

Full Length Research Paper

Antitumor activity of *Scutellaria baicalensis* Georgi total flavonoids on mice bearing U14 cervical carcinoma

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Some studies have shown that *Scutellaria baicalensis* Georgi total flavonoids (STF) could inhibit the growth of carcinoma cells. However, the effect and mechanism of STF on cervical cancer *in vivo* was not studied in detail presently; so, our study was to investigate the effect and mechanism of STF on cervical cancer. The mice models bearing U14 cervical cancer were established in our study and STF were orally administered to mice at a dose of 1000, 500 and 250 mg/kg body weight, respectively. The rate of tumor inhibition was studied, and the cell morphology of tumor, liver and kidney were observed after HE staining. The cell cycle and the percentage of apoptotic cells were detected by flow cytometer, and the content of serum proliferating cell nuclear antigen (PCNA) and the expression of Bcl-2 and Bax gene were assayed by enzyme linked immunosorbent assay (ELISA) and standard immunohistochemical SP method, respectively. These results showed that the highest tumor inhibition rate was 55.71%, the cell cycle of tumor could be arrested in G₀/G₁ phase, and the content of serum PCNA reduced ($P < 0.01$), the apoptosis of tumor could be induced by STF treatment with the percentage of $19.15 \pm 0.98\%$. Moreover, compared with the negative control group, the expression of Bcl-2 was reduced and the expression of Bax was increased after STF treatment ($P < 0.05$). So, we conclude that the growth of U14 cervical cancer could be inhibited by STF, the cell proliferation inhibited by arresting cell cycle, and the cell apoptosis induced by regulating the expression of Bax and Bcl-2 gene by the treatment of STF.

Key words: *Scutellaria baicalensis* Georgi total flavonoids, U14 cervical cancer, cell cycle, cell apoptosis, Bax, Bcl-2, PCNA.

INTRODUCTION

Scutellaria baicalensis Georgi total flavonoids (STF) are the main active ingredients of *S. baicalensis* Georgi. About 40 flavonoids have been separated from *S. baicalensis* Georgi presently, such as baicalin (Xu et al.,

2011), baicalein (Lin et al., 2007), wogonin (Lee et al., 2007) and wogonoside. STF that were used for some studies were extracted from the stem and leaf of *S. baicalensis* Georgi in some reports, while STF studied in our experiments were from the roots of *S. baicalensis* Georgi.

It has been reported that STF had a variety of effects, including the anti-inflammatory (Liu et al., 2002), anti-pyretic, analgesic, anti-microbial, anti-tumor, anti-oxidant, scavenging oxygen free radicals effects, regulating immune function, protecting liver, neuron (Gasiowski et al., 2011), cardiovascular, cerebrovascular and so on.

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Abbreviations: STF, *Scutellaria baicalensis* Georgi total flavonoids; PCNA, proliferating cell nuclear antigen; ELISA, enzyme linked immunosorbent assay.

Now STF have been used in studying its effects on some kinds of cancer cell lines (Sheng et al., 2008), such as bladder cancer (Ikemoto et al., 2000), liver cancer (Chang et al., 2002), prostate cancer (Miocinovic et al., 2005), lung cancer (Lee et al., 2005) and breast cancer (Wang et al., 2010). These reports demonstrated that STF could inhibit cell proliferation and induce apoptosis of cancer in a variety of ways, moreover, STF have the strong inhibition effect on human cervical cancer Hela cell line.

Wang et al. (2005) indicated that *S. baicalensis* stem-leaf total flavonoid could inhibit the growth of Hela cell line *in vitro*, and the possible mechanism was that it could inhibit DNA mutation induced by free radicals. Furthermore, Li et al. (2009) indicated that oroxylin A (a flavonoid extracted from the roots of *S. baicalensis* Georgi) could inhibit the growth of human Hela cells *in vitro* and *in vivo* strongly, and the apoptosis induction might be the potential mechanisms. However, the effects and mechanisms of STF on U14 cervical cancer *in vivo* have not been distinct up to now. So the anti-cancer activities and mechanisms of STF *in vivo* as plant amedica were studied in this research.

After STF were extracted and purified from the dry roots of *S. baicalensis*, the mice models bearing U14 cervical cancer were established. Then STF of high, medium and low dose were orally given to the mice for the observation of the influence on the growth of mice bearing U14 cervical cancer. The purpose of our study was to investigate the anti-cancer activity and the possible mechanism of STF *in vivo* on mice bearing U14 cervical cancer.

