International Journal of Biology; Vol. 10, No. 2; 2018
ISSN 1916-9671 E-ISSN 1916-968X
Published by Canadian Center of Science and Education

Study on the Extraction and Antitumor Activity of Achyranthes Bidentata Polysaccharide

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Received: February 1, 2018 Accepted: February 22, 2018 Online Published: March 3, 2018

doi:10.5539/ijb.v10n2p23 URL: https://doi.org/10.5539/ijb.v10n2p23

Abstract

Achyranthes bidentata polysaccharides (ABPS), a major active ingredient of Achyranthes bidentata, has many kinds of well-known pharmacological activity, such as anti-coagulation, indirect anti-tumor, anti-aging and hypoglycemic effect, and also have certain effects on the immune system. However, the direct anti-tumor activity of ABPS remain unclear. In this paper, ABPS was efficiently isolated from Achyranthes bidentata adopted orthogonal experimental method, and then its anti-tumor effect on LTEP-a-2 cells was investigated. LTEP-a-2 cells were treated with various concentrations of ABPS (0, 0.2, 0.4, 0.6, 0.8 and 1 mg/mL) for 48h. Subsequently, cell viability was analyzed by MTT method and the effects of ABPS on the apoptosis of LTEP-a-2 cells was explored by DNA ladder, PI and Annexin V FITC/PI double staining. The results showed that the optimum extraction conditions of ABPS was that the extraction temperature at 90℃, 1:40(W/V) of solid to liquid, and extraction time 4 h. In addition, the yield and purity of ABP was measured up to 15.52% and 97.21% respectively. Furthermore, ABPS significantly inhibited the proliferation and induced the apoptosis of LTEP-a-2 cells. So ABPS might be utilized as a new potential therapeutic agent against Lung Adenocarcinoma.

Keywords: Achyranthes bidentata polysaccharide, Antitumor activity, Lung adenocarcinoma, Apoptosis

1. Introduction

At present, lung cancer has become a malignant tumor with the highest morbidity and mortality in the world, which is a serious threat to human health (Siegel, Miller, & Jemal, 2018). Lung adenocarcinoma is one of the most common histological types of lung cancer. In recent years, the incidence of lung adenocarcinoma has increased significantly, meanwhile its survival rate is very low and its prognosis is very poor (Little, Gay, Gaspar, & Stewart, 2007). Cancer is a complex and refractory disease, in recent years, anti-tumor therapy of traditional Chinese medicine has been a hot topic of clinical research because it has the characteristics of effectively remedy the shortcomings of traditional treatment, improving the quality of patients' life, low cost and small side effect（Zhang, Zhou, & Wang, 2012). Achyranthes bidentata, containing saponins, polysaccharides, flavonoids, steroids and other compounds, has the effects of promoting blood circulation, anti-aging, anti-inflammatory and so on (Meng & Li, 2001). Achyranthes bidentata polysaccharides (ABPS) is an immunomodulator（Zhang, Zhou, & Wang, 2012) and inhibit the growth of tumor cells by promoting cellular immunity and humoral immunity. Therefore, ABPS has good prospects in the field of anti-tumor application. A large number of pharmacological and clinical studies have shown that previous research about ABPS mainly focus on increasing organ immunity and few on the direct anti-tumor effect（Jin et al., 2007). In this study, the extraction conditions of ABPS was optimized by orthogonal experiment, then the effects of ABPS on proliferation, apoptosis and migration of lung adenocarcinoma LTEP-a-2 cells were observed in vitro in order to elucidate its possible antitumor mechanism.

2. Materials and methods

2.1 Regents

Trypsin, fetal calf serum (FCS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), RPMI1640, penicillin, streptomycin and Annexin V-FITC/PI Apoptosis Detection Kit all were purchased from

Sigma Chemical Co. (St. Louis, MO, USA). Cell cycle staining kit was bought fron Multi Sciences (Hangzhou, China). Achyranthes bidentata collected from mid October to early November was bought from Changzhou TCM Hospital.

2.2 Extraction of ABPS

Using the orthogonal method to find the optimum extraction condition of ABPS, the extraction temperature, liquid-material ratio and extraction time were considered as the variables for optimization and each factor contained three levels (Table 1).

