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BASIC INVESTIGATION

In vitro inhibitory and pro-apoptotic effect of Stellera Chamaejasme L Extract on human lung cancer cell line NCI-H157

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Abstract

OBJECTIVE: To investigate the inhibitory and pro-apoptotic effect of Stellera Chamaejasme L extract (ESC) in vitro.

METHODS: ESC was first extracted with ethanol, and then washed using a polyamide column with 60% ethanol. ESC was then decompressively recycled and vacuum dried at room temperature to obtain active fractions. Subsequently, the cytotoxic and apoptotic effects of ESC on NCI-H157 human lung cancer cells were determined.

RESULTS: The results showed that ESC was rich in isomers of Chamaejasminor, neochamaejasmine and Sikokianin. ESC had significant cytotoxicity against NCI-H157 cells, with an IC_{50} of approximately 18.50 μg · mL^{-1}. ESC caused significant increase in total apoptotic rate, the activity of caspase 3 and 8, and Fas protein expression (P<0.05).

CONCLUSION: The inhibitory effect of ESC on NCI-H157 tumor cells might partly be attributed to its apoptotic induction through activation of the Fas death receptor pathway.

Key words: Stellera chamaejasme L; Lung neoplasms; Apoptosis; Antigens; CD95; Lissamine rhodamine B

INTRODUCTION

Pharmacological screening in vitro is fast, sensitive and specific, as conditions are easy to control. These advantages play important roles in research of drug activity screening, toxicity evaluation and in-depth study of cellular and molecular pharmacology. It is particularly important for early anti-tumor activity screening of plant extracts or compounds.[1] Sulforhodamine B (SRB) is a protein binding dye, which combines with basic amino acids of biological macromolecules, causing color change proportional to live cell protein. This staining technique has been widely used for testing cell proliferation and chemosensitivity.[2] SRB staining has similar sensibility compared to other cytotoxicity assays such as the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) or clonogenic assay. The SRB assay possesses a colorimetric end point and is nondestructive and indefinitely stable. These practical advances make the SRB assay an appropriate and sensitive assay to measure drug-induced cytotoxicity, even on large-scale application.[3] In fact, SRB has been adopted by the National Cancer Institute of the USA for large-scale screening of new drugs.[4]

Apoptosis is a cell self-destructive process under genetic control.[5] A variety of human malignant tumor cells lose some of the capacity to respond to apoptotic stimuli. This phenomenon is most prominent in tumor tissue. As a tumor is the disease not only of abnormal cell proliferation and differentiation, but also of abnormal apoptosis,[6] enhancing the study of drug-induced apoptosis and its mechanism, will provide new ideas and approaches in the prevention and treatment of cancer. In 1992, Hickman first proposed that inducing apoptosis of tumor cells could act as the main objective and means in cancer therapy. Thereafter, inducing tumor cell apoptosis became a hot topic in international cancer treatment research.[5] Recent studies have shown that many active ingredients or extracts from traditional Chinese medicine can induce apoptosis of tumor cells.[7-10] Deepening investigations have demonstrated that inducing apoptosis of tumor cells may be an important target for anti-tumor properties found in traditional medicines.

The dry roots of Stellera chamaejasme L, known as "Rui xiang lang du" in Chinese, are believed to possess a pungent and warm-nature. Their effects are clearing heat and detoxication, edema alleviation, rot removal, and myogenicity, and have long been used by the Chinese for the treatment of various tumors. The chemical composition of the herb includes diterpenoid, lignans, coumarin and other phenylpropanoids, flavonoids, and volatile oils.[11] Much experimental evidence has also confirmed that several ingredients (e.g., neochamaejasmin A and daphnane-type diterpene gnidimacrin) separated from Stellera chamaejasme L possess anti-cancer effects. These effects include an influence on apoptosis related protein expression (e.g., Fas-procaspase 8-procaspase 3 and p21-procaspase 3), that ultimately induced apoptotic events in tumor cells.[12-17] It is suggested that Stellera chamaejasme L has an important potential antineoplastic value. Our previous experimental studies have also shown that extract of Stellera Chamaejasme (ESC) significantly inhibited growth of H_{22} tumor cells in mice.[18]

In this study, we observed the inhibitory effect of ESC on the human lung cancer cell line, NCI-H157. We used the SRB method to determine whether ESC can induce apoptosis in tumor cells, and also to investigate the mechanism by which this induction may occur.

