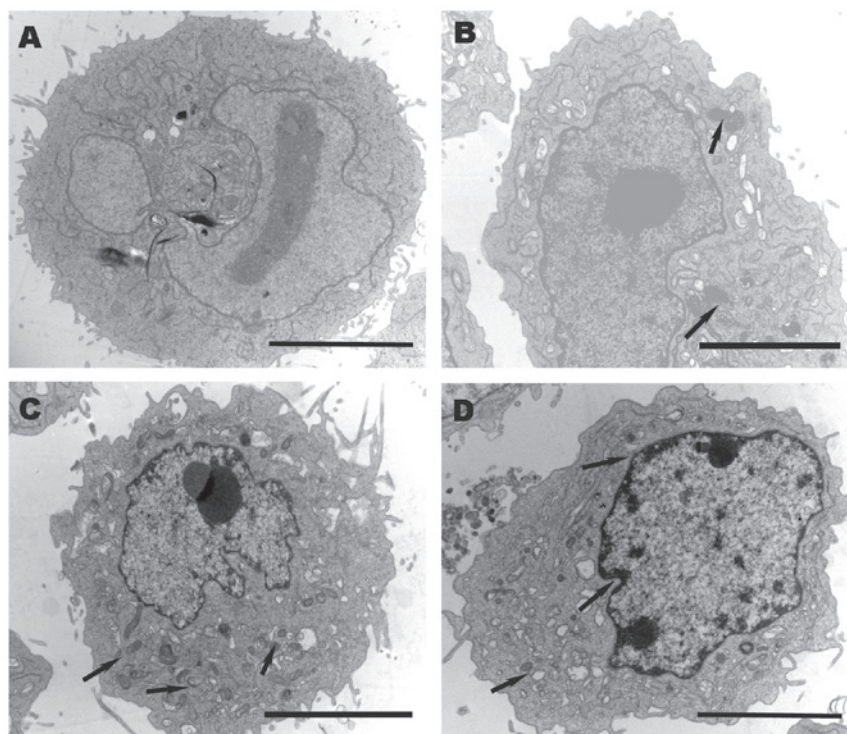


Figure 4. Effect of EETEB on the morphology of SGC-7901 cells, observed using a transmission electron microscope. Cells were grown and treated with increasing concentrations of EETEB (up to 1.5 mg/ml) for 24 h. Cells treated with (A) 0 (control group), (B) 0.75, (C) 1.0 and (D) 1.5 mg/ml EETEB. Arrows indicate apoptotic phenomena in the mitochondria and karyotheca of the SGC-7901 cells. Scale bars, 5 μ M. EETEB, ethanolic extract of *Tulipa edulis* Bak.

followed by FACS analysis. As indicated in Fig. 2A and B, following treatment with 0, 0.75, 1.0 and 1.5 mg/ml EETEB, the percentage of cells undergoing early or late apoptosis

was 8.90 ± 0.81 , 14.31 ± 1.13 , 18.28 ± 2.32 and $50.68 \pm 2.05\%$, respectively. EETEB concentrations of 1.0 and 1.5 mg/ml resulted in a significant increase in apoptosis compared with

