

Full Length Research Paper

# Antitumor effect of the ethanol extract of *Scutellaria baicalensis* on the mice bearing U14 cervical cancer

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*Scutellaria baicalensis* which is a traditional plant amedica in China possesses a wide anti-cancer effect. However, the inhibition effect and mechanism of *S. baicalensis* on cervical cancer is not clear up to now. In our study, two kinds of ethanol extract of *S. baicalensis* were used in U14 cervical cancer mice. The rate of tumor inhibition was detected, and the tumor cell morphology was observed by hematoxylin and eosin (H.E.) staining method. The cell cycle and apoptosis rate were examined by flow cytometry and the content of tumor necrosis factor-alpha (TNF- $\alpha$ ) in serum was determined by enzyme-linked immunosorbent assay (ELISA) kit. Furthermore, the expression of B-cell lymphoma 2 (Bcl-2) and Bax gene was detected by immunohistochemistry method. The results showed that the tumor growth could be inhibited with the highest inhibition rate of 59.86% and the apoptosis of tumor cells could be induced and cell cycles were arrested at S phase in the ethanol extract of *S. baicalensis* groups. Besides, the content of TNF- $\alpha$  in serum was significantly increased ( $P < 0.05$ ). The Bcl-2 positive cells got significance reduction and Bax positive cells were increased. So we conclude that the ethanol extract of *S. baicalensis* can inhibit the growth of tumor cells, arrest the cell cycle and induce the cells apoptosis and increase the content of tumor necrosis factor-alpha TNF- $\alpha$  in serum. The mechanism of anti-tumor activity might be associated with down regulating the level of Bcl-2 gene and up regulating the level of Bax gene.

**Key words:** *Scutellaria baicalensis*, cervical cancer, cell cycle, cell apoptosis, TNF- $\alpha$ , Bcl-2, Bax.

## INTRODUCTION

Cervical cancer is one of the most common woman cancers in the world, there were about 500,000 new cases of cervical cancer reported worldwide annually and cervical cancer has become the main reason of women death in the developing countries. People discover that the treatment of cervical cancer with plant amedica can

not only markedly lessen the clinical symptoms, but also lessen the toxicity and enhance the therapeutic effect when coordinating with radiotherapy and chemotherapy. Studies have shown that some plant amedica ingredients can inhibit the proliferation of cervical cancer cells and promote the apoptosis of cancer cells. The dry root of *Scutellaria baicalensis* Georgi has been one of the most popular plant amedica in China for a long time. It can be used as an ingredient in clinical formulation; moreover, it is usually used as the raw material in traditional Chinese plant medicines. Modern research shows that *S.*

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*baicalensis* Georgi is a multi-purpose plant amedica, and its main bioactive ingredients can inhibit the proliferation of many cancer cell lines (Min, 2009). The combination of baicalein and baicalin, which were the main flavones of *S. baicalensis* Georgi, could induce apoptosis of human breast cancer MCF-7 cells, and activate caspase-3 and -9, meanwhile, down-regulate the expression of Bcl-2 gene and up-regulate the expression of Bax gene and p53 (Zhou et al., 2009). Wogonin could inhibit the proliferation of ovarian A2780 cells and induce the cell apoptosis, and the anti-cancer mechanism might be related with inducing apoptosis (Li et al., 2003). Gao et al. (2011) observed that the absolute ethanol extract of *S. baicalensis* could induce the apoptosis of lung cancer cells and meanwhile increase the expression of p53 gene and Bax (Gao et al., 2011).

So far little about the anti-cancer effects of *S. baicalensis* Georgi on cervical cancer has been reported, in order to discover the anti-cancer mechanism of ethanol extract of *S. baicalensis* Georgi and find a new herb component to treat cervical cancer, the ethanol extract (30% ethanol extract) of *S. baicalensis* Georgi (ESBI) and the ethanol extract II (50% ethanol extract) of *S. baicalensis* Georgi (ESBII) were isolated and treated on the cervical cancer U14 mice tumor model. The effects of ESBI and II on anti-tumor, cell apoptosis and cell cycle, and tumor necrosis factor-alpha (TNF- $\alpha$ ) were determined, respectively, moreover, the expression of anti-apoptotic protein Bcl-2 and pro-apoptotic protein Bax was also detected in our study.