MATERIALS AND METHODS

Preparation of ESC

Stellera chamaejasme L herbal medicine was extracted 3 times with ethanol and the concentrated liquid (volatile to non-alcohol taste) was washed on a polyamide column with 60% ethanol, and then decompressively recycled and vacuum dried at room temperature. The final compound obtained was ESC.

Analysis of ESC

ESC was dissolved with 1 mL of ethanol. Chromatographic equipment included a Waters Alliance 2695-996 HPLC and Zorbax column (4.6 mm × 250 mm). The mobile phase included acetonitrile, water, and 1% acetic acid-water. Column temperature was 25° C, injection volume was 20 l, and detection wavelength was 290 nm. Gradient separation conditions are shown in Table 1. Mass spectra conditions incorporated a Finnigan TSQ mass spectrometer, ESI interface; spray voltage of 4.5kV, capillary temperature of 320° C, purge gas 40psi, auxiliary gas 20au, and m/z 120-1400 positive and negative ion scan.

Table 1 Gradient isolation conditions

Time (min)	Acetonitrile (%)	Water (%)	1% acetic acid-water (%)
0	10	80	10
40	50	40	10
60	90	0	10
65	100	0	0
70	100	0	0

Cell line and culture

NCI-H157 cell line was purchased from the Cell Culture Center of the Chinese Academy of Medical Sciences (Beijing, China). Cells were grown in RPMI 1640 medium (GIBCO, Grand Island, NY, USA) containing 10% fetal bovine serum (Hyclone Laboratories, Logan, UT, USA) and 2 mM of glutamine (Sigma, St. Louis, MO, USA) in a humidified incubator (Mco-15AC type, SANYO Electric Co Ltd, Osaka, Japan) at 37°C in 5% CO_2. Cells were split twice weekly and logarithmic growth cells were selected for experiments.

Cytotoxicity assay

Cells were seeded into 96-well microtiter plates in 100 μL medium at a plating density of approximately 5000 cells/well. After cells were inoculated, the microtiter plates were incubated at 37° C in 5% CO_2, 95% air and 100% relative humidity for 24 h prior to the addition of experimental drugs.

Groups were divided into control (only complete medium added), dimethyl sulfoxide (DMSO, final concentration, 0.1%), 5-fluorouracil (5-FU) injection (final concentration, 100 μg/mL, Shang Hai XuDong HaiPu Pharmaceutical Co. Ltd, Shanghai, China), increasing concentrations of ESC (final concentrations: 12.5, 25, 50, 75, 100 μg/mL). Every group had 4-6 double wells. ESC was dissolved in DMSO and stored frozen prior to use. At the time of drug addition, different concentrations were thawed and diluted to twice the desired final maximum test concentration with complete medium. These ESC dilutions (100 μL) were then added to

wells already containing 100 μL of medium, resulting in the required final drug concentration.

Following the addition of ESC, plates were incubated for a further 48 h. The assay was terminated by adding cold trichloroacetic acid (TCA). Cells were then fixed in situ by gently adding 50 μL of cold 50% (w/v) TCA (final concentration, 10%) and incubated for 1 h at 4°C. Then, 100 μL of sulforhodamine B solution (SRB; Sigma, St. Louis, MO, USA) at 0.4% (w/v) in 1% acetic acid was added to each well, and plates were incubated for 10 min at room temperature. After staining, unbound dye was removed by washing five times with 1% acetic acid and plates were dried in air. Bound stain was subsequently solubilized with 10 mM triama base and the absorbance was read on an automated plate reader (ELX800 type, BIO-TEX Instruments, INC, Winooski, VT, USA) at a wavelength of 515 nm.

Inhibition rate (%) was calculated as follows: Inhibition rate (%) =1-Mean absorbance of treatment group/ Mean absorbance of vehicle control group. IC_{50} was calculated using a logistic method.

Flow cytometry assay

Cells (1 mL of 5×10^5 cells) were inoculated into 6 well plates. Groups were divided into control, DMSO, 5-FU, and ESC (final concentrations 25, 50, 100 μg/mL). Each group had 3 double wells. After 24 h incubation, drugs were added to plates and incubated for another 24 h.

