

# Apoptosis and antitumor effects between $\beta$ -elemene and astragaloside and drug mechanism analysis

Shi Liping<sup>1,2</sup>, Chen Tingting<sup>1</sup>, Yuan Zheng<sup>1,3</sup>, Xie Tian<sup>4\*</sup> and Cheng Haibo<sup>1</sup>

<sup>1</sup>First Clinical Medical Institute, Nanjing University of Chinese Medicine, Nanjing, Jiangsu, China

<sup>2</sup>Nanjing Yuhua Hospital, Nanjing, Jiangsu, China

<sup>3</sup>Jiangsu Provincial Hospital of Traditional Chinese Medicine, Nanjing, Jiangsu, China

<sup>4</sup>Hangzhou Normal University, Hangzhou, Zhejiang, China

**Abstract:**  $\beta$ -elemene is an effective anticancer drug extracted from *Rhizoma curcumae*. It is a non cytotoxic antineoplastic agent, which can obviously inhibit the proliferation of tumor cells. In this paper, we observed the proliferation inhibition and apoptosis of  $\beta$ -elemene and Astragaloside on human hepatoma cell HepG2 and mouse hepatoma H22 cells, and provide a reference for further proof that  $\beta$ -elemene and astragaloside can induce tumor cell apoptosis. The results showed that after 24 h, group astragaloside,  $\beta$ -elemene group and combined treatment group had inhibitory effect on the proliferation of HePG2 cells, in which the combined treatment group had the best effect and the inhibition rate reached 66.71%. The apoptosis rates of Hep G2 cells in the drug treatment group were 0.9%, 22.4% and 45.8%, respectively, and there was statistical significance in each drug group compared with the control group ( $P < 0.05$ ). It can be seen that *Astragalus membranaceus* and  $\beta$ -elemene have obvious inhibitory effects on the growth of liver cancer cells and their combination has synergistic effect.

**Keywords:**  $\beta$ -elemene, drug mechanism, apoptosis, astragaloside, protein expression.

## INTRODUCTION

Liver cancer is the fourth most lethal malignant tumor in the world, and tens of millions of people are diagnosed with it every year (Anna *et al.*, 2017). At present, the main clinical treatment of the disease is surgery, chemotherapy and radiotherapy, but these treatments will bring serious adverse reactions (Balasubramaniam *et al.*, 2014). Therefore, a drug with low toxicity and side effects is urgently needed.  $\beta$ -elemene is an effective anticancer drug extracted from zedoary Chinese medicine. It is a non cytotoxic antitumor drug, which can obviously inhibit the proliferation of tumor cells (Chen *et al.*, 2014). It can be realized by preventing the cell cycle of tumor cells and promoting the apoptosis of tumor cells. In this paper, we observed the proliferation inhibition and apoptosis of human hepatoma cells HepG2 and murine hepatoma H22 cells by observing  $\beta$ -elemene and astragaloside (Devaux *et al.*, 2017). In order to further confirm that  $\beta$ -elemene and astragaloside can provide anti-tumor evidence by inducing apoptosis of tumor cells.

The occurrence and development of malignant tumors are closely related to the impairment of the body's defense function, especially the cellular immune function and the expression of related cytokines, such as dendritic cells (DC), auxiliary T cells (Th1, Th2, etc.), tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin (IL-12, IL-1 $\beta$ ) and interferon (IFN). The abnormal expression has a significant effect on the occurrence and development of

tumor (Dan *et al.*, 2015). The sustained growth of tumor also inhibits the immune function of the body and further develops the tumor. Studies have shown that Astragalus and its chemical components can enhance immune function by regulating immune cells and cytokines (Ferlay *et al.*, 2015). Apoptosis is a programmed cell death regulated by many genes. It plays an important role in the development and development of tumor (Gaopeng *et al.*, 2015). The induction of tumor cell apoptosis has become one of the hotspots of cancer treatment. Studies have shown that astragaloside can induce tumor cell apoptosis by regulating the expression of these genes and proteins, thereby inhibiting tumor growth (Chen *et al.*, 2017). The current research suggests that the mechanisms of astragaloside to promote tumor cell apoptosis include: (1) Blocking the proliferation cycle of tumor cells; (2) the effect of apoptosis signal transduction pathway; (3) regulating the expression of oncogene and tumor suppressor gene. In this paper, we analyzed the effect of combined use of  $\beta$ -elemene and astragaloside on inducing apoptosis of tumor cells.