MATERIALS AND METHODS

Chemicals and reagents

S. baicalensis Georgi was purchased from Minle Drugstore of Qinhuangdao in China, which grew in Inner Mongolia Autonomous Region in China. AB-8 macropore absorbed resin was obtained from Beijing Huayangtiany Science and Technique Co., Ltd in China. Cyclophosphamide (CTX) was obtained from Shanxi Powerdone Pharmaceuticals Co., LTD. in China (the Lot number was 1008421), and diluted to a concentration of 25 mg/kg by physiological saline before using. Hematoxylin and Eosin (HE) staining kit was obtained from Beyotime Institute of Biotechnology in China. Annexin V-FITC apoptosis detection kit was obtained from Nanjing KeyGen Biotech. Co., LTD. in China. Cell cycle and apoptosis analysis kit was purchased from Beyotime Institute of Biotechnology in China. Mouse PCNA ELISA kit was bought from Research and Diagnostic Systems, Inc., America. SP kit (Rabbit), DAB, rabbit anti-Bcl-2 and rabbit anti-Bax were purchased from Beijing Biosynthesis Biotechnology Co. Ltd in China. All other chemicals used were of analytical reagent grade.

Cell lines

Mouse U14 cervical cancer cell line was purchased from Peking Cancer Hospital Cell Bank of Chinese Academy Medical Sciences, and passaged in Kun Ming mice abdomen.

Animals

Female Kun Ming mice of SPF (Specific pathogen free, 6 weeks old, 18 to 22 g) were provided by the Experimental Animal Center of Chinese Military Academy of Medical Sciences, the Lot Number was SCXK-(Army) 2007-004. Mice were kept in a clean room with appropriate temperature ($20 \pm 1^\circ\text{C}$) 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 was approved by the animal Ethics Committee of the University.

Extraction and purification of STF

500 g, *S. baicalensis* Georgi was extracted with 50% ethanol twice by heat circumfluence (80°C) for 2 h. The filtrate was collected and evaporated. STF could dissolve in alkaline solution, while precipitate could dissolve in acidic solution, so the pH of the concentrated solution was adjusted by adding 2 mol/L HCl and 20% NaOH repeatedly to separate STF (Du et al., 2008). The collected mixture was purified further with AB-8 macropore absorbed resin column, and the eluent was collected, dried and weighed after eluting it with 70% ethanol (Xu et al., 2006).

Establishment of the mice models bearing U14 cervical carcinoma

The mice were starved for 12 h before the establishment of tumor model and U14 cervical cancer cells (5×10^6 cells/ml) were passaged in abdomen. On the 7th day, ascites was extracted, and the cell concentration was adjusted to 5×10^6 cells/ml. 0.2 ml cancer cell suspension was injected into the left axilla s.c. of each mouse. The successful rate of vaccination was 100%.

Effect of STF on U14 solid tumor growth in mice

Mice were randomly divided into 5 groups (10 mice /group). The 5 groups were designated as tumor control group, CTX control group, STF high, medium and low dose groups. After 24 h of tumor inoculation, STF were given orally to the mice of STF high, medium and low dose (1000, 500 and 250 mg/kg body weight, respectively) groups according to pre-test and the dose of active screening. The group administered with vehicle alone (distilled water, 0.2 ml/day, p.o.) was taken as control and the group treated with CTX (25 mg/kg, 0.1 ml/day, i.p.) was considered as the standard reference drug. All the groups were treated for 14 days continuously. The animal living conditions were observed every day, and the changes of tumor volume and body weight were monitored. On the 15th day, all animals were executed and the transplanted tumors were harvested and weighed. The rate of tumor inhibition was calculated by the formula:

$$\frac{C - T}{C} \times 100\%$$

Where, "T" and "C" is the average tumor weight of treated groups and control group.

Effect of STF on the morphology of tumor, liver and kidney cell

The tumor, liver and kidney of executed mice were excised and

Table 1. Effect of STF on U14 solid tumor growth in mice.

Group	Treatment (mg/kg)	Body weight (g)		Tumor weight (g)	Inhibition rate (%)
		Beginning	End		
Control	Vehicle	23.79 ± 0.93	22.90 ± 1.47	2.89 ± 0.19	—
CTX	25	23.89 ± 1.02	23.98 ± 1.60	1.07 ± 0.08*	62.98
High dose of STF	1000	23.48 ± 0.76	24.70 ± 1.04	1.28 ± 0.11*	55.71
Medium dose of STF	500	23.27 ± 0.80	25.18 ± 1.79	1.67 ± 0.10*	42.21
Low dose of STF	250	23.35 ± 0.85	25.52 ± 1.32	1.90 ± 0.15*	34.26

n = 10, (mean ± S.D., g), *P < 0.05 as compared with the control group.

fixed in 10% neutral formalin at 4°C, embedded in paraffin, and cut into 4 µm sections with RM2235 Rotary Microtome (Leica Company, Germany) for histological study. The sections were stained with HE staining kit and then examined for cell morphology with light microscope. Ten fields were selected randomly to count the number of tumor cells for each section under 400 × microscope, and the mean number calculated represented the number of tumor cells of this section.