The process for the flow cytometry assay was conducted according to Annexin V-FITC Apoptosis Detection Kit protocol (Abcam, Cambridge, UK). Cells were routinely digested in each well, washed with 500 μL of PBS and centrifuged at 200 g for 5 min. The supernatant was then aspirated, whereupon 100 μL of annexin-V-FITC (at 1g/mL in HEPES buffer with 1.8 mM of $CaCl_2$) was added to the cells and incubated for 5-10 min at room temperature in the dark. Finally, 1 ml of HEPES containing 10 g/ml propidium iodide was added. Samples were analyzed immediately using flow cytometry (Becton, Dickinson and Company, Franklin Lakes, NJ, USA).

Caspase activity assay

The methods used for cell treatment were the same as those used in the flow cytometry assay.

The activity of caspase 3, 8, and 9 were measured with caspase assay kits (Chemicon International, Inc., Temecula, CA, USA). Methods were based on the kit protocol. First, a pNA standard curve was generated. 1×10^6 cells were collected (1500 rpm for 10 min). Cells were resuspended in 50-500 μL of chilled 1× cell Lysis Buffer. Cells were incubated on ice for 10 minutes and then centrifuged for 5 minutes in a microcentrifuge (10,000 × g). Supernatant (cytosolic extract) was transferred to a fresh tube and put on ice. The protein concentration for each sample set was detected. Assay mixture was prepared in a 96-well plate or standard microcentrifuge tubes, according to assay kits. Samples were incubated for 1-2 h at 37°C and read at 405 nm in a microtiter plate reader. Fold-increase in caspase-3, 8, 9 activity was determined by comparing the optical density (OD) reading from the induced apoptotic sample with the level of the uninduced control.

Fas and Fas Ligand ELISA

Cells were prepared as described above. The quantitative detection of Fas and Fas Ligand in cells were determined with an enzyme ligand immunosorbent assay kit (R&D Systems, Inc., Emeryville, CA, USA). Briefly, cell supernatant together with 9 × Sample Buffer (100μL) was added to the corresponding sample well of the microtiter plate. Plates were incubated at 37°C for 2 h. After the antigens present in the sample were bound to the coating antibodies adsorbed to the microwells, plates were washed with 100 μL of wash buffer for 5 times. Biotinylated antibody (100 μL) was then added to the microtiter plates and incubated at 37°C for 1 h. Plates were washed again. Conjugated enzyme (100 μL) was then added to the wells to bind to antigens captured by the biotinylated antibody. After removing unbound conjugated enzyme antibody by washing, TMB Solution (100 μL) was then added to each well. After incubation at room temperature in the dark for 15 minutes, the reaction was stopped by adding 100 μL of Stop Solution, and the absorbance was measured at 450 nm with a microplate reader (Wellscan MK3, Helsinki, Finland). Fas and Fas Ligand sample concentrations were determined on the base of a standard curve prepared from seven standard dilutions. All samples were analyzed in duplicate, and the limit of detection was determined to be 15 $pg\cdot mL^{-1}$.

Statistical analysis

All results were expressed as mean±SD. One way analysis of variance (ANOVA) with multiple comparison tests were used. $P<0.05$ was considered significant. Statistical tests were performed using SPSS version 12.0 (IBM, Armonk, NY, USA).

RESULTS AND DISCUSSION

Chinese medicines are made from complex ingredients. Extracting the active ingredient from Chinese medicines demands a huge workload and it is easy to lose potential active ingredients during the extracting process. Our research experience showed that a mixture of traditional Chinese medicine often has better efficacy than a single component compound. We therefore preliminarily adopted a LC-MS method for the analysis of ingredients within the ESC.

Results showed that ESC was still a mixture of compounds. Figure 1 shows actual analysis of chromatograms. Part of the peak components containing the ingredients of Chamaechromone, neochamaejasmine and Sikokianin were identified (Table 2).