## MATERIALS AND METHODS

### *Animal and cell lines*

40 mice, male and 14–16 g, were provided by Nanjing University of Chinese Medicine laboratory animal research center. Fully enclosed feeding facilities are adopted with temperature between 18 and 22 degrees C, relative humidity between 50 and 60%, noise below 85 dB, ammonia concentration of 20 PPM, ventilation for 8 to 12 times per hour. The cell line is mainly H22 cell line

\*Corresponding author: e-mail: iccc2013@yeah.net

of mouse liver cancer. HepG2 cells are derived from liver cancer tissue, which secrete a variety of plasma proteins, such as albumin,  $\alpha$ 2-globulin, blood fibrinolytic enzyme, Iron transferrin and so on. The cells are provided by Nanjing University of Chinese Medicine. The animal experiment program has been approved by the experimental animal ethics committee, which conforms to the principles of animal protection, animal welfare and ethics, and conforms to the relevant provisions of the national laboratory animal welfare ethics, No. PTSF14.

Reagents include  $\beta$ -elemene milk, astragaloside, fetal bovine serum, MTT kit, Annexin V-FITC cell apoptosis detection kit, BCA protein quantitative kit, ECL luminous box.

### **Cell culture**

The human hepatoma HepG2 cells and mouse liver cancer H22 cells were used as the research object. The 1640 culture medium containing 10% calf serum and the incubator of 37°C were cultured to collect the cells in the logarithmic growth period. The HepG2 cells were inoculated with  $5 \times 10^4$  density of each pore to 96h and incubated for 24 h and 48h respectively, with 6 parallel holes in each group. Each of them was cultured 24, 48 h, and 20  $\mu$ l MTT working liquid was added to the instructions per hole. 4 h was incubated at 37 centigrade, and the liquid was sucked to each hole, supernatants were removed. 150 $\mu$ l DMSO was added to the oscillator to shake 10 min (centrifugation rate as 800rpm) and the absorbance (A) was measured at 490 nm by an enzyme scale. Repeated these experiments for 3 times, the cell growth inhibition rate formula is as follows: survival rate =  $(A_{\text{blank group}} - A_{\text{drug group}}) / A_{\text{blank group}} \times 100\%$ .

### **Detection of apoptosis rate**

The cultured HepG2 cells were inoculated into 6 hole plates at  $5 \times 10^5$  density per pore and divided into blank control group and drug group. After 24 h culture, the cells were collected and washed 2 times with PBS, 500  $\mu$ l Binding Buffer resuspension cells were added and FITC and PI dyes were added to 5  $\mu$ l to be mixed gently and the light response of the ice on the ice was detected by the upper flow cytometry after 15 min.

### **Experimental grouping and drug delivery scheme**

By intraperitoneal injection of H22 cell suspension, H22 cells were pass aged in Kunming mice. After 6 days, mouse ascites was extracted and taking 1500 rpm centrifugation for 5 min. The cell ascites were diluted with  $1 \times 10^6$  cells/mL and then inoculated via subcutaneous injection, at the axillary right armpit of Kunming mice (0.2 mL each), and the whole process was strictly aseptic operation. 40 tumor bearing mice were randomly divided into 4 groups, 10 in each group, which were the control group, the astragaloside group, the  $\beta$ -elemene group and

the combined treatment group (astragaloside+ $\beta$ -elemene). After 24 h, the mice were treated for 14 days of gavage according to the body mass of the mice. The blank control group was treated with saline, 500 $\mu$ l each time, and the dosage of each group in group astragaloside and group  $\beta$ -elemene was 100 $\mu$ g/ml and 500 $\mu$ l each time. Each of the combined treatment group was given the astragaloside group, the  $\beta$ -elemene group 100 $\mu$ g/ml, 250 $\mu$ l.