Effect of STF on cell cycle and apoptosis of tumor cells

The tumor tissues of every group were made into single cell suspension by filtering through 300 mesh nylon net. The cell concentration was adjusted to 1×10^6 cells/ml after being centrifuged and washed 3 times. The cells were fixed with cold 70% ethanol overnight, finally stained with PI solution containing RNase for 30 min. Cell Cycle and Apoptosis Analysis kit was purchased from Beyotime Institute of Biotechnology (China). The percentage of cells in each phase of cell cycle and apoptotic cells was detected by FACSCalibur Flow Cytometry (Becton Dickinson Company, America) with ModFIT, and the excitation wavelength was 488 nm.

Effect of STF on the proliferating cell nuclear antigen (PCNA)

The concentration of serum PCNA was measured by mouse PCNA ELISA kit. On the 15th day after model establishment, the blood was collected by excising the mice eyeballs. After coagulating, the blood was centrifugated at 2000 r/min for 20 min to separate serum. ELISA means was performed to determine the concentration of serum PCNA. OD values were read with MULTISKAN MK3 Microplate Reader (Thermo Company, America) at 450 nm after adding the stop solution.

Effect of STF on the expression of Bcl-2 and Bax gene in tumor tissue

Tumor tissue sections were stained with the standard immunohistochemical SP (streptavidin peroxidase conjunction) method and stained with DAB, and then the sections were mounted with neutral gum and observed with light microscope. The cells whose membrane or cytoplasm turned distinct brown were regarded as positive cells and those whose nucleus turned blue were regarded as negative cells. The numbers of positive cells were counted with a hemacytometer under 400 × microscope and the mean number was calculated.

Statistical analysis

The statistical analysis was performed by one-way analysis of

variance, and the differences between means were tested with Duncan's multiple range tests. P-values of less than 0.05 were considered significant in statistics. Data were expressed as mean ± S.D. The statistical software SPSS13.0 was used for the data processing.

RESULTS

Extraction and purification of STF

The 11.38 g of STF was extracted from 500 g *S. baicalensis* Georgi, and the extraction percentage of STF was 2.28%.

Effect of STF on U14 solid tumor growth in mice

Compared with the control group, the tumor weight of the CTX group and the STF groups reduced significantly (P < 0.05). The tumor inhibition rates of the STF groups were lower than that of the CTX group but higher than 30%. STF inhibited the growth of solid tumor in a dose-dependent manner (P < 0.05), and the anti-tumor activity of the high dose STF group was the strongest among them (Table 1). The mice in the control group were all in bad condition, and body weight free tumor of them decreased. The body weight of the CTX group did not change basically, but body weight of the STF groups increased to some extent. While the mice of the STF groups were all in good spirits, the color and smoothness of mice fur were better than those of the control group.

Effect of STF on the morphology tumor, liver and kidney cell

The forms of tumor cells in all groups were observed after HE staining. Compared with the control group, tumor cells in the CTX group and STF groups appeared nucleus shrunk, cells dwindled and turned round as well as detached from surrounding cells evidently (Figure 1).

The color and texture of liver and kidney were observed with the eyes, and then tissue examinations of liver and kidney were conducted by means of HE staining. Compared with the control group, there were no