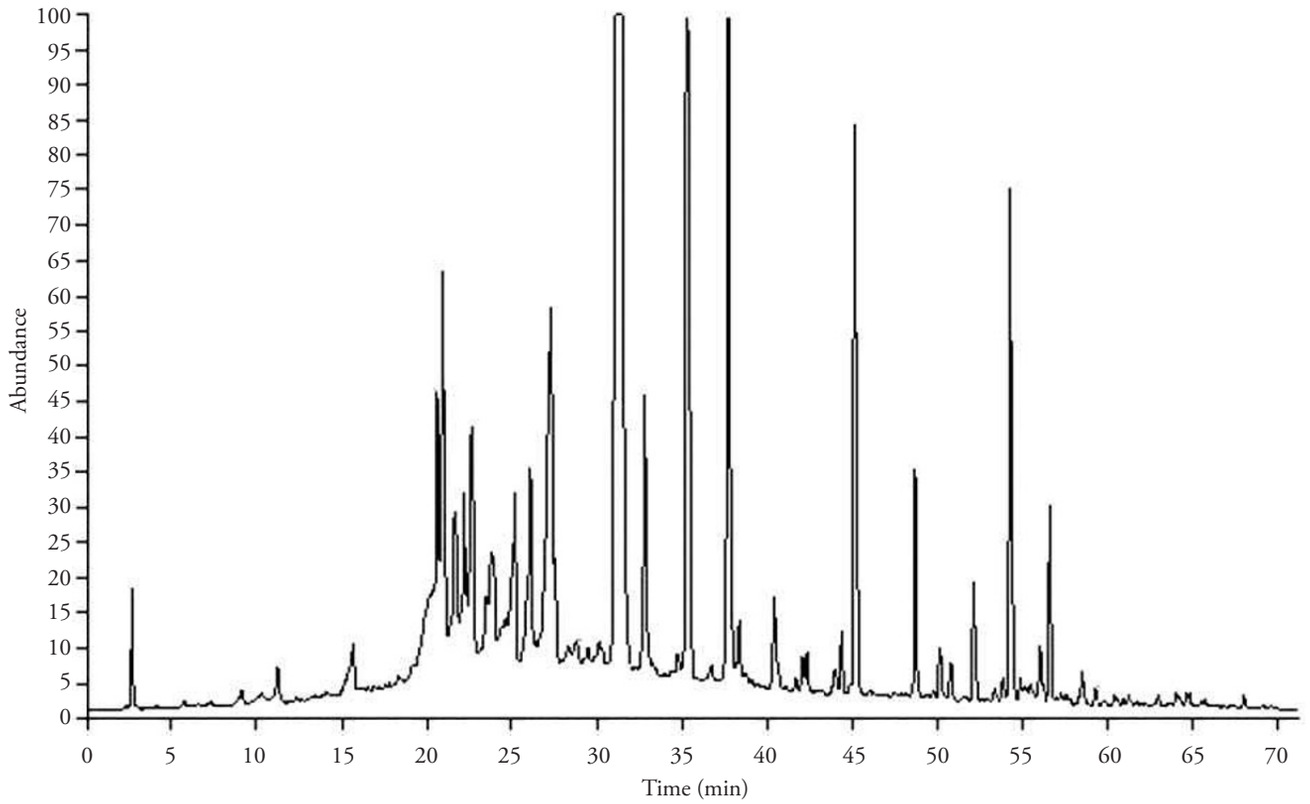


Figure 1 Liquid chromatography of ESC

Table 2 Identification of ESC components by mass spectrometry

Peak number	Time	Molecular Weight	Component identification results
1	31.17	542	Chamaechromone or isomer
2	31.43	542	Chamaechromone or isomer
3	32.75	542	One Isomer of Chamaejasminor, neochamaejasmine, etc
4	35.20	542	One Isomer of Chamaejasminor, neochamaejasmine, etc
5	35.38	542	One Isomer of Chamaejasminor, neochamaejasmine, etc
6	37.60	542	One Isomer of Chamaejasminor, neochamaejasmine, etc
7	38.33	542	One Isomer of Chamaejasminor, neochamaejasmine, etc
8	40.28	556	One Isomer of Sikokianin A,C, etc
9	45.15	unknow	unknow
10	48.63	570	Chamaejasminor B or isomer
11	54.24	394	unknow

In this study, we adopted the SRB assay to determine the cytotoxicity of ESC. Our study showed that the SRB method has good sensitivity, stability and reproducibility. SRB results showed that the OD values of 50, 100, 200 μg • mL^{-1} of ESR decreased greatly compared to the DMSO group (P<0.01). Inhibition rates from the 50, 100, 200 μg • mL^{-1} ESC groups were 64.31%, 84.68% and 84.37%, respectively, and the IC_{50} of ESC was approximately 18.5μg • mL^{-1}. According to Evaluation Criteria of anti-cancer drugs in vitro [19] (plant extract IC_{50} < 20 μg/mL, the highest inhibitory rate > 80%), ESC had strong inhibitory effect against NCI-H157 cancer cells (Figure 2).

