

Full Length Research Paper

Purified alkaloid extract of *Scutellaria barbata* inhibits proliferation of hepatoma HepG-2 cells by inducing apoptosis and cell cycle arrest at G₂/M phase

Tie-shan Wang^{1,2#}, Li-jing Chen^{2#}, Zhao-yu Wang^{3#}, Sheng-tan Zhang¹ and Jing-ming Lin^{1*}

¹Department of Pharmacy, Zhujiang Hospital, Southern Medical University, Guangzhou 510282, China.

²Department of Pharmacy, Guangdong Women and Children's Hospital and Health Institute, Guangzhou 510010, China.

³College of Life Science and Bio-Pharmaceutical, Guangdong Pharmaceutical University, Guangzhou 510006, China.

Accepted 16 June, 2016

Scutellaria barbata was widely used as an antitumor agent in traditional Chinese medicine. However, its antitumor components and mechanism remained unclear. In the present study, the effects of purified alkaloid extract of *Scutellaria barbata* (PAESB) on the cultured hepatoma HepG-2 cells were investigated. 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay showed that PAESB could inhibit the proliferation of HepG-2 cells in a dose- and time-dependent manner. Annexin V-FITC and propidium iodide (PI) staining showed that PAESB had a positive effect on apoptosis of HepG-2 cells. After treatment by PAESB (0.75 mg/ml) for 48 h, the HepG-2 cells apoptosis rate (25.12±0.91%) had statistical significance as compared with the negative group (2.97±0.49%) (P<0.01). Among the various phases of cell cycle, significant increase in the percentage of G₂/M phase occurred after treated with PAESB (0.75 mg/ml) for 48 h by comparison with the corresponding values for HepG-2 cells without PAESB (P<0.01). These results indicated that PAESB exhibited potential anticancer activity against hepatoma HepG-2 cells through induction of apoptosis and G₂/M phase cell cycle arrest.

Key words: *Scutellaria barbata*, purified alkaloid extract, apoptosis, cell cycle arrest, hepatoma HepG-2 cells.

INTRODUCTION

Natural products have been considered as a valuable source for the discovery of novel drugs including anticancer agents (Schwartzmann et al., 2002). *Scutellaria barbata*, as a popular traditional medicinal herb "Ban-Zhi-Lian" listed in the Chinese pharmacopoeia, has a long therapeutic history for cancers, inflammation and urinary disease. *S. barbata* is a perennial herb which is distributed natively throughout southern China.

Previous phytochemical studies of this plant have revealed presence of a large number of flavonoids (Sato et al., 2000), volatile oil (Yu et al., 2004), unique neo-clerodane type diterpenoids and alkaloids (Dai et al., 2007, 2008, 2009; Lee et al., 2010; Wang et al., 2010) as well as other organic acids, sterides and polysaccharides. Due to the different components in a herb, mixtures or extracts of herbs might have more therapeutic or preventive activity than alone (Vickers, 2002). Recent, several studies have demonstrated that extracts from *Scutellaria barbata* (ESB) can inhibit cell proliferation, induce apoptosis and cell cycle arrest in kinds of human cancer, including gynecological tumor (Lee et al., 2004, 2006; Kim et al., 2005, 2008), leukemia (Cha et al., 2004; Yu et al., 2004), colon cancer (Goh et al., 2005), hepatoma (Dai et al., 2008; Lin et al., 2006), lung cancer (Yin et al., 2004), skin cancer (Suh et al., 2007) and so on. Thus, it can be assumed that ESB potently exert anticancer activity in addition to its other useful pharmacological activities. In spite of these therapeutic effects of ESB, little is known about the active chemical

*Corresponding author. E-mail: linjm1231@21cn.com. Tel: 86-20-61643557. Fax: 86-20-61643010.

#These authors contributed equally to this work.