## MATERIALS AND METHODS

### Instruments and chemicals

The powder of *S. baicalensis* Georgi was bought from MinLe Pharmacy of Qinhuangdao in Hebei province of China (which grows in Inner Mongolia Autonomous Region in China). Cyclophosphamide (CTX) was purchased from Shanxi Powerdone Pharmaceuticals Company Limited of China, and prepared for the concentration of 25 mg/kg by physiological saline. Hematoxylin and eosin (H.E.) staining kit and cell cycle and apoptosis analysis kit were obtained from Beyotime Institute of Biotechnology of China. Mouse TNF- $\alpha$  enzyme-linked immunosorbent assay (ELISA) kit was purchased from Becton, Dickinson and Company of America. Immunohistochemical staining kit and concentrated diaminobenzidine (DAB) kit were bought from Zhongshan Goldenbridge Biotechnology Company Limited of China. Rabbit anti-Bcl-2 and rabbit anti-Bax were purchased from Beijing biosynthesis biotechnology Company Limited of China. All other chemicals used were of analytical reagent grade.

### Animals

Female Kun Ming mice of six weeks old (21 to 23 g) were provided by the Experimental Animal Center of Chinese Military Academy of Medical Sciences, the Lot Number was SCXK-(Army) 2007-004. The mice were kept in plastic cages in a clean room with the appropriate temperature (20 $\pm$ 1°C) and humidity, and provided 8 to 10 h illumination every day. They were fed with a standard pellet

diet and water ad libitum. The animals were treated according to the National Institute of Health Guide for the care and use of laboratory animals and their experimental use were approved by the animal ethics committee of the university.

### Cell lines

Mouse cervical cancer U14 cell line was obtained from the Peking Cancer Hospital Cell Bank of Chinese Academy Medical Sciences and adjusted to 5 $\times$ 10<sup>6</sup> cells/ml by saline.

### Preparation of ethanol extract of *S. baicalensis* Georgi

The powder of *S. baicalensis* Georgi was exhaustively extracted with 90% ethanol of five time volumes (g/ml) and got heat circumfluence for 2 h twice at 80°C. The concentrated liquid was collected after removing the solvent by evaporation under reduced pressure, and then got through the silica gel column chromatography. The collected liquid (50 ml) was ESBI while the eluent were 30% ethanol and then the collected liquid (50 ml) was ESBII while the eluent were 50% ethanol. ESBI and II liquid were put in drying oven to cryodry.

### Establishment of cervical cancer model in mice

The mice were fed with water but without food for 12 h before the model establishment. Under the sterile condition, 0.2 ml U14 cells (5 $\times$ 10<sup>6</sup> cells/ml) were injected into the left axilla s.c. per mouse. The rate of successful vaccination was 100%. After 24 h of vaccination, the mice were randomly divided into eight groups, and each group got 10 animals. The mice of the ESBI treated groups (1000, 500 and 250 mg/kg) and the ESBII treated groups (1000, 500 and 250 mg/kg) were given orally of 0.2 ml extract liquid every day. The group administered with 0.2 ml distilled water every day was taken as control and the group treated with cyclophosphamide (CTX, 25 mg/kg/day, i.p.) was considered as the standard reference drug. All groups were continuously treated for 14 days. The living conditions of mice were observed every day, meanwhile, the changes of tumor volume and body weight were monitored.

### Effect of ESBI and II on solid tumor growth

On day 15, all of the mice were killed, and then transplanted tumors of mice were harvested and weighted. The rate of tumor inhibition was calculated by the formula: (C-T)/C $\times$ 100%, where "T" and "C" meant average tumor weight of treated groups and control group.

### Effect of ESBI and II on tumor cell morphology

The tumor of mice in all groups were excised, and fixed in 10% neutral formalin at 4°C, embedded in paraffin, cut into 4  $\mu$ m sections for histology study. The sections were stained with H.E. staining method and histological examinations were carried out under light microscopy.

### Effect of ESBI and II on cell cycle and cells apoptosis of tumor tissues

The tumor tissues (about 1 to 2 mm<sup>3</sup>) of all groups were cut into pieces, and made into single cell suspension by filtered through 200 mesh nylon net. The cell concentration was adjusted to 1 $\times$ 10<sup>6</sup> cells/ml after being centrifugated and washed three times. The cells

**Table 1.** Effect of ESBI and II on solid tumor growth.

Group	Dose (mg/kg)	Tumor weight (g)	Inhibition rate (%)
Control	Vehicle	2.89±0.17	—
CTX	25	1.07±0.08*	62.98%
ESBI	1000	1.16±0.11*	59.86%
	500	1.54±0.14*	46.71%
	250	1.65±0.08*	42.91%
ESBII	1000	1.46±0.12*	49.48%
	500	1.64±0.13*	43.25%
	250	1.81±0.16*	37.37%

\*Represent significant differences compared with control group, \* $P < 0.05$  (means  $\pm$  S.D., n=10)

were overnight fixed with cold 70% ethanol, and then stained with propidium iodide (PI) solution consisting of RNase for 30 min in dark. The percentage of cells in each phase of the cell cycle and apoptosis were detected in an EPICS XL flow cytometry equipped with a 488 nm argon laser using ModFIT.