Table 1 Factor levels for orthogonal test

Experiment number	Level 1	Level 2	Level 3
Temperature（℃）	70	1:20	2
Liquid ratio (g/mL)	80	1:30	3
Extraction time（h）3	90	1:40	4

According to ABPS is soluble in water, the water extraction of ABPS was adopted. 10 g of Achyranthes bidentata root was weighed, cut, and put into the round-bottomed flask. Then 60% ethanol was also added into the flask and heated at 90 ℃ for 3h. After that, content in the flask was filtered and filter residue was put into the flask and heated to reflux according to the orthogonal experiment conditions (Table 1). The content in the flask was filtered and the filtrate was collected and centrifugated. Then the supernatant was concentrated by rotary evaporation apparatus until the liquid in the circular flask decreased 5 time. Concentrated liquid was transferred into beaker, and 95% of ethanol was also added, then the content in the beaker was stirred until there was no precipitation generated. The liquid in the beaker was centrifugated and the supernatant was removed, then deionized water was added into the precipitation to dissolved the polysaccharide. Polysaccharide solution was centrifugated and the supernatant was retained, then the protein in the supernatant was removed by Sevag method. Finally, polysaccharide was vacuum dried and weighted.

2.3 The Purity of ABPS was Analyzed by Phenol Sulfuric Acid Assay

Using phenol sulfuric acid method to calculate the purity of ABPS. First, glucose standard curve was drawn. 0, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4 and 2.0 mL of 1mg/ml glucose standard solution was added into 8 tubes respectively, then added water into every tube up to the final volume of 2 mL. 1ml of 5% phenol and 5ml of H_2SO_4 were added and immediately mixed. When the solution temperature came to room temperature, the absorbance of every tube was detected by UV spectrophotometer at 490 nm. Finally, glucose standard curve was drawn with glucose concentrations as the abscissa and light absorption as the ordinate. Second, total content of soluble sugar was determined. 2 mL of polysaccharide solution was put into a tube, then 1 mL of 5% phenol and 5mL of concentrated sulfuric acid were added. The contents in the tube were mixed immediately and stood to room temperature. Finally, the absorbance was measured at 490 nm. This step was repeated 3 times. According to the glucose standard curve, the content of the total soluble sugar (denoted c) was calculated. Third, the purity of polysaccharide was analyzed. Based on the content of total soluble sugar, polysaccharide purity was calculated according to the following formula.

$$\text{Purity}(\%) = (c\times100\times100\times10^{-6})/0.0038$$

2.4 Cell Lines and Culture

LTEP-a-2 cells was kindly donated by Professor Zhigang Tu (Institute of Life Sciences, Jiangsu University).The cells were grown in RPMI1640 supplemented with 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/ml streptomycin in a humidified incubator at 37 °C and 5% CO_2.

In this experiment, six groups of LTEP-a-2 cells were designed in accordance with the concentration of ABPS which stimulated the cells for 48 h: 0 mg/mL, 0.2 mg/mL, 0.4 mg/mL, 0.6 mg/mL, 0.8 mg/mL and 1 mg/mL.

2.5 Cell Viability was Tested by MTT Assay

The concentration of cells was adjusted to 5×10^4 mL^{-1}. An amount of 100 μL of cell solution was added into each well of the flat-bottom 96-well microculture plates overnight, then the medium was replaced by RPMI-1640 with or without ABPS and cultured for 48 h. 25 μL of 0.5% MTT was added into each well and incubated at 37 ℃ for 4 h, then the supernatant was removed and 100 μL of DMSO was put into every well. Finally, the plate was shaken slightly for 10min and OD values were read at 490 nm on a Microplate Reader Bio-Rad 550.

2.6 DNA Degradation was Determined by DNA Ladder Test

For DNA preparation, the cells were pelleted by centrifugation at 200g for 10 min. Then, lyses buffer were added to the cell pellet and incubated for 15 min. The cell lysate were centrifuged at 13,000 g for 15min, and the

DNA was pelleted overnight with the same volume of isopropanol. After the elution by addition of 1 mL washing buffer (70% ethanol) and centrifugation at 7600 g for 5 min, the DNA pellet was dissolved in TE buffer (10 mM Tris–HCl, 0.1 mM EDTA, pH 8.8). Electrophoresis was performed on 1.2% agarose gel. The DNA was visualized by UV illumination after standard ethidium bromide staining.