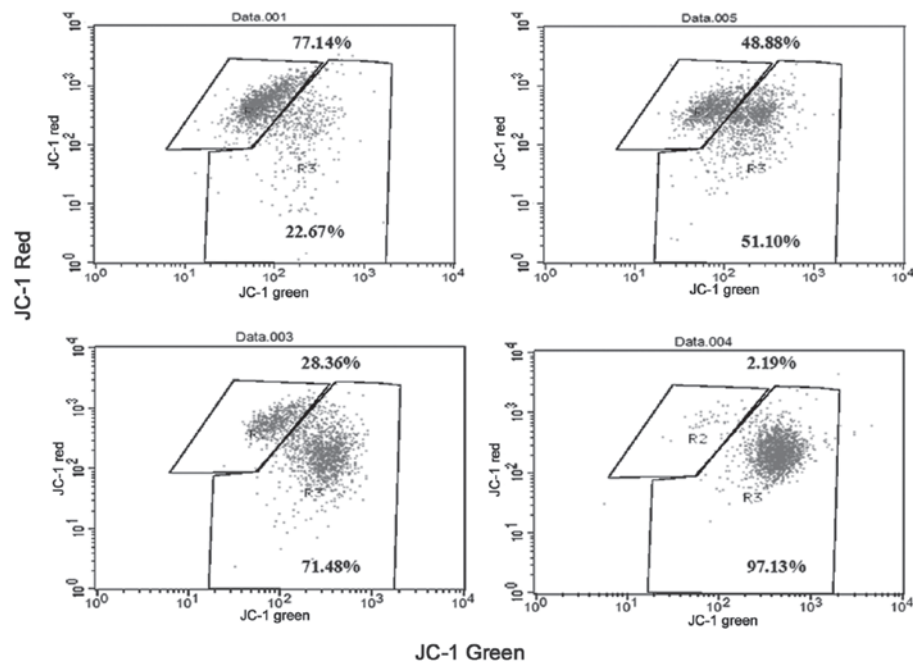


Figure 5. Effect of EETEB on the mitochondrial membrane potential in SGC-7901 gastric cancer cells. Cells were grown and treated with increasing concentrations of EETEB (up to 1.5 mg/ml) for 24 h. JC-1 fluorescent staining intensity was detected using fluorescence-activated cell sorting. EETEB, ethanolic extract of *Tulipa edulis* Bak.

the control group ($P < 0.05$), indicating that EETEB promoted SGC-7901 cell apoptosis in a dose-dependent manner.

The promotion of cellular apoptosis by EETEB treatment was verified by examining nuclear morphological changes following staining of cell nuclei with the DNA-binding dye, DAPI. As indicated in Fig. 3, EETEB-treated cells (Fig. 3B-D) exhibited typical apoptotic morphological features, such as condensed chromatin and fragmented nuclei. By contrast, the untreated cell nuclei (Fig. 3A) exhibited homogenous and less intense staining.

The morphological effect of EETEB treatment on SGC-7901 cells was also evaluated using TEM. As demonstrated in Fig. 4A, untreated control cells did not display any morphological changes. However, EETEB treatment resulted in a decrease in the number of microvilli, shrinkage of the cytoplasmic organoid volume (as evidenced by the promotion of electron density), shrinkage of the nucleolus and margination of the heterochromatin (Fig. 4B-D).

EETEB induces the loss of mitochondrial membrane potential in SGC-7901 cells. Changes in the mitochondrial membrane potential were examined using FACS analysis with JC-1 staining. The membrane-permeable JC-1 dye exhibits potential-dependent accumulation in the mitochondria, indicated by a fluorescence emission shift from green (wavelength, ~525 nm) to red (wavelength, ~590 nm) (20). Therefore, a collapse in the mitochondrial membrane potential during apoptosis can be indicated by a decrease in the ratio of red/green fluorescence intensity. As demonstrated in Fig. 5, following treatment with 0, 0.75, 1.0 and 1.5 mg/ml EETEB, the JC-1 red/green fluorescence ratio in SGC-7901 cells was 22.67 ± 0.56 , 51.10 ± 1.25 , 71.48 ± 1.18 and $97.13 \pm 3.21\%$, respectively. This indicated that EETEB treatment induced the loss of mitochondrial membrane potential in SGC-7901 cells in a dose-dependent manner.