#### Effect of ESBI and II on TNF- $\alpha$ in serum

The blood samples of all animals were collected via eyeball before the mice were killed. The blood samples were kept at room temperature for 20 min, and then centrifugated at the speed of 2000 rpm for 20 min, finally the supernatant was collected. The content of serum TNF- $\alpha$  was determined by following the procedure of Mouse TNF- $\alpha$  ELISA Kit.

#### Effect of ESBI and II on expression of Bcl-2 and Bax protein in tumor tissues

The harvested tumors of all groups were fixed in 10% formalin, embedded in paraffin and cut into 4  $\mu$ m sections. The tumor tissue sections were stained with standard immunohistochemical streptavidin/peroxidase (SP) method and observed with light microscope. The cells with distinct brown color in cytoplasmic or membrane were considered to be positive cells. The number of positive cells and negative cells in five random selected eyesight of each section was counted. The rates of Bcl-2 and Bax positive cells were calculated by the following formula: The rate of positive cells (%) = the number of positive cells/(the number of positive cells + the number of negative cells)  $\times$  100%.

#### Statistical analysis

The statistical software SPSS13.0 was applied in the data processing. The data were shown as mean  $\pm$  standard deviation (SD). Statistical comparison among treatments was carried out using one-way analysis of variance (ANOVA). The statistical significances between control and drug treatment groups were calculated by the Student's *t*-test. Data were taken as significant where  $P < 0.05$ .

## RESULTS

### Effect of ESBI and II on solid tumor growth

On day 15, the mice were killed and the tumors of all

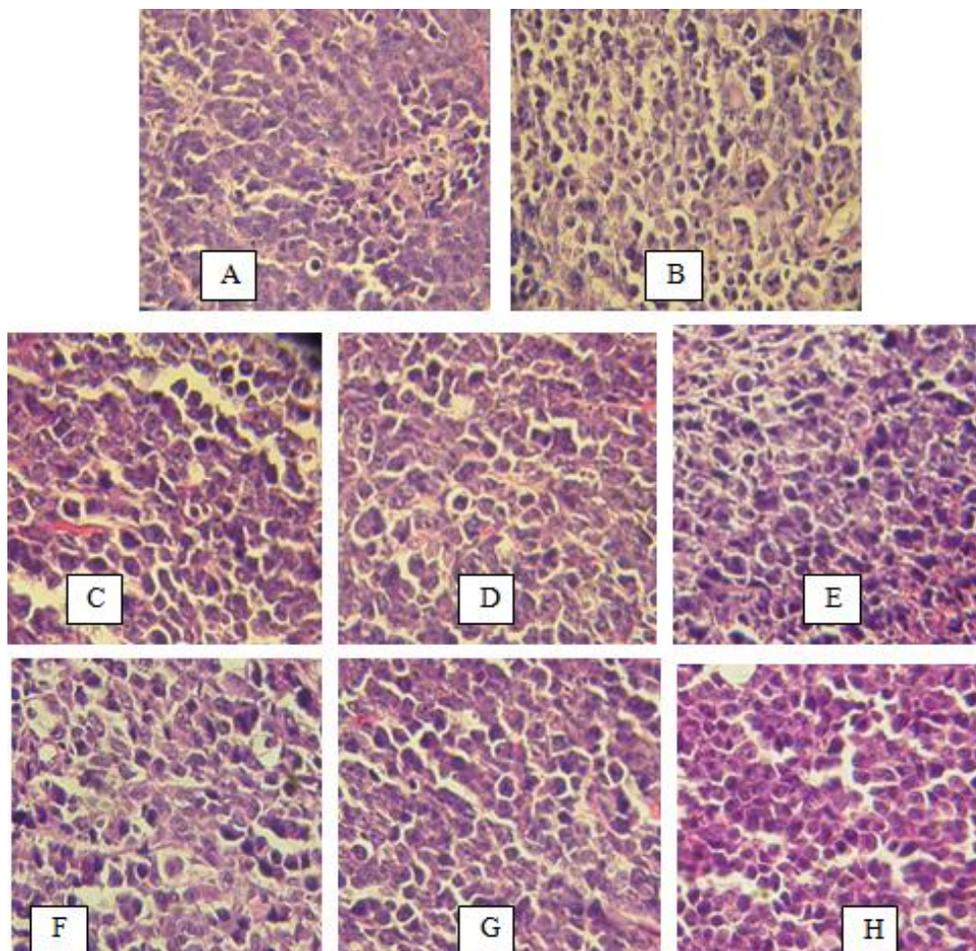
groups were harvested, and then the tumor inhibition rates were calculated. As we can see from Table 1, the tumor weights of the CTX group, and the ESBI and II groups were decreased significantly ( $P < 0.05$ ) compared with the control group. The tumor weights of the ESBI groups (1000, 500 and 250 mg/kg) were less than those of the ESBII groups. The results show that both of the tumor inhibition rates in the ESBI groups and the ESBII groups were less than that of CTX group, but bigger than 30%. There was a reduction in the tumor inhibition rate of the ESBII groups compared with the ESBI groups. However, the tumor inhibition rates of the ESBI groups and the ESBII groups became to reduce with the dose reducing from 1000 to 250 mg/kg.