The day of tumor transplantation was recorded for 0 days. After sixth days after transplantation, the tumor could be touched in the armpit of mice. After 14 days of gavage, the mice were killed and the tumor tissue was stripped. The tumor mass was called and the tumor suppressor rate was calculated. The formula is: tumor inhibition rate =  $(\text{model group tumor quality} - \text{drug group tumor quality}) / \text{model group tumor mass} \times 100\%$

### **Detection of T lymphocyte phenotype by flow cytometry analysis**

The expression of CD4 and CD8 was detected by flow cytometry: (1) In each group,  $1 \times 10^8$  cells were added, 0.5% paraformaldehyde was fixed at 4°C, 1h, 1500 rpm was centrifugally 5min, and the supernatant was discarded. (2) Use cold PBS to wash cells 2 times, 1500rpm centrifuge 5min and discard supernatants. (3) PE labeled CD4 and CD8 monoclonal antibodies were added to the cell samples, and 30min was incubated at 4°C. The cells were washed with ice cold PBS for 2 times, 1500rpm centrifugally 5min, and the supernatant was discarded. (4) Single cell suspension was prepared using 600 $\mu$ l cold PBS suspension cells. (5) Samples were determined by flow cytometry at Beckman Coulter.

### **Detection of protein expression by Western Blot**

The tissues were weighed in each treatment group, and the EP tube was lapping tissue in the ball mill. The corresponding amount of lysate was added, the ice reaction was 30 min, and the supernatant was taken after 4 centigrade 14000 RPM centrifugation 15 min. The protein concentration was measured according to the reagent instructions, A570 was measured and the standard curve was drawn. The corresponding quantity of protein samples, SDS-PAGE electrophoresis, electrophoretic transfer to PVDF membrane, after TBST rinse and 5% degreased milk powder closed 3 h after the addition of one anti, 4 C overnight, after TB ST rinsing, adding two anti, room temperature 3 h and then TBST rinse and finally color developer color, exposure.

## **STATISTICAL ANALYSIS**

The data were expressed by mean $\pm$ standard deviation ( $\bar{x} \pm s$ ), and variance analysis was used for comparison between the groups. All quantitative results and data are analyzed according to SPSS 18 software.

**Table 1:** Effect of HePG2 cell proliferation inhibition

Group	24h		48h	
	Optical density value	Inhibition rate /%	Optical density value	Inhibition rate /%
Control group	0.264±0.013	-	0.415±0.024	
Astragaloside group	0.196±0.008	22.32±8.451	0.327±0.031	24.12±4.164
β-elemene group	0.154±0.015	45.74±4.128	0.175±0.016	58.47±3.145
Combined treatment group	0.093±0.006	66.71±1.547	0.085±0.004	79.15±1.342