A method proposed in recent years to detect apoptosis is the Annexin V method. Annexin V selectively binds the inner membrane phospholipids, phosphatidylserine (PS). In early apoptosis, PS moves to the cell surface, allowing this important feature of apoptosis to be directly detected by flow cytometry combined with a green fluorescent probe FITC-Annexin V. Propidium iodide (PI) can stain the late apoptotic cells to show red fluorescence.[8] We used Annexin V/PI combined double labeling to observe the apoptosis percentage of ESC in NCI-H157 cells.[20] Our results showed that the early apoptosis rate of 25, 50, and 100 μg • mL^{-1} of ESC were (3.35 ± 0.83)%, (4.64 ± 0.54)% and (7.38 ± 2.12)%, respectively. The late apoptosis rate of 25, 50, 100μg • mL^{-1} of ESC were (10.08±1.96)%, (11.63±0.40)% and (18.07 ± 1.45)%, respectively. The total apoptosis rate of 25, 50, 100 μg • mL^{-1} AESC were (13.43 ± 2.79)%, (16.27 ± 0.94)% , and (25.45 ± 3.57)% , respectively. Compared to the DMSO group, the early, late and total apoptosis rate of 25, 50, 100μg • mL^{-1} AESC increased greatly (P<0.05, P<0.01 and P<0.01, respectively). ESC can, therefore, induce the apoptosis of NCI-H157 cells in vitro (Figure 3).

There are at least two broad pathways that lead to apoptosis, an "extrinsic" and an "intrinsic" pathway. In both pathways, signaling results in the activation of a

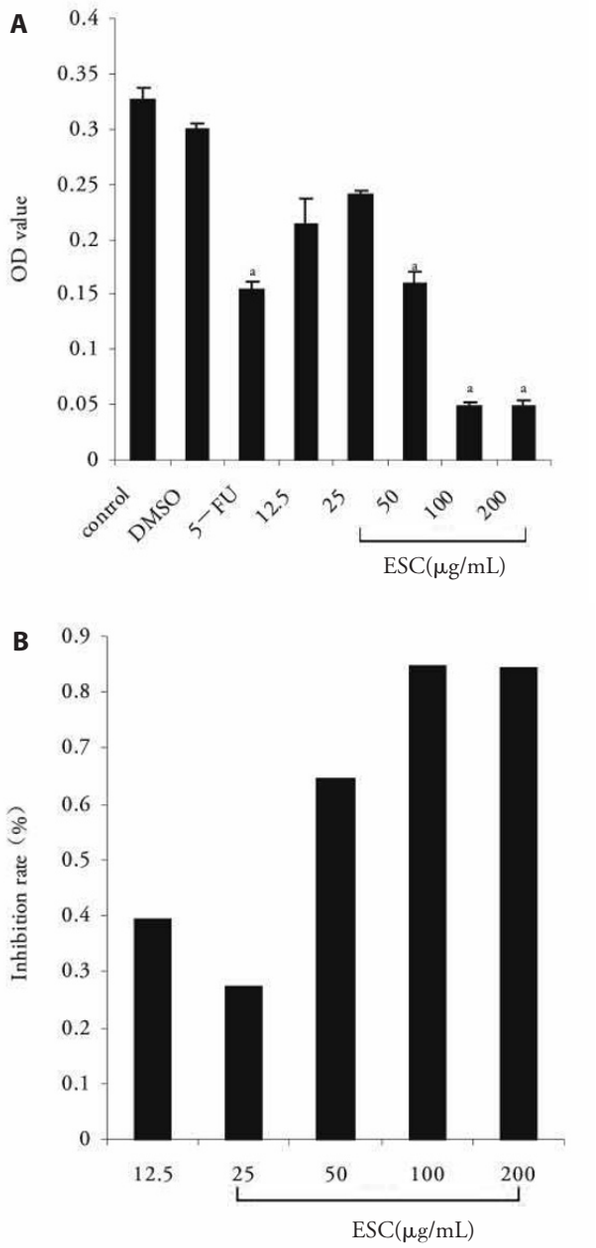


Figure 2 Cytotoxicity of ESC against NCI-H157 cancer cells. (A) Absorbance value of different ESC groups are shown (mean±SD). (B) Inhibition rates of different concentrations of ESC were calculated. [a]*P*<0.01, significant versus control group.