### Effect of ESBI and ESBII on tumor cell morphology

The apoptotic cells in tumor tissues of drug treated groups were increased significantly compared with the control group, and the tumor cells were getting shrinkage and separated with the surrounding cells. Besides, the nuclear chromatin of the ESBI and II groups were agglomerated and showed the characteristics of apoptosis. The number of apoptotic cells in the CTX group was more than those of the rest groups, and the number of apoptotic cells in the high dose ESBI group was just less than that of the CTX group but more than those of the rest groups (Figure 1).

### Effect of ESBI and II on cell cycle and cells apoptosis of tumor tissues

The cell cycle and apoptosis of U14 tumor tissues were detected by flow cytometry. Compared with the control group, the tumor cells of the CTX, ESBI and II groups got reduced at  $G_0/G_1$  phase significantly ( $P < 0.05$ ), while increased at S phase ( $P < 0.05$ ), and the tumor cells remained almost unchanged at  $G_2/M$  phase. The results prompted that the cell cycles could be arrested at S



**Figure 1.** Tumor tissue section of H.E.staining (10×40); A, tumor tissue section of control group; B, tumor tissue section of CTX group; C, tumor tissue section of high dose ESBI group; D, tumor tissue section of middle dose ESBI group; E, tumor tissue section of low dose ESBI group; F, tumor tissue section of high dose ESBII group; G, tumor tissue section of middle dose ESBII group; H, tumor tissue section of low dose ESBII group.

phase by ESBI and II groups. What is more, the percentage of apoptosis cells could be significantly increased ( $P<0.05$ ) by the ESBI and II groups in contrast to the control group. The percentage of apoptosis cells in the ESBII groups was less than that of the ESBI groups. All of the results indicated that ESBI and II could induce the apoptosis of tumor, but the effects of inducing apoptosis in the ESBI groups were stronger than those of the ESBII groups (Table 2).

#### Effect of ESBI and II on TNF- $\alpha$ in serum

The content of TNF- $\alpha$  in serum was detected by ELISA kit. The content of TNF- $\alpha$  in the CTX group and ethanol extract of *S. baicalensis* groups got a significant addition compared with the control group ( $P<0.05$ ). In addition, the content of TNF- $\alpha$  in the ESBI groups were bigger than those of the ESBII groups between the same does. The

results suggested that the content of TNF- $\alpha$  in serum could be increased by the ethanol extract of *S. baicalensis*, and the effects of increasing the content of TNF- $\alpha$  in the ESBI groups were better than those of the ESBII groups (Figure 2).