2.7 Cell Cycle was Tested by PI Staining

10^6 cells treated with various concentration of ABPS were collected, washed by cold PBS, and cultured with 1 mL DNA staining solution and 10 μL permeabilization solution for 30 min in darkness. The cell cycle was detected by Beckman counter cell.

2.8 Cell Apoptosis was Analyzed by Annexin V-FITC /PI Double Staining

Annexin-V FITC/PI double stain assay was performed to measure apoptosis by flow cytometry according to the manufacturer's protocol. Briefly, 1×10^5 cells cultured in the presence or absence of ABPS were collected, washed with cold PBS and resuspended in 500 μL binding buffer, followed by addition of 5 μL FITC-labeled Annexin V and 10 μL PI for 5 min at room temperature in darkness. Finally, each sample was analyzed with Beckman counter cell. Flow cytometry was performed on LTEP-a-2 cells gated on the basis of their forward and side light scatter with any cell debris excluded from analysis. Annexin V-FITC (Ex = 488 nm, Em = 530 nm) was detected through FITC detection channel (usually FL1) and PI was detect by PE detection channel (usually FL2). Apoptotic cells were defined as $FITC^+/PI^-$ cells. The gated LTEP-a-2 cells were then plotted for Annexin V-FITC and PI in a 2-way dot plot to assess percentage of apoptotic LTEP-a-2 cells.

2.9 Statistical Analysis

The data are presented as mean ± standard deviation (S.D.). Differences between control and test groups were assessed by one way analysis of variance (ANOVA) and Student's t-test. A probability (P) value of <0.05 and 0.01 is considered to be significant and very significant. Each experiment was repeated for at least three times.

3. Results

3.1 The Optimization of ABPS Extraction Process Conditions

The results showed that main factors affected the extraction of ABPS were extraction temperature (A), liquid-material ratio (B) and time of extraction (C). Moreover, the influence degree of each factor was A>B>C, and the order of each factor level was A3>A2>A1, B3>B2>B1, and C3>C2>C1 (Table 2). Therefore, it can be concluded that the optimal extraction process conditions were water extraction time 4h, liquid-material ratio of 40:1 (mL:g) and extraction temperature 90 ℃. ABPS was extracted according to these conditions, its yield was 15.52% (G/G).

Table 2. The results of orthogonal test for extraction of ABPS

Test number	Temperature A	Liquid-material ratio B	Time of extraction C	The concentration of ABPS (mg/ml)
1	70	1：20	2	0.00105
2	70	1：30	3	0.00448
3	70	1：40	4	0.03934
4	80	1：20	3	0.02230
5	80	1：30	4	0.02543
6	80	1：40	2	0.03742
7	90	1：20	4	0.03713
8	90	1：30	2	0.03943
9	90	1：40	3	0.05993
T1	0.04487	0.06048	0.07790	
T2	0.08515	0.06934	0.08671	
T3	0.13694	0.13669	0.10190	
X1	0.01496	0.02016	0.02596	
X2	0.02838	0.02311	0.02890	
X3	0.04550	0.04556	0.03397	
R	0.03054	0.02540	0.00801	

Note: T1, T2 and T3 values indicated the same level of each factor; X1, X2 and X3 values meant the average of the same level of each factor; The R value indicated extreme difference, and determined the primary and secondary order of each factor, the greater the R value, the larger the influence.

3.2 The Concentration of ABPS was Analyzed

3.2.1 Drawing of Standard Glucose Curve

Different concentrations of glucose solution were prepared, then the light absorption value at 490nm was detected by spectrophotometer, and the standard curve of glucose was drawn (Table 3).

Table 3. The absorbance of different concentration of glucose solution

Concentration (ug/mL)	0	8	12	16	20	24	28	40
Absorbance value(A)	0	0.121	0.203	0.284	0.378	0.456	0.559	0.839

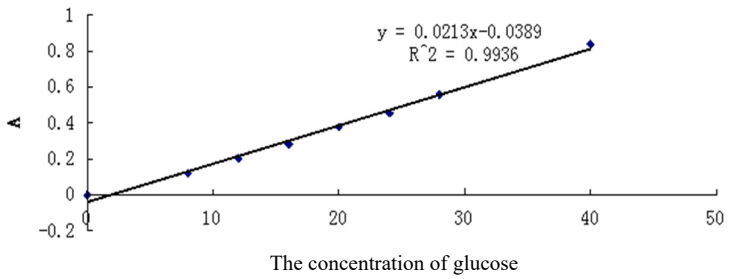


Figure 1. Standard curve of glucose

Polysaccharide of 38 μg/ml was prepared and its average absorbance was 0.748 measured by phenol-sulfuric acid method. Then soluble polysaccharide content calculated from the standard equation was 36.94 μg/ml.