**Table 2:** Effect of T lymphocyte in mice

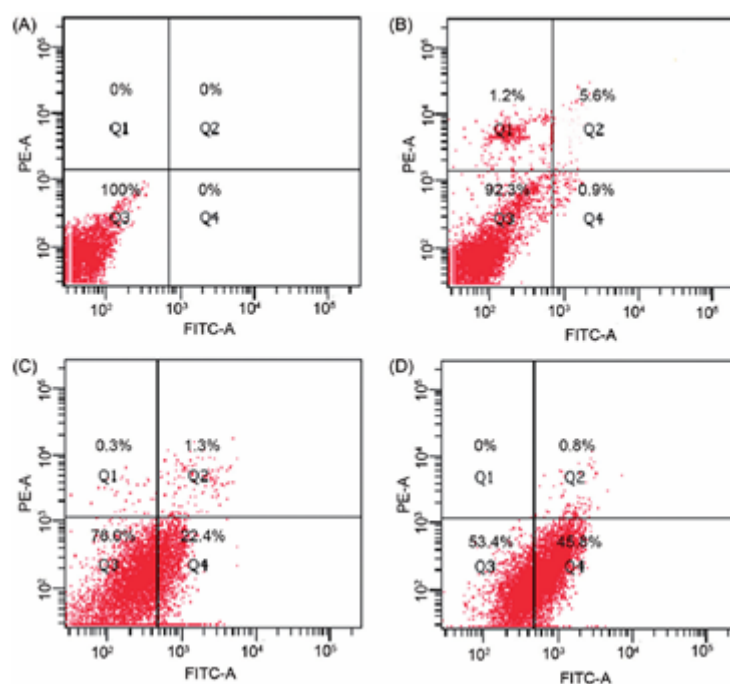
Group	CD4	CD8	CD4+/CD8+
Control group	20.1±2.01	16.2±1.91	1.23±0.54
Astragaloside group	11.5±0.45	13.7±0.82	0.84±0.13
β-elemene group	13.1±0.83	15.7±0.95	0.86±0.08
Combined treatment group	15.2±1.52	14.8±1.22	1.02±0.38

**Table 3:** The effect of tumor suppressor rate in H22 tumor bearing mice

Group	Tumor weight/g	Tumor suppressor rate %
Control group	1.126±0.204	-
Astragaloside group	0.972±0.184	19.3
β-elemene group	0.731±0.058	36.7
Combined treatment group	0.641±0.126	42.9

**Table 4:** Changes in the expression of related proteins

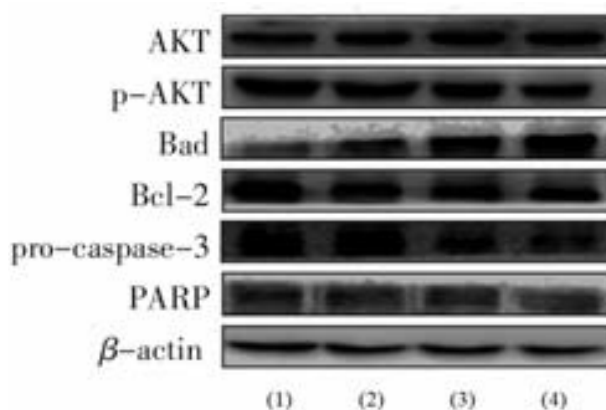
Group	KAT	Bcl-2	Pro-caspase-3	PARP
Control group	0.924±0.137	0.613±0.013	0.204±0.021	0.817±0.017
Astragaloside group	0.872±0.115	0.508±0.028	0.382±0.017	0.741±0.051
β-elemene group	0.856±0.128	0.427±0.016	0.425±0.033	0.712±0.062
Combined treatment group	0.741±0.096	0.134±0.007	0.491±0.052	0.583±0.024

**Fig. 1:** Apoptosis rate changes of Hep G2 cells

## RESULTS

### *Inhibition of proliferation of hepatoma cells in vitro*

After the action of 24h and 48h respectively, the proliferation inhibition of human hepatocellular carcinoma HepG2 cells was observed by different treatments. After 24h, astragaloside,  $\beta$ -elemene and combined treatment groups, the proliferation inhibition of HePG2 cells was produced. The combined treatment group had the best effect and the inhibition rate reached 66.71%. With the prolongation of action time, the inhibition rate of each group increased, and the inhibition rate of the combined treatment group was up to 79.15%. Compared with 24 h, the inhibition rate had significant difference ( $P < 0.01$ ).



**Fig. 2:** Changes in the expression of related proteins: 1) control group, 2) astragaloside group, 3)  $\beta$ -elemene group, 4) combined treatment group.