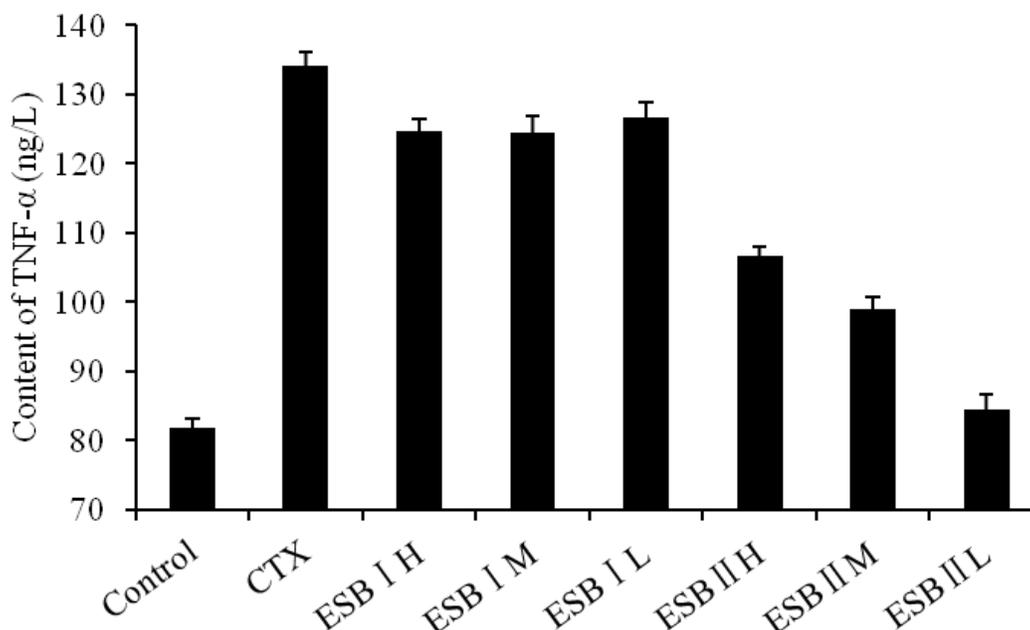
#### Effect of ESBI and II on expression of Bcl-2 and Bax protein in tumor tissues

In order to further study the mechanism of the ethanol extract of *S. baicalensis* on U14 cervical cancer mice, the expression of Bcl-2 and Bax protein in tumor tissues was detected by immunohistochemical and shown in Figures 3 and 4, the statistical results of Bcl-2 and Bax expression were shown in Table 3. There was a significant reduction on Bcl-2 positive cells in the tumor tissues of the CTX group and ethanol extract of *S. baicalensis* groups compared with the control group ( $P<0.05$ ). The

**Table 2.** Effect of ESBI and II on cell cycle and apoptosis of tumor cells.

Group	Treatment (mg/kg)	G <sub>0</sub> /G <sub>1</sub> (%)	S (%)	G <sub>2</sub> /M (%)	Percentage of apoptosis cell (%)
Control	Vehicle	63.96±3.34	28.49±2.01	7.54±1.21	10.86±0.37
CTX	25	57.82±2.62*	32.43±1.85*	9.75±0.87 <sup>#</sup>	27.52±0.32*
	1000	50.20±2.01*	42.01±1.47*	7.79±1.01 <sup>#</sup>	21.68±0.91*
ESBI	500	52.61±1.77*	39.90±1.62*	7.49±0.52 <sup>#</sup>	17.50±1.02*
	250	54.73±1.89*	37.89±2.09*	7.38±0.18 <sup>#</sup>	13.31±0.83*
ESBII	1000	51.20±1.76*	40.40±3.01*	8.40±0.54 <sup>#</sup>	15.73±1.41*
	500	57.18±2.10*	33.80±2.42*	9.02±0.65 <sup>#</sup>	13.96±1.22*
	250	62.34±2.24*	29.53±1.59*	9.13±0.48 <sup>#</sup>	12.60±1.25*

\* $P < 0.05$  and <sup>#</sup> $P > 0.05$  as compared with control group. (means ± S.D., n=10)