Abbreviations: PAESB, Purified alkaloid extract of *Scutellaria barbata*; PI, propidium iodide; ESB, extracts from *Scutellaria barbata*; HCl, hydrochloric acid; NH₄OH, ammonium hydroxide; NaOH, sodium hydroxide; CH₃Cl, chloromethane; MTT assay, 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide; PBS, phosphate-buffered saline; DMSO, dimethylsulfoxide.

constituents of ESB for antitumor. Its antitumor mechanism still remains unclear. Therefore, in the course of our search for new and safe anti-cancer agents, the present study was carried out to evaluate the anti-cancer activity of PAESB in cultured hepatoma HepG-2 cells. Our study clearly demonstrated that PAESB exhibited potential anticancer activity and could inhibit proliferation of hepatoma HepG-2 cells via induction of apoptosis and G₂/M cell cycle arrest.

MATERIALS AND METHODS

Plant material

S. barbata was collected in Henan Province, China, in September, 2009 and identified by Prof. Shou-yao Zhang, Department of Pharmacy, Zhujiang Hospital, Southern Medical University, Guangzhou, China. A voucher specimen, number 090912, was deposited there. The collected sample was air-dried, pulverized using a mill hammer and stored in polythene bags for use.

Preparation of PAESB

The air-dried aerial parts of *S. barbata* (3.59 kg) was finely cut and soaked in acid ethanol (12 mol/L HCl-95% EtOH, 2:100, v/v) for 72 h at room temperature, then extracted three times in an ultrasonic bath with acid ethanol for 45 min. After evaporating the solvent under reduced pressure, the extract was dissolved and suspended in 2% hydrochloric acid (HCl) solution, stood overnight and filtrated. The acidic solution was basified to pH=10 with ammonium hydroxide (NH₄OH) solution and exhaustively extracted with chloromethane (CH₃Cl). The chloroform phase was combined and dried using a speed vacuum centrifuge to yield the alkaloidal extract which was dissolved in 2% HCl solution again and basified to pH=10 with NH₄OH solution, exhaustively extracted with CH₃Cl. The chloroform phase was combined and dried using a speed vacuum centrifuge to yield PAESB. 300 mg PAESB was dissolved in 2% HCl solution and basified to pH=7.4 with sodium hydroxide (NaOH) solution, then diluted with saline to obtain PAESB solution (30 mg/ml) which was sterilized using a 0.22 μm filter and stored at 4°C until use. The final concentrations of PAESB which were freshly diluted with culture medium for each experiment were 0.75, 1.0, 1.5 and 2.0 mg/ml, respectively.

Cell lines and culture

Human hepatoma HepG-2 cells were kindly given by Prof. Sheng-jun Liu, Nanfang Hospital, Southern Medical University, Guangzhou, China. The cell culture was maintained in 90% RPMI-1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin in 25 cm² culture flasks at 37°C in a humidified incubator with 5% carbon dioxide supplementation. The cells were fed every two days and subcultured once they reached 70-80% confluence.

3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay measured HepG-2 cells proliferation inhibition rate

HepG-2 cells (1×10⁵ cells/ml) in exponential growth stage were suspended in medium and seeded in 96-well plates with 100 μl/well. After culturing for 24 h to obtain adherent monolayer cells, the

medium was discarded. Then cells were washed with phosphate-buffered saline (PBS) twice and incubated in the fresh medium containing various concentrations of PAESB solution (the final concentrations were 0.75, 1.0, 1.5 and 2.0 mg/ml, respectively) for 24, 48, 72 and 96 h, respectively. Positive group was treated with 5-FU solution (the final concentration was 250 μg/ml). Wells added the same volumes of sterilized saline were set as negative group. At the end of each time point, the drug-containing medium was replaced by fresh medium. Then MTT was added to each well. The plates were incubated for additional 4 h at 37°C. After removing the supernatant solution, dimethylsulfoxide (DMSO) solution was added to each well, gently vibrated for 10 min. The absorbency at a wavelength of 570 nm of the dissolved solution was measured with a microplate reader. Results are expressed as the percentage growth inhibition with respect to the untreated cells. The growth inhibition rate (%) = (negative group's OD-test group's OD)/negative group's OD×100%. The morphologic changes of HepG-2 cells were observed with an inverted microscope at 200×.