family of Cys (cysteine) proteases, named caspases. In general, the extrinsic pathway activates caspase 8 and the intrinsic pathway activates caspase 9. Finally, both pathways activate caspase 3 that acts in a proteolytic cascade to dismantle and remove the dying cells.[21] In this study, we used spectrophotometry to detect the activities of these caspases. The results showed that the activity of caspase 3, and 8 in NCI-H157 cells treated by ESC were increased greatly (P<0.05 and P<0.01, respectively), compared with the DMSO group. The growth rates of caspase 3 activity in 25, 50, 100μg • mL^{-1} ESC groups were 49.47%, 33.74% and 52.58%, compared with the DMSO group. The growth rates of caspase 8 activity in 25, 50, 100 μg • mL^{-1} of ESC groups were 64.84%, 67.57% and 47.62%, compared

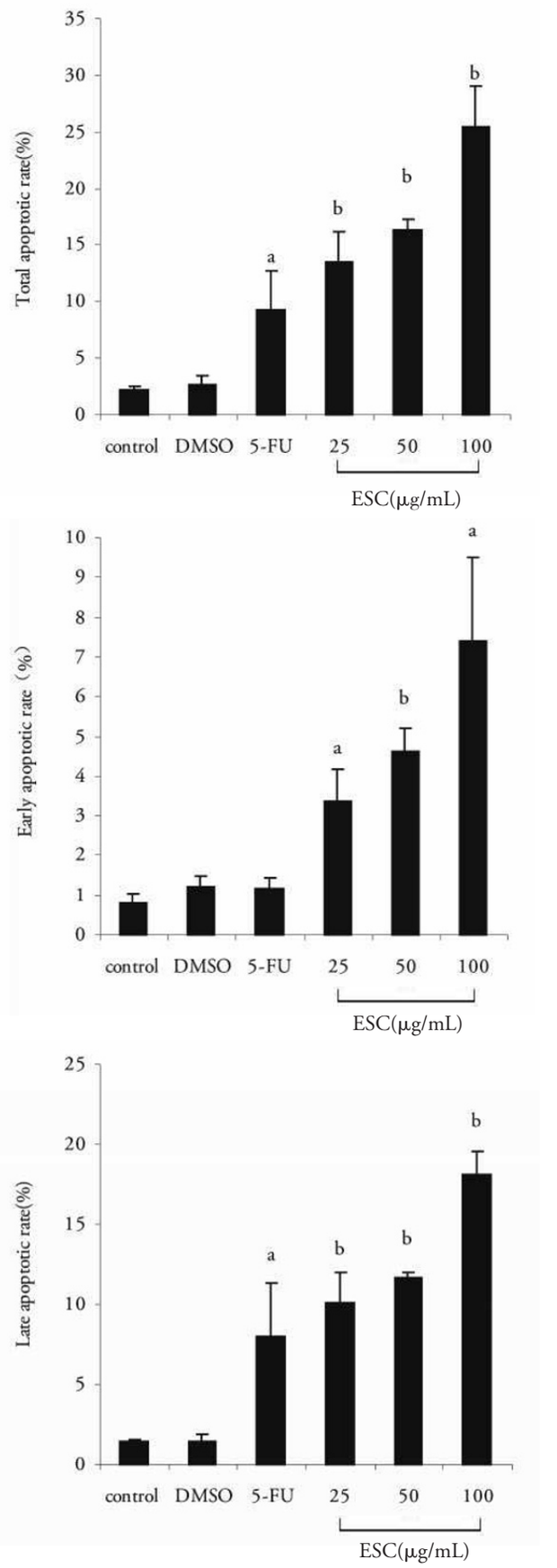
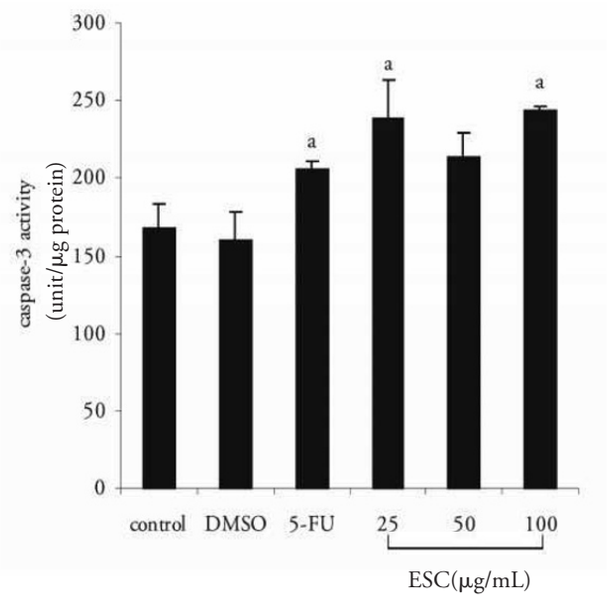
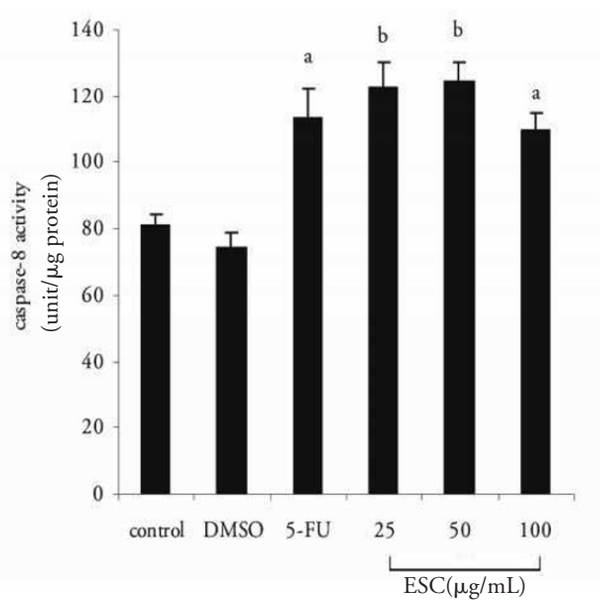