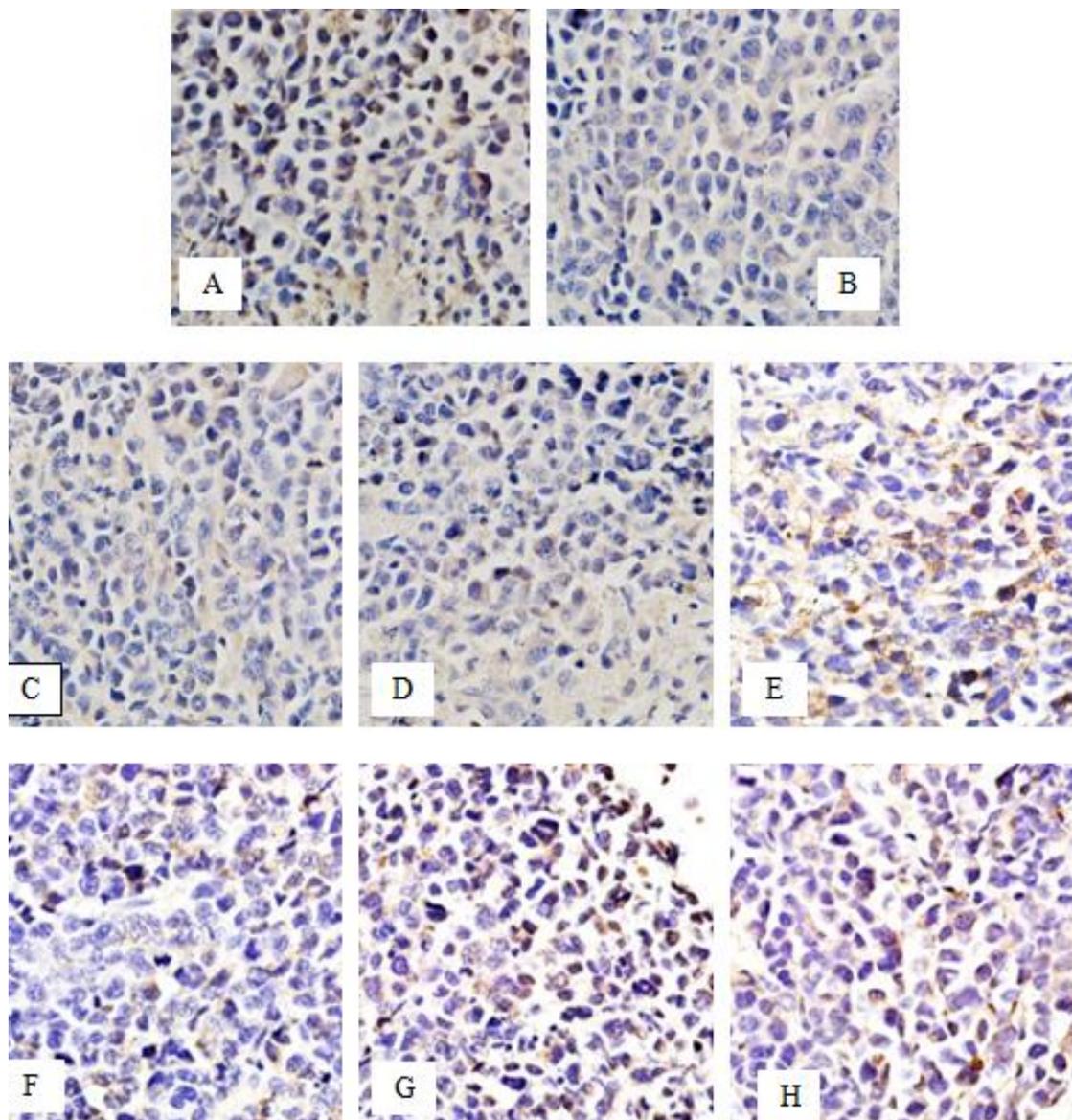
**Figure 2.** Effect of ESBI and II on TNF- $\alpha$  in serum; ESB I H, ESB I M and ESB I L: represent, respectively for the dose of 1000, 500 and 250 mg/kg of ESBI. ESB II H, ESB II M and ESB II L: represent, respectively for the dose of 1000, 500 and 250 mg/kg of ESBII.

Bax positive cells were increased significantly in the CTX group and the ESBI groups compared with the control group ( $P < 0.05$ ). However, there was no significant difference between the ESB II groups and control group ( $P > 0.05$ ). All of the ratios of Bax/Bcl-2 in the ethanol extract of *S. baicalensis* groups were bigger than that of the control group.

## DISCUSSION

Apoptosis is a kind of programmed cell death, and many kinds of mechanism such as gene regulation and signal

transduction were involved. Inducing cell apoptosis is an effective way of cancer treatment, and many kinds of plant amedica can induce the apoptosis of cancer cells. The mechanisms of plant amedica inducing apoptosis include that arresting the cell cycle and inhibiting the proliferation of cancer cells (Wang et al., 2011), regulating the expression of anti-apoptotic protein and pro-apoptotic protein (Wang et al., 2006; Hiroki et al., 2011), affecting the signal pathway of cell apoptosis (Li et al., 2008), regulating the expression and activity of telo-merase (Cheng et al., 2003), improving the body immune (Li et al., 2008) and so on. However, the composition in plant amedica is very complex, and the active