Annexin V-FITC/PI double staining measured HepG-2 cells apoptosis rate

HepG-2 cells (2×10⁵ cells/ml) in exponential growth stage were suspended in medium and seeded in 6-well plates with 2 ml/well. After cultured for 24 h to obtain adherent monolayer cells, the medium was removed and washed with PBS twice. Then the fresh medium containing PAESB (0.75 mg/ml) was added. Positive group was treated with 5-FU solution (the final concentration was 250 μg/ml). Wells added the same volumes of sterilized saline were set as negative group. After incubating for 48 h, the detached and attached cells were harvested and washed with PBS twice and centrifuged at 1000 rpm for 5 min to remove PBS. Then the cells were treated according to the Annexin V-FITC/PI apoptosis detection kit. The early apoptosis rate of cells was analysis by flow cytometer. Cells positive for Annexin V but negative for PI fluorescence were identified as apoptotic.

Flow cytometer analyzed HepG-2 cells cycle distribution

HepG-2 cells (2×10⁵ cells/ml) in exponential growth stage were suspended in medium and seeded in 6-well plates with 2 ml/well. After cultured for 24 h to obtain adherent monolayer cells, the medium was removed and washed with PBS twice. Then the fresh medium containing PAESB (0.75 mg/ml) was added. Positive group was treated with 5-FU solution (the final concentration was 250 μg/ml). Wells added the same volumes of sterilized saline were set as negative group. After incubating for 48 h, the detached and attached cells were harvested, then washed with PBS twice and fixed in 70% ice-cold ethanol at -20°C overnight. After fixation, the ethanol was removed. Cells were washed with PBS twice and resuspended in 0.5 ml PBS. Then 50 μl RNase and 450 μl PI (50 μg/ml) was added. After incubated at 37°C for 15 min in the dark, the samples of cells were then analyzed for DNA content by flow cytometer. The cell cycle distributions were analyzed by ModFit LT 2.0 software.

Statistical analysis

Experimental data were expressed with mean±standard deviation ($\bar{x} \pm S$). Statistical analysis was performed with analysis of variance of factorial design and One-Way ANOVA using the statistical software SPSS 13.0. Significant level α=0.05, P<0.05 was considered statistically significance.

Table 1. Inhibition rate of HepG-2 cells after treated with different concentrations of PAESB for 24 to 96 h by MTT assay.

Groups (mg/ml)	Inhibition rate (%)				F	P
	24 h	48 h	72 h	96 h		
0.75	32.88±3.37	40.50±2.28	53.35 ±1.88	51.92±6.91	16.764	0.001
1.0	40.08±5.41	61.52±2.59	70.09 ±1.04	66.39±8.34	20.310	0.000
1.5	47.85±9.07	81.79±2.05	86.69 ±1.17	78.55±5.86	30.324	0.000
2.0	54.34±1.72	81.94±0.92	87.99±0.43	81.09±0.40	656.346	0.000
F	8.283	276.590	515.382	14.100	F = 3.654 ^a	
P	0.008	0.000	0.000	0.001	P = 0.003 ^a	

^A: interaction effect. The values were expressed as mean ± standard deviation of triplicate measurements.