Figure 3 Annexin V-FITC staining of apoptosis induced by ESC in NCI-H157 cancer cells. [a]*P*<0.05, [b]*P*<0.01, significant versus control group.

with the DMSO group. Activity of caspase 9 in NCI-H157 cells treated by ESC did not change signifi-





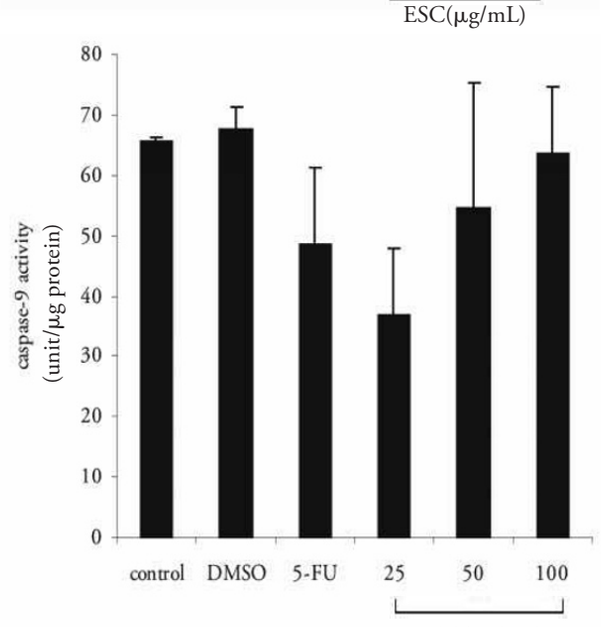
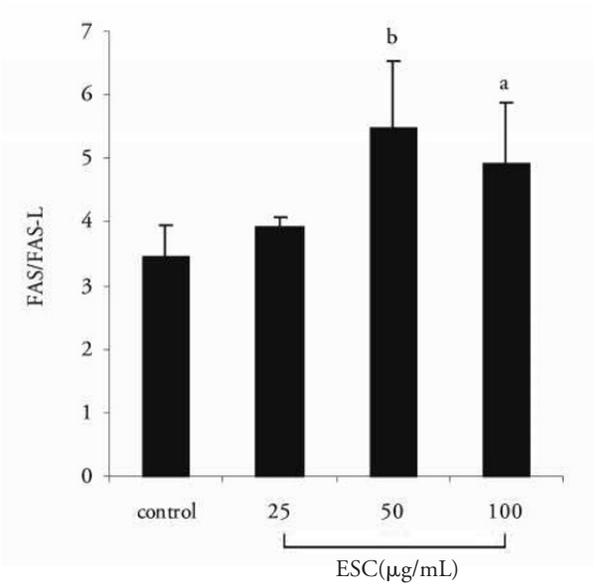
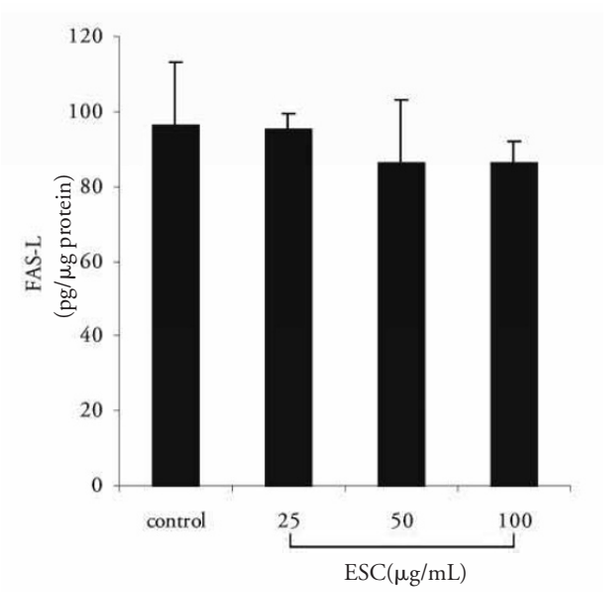