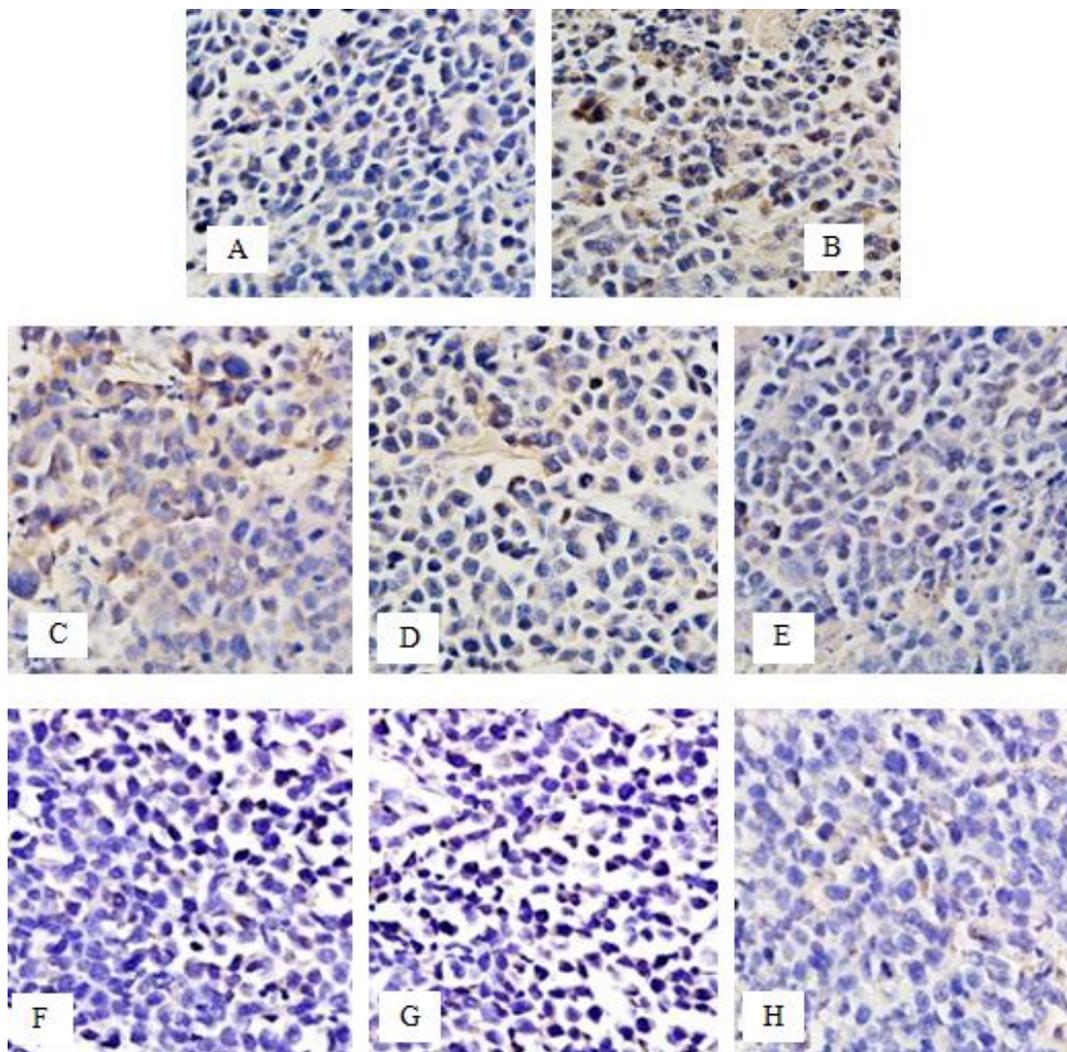


**Figure 3.** Effect of ESBI and II on expression of Bcl-2 protein in tumor tissues; A, tumor tissue section of control group; B, tumor tissue section of CTX group; C, tumor tissue section of high dose ESBI group; D, tumor tissue section of middle dose ESBI group; E, tumor tissue section of low dose ESBI group; F, tumor tissue section of high dose ESBI group; G, tumor tissue section of middle dose ESBI group; H, tumor tissue section of low dose ESBI group.

ingredients and concentration may be different due to the difference of extraction method and condition, so the effect and mechanism of plant amedica extract on cancer cells may be different. In order to discover the effect of different herb extraction on pharmacological effect, we further separated the ethanol extract of *S. baicalensis* George and the 30% ethanol and 50% ethanol were used as the eluent of silica gel column chromatography and ESBI and II were gotten respectively from the ethanol extract of *S. baicalensis*.

The definite component of ESBI and II might not be same because of the different concentration of eluent.