$$\text{polysaccharide purity (\%)} = (36.94\times100\times100\times10^{-6})/0.0038=97.21\%$$

3.3 Cell Viability was Inhibited by ABPS

The effect of ABPS on the viability of LTEP-a-2 cells detected by MTT assay is shown in Figure 2. The viability of LTEP-a-2 cells treated with different concentrations of ABPS for 48h all remarkably decreased compared with non-ABPS-treated group.

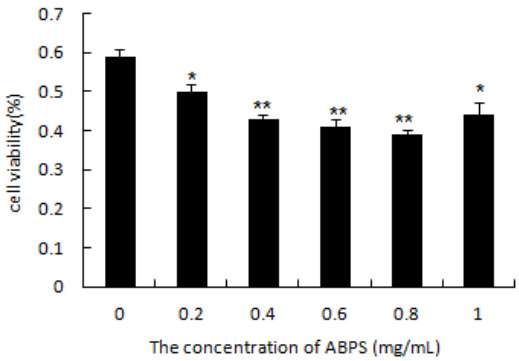
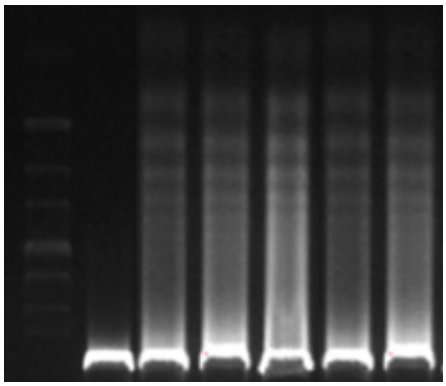


Figure 2. Effect of ABPS on the viability of LTEP-a-2 cells. LTEP-a-2 cells were cultured with various concentrations of ABPS (C, 0.2mg/mL, 0.4mg/mL, 0.6mg/mL, 0.8mg/mL and 1mg/mL) for 48h. Then, MTT assay was used to detect the viability of each group. Data represent the mean ± S.D. of 5 parallel wells from three independent experiments

3.4 DNA Laddering Detection

We then checked whether the decreasing of the viability of LTEP-a-2 cells underwent apoptosis which appeared to clear their DNA into very precise fragments of 180–200 bp. DNA fragmented in this way forms a ladder which can generally be detected by agarose gel electrophoresis of DNA isolated from apoptotic cells. Our study revealed that all ABPS-treated LTEP-a-2 cells showed typical ladders (Figure 3). These data suggested that ABPS induced the apoptosis of LTEP-a-2 cells.



Marker 0 0.2 0.4 0.6 0.8 1

Figure 3. Agarose gel electrophoresis of DNA in LTEP-a-2 cells cultured for 48 h. LTEP-a-2 cells were treated with different concentrations of ABPS. Fragmented DNA was collected and assessed by agarose gel electrophoresis and ethidium bromide. Data shown are representative of three separate experiments. Lane 1: DNA marker. Lane 2: LTEP-a-2 cells were treated without ABPS. Lanes 3 to 7: the concentrations of ABPS were 0.2, 0.4, 0.6, 0.8 and 1 mg/mL, respectively. The blots shown are representative of three independent experiments