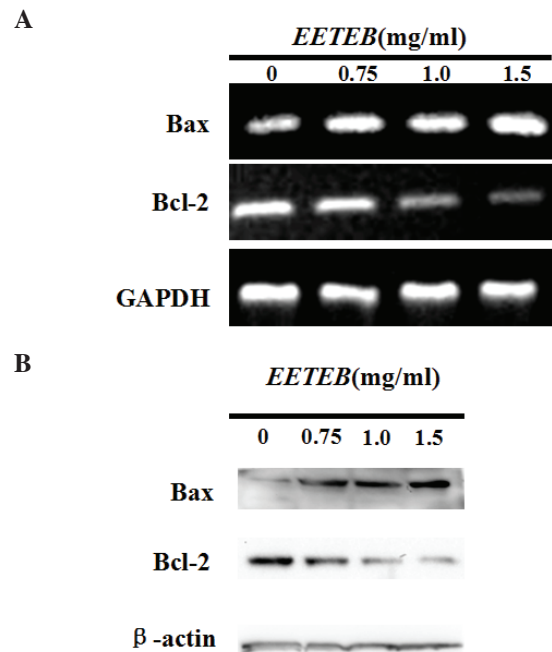


Figure 6. Effect of EETEB treatment on SGC-7901 cell apoptosis, based on the Bax and Bcl-2 expression levels. (A) Reverse transcription-polymerase chain reaction (RT-PCR) detection of Bax and Bcl-2 mRNA expression levels. The cells were grown and treated with increasing concentrations of EETEB (up to 1.5 mg/ml) for 24 h prior to RT-PCR. GAPDH was used as the internal control. (B) Western blot analysis of Bax and Bcl-2 protein expression levels. The cells were grown and treated with increasing concentrations of EETEB (up to 1.5 mg/ml) for 24 h prior to western blotting. β -actin was used as the internal control. EETEB, ethanolic extract of *Tulipa edulis* Bak; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X protein.

EETEB regulates the expression of Bcl-2 family members in SGC-7901 cells. The underlying mechanism mediating

the pro-apoptotic activity of EETEB was investigated by performing RT-PCR to detect the mRNA expression levels and western blotting to determine the protein expression levels of Bcl-2 and Bax in SGC-7901 cells. As shown in Fig. 6, EETEB treatment markedly reduced the mRNA (Fig. 6A) and protein (Fig. 6B) expression levels of anti-apoptotic Bcl-2. By contrast, the expression of pro-apoptotic Bax appeared to be markedly increased following EETEB treatment, indicating that EETEB promoted SGC-7901 cell apoptosis by increasing the pro-apoptotic Bax/Bcl-2 ratio.

Discussion

An abnormal increase in cell proliferation or a reduction in cell apoptosis are two important characteristics of cancer cells (21). However, numerous currently used anticancer agents contain compounds that directly or indirectly damage healthy cells as well as cancer cells, limiting their long-term use and reducing their therapeutic efficacy (22,23). These problems highlight the requirement for the development of novel therapeutic cancer agents. Traditional Chinese herbal medicine compounds, which exhibit relatively less side-effects, have been used in clinical settings to treat various types of disease, including GC. TEB has been used previously in numerous Traditional Chinese medicine herbal formulas for the treatment of cancer, thyreitis and lymphadenitis without any toxic effects (24-27). Therefore, we hypothesized that TEB is not cytotoxic to healthy cells and thus healthy gastric cells were not used for comparison in the present study.

To the best of our knowledge, the present study is the first to report that EETEB appears to reduce viability and inhibit growth in human gastric carcinoma (SGC-7901) cells in a dose-dependent manner. Furthermore, the current study demonstrated that EETEB may induce SGC-7901 cell apoptosis. Thus far, the Bcl-2 family has been identified to mediate an apoptotic signaling pathway, the mitochondrial pathway (28). Bax and Bcl-2 are members of the Bcl-2 family of proteins regulates apoptosis (29). Bax is pro-apoptotic, and Bcl-2 is considered to be anti-apoptotic (30). Therefore, the expression of Bcl-2 or Bax may determine whether cancer cells progress towards apoptosis (9). In the present study, a marked change in the mRNA and protein expression patterns of Bcl-2 and Bax was observed following EETEB treatment. Furthermore, flow cytometry was performed, identifying that EETEB treatment caused a significant increase in cell apoptosis in a dose-dependent manner. Therefore, EETEB is hypothesized to result in cell apoptosis through a process that involves the opening of the mitochondrial permeability transition pore complex (31). In addition, the present study demonstrated that EETEB caused the loss of mitochondrial transmembrane potential, a factor that is considered to be a major determinant in cellular commitment to cellular apoptosis or death (32). Finally, TEM was used to observe the morphology of SGC-7901 cells treated with the EETEB. The results indicated that the number of cell microvilli and the cytoplasmic volume decreased, the electron density was promoted, the nucleolus decreased in size and the heterochromatin was margined. Thus, the morphological changes detected in the SGC-7901 cells demonstrated apoptosis.

In conclusion, the present results of the present study indicated that EETEB treatment may effectively kill the human GC cells, SGC-7901. Furthermore, the current data demonstrates, for the first time, that EETEB inhibits proliferation and induces apoptosis via the mitochondrial signaling pathway. However, it is important to note that the present study only examined the *in vitro* antitumor effects of EETEB. Therefore, the *in vivo* antitumor effects of EETEB, as well as potential molecular mechanisms, require further investigation.

Acknowledgements

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