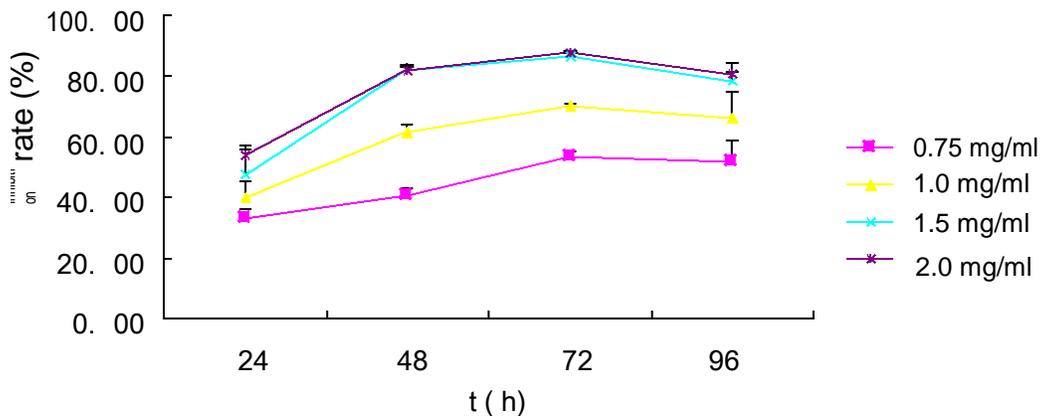


Figure 1. Inhibition of HepG-2 cells proliferation by PAESB. Cells were treated with different concentrations of PAESB. The optical densities of cells were determined at 24, 48, 72 and 96 h, respectively by MTT assay. Analysis of variance of factorial design showed that the growth of HepG-2 cells was inhibited by PAESB in a dose- and time- dependent manner ($P < 0.05$).

RESULTS

Inhibition of HepG-2 cells proliferation by PAESB

The growth inhibition of PAESB in the cultured HepG-2 cells was evaluated by MTT assay. It was shown that a shorter period of treatment with 0.75 µg/ml PAESB reduced growth inhibition only slightly. Further increases in the concentration of PAESB resulted in greater increases in the proliferation inhibition rate of HepG-2 cells. The growth inhibition of cells in high concentration of PAESB (1.5 and 2.0 mg/ml) treatment groups was not significantly higher than that in positive group (Table 1). MTT assay showed that PAESB could inhibit growth of HepG-2 cells in a time and concentration dependence manner (Figure 1).

Morphological monitoring

The number of HepG-2 cells was decreased after treatment with PAESB when compared with negative group.

The higher the concentration of PAESB used to treat HepG-2 cells, the fewer cells were observed. Cellular morphology progressively changed with increasing duration of exposure to PAESB. Figure 2 showed the representative photos of cells treated with either 0.75 or 1.5 mg/ml PAESB for 48 h.

Effect of PAESB on HepG-2 cells apoptosis

To further confirm that PAESB induced cell apoptosis, HepG-2 cells were treated with PAESB to study the apoptosis by staining with Annexin V-FITC and PI and subsequently analyzed by flow cytometer. The dual parameter fluorescent dot plots showed the viable cell population in the lower left quadrant (annexin V-PI-). The cells at the early apoptosis are in the lower right quadrant (annexin V+PI-). As indicated in Table 2, after 0.75 mg/ml PAESB treating HepG-2 cells for 48 h, the cells apoptosis rate (25.12±0.91%) had statistical significance as compared with the negative group (2.97±0.49%)($P < 0.01$). These results suggested that PAESB (0.75 mg/ml) had