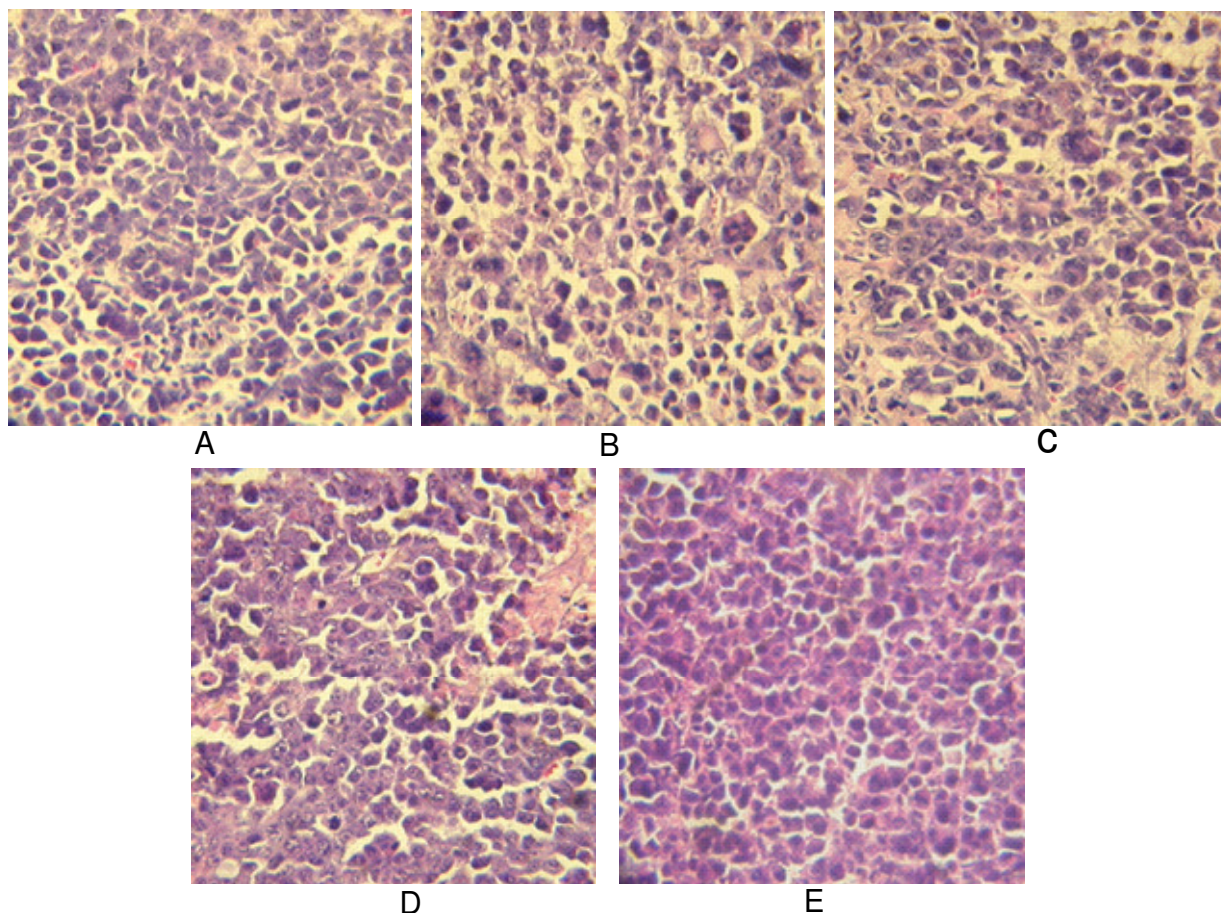


Figure 1. Tumor tissue sections of H E staining (10 × 40). (A): Control group, (B): CTX group, (C): High dose STF group, (D): Medium dose STF group, (E): Low dose STF group.

obviously pathological changes in liver and kidney tissues of the STF groups; the color, gloss and texture were normal. The tissue sections of liver and kidney showed that central vein and hepatic lobule were clear, glomerular and renal tubular were also evident (Figure 2).

Effect of STF on cell cycle and apoptosis in tumor tissue

In contrast to the control group, the percentage of apoptotic cells in the STF groups increased ($P < 0.05$, $P < 0.01$). With the increasing of STF concentration, the numbers of cells in G_0/G_1 phase increased, while cells in S and G_2/M phases decreased (Figure 3).

The percentage of apoptotic cells in the control group was (5.22 ± 0.36)%, and the percentage of apoptotic cells in the CTX group and high, medium and low dose STF groups were increased to (23.01 ± 1.15), (19.15 ± 0.98), (16.19 ± 0.96) and (10.24 ± 0.81)%, respectively. However, there was a significant reduction in the percentage of apoptotic cells of the STF groups compared to the CTX

group ($P < 0.05$) (Figure 4).

Effect of STF on the concentration of serum PCNA

Compared with the control group (141.23 ± 6.46) ng/L, the concentrations of serum PCNA decreased by the administration of the STF groups to (102.29 ± 4.73), (116.83 ± 4.52) and (131.74 ± 6.16) ng/L, respectively ($P < 0.01$), and the concentration of PCNA in the CTX group reduced to (129.07 ± 5.91) ng/L ($P < 0.05$) (Figure 5).

Effect of STF on the expression of Bcl-2 and Bax gene in tumor tissue

In contrast to the control group, both CTX and STF could reduce the expression of Bcl-2 gene and increase the expression of Bax gene in a dose dependent manner ($P < 0.05$), and the proportion of Bax/Bcl-2 in the CTX group and STF groups increased ($P < 0.05$) (Figures 6 and 7, Table 2).