### *Apoptosis rate changes of Hep G2 cells*

After 24 h, the early apoptosis rate of control group, astragaloside group and  $\beta$ -elemene group was 0.9%, 22.4% and 45.8% respectively. The concentration group of each drug was statistically significant compared with that of the blank control group ( $P < 0.05$ ), such as fig. 1. The effect of  $\beta$ -elemene on the proliferation inhibition of hepatoma cells was observed. The results showed that the proliferation inhibition rate of Hep G2 cells was up to 45.74% after the intervention of 24 h, indicating that  $\beta$ -elemene could significantly inhibit the proliferation of hepatoma cells. The combined treatment group had a more significant effect, reaching 66.71%, indicating that astragaloside combined with  $\beta$ -elemene could make the proliferation of hepatoma cells more effective. The results showed that after the action of 24h, the hepatoma cells in group  $\beta$ -elemene had been apoptotic. This indicates that  $\beta$ -elemene can obviously inhibit the growth of human hepatoma Hep G2 cells and induce a large number of apoptosis in early stage.

### *Effect of T lymphocyte*

The expression of CD4 and CD8 was detected by flow

cytometry, as shown in table 2. Compared with the control group, the number of cells in the experimental control group and the experimental group decreased significantly. Compared with the single drug group, the number of mice in the combined group increased. At the same time, the proportion of peripheral cell subsets in normal mice and tumor bearing mice changed significantly. The combination group increased significantly,  $CD4^+ / CD8^+ > 1$ .

### *Tumor suppressor rate*

The tumor size was significantly reduced, and the tumor inhibition rates were 19.3%, 36.7% and 42.9%, respectively. Compared with the control group, the tumor weight of each group decreased significantly ( $P < 0.05$ ). Among them, the tumor inhibition rate of  $\beta$ -elemene group was higher than that of astragaloside group, and the combined treatment group had the most significant effect, as shown in table 3.

### *Changes in the expression of apoptosis related proteins in cells*

Compared with the control group, the expression of Caspase-3 protein in each group was significantly increased ( $P < 0.05$ ) and the difference between the astragaloside group and the combined treatment group was statistically significant ( $P < 0.05$ ), showed in table 4, fig. 2. The expression of spase-3 protein in group astragaloside,  $\beta$ -elemene group and combined treatment group was higher than that of control group, suggesting that  $\beta$ -elemene induced apoptosis of hepatoma cells may activate the cascade reaction of the family and activate cell apoptosis by up regulation of Caspase-3 expression.

The expression of the upstream regulatory protein AKT decreased ( $P < 0.001$ ), the corresponding Bcl-2 expression decreased significantly ( $P < 0.001$ ), and the expression of pro-caspase-3 protein in the downstream and the final downstream executive protein PARP in the downstream was also significantly decreased ( $P < 0.001$ ).

## DISCUSSION

Apoptosis is a process of maintaining programmed cell death controlled by genes (Ghoneum *et al.*, 2015). In recent years, the mechanism of tumor apoptosis has been inhibited, leading to the formation and development of tumors. Cell apoptosis has its unique biological characteristics (Hsuan *et al.*, 2016; Gwak *et al.*, 2017). One is the final formation of apoptotic body. By maintaining the integrity of the cell membrane, the volume of the cell is reduced, the nucleolus is concentrated and the nucleolus is broken; one is the production of different lengths of oligomeric nucleosome fragments, mainly by activating endogenous endonuclease, and simultaneously in the cell, the specific ladder like Ladder atlas appeared in the electrophoresis

(Luo *et al.*, 2015). Nowadays, many anti-tumor drugs achieve anti-tumor effect by inducing apoptosis of tumor cells.