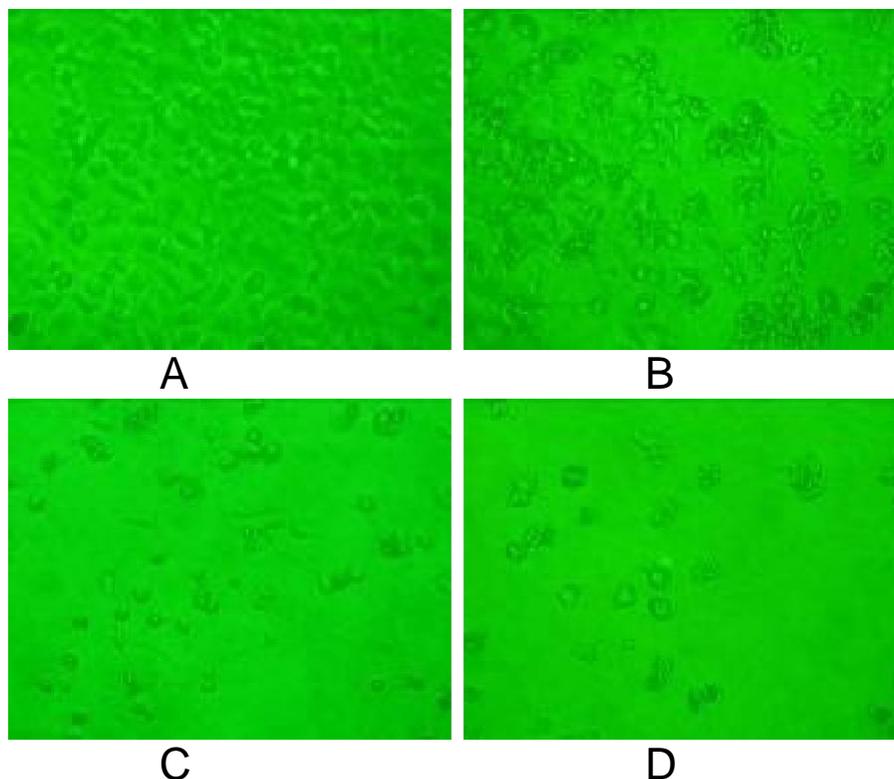


Figure 2. Morphological changes of HepG-2 cells after treatment with PAESB for 48 h. (A) cells untreated; (B) cells treated with 0.75 mg/ml PAESB; (C) cells treated with 1.5 mg/ml PAESB; (D) cells treated with 250 µg/ml 5-FU. Cell photos were observed by an inverted microscope at 200x.

Table 2. Apoptosis rate of HepG-2 cells treated with PAESB for 48 h.

Group	Apoptosis rate (%)
Negative	2.97±0.49
Positive	1.28±0.31
Test	25.12±0.91 ^a
F	1358.991
P	0.000

^aP< 0.01 VS negative group; Apoptosis rates of negative group, positive group and 0.75 mg/ml PAESB-treated cells group were determined by Annexin V-FITC/PI staining and flow cytometric analysis. The values were expressed as mean ± standard deviation of triplicate measurements.

positive effects on apoptosis of HepG-2 cells (Figure 3).

Effect of PAESB on cell cycle distributions of HepG-2 cells

To determine if cell growth inhibition involves cell cycle changes, we examined cell cycle phase distribution by flow cytometer. As indicated in Table 3, when cells were

treated with 0.75 mg/ml PAESB for 48 h, significant increase in the percentage of G₂/M phase occurred by comparison with the corresponding values of negative group (P<0.01), while the percentage of cells at S and G₀/G₁ phases were decreased compared with negative G₀/G₁ phases were decreased compared with negative group (P>0.05). These results showed that PAESB (0.75 mg/ml) could arrest cell cycle progression in G₂/M phase (Figure 4).

DISCUSSION

Hepatocellular carcinoma is one of the most common causes of malignancy-related deaths worldwide. Currently the main treatment of hepatocellular carcinoma is still chemotherapy. And the development of new anticancer drugs has been a key issue in cancer chemotherapy because more cancer cells can resist to current chemotherapy and the chemotherapy will eventually dominate the normal cell population, resulting in much higher mortality (Kang et al., 2005). Recently, there is a growing interest in the use of plant materials for the treatment of various human diseases including cancer. Natural products or herbal recorded in Chinese pharmacopoeia have been prescribed in many diseases