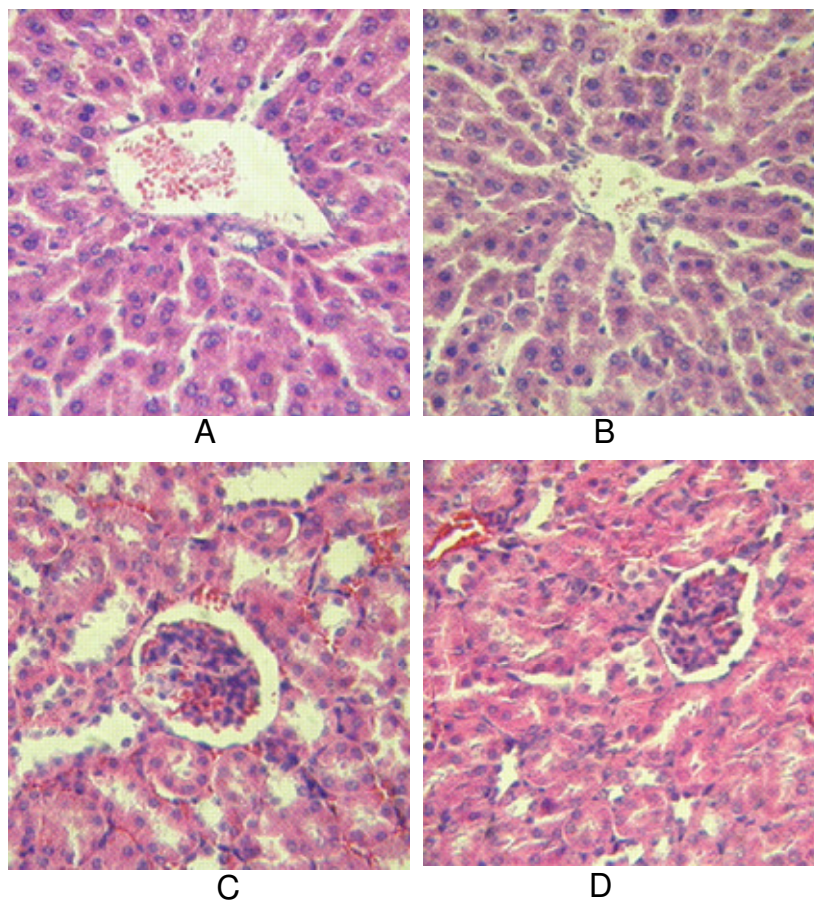


Figure 2. Liver and kidney tissues sections of H E staining (10 × 40).(A): Liver tissue of control group,(B): Liver tissue of high dose STF group,(C): Kidney tissue of control group,(D): Kidney tissue of high dose STF group.

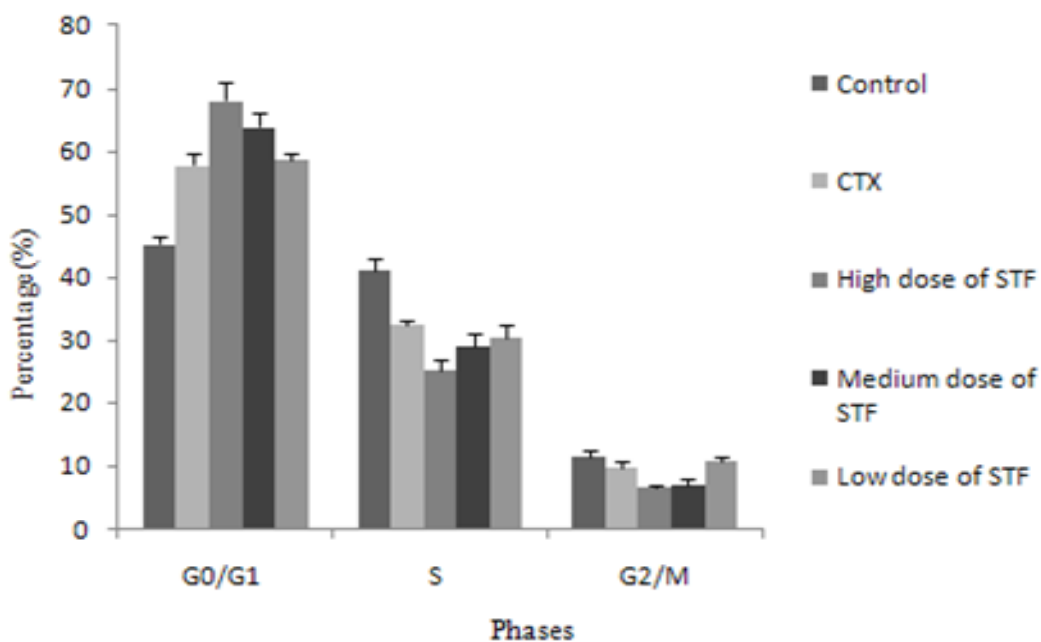


Figure 3. Effect of STF on cell cycle in tumor tissue.