Figure 4 Changes in activity of caspase 3, 8, and 9 influenced by ESC

[a]*P*<0.05, [b]*P*<0.01, significant versus control group.





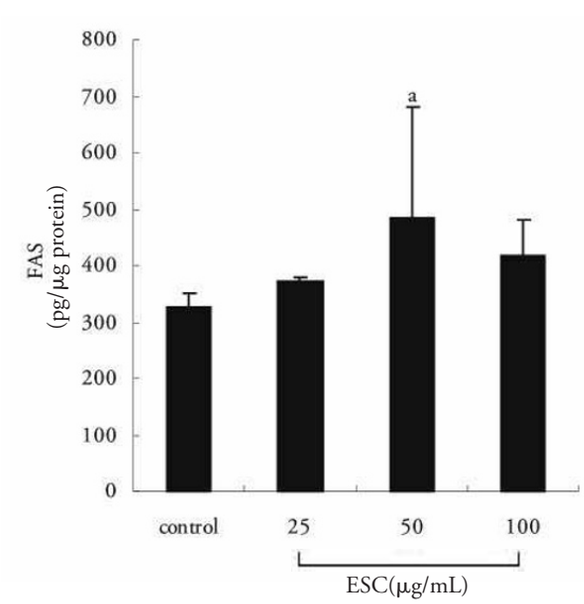


Figure 5 Influence of ESC on Fas, Fas-L expression and Fas/Fas-L ratio in NCI-H157 cancer cells

[a]*P*<0.05, [b]*P*<0.01, significant versus control group.

cantly. These results suggested that ESC induces apoptosis in NCI-H157 cancer cells via the extrinsic pathway (Figure 4).

The Fas (CD95) receptor is a cell surface protein that mediates apoptotic cell death when triggering by Fas ligand (FasL). This interaction causes Fas receptor homo-oligomerization and this leads to activation of the caspase cascade (apoptotic extrinsic pathway).[22] There have been multiple reports in the literature measuring basal levels of Fas and FasL in different neoplasms. Whereas the proapoptotic role of Fas and FasL are well known, conflicting data are available concerning the functionality of these two proteins. The increase of Fas expression in cancer cells has shown proapoptotic effects, and the increase of FasL expression in a variety of tumors has been proposed to play a role in tumor formation and metastasis.[23,24] Our results demonstrated that 50μg • mL^{-1} of ESC can upregulate Fas expression in NCI-H157 cells, but had no effect on FasL expression. Probably, apoptosis was induced by ESC in NCI-H157 cells through the Fas receptor pathway. However, this observation needs to be confirmed in further studies with a specific Fas inhibitor to determine whether this effect is indeed through the death receptor Fas pathway.

Fas/FasL ratio can be useful as a dynamic response predictor in cancer patients following chemotherapy. Clinical data has strongly indicated that an increment of FasS/FasL ratio after treatment can be an excellent marker of chemosensitivity in cancer patients. By contrast, a decreased ratio after treatment can be a predictor of chemoresistance despite an initial response.[24] Our data showed that the Fas/FasL ratio in the 50 and 100 μg • mL^{-1} groups were greatly increased. The results suggested that NCI-H157 cancer cells were sensitive to ESC treatment (Figure 5).

ESC exhibited a high inhibitory effect against NCI-H157 tumor cells in vitro. This modulatory property could be attributed to its activation of the Fas death receptor pathway and induction of apoptosis.

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