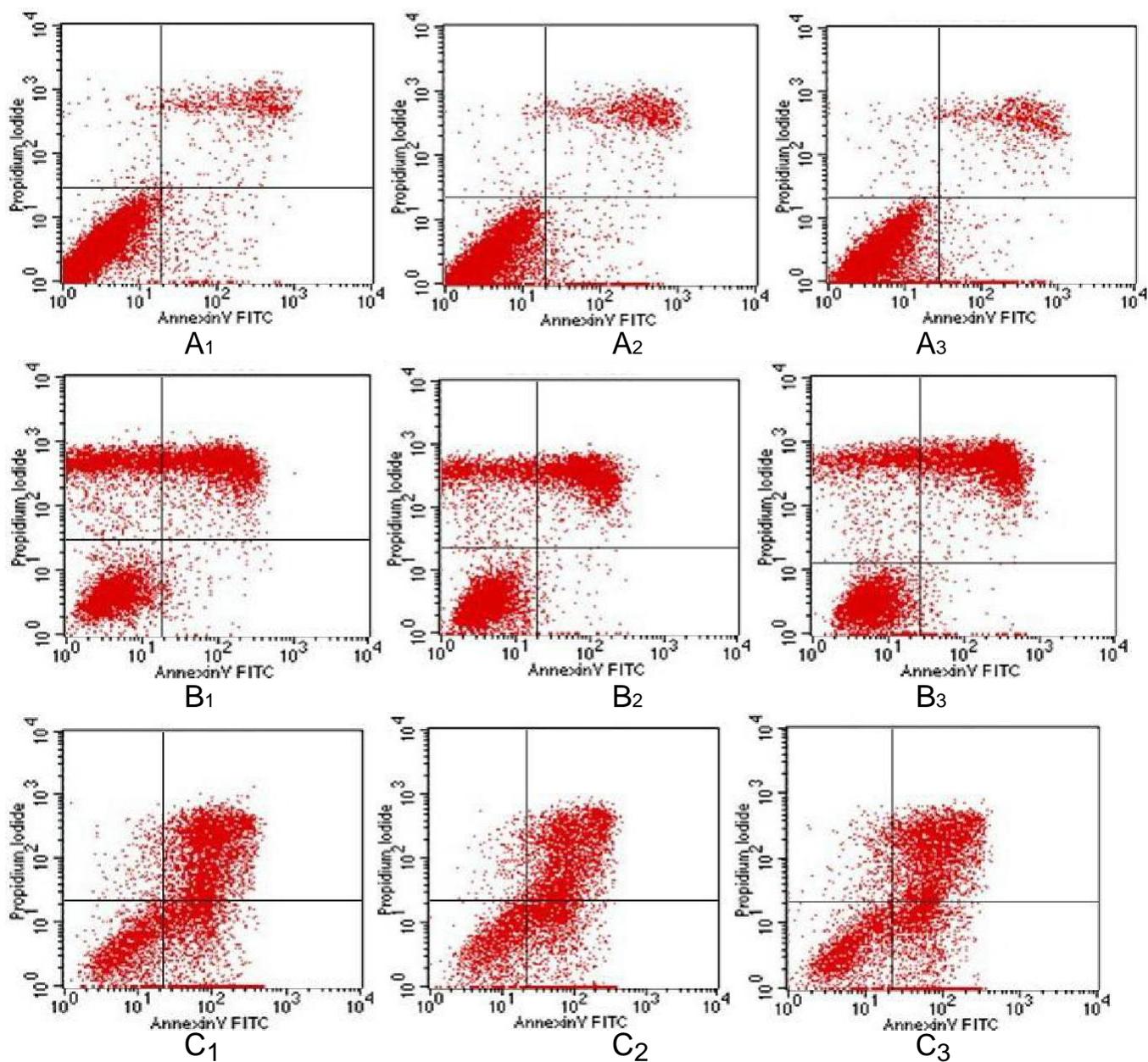


Figure 3. The apoptosis of HepG-2 cells after treated with PAESB for 48 h. (A) negative; (B) positive; (C) cells treated with 0.75 mg/ml PAESB.

Table 3. The cycle distributions of HepG-2 cells after treated with PAESB for 48 h.

Group	Cell cycle distribution (%)		
	G ₀ /G ₁	S	G ₂ /M
Negative	58.19±1.01	33.57±1.94	8.24±0.95
Positive	66.02±6.01 ^a	33.28±2.39	0.70±1.14 ^a
Test	43.69± 1.76	41.46±7.52	14.85±2.24 ^a
F	28.743	2.935	62.558
P	0.001	0.129	0.000

^aP<0.05 VS negative group; Cell cycle distributions in negative group, positive group and 0.75 mg/ml PAESB-treated cells were determined by PI staining and flow cytometric analysis. The values expressed were mean ± standard deviation of triplicate measurements.