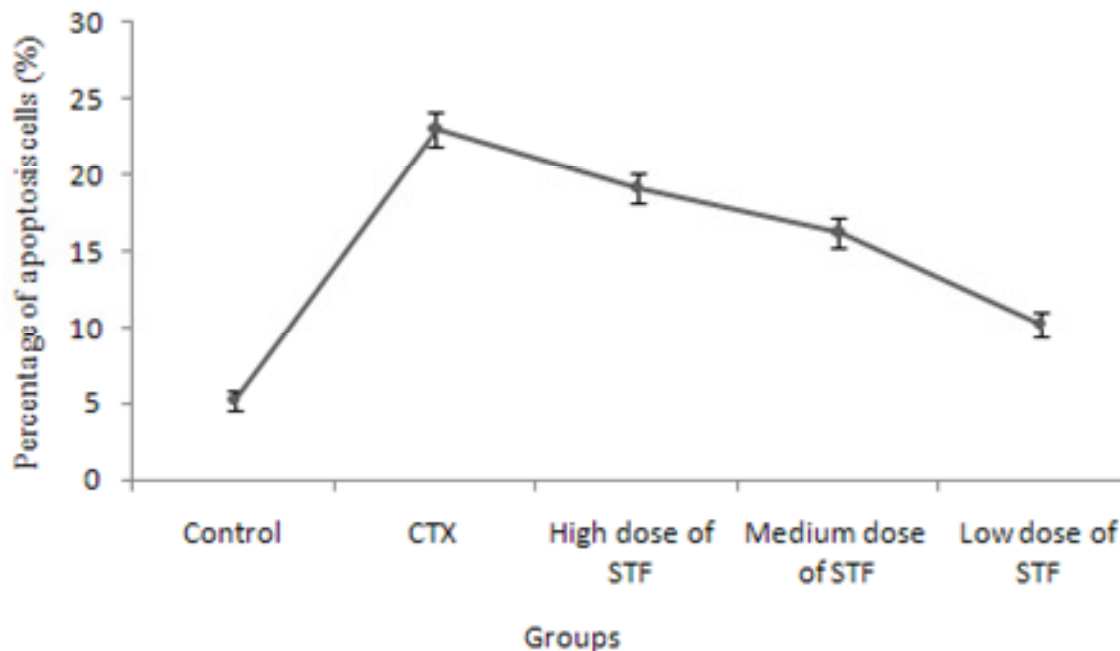


Figure 4. Effect of STF on cell apoptosis in tumor tissue.

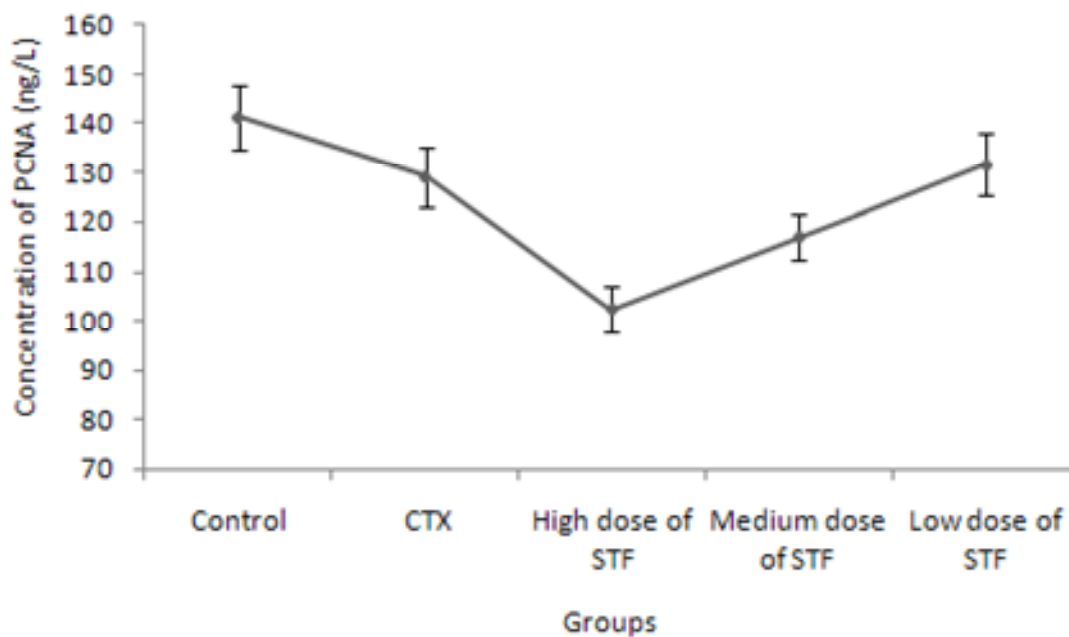


Figure 5. Effect of STF on the concentration of serum PCNA.

DISCUSSION

Some researches show that flavonoids have an important effect on cancer chemoprevention and chemotherapy. The anti-tumor mechanisms include carcinogen inactivation, cell cycle arresting, anti-proliferation, apoptosis induction and differentiation, angiogenesis inhibition, and anti-oxidation or a combination of these mechanisms

(Ren et al., 2003).

STF are the main active ingredients extracted from *S. baicalensis* Georgi, which are widely used in anti-tumor studies. When the tumor inhibition rate is higher (30%) after treatment of medicine, it is commonly deemed that this medicine has the effect of anti-tumor. Our study shows that the tumor inhibition rate in the STF groups were higher than 30%, and indicated that STF could