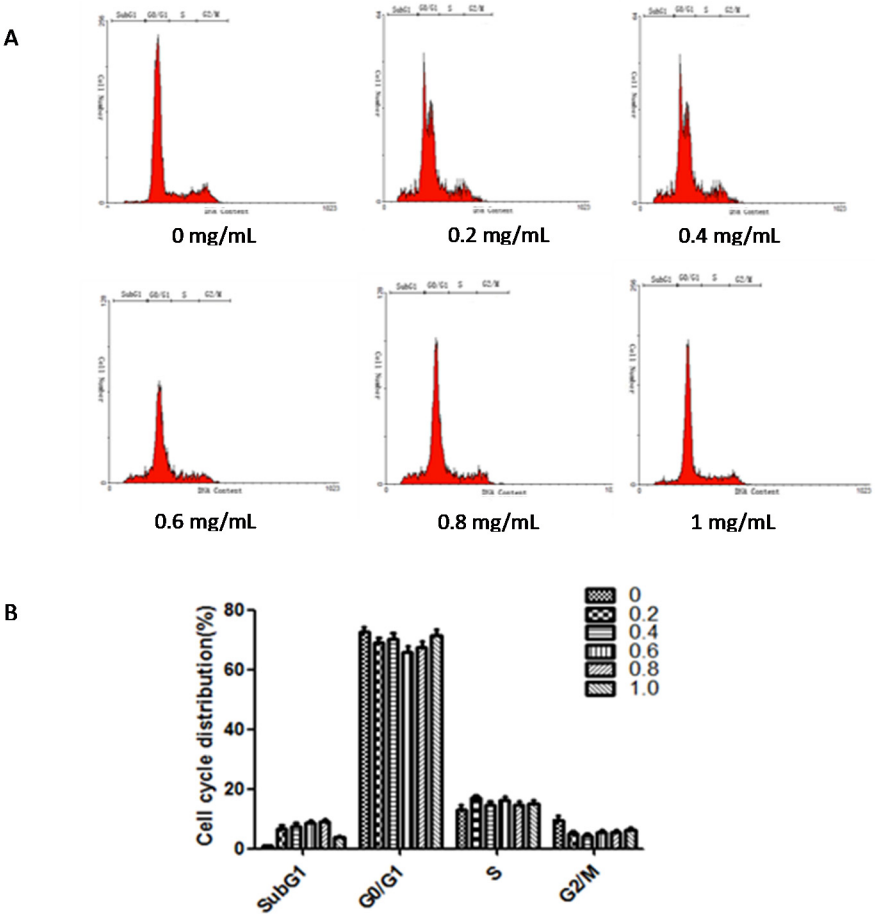


Figure 4. Cell cycle was analyzed by flow cytometry. LTEP-a-2 cells were incubated with various concentrations of ABPS for 48h as indicated. Cell cycle was assayed by flow cytometry after staining ethanol-fixed cells with PI. For each sample, 20,000 cells were analyzed. (A) Representative plot of PI staining of LTEP-a-2 cells treated with different concentration of ABPS. (B) The DNA contents (%) in each phase of cell cycle of LTEP-a-2 cells treated with indicated concentrations of ABPS for 48h are plotted

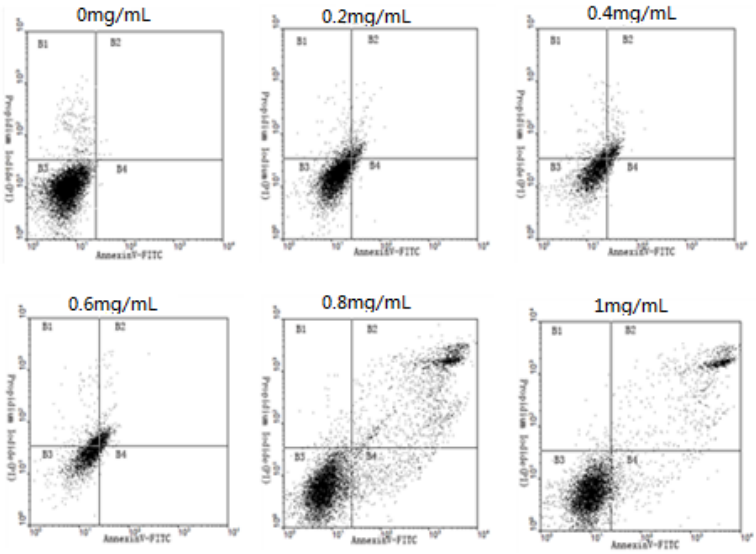
3.5 Analysis of Cell Cycle

To further verify that ABPS induced apoptosis, the cells were stained with PI and analyzed for cell cycle by flow cytometry. The fraction of cells in apoptosis was identified in a DNA histogram as a subG1 hypodiploid population. As shown in Figure 4, LTEP-a-2 cells of control group showed less subG1 hypodiploid population while cells treated with ABPS showed more. When the concentration of ABPS was 0.8mg/mL, the DNA contents of SubG1 phase was 9.21%. Moreover, the DNA contents of G0/G1 and S phases of LTEP-a-2 cells treated with ABPS changed little compared with the control group, while the proportion of G2/M phase was relatively lower. G2/M phase is a period of time from the completion of DNA replication to the end of mitosis. These data indicated that the ABPS inhibit cell division and promoted cell apoptosis.