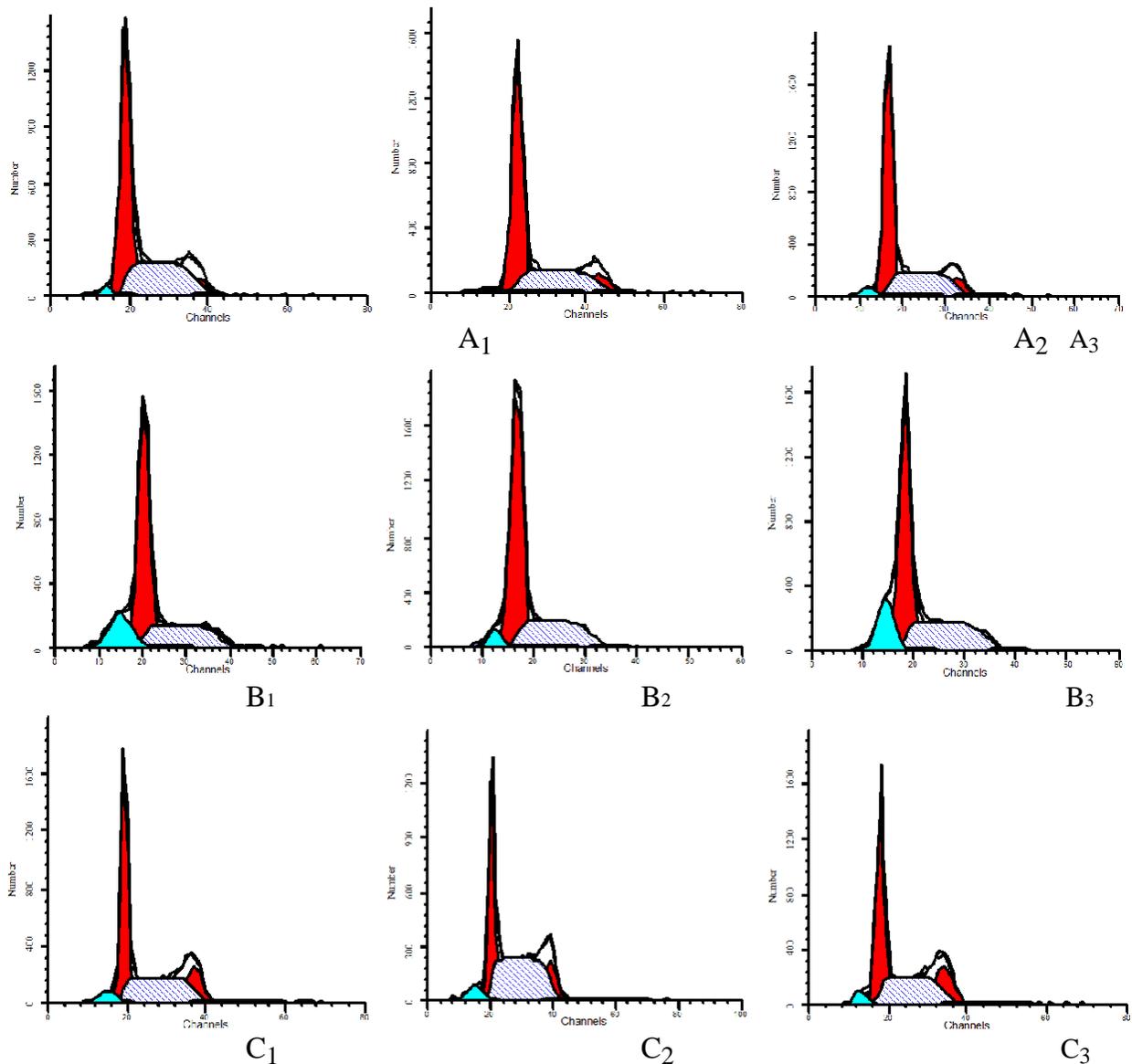


Figure 4. Flow cytometric analysis of HepG-2 cells cycle distribution after treated with PAESB for 48 h. (A) negative; (B) positive; (C) cells treated with 0.75 mg/ml PAESB.

for a long time. Many of them have also been considered as a valuable source for anticancer drug discovery (Schwartzmann et al., 2002). Due to the different components in a herb, mixtures or extracts of herbs may have synergistic activities or buffering toxic effects, more therapeutic or preventive activity than alone (Vickers., 2002). From this point of view, it is important to study the anticancer activity of a whole extract of herbal and its mechanism.