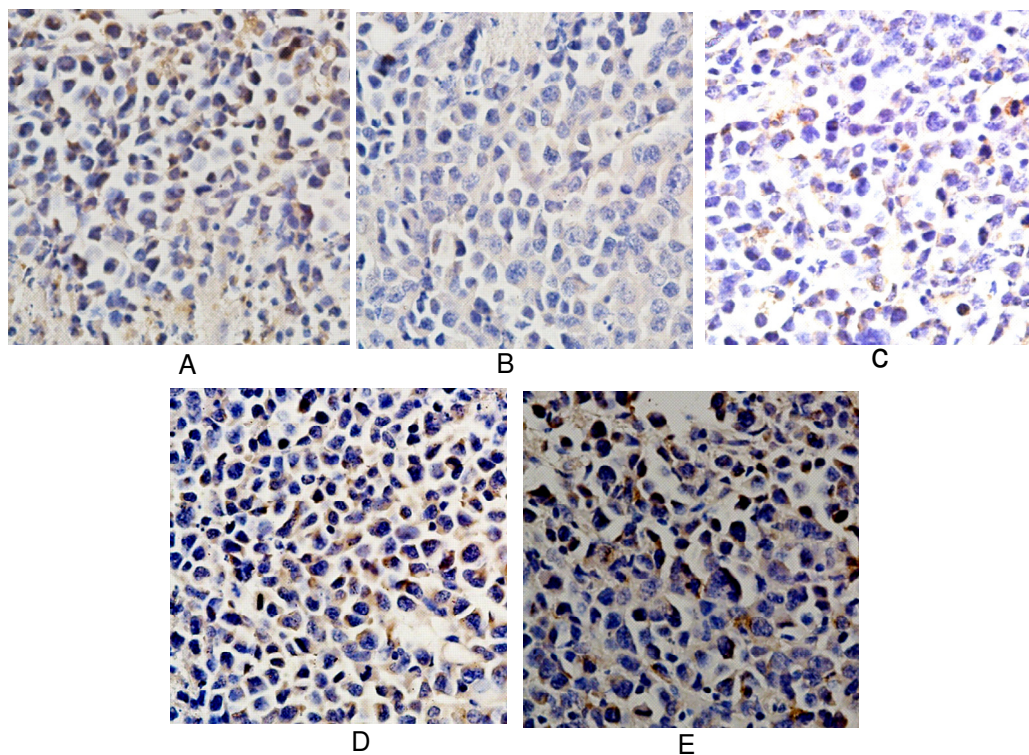


Figure 6. Effect of STF on the expression of Bcl-2 gene (10 × 40). (A): Control group; (B): CTX group; (C): High dose STF group; (D): Medium dose STF group; (E): Low dose STF group.

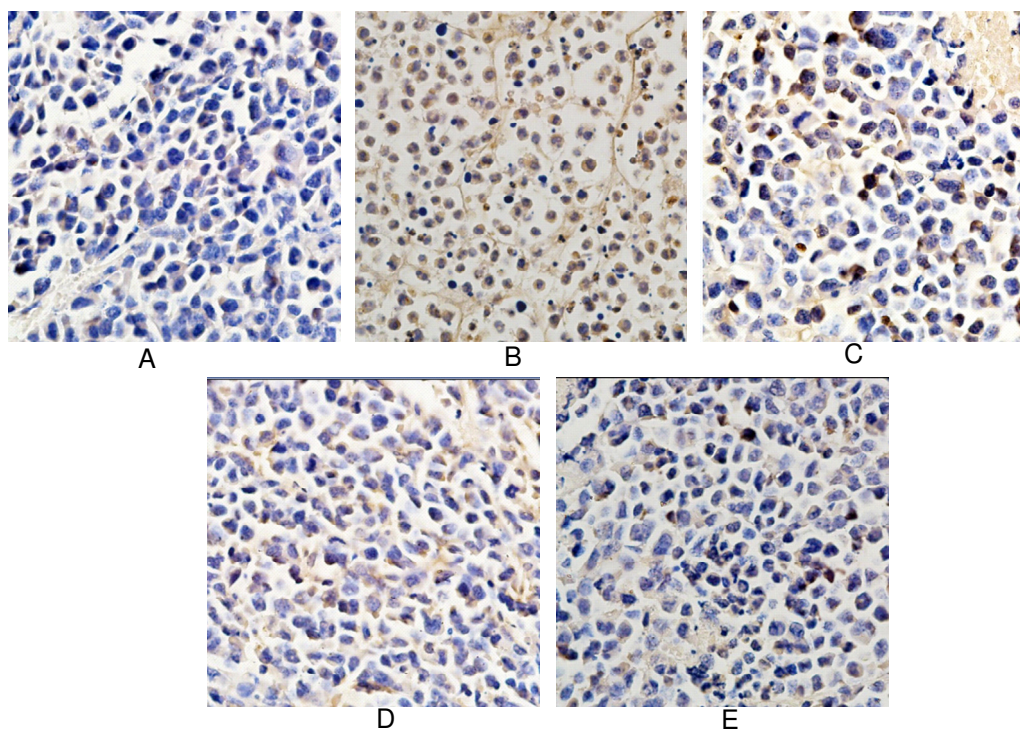


Figure 7. Effect of STF on the expression of Bax gene (10 × 40). (A): Control group; (B): CTX group; (C): High dose STF group; (D): Medium dose STF group; (E): Low dose STF group.

Table 2. Effect of STF on the expression of Bcl-2 and Bax gene.