The mice with U14 tumor were treated respectively with ESBI and II, and the results showed that ESBI and II could inhibit the growth of U14 tumor with the highest inhibition rate of 59.86%. After detecting by H.E. dyeing and flow cytometry, both ESBI and II could induce the apoptosis of tumor, but the induction extent was different between the two kinds of ingredient, and the inhibition rates in the ESBI groups were bigger than those of the same dose of the ESBI groups. The abnormality of cell cycle regulation is one of the important reasons in tumor development, and cell apoptosis could be induced by arresting the cell cycle. According to our research,



**Figure 4.** Effect of ESBI and II on expression of Bax protein in tumor tissues; A, tumor tissue section of control group; B, tumor tissue section of CTX group; C, tumor tissue section of high dose ESBI group; D, tumor tissue section of middle dose ESBI group; E, tumor tissue section of low dose ESBI group; F, tumor tissue section of high dose ESBI group; G, tumor tissue section of middle dose ESBI group; H, tumor tissue section of low dose ESBI group.

**Table 3.** Effect of ESBI and II on expression of Bcl-2 and Bax protein.

Group	Treatment (mg/kg)	Bcl-2 positive cells (%)	Bax positive cell (%)	Bax/Bcl-2
Control	Vehicle	53.28±4.46	13.33±0.62	0.25
CTX	25	35.42±2.82*	35.12±2.93*	0.99
ESBI	1000	37.51±1.27*	30.27±1.52*	0.81
	500	39.80±2.21*	28.66±2.41*	0.72
	250	46.38±1.19*	24.73±2.03*	0.53
ESBII	1000	40.62±1.04*	16.90±2.36	0.41
	500	47.84±2.06*	14.06±1.20	0.29
	250	48.26±2.42*	15.63±1.78	0.32

\*Represent significant differences compared with control group, \* $P < 0.05$ . (means  $\pm$  S.D., n=10)

the tumor cell cycle could be arrested at S phase by ESBI and II. So we supposed that the ethanol extract of *S. baicalensis* Georgi could induce the apoptosis of tumor cells by arresting the cell cycle.

TNF- $\alpha$  is an important immune response-modifying cytokines produced primarily by the cells of monocytic lineage and can enhance the immune function, meanwhile it also can enhance the anti-tumor response and promote tumor regression (El-Obeid et al., 2006). The content of TNF- $\alpha$  in serum of the ESBI and II groups was bigger than that of the control group in our experiment; the consequence indicated that the immune function of mice could be enhanced by ESBI and II.

So far the mechanisms of apoptosis are becoming clear and the key protein and apoptosis-related genes in the apoptosis pathway have become a molecular target of anti-cancer drug. In cancer therapy, increasing the activity of pro-apoptotic genes selectively and/or inhibiting the expression of anti-apoptotic genes can not only enhance the efficacy of chemotherapy of radiotherapy, but also reduce the toxic side-effects of drug (Zhou and Huang, 2004). Therefore, inducing the apoptosis of tumor and reducing the apoptosis of healthy tissue cells have become a hot topic in cancer treatment. Pro-apoptotic gene Bax and anti-apoptotic gene Bcl-2 play an important role in regulating the apoptosis of tumor as the most representative members of Bcl-2 family. The expression of Bcl-2 and Bax in tumor cells was examined by immunohistochemistry in our research. The results showed that the Bcl-2 positive cells in tumor tissues of the ESBI and II groups were decreased significantly and the Bax positive cells in tumor tissues of the ESBI groups showed a significant increase, meanwhile the ratios of Bax/Bcl-2 in the ESBI and II groups were bigger than that of the control group. Our study indicated that the two ingredients ethanol extract from *S. baicalensis* could induce the apoptosis of tumor by down regulating the level of Bcl-2 gene and up regulating the level of Bax gene in tumor tissues.

In summary, ESBI and II which were extracted from *S. baicalensis* could inhibit the growth of U14 cervical cancer cells of mice and induce the cells apoptosis by arresting cell cycle at S phase and regulating the expression of Bcl-2 and Bax gene. Besides, the content of TNF- $\alpha$  in serum could be increased by ESBI and II, thus the immune function of mice was also strengthened. However, the chemical composition and the concentration of ingredients in ESBI and II may be different due to the difference of extraction method, so the effect of ESBI and II on cervical cancer was significantly different, the effect of ESBI on inducing cells apoptosis and enhancing the immune function was better than that of ESBI. The anti-cancer mechanism of the ethanol extract from *S. baicalensis* and the difference reasons of the anti-tumor effects of ESBI and II remain to be further studied.

## Abbreviations:

**ESBI**, ethanol extract I of *Scutellaria baicalensis* Georgi; **ESBII**, ethanol extract II of *Scutellaria baicalensis* Georgi; **CTX**, cyclophosphamide; **H.E**, hematoxylin and eosin; **TNF- $\alpha$** , tumor necrosis factor alpha; **ELISA**, enzyme-linked immunosorbent assay; **PI**, propidium iodide; **SP**, streptavidin/peroxidase.

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