S. barbata is a perennial herb which is natively distributed throughout Korea and southern China. This herb is known in traditional Chinese medicine as “Ban-Zhi-Lian” and traditional Korean medicine as “Ban-Ji-Ryun”, respectively. *S. barbata* has been traditionally used as an antitumor agent, an anti-inflammatory agent

and a diuretic in China. In the clinic, it often can be used to treat kinds of tumors in combination with other traditional Chinese medicines. This plant contains a large number of flavonoids, volatile oil, unique neo-clerodane type diterpenoids, alkaloids as well as other organic acids, sterides and polysaccharides. Recent studies indicate that its extract have growth inhibitory effect on a number of human cancer cells, including tumor, leukemia, colon cancer, hepatoma, lung cancer, skin cancer and so on. However, the chemical constituents of *S. barbata* for antitumor activity have not been fully determined. The underlying mechanism of anti-cancer remains unclear. Thus, the present study investigated the effect of PAESB on the proliferation, apoptosis or cell cycle of cultured hepatoma HepG-2 cells. The MTT assay showed that

PAESB could inhibit the proliferation of HepG-2 cells in a dose- and time-dependent manner. The Cellular morphology progressively changed with increasing duration of exposure to PAESB had also shown it had potent cytotoxicity on the HepG-2 cells.

Apoptosis or programmed cell death is a normal physiological process serving to eliminate unwanted cells and maintain homeostasis in healthy tissue. Tumor growth is regulated by the balance between cell proliferation and apoptosis. Induction of apoptosis, programmed cell death is one approach to cancer therapy (Los et al., 2003). AnnexinV-FITC and PI staining showed that after 0.75 mg/ml PAESB treating HepG-2 cells for 48 h, the cells apoptosis rate ($25.12 \pm 0.91\%$) had statistical significance as compared with the negative group ($2.97 \pm 0.49\%$). This finding suggested that it may have potential as a cancer-prevention agent because the therapeutic application of apoptosis had been regarded as a model for developing anti-tumour drugs (Hong et al., 2003). Cancer is characterized by proliferation disorders and apoptosis obstacles. Inhibiting cell proliferation and increasing apoptosis in tumors are effective tactics for preventing tumor growth. What's more, many anti-cancer agents arrest the cell cycle at the G₀/G₁, S or G₂/M phase and then induce apoptotic cell death (Tian et al., 2006). In the present study we found that significant increase in the G₂/M phase of the cell cycle occurred after 0.75 mg/ml PAESB treatment HepG-2 cells for 48 h. This suggested that the anti-proliferative effect of PAESB on human hepatoma HepG-2 cells was related to arrest at G₂/M phase of the cell cycle.

The present study did not identify or characterize the active chemical components existing in PAESB. Therefore, we had not known whether the antitumor activity of the PAESB was due to the effect of an individual active compound or the combined effects of multiple compounds contained in the extract. But our qualitative chemical analysis indicated that alkaloids might be the most possible ingredients responsible for the growth inhibition, apoptosis induction and cell cycle arrest of HepG-2 cells.

Conclusion

We had demonstrated that PAESB could inhibit the growth of the HepG-2 cells, induce apoptosis and cell cycle arrest at G₂/M phase. These results suggested that the PAESB had anticancer activity *in vitro*. It could be considered as a valuable source for anticancer drug discovery. However, it had potential for further investigations including elucidation of active compounds and evaluating its anticancer activity *in vivo*. The specific molecular signaling pathways for induction of apoptosis remained to be identified.

ACKNOWLEDGEMENTS

This research was supported by Guangdong natural

science fund (Project No.10151051501000029), Tie-shan Wang, Li-jing Chen and Zhao-yu Wang.

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