Group	Treatment (mg/kg)	The percentage of Bcl-2 positive cells (%)	The percentage of Bax positive cells (%)	Bax/Bcl-2 proportion
Control	Vehicle	53.28 ± 3.06	13.33 ± 0.88	0.25
CTX	25	35.42 ± 2.02*	35.12 ± 2.33*	0.99
High dose of STF	1000	39.31 ± 2.38*	32.04 ± 1.87*	0.82
Medium dose of STF	500	40.97 ± 2.61*	29.41 ± 1.94*	0.72
Low dose of STF	250	43.83 ± 2.98*	25.41 ± 1.27*	0.58

n = 10; (mean ± S.D., %), *P < 0.05 as compared with control group.

inhibit the growth of U14 cervical cancer cells in mice in a dose-dependent manner, yet it was lower than the treatment of CTX.

The tissue sections showed the cells of the control group had larger nucleus and less cytoplasm, moreover, cancer cells involved circumambient tissues widely. The number of tumor cells of the STF groups was clearly reduced in contrast to the control group. The shrinkage of cell nucleus, the formation of apoptotic bodies, and the production of massive necrotic areas were found. The tissue sections also indicated that liver and kidney were not affected by the treatment of STF and there were no toxic effects on liver and kidney.

Cell cycle and apoptosis were analyzed by flow cytometry. Apoptotic peak appeared before G₀/G₁ phase. Compared with the control group, the percentage of apoptosis cells in the CTX group and STF groups overtly increased. Eukaryotic cell cycle includes G₁, S, G₂ and M phases, and only G₁ phase can accept the external information stimulation, thus the restriction point of G₁ phase is a critical point of transition from G₁ to S phase. So this point transition can determine whether or not cells are proliferated, arrested and dead. Compared with the control group, the percentages of cells in G₀/G₁ phase of the STF groups were increased and the cells in S and G₂ / M phase decreased so that the tumor cells accumulated in G₀/G₁ phase. DNA replication was stopped by preventing cell transition from G₁ to S phase. The conclusion might be that STF could forbid cell mitosis and proliferation, and induce apoptosis. Zhang et al. (2003) investigated the anti-tumor activities of the extract from *S. baicalensis* on the head and neck squamous cell carcinoma (HNSCC) SCC-25 and KB cells, they also found that tumor cells were arrested in G₁ phase. These results were similar to ours.

In contrast to the control group, the contents of PCNA in the STF groups decreased indicating that STF could restrain cell proliferation. This result was consistent with those of the tumor inhibition rate, cell cycle and apoptosis in our study. This study shows that STF could arrest cell cycle, inhibit cell proliferation and trigger apoptosis. PCNA is the adjunctive protein of DNA polymerase δ , which is closely related with DNA synthesis and also plays an important role on priming cell proliferation, so PCNA is a valid index of reflecting cell proliferation. The

content of PCNA was very low in G₀ phase, increases in late G₁ phase, reaches peak in S phase, decreased markedly in G₂/M phase. The changes of PCNA content were consistent with that of DNA replication.

The decrease of Bcl-2 gene and increase of Bax gene in the STF groups in a dose-dependent manner by the standard immunohistochemical SP method were examined. Bcl-2 is the main gene of inhibiting apoptosis and increasing cell lifetime, and a complex network is build by Bcl-2 with its family members together to regulate cell apoptosis. This report (Reed et al., 1996) shows that Bcl-2 is a major gene for inhibiting apoptosis, while Bax promotes cell apoptosis, so Bcl-2 and Bax are two opposite function genes. Bax is a gene that promote apoptosis directly and antagonize the effect of Bcl-2 gene. Bax/Bcl-2 proportion of the STF groups also increased in a dose-dependent manner. Bax/Bcl-2 ratio is regarded as the key factor that affect apoptosis, the heterodimers of Bcl-2 and Bax gene are increased when Bcl-2 gene increases, and the trends to apoptosis are getting weaken. But when Bax gene increases, the homodimer formatted by Bax gene will take charge, and the cells will go to apoptosis easily. So Bax/Bcl-2 proportion is important in determining whether cells are going into the state of apoptosis (Miao et al., 2005). Therefore, the anti-tumor mechanism of STF might be that STF acted on the mitochondrial pathway by down-regulating the expression of Bcl-2 gene and up-regulating the expression of Bax gene to induce apoptosis.

Conclusion

In conclusion, our results showed that STF could inhibit the growth of U14 cervical cancer *in vivo*, and the anti-tumor effects were in a dose-dependent manner. The authors thought that the anti-tumor mechanisms of STF might be that it could arrest cell cycle in G₀/G₁ phase, inhibit cell proliferation by decreasing the contents of PCNA, and induce apoptosis by acting on the mitochondrial pathway by way of regulating the expression of Bcl-2 and Bax gene. However, anti-tumor effects of plant amedica have many characteristics, such as multi-target, multi-link and multi-effect. Thus, further studies will be required to investigate the detailed mechanisms of STF

on cervical cancer.

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