3.6 Annexin V FITC/PI Assay

In the early stages of apoptosis, phosphatidylserine (PS) is translocated from the inner side of the plasma membrane to the outer layer. Annexin V, a calcium dependent phospholipid-binding protein with a high affinity for PS, can therefore be used as a sensitive probe for the exposure of PS on the cell membrane and hence as a marker of apoptosis. AnnexinV^- PI^- cells were used as controls, while Annexin V^+ PI^- cells and Annnexin V^+ PI^+ cells were designated as apoptotic. Results showed that LTEP-a-2 cells in all ABPS–treated groups appeared significant apoptosis compared with control group (Figure 5).

A



B

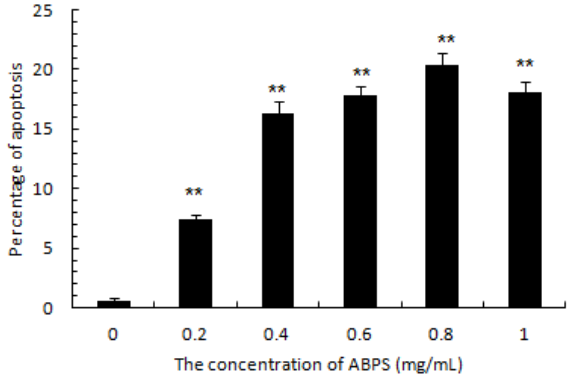


Figure 5. Effects of ABPS on LTEP-a-2 cells discriminated by Annexin-V-FITC and propidium iodide double stain. (A) Representative dot plots of Annexin V/PI staining are shown in each group cells. The lower left quadrant contains the vital (double negative) population. The lower and upper right quadrant contains the apoptotic (Annexin V^+/PI^- and Annexin V^+/PI^+) population. (B)The percentage of apoptotic LTEP-a-2 cells with ABPS treatment is plotted

4. Discussion

ABPS is one of the important active components in dried root of traditional Chinese medicine Achyranthes bidentata. It has the characteristics of small molecular weight, excellent water solubility, nonantigenicity and easy absorption. Therefore, ABPS are attracting more and more attention. Previous studies have shown that ABPS has many pharmacological effects, Such as anti-aging (Zhang & Ye, 2007), Anticoagulation (Mao, Xia, Yuan, & Ye, 2000), antioxidant (Wang & Zhang, 2006), antibacterial (Mao, Xia, Yuan, & Ye, 2000), and improve the role of immune (Zhu, Pan, Zheng, Cui, Cao, & 2012) and so on.

In this paper, the extraction process for soluble polysaccharides from Achyranthes bidentata was developed by orthogonal experiments. The factors affecting the process, such as extraction temperature, ratio of solid to liquid, and treatment time were investigated. The optimal conditions of the extraction were as follows: temperature at 90 ℃, 1:40(W/V) of solid to liquid, extracting 4 h. Under these conditions the yield and purity of polysaccharides reached to 15.52% and 97.21% respectively.

The high purity of ABPS made it possible to study its antitumor effect. Our data showed that ABPS inhibit the proliferation of LTEP-a-2 cells and promote its apoptosis, and these findings were in accordance with previous research. Song Jun and others (Song & Yang, 2001) showed that ABPS also had inhibitory effect on mouse hepatoma cell H22. In fact, ABPS exerted antitumor effect through many mechanisms, such as anticoagulation effect (Mao, Xia, Yuan, & Ye, 2000), changes in the biochemical characteristics of the cell membrane (Yu & Zhang, 1995), improving TNF generation of spleen cells of, enhancing the activity of (Shao & Liu, 2002) killer cells and promoting the maturation of dendritic cells (Chen, Wang, Jia, Cong, & Sun, 2015).

In conclusion, as a well known traditional Chinese medicine, ABPS promoted the notable apoptosis of lung adenocarcinoma cell line which may provide a theoretical basis for its clinical application.

Acknowledgements

This work was supported by National Natural Science Foundation of China (Grant No. 81603336) and Training Program for University Prominent Young and Middle-aged Teachers and Presidents.

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