At present, the anti tumor of Chinese medicine has become a hot spot in clinical and experimental research, and great progress has been made (Pathil *et al.*, 2011). Many anti tumor drugs and compound compounds have appeared on the basis of different pathogenesis and clinical manifestations (Nikitopoulou *et al.*, 2012). At present, many effective monomers, single drug and compound compatibility have significant inhibitory effects on tumor. Modern pharmacology has many studies on Astragalus and Rhizoma Curcumae. Astragalus polysaccharides can significantly enhance the immune system, expand coronary artery, improve cardiac function, and enhance antioxidant capacity (Reber *et al.*, 2013). Astragalus can also prevent lipid peroxidation, improve renal function, prevent liver glycogen reduction, anti-aging and other functions and pharmacological effects (Schmidt *et al.*, 2013). The antitumor effect of Astragalus polysaccharides may be enhanced by regulating the immune function of the whole cell to enhance the anti-tumor effect. It can promote the anti-tumor effect by promoting the secretion of anti-cancer cytokine, the expression of one receptor and the proliferation of the precursor cells (Umar *et al.*, 2017). Astragalus polysaccharides have direct killing effect on tumor cells.  $\beta$ -elemene is an effective extract of zedoary Chinese medicine (Zhu *et al.*, 2015). It can effectively inhibit the proliferation of tumor cells, block cell cycle, induce apoptosis of tumor cells, inhibit angiogenesis, inhibit tumor metastasis, reverse the multidrug resistance of tumor cells, and can assist other chemotherapeutic drugs to improve the curative effect of chemotherapy. It has unique characteristics in promoting apoptosis of tumor cells (Vekov *et al.*, 2015).

$\beta$ -elemene can significantly inhibit the proliferation of BNL cells, and this inhibition is related to the promotion of cell apoptosis and also inhibits the proliferation of lung cancer cells (Schmidt *et al.*, 2013). In addition,  $\beta$ -elemene also has a proliferation inhibition effect on bladder cancer 5637 cells and induces apoptosis of bladder cancer cells through caspase-3 mediated apoptosis pathway. Although the sensitivity of different tumor cells to  $\beta$ -elemene inhibition is different;  $\beta$ -elemene inhibits the proliferation of tumor cells with obvious selectivity compared to normal cells (Nikitopoulou *et al.*, 2012). At present, the research shows that many gene products are involved in the process of cell apoptosis, and Bcl-2 is the core molecule to prevent cell apoptosis, and the occurrence of tumor is closely related to its over-expression. The analysis of Bcl-2 protein level showed that  $\beta$ -elemene was accompanied by apoptosis of A375 cells, accompanied by down regulation of apoptosis related protein Bcl-2 expression, and the content of Bcl-2 was negatively

correlated with the rate of apoptosis. In the process of inducing apoptosis,  $\beta$ -elemene can inhibit the expression of Bcl-2 through a certain way, thereby initiating or promoting cell apoptosis (Yanyan *et al.*, 2017).

A large number of studies have shown that Astragalus has a significant antitumor effect (Liu *et al.*, 2015), and its mechanism includes enhancing the immune function of the body, inhibiting the proliferation of tumor cells, promoting the apoptosis of tumor cells, inhibiting the metastasis of tumor cells and eliminating free radicals (Xiong *et al.*, 2015). Studies have shown that *Astragalus membranaceus* can induce tumor cell apoptosis by regulating the expression of these genes and proteins, thereby inhibiting tumor growth (Stephen *et al.*, 2016). The current research suggests that the mechanisms of *Astragalus membranaceus* to promote tumor cell apoptosis are: blocking the proliferation cycle of tumor cells, affecting the signal transduction pathway of cell apoptosis, and regulating the expression of oncogene and tumor suppressor gene.

## CONCLUSION

The results of this study showed that all drugs had significant inhibitory effects on HCC cells, and the combination of Astragalus and  $\beta$ -elemene had the best effect. At the same time, the study showed that with the increase of drug concentration, the inhibition rate of cell growth also increased. According to the above results, we believe that *Astragalus membranaceus* and  $\beta$ -elemene have obvious inhibitory effects on the growth of hepatocellular carcinoma cells and their combination has synergistic effect.

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