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*Full Length Research Paper*

# Purified alkaloid extract of *Scutellaria barbata* inhibits proliferation of nasopharyngeal carcinoma CNE-1 cells by inducing apoptosis and cell cycle arrest at S phase

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***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 *S. barbata* (PAESB) on the cultured nasopharyngeal carcinoma CNE-1 cells were investigated. MTT assay showed that PAESB could inhibit the proliferation of CNE-1 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 CNE-1 cells. After treated by PAESB (0.75 mg/ml) for 48 h, the CNE-1 cells apoptosis rate (5.13 ± 0.70%) had statistical significance as compared with the negative group (1.42 ± 0.26%) ($P < 0.05$). Among the various phases of cell cycle, significant increase in the percentage of S phase occurred after treated with PAESB (0.75 mg/ml) for 48 h while the percentage of cells at $G_0/G_1$ and $G_2/M$ phases were decreased by comparison with the corresponding values for CNE-1 cells without PAESB ($P < 0.05$). These results indicated that PAESB exhibited potential anticancer activity against nasopharyngeal carcinoma CNE-1 cells through induction of apoptosis and S phase cell cycle arrest.**

**Key words:** *Scutellaria barbata*, purified alkaloid extract, apoptosis, cell cycle arrest, nasopharyngeal carcinoma CNE-1 cells.

## INTRODUCTION

Herbal recorded in Chinese Pharmacopoeia have been prescribed in many diseases for a long time. Many of them have also been discovered to be potential sources of antitumor drugs (Vickers, 2002). *Scutellaria barbata* (Lamiaceae) is a perennial herb, also known as a popular traditional medicinal herb “Ban-Zhi-Lian” listed in the Chinese Pharmacopoeia. It is distributed natively throughout Southern China and has been traditionally used in treatment of tumor, inflammation, hepatitis and gynecological diseases in China. Phytochemical studies had shown that *S. barbata* contained 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. Pharmacology studies indicated that extracts from *S. barbata* (ESB) had antitumor activitiy on gynecological tumor cells (Lee et al., 2004, 2006; Kim et al., 2005, 2008), leukemia cells (Cha et al., 2004; Yun et al., 2004), colon cancer cells (Goh et al., 2008), hepatoma cells (Dai et al., 2008; Lin et al., 2006), lung cancer cells (Yin et al., 2004), skin cancer cells (Suh et al., 2007) and can be used to treat other tumors in combination with other traditional Chinese medicines. Thus, it can be assumed that ESB potently exert anticancer activity in addition to its other useful pharmacological activities. However, the active chemical constituents in ESB for anticancer activity have not been fully determined. And its antitumor mechanism still remains unclear. Therefore, the objective of this study was to investigate the antitumor activity of PAESB on human nasopharyngeal carcinoma CNE-1 cells. Our study clearly demonstrated that PAESB exhibited potential anticancer activity and could inhibit proliferation of CNE-1 cells via induction of apoptosis and S cell cycle

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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% HCl solution, stood overnight and filtrated. The acidic solution was basified to pH = 10 with $NH_4OH$ solution and exhaustively extracted with $CH_3Cl$. The chloroform phase were 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 $NH_4OH$ solution, exhaustively extracted with $CH_3Cl$. The chloroform phase were combined and dried using a speed vacuum centrifuge to yield PAESB. 300 mg of PAESB was dissolved in 2% HCl solution and basified to pH = 7.4 with NaOH solution, then diluted with saline to obtaine PAESB solution (30 mg/ml) which was sterilized using a 0.22 μm filter and stored at 4℃ 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 nasopharyngeal carcinoma CNE-1 cell was kindly given by Ting Wei, Zhujiang 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 $cm^2$ culture flasks at 37℃ in a humidified incubator with 5% carbon dioxide supplementation. The cells were fed every two days and subcultured once they reached 70 to 80% confluence.

### MTT assay measured CNE-1 cells proliferation inhibition rate

CNE-1 cells ($1 \times 10^5$ 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 PBS twice and incubated in the fresh medium containing various concentrations of PAESB solutions (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 volume 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℃. After removing the supernatant solution, DMSO solution (150 μl) was added to each well, gently vibrated for 10 min. The absorbency at a wavelength of 570 nm of the dissolved solutions were measured with a microplate reader. Results are expressed as the percentage growth inhibition with respect to the untreated cells. The growth inhibition rate was determined using the following formula. Growth inhibition rate (%) = (negative group's OD-test group's OD)/negative group's OD × 100%. The morphologic changes of CNE-1 cells were observed with an inverted microscope.

### Annexin V-FITC/PI double staining measured CNE-1 cells apoptosis rate

CNE-1 cells ($2 \times 10^5$ 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. And then, the fresh medium containing 0.75 mg/ml PAESB was added. Positive group was treated with 5-FU solution (The final concentration was 250 μg/ml). Wells added the same volume 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 CNE-1 cells cycle distribution

CNE-1 cells ($2 \times 10^5$ 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. Cells were washed with PBS twice, then 2 ml of the fresh medium containing 0.75 mg/ml PAESB was added. Positive group was treated with 5-FU solution (The final concentration was 250 μg/ml). Wells added the same volume 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℃ 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) were added. After incubated at 37℃ 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 ($\overline{X} \pm S$). Statistical analysis was performed with analysis of variance of factorial design and One-Way ANOVA using the statistical software SPSS 13.0. Significant level $\alpha = 0.05$, $P < 0.05$ was considered statistically significance.

## RESULTS

### Inhibition of CNE-1 cells proliferation by PAESB

The growth inhibition of PAESB in the cultured CNE-1 cells was evaluated by MTT assay. It was shown that treatment with PAESB (0.75 μg/ml) for 24 h reduced CNE-1 cells growth inhibition only slightly. Further increases in the concentration of PAESB resulted in

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

| Groups | Inhibition rate (%) | | | | Sum | F | P |
|---|---|---|---|---|---|---|---|
| | 24h | 48h | 72h | 96h | | | |
| 0.75 mg/ml | 12.39±1.91 | 36.32±2.81 | 42.18 ±5.13 | 54.14±1.29 | 36.26±16.10 | 93.641 | 0.000 |
| 1.0 mg/ml | 19.67±6.33 | 46.65±3.10 | 58.03 ±2.72 | 77.96±0.44 | 50.58±22.24 | 124.043 | 0.000 |
| 1.5 mg/ml | 36.38±5.87 | 71.05±8.50 | 87.36 ±1.37 | 90.47±0.59 | 71.32±22.87 | 67.589 | 0.000 |
| 2.0 mg/ml | 62.72±3.48 | 85.67±4.83 | 91.68±2.57 | 93.32±0.62 | 83.35±13.09 | 56.482 | 0.000 |
| Sum | 32.80±20.61 | 59.92±20.86 | 69.81±21.63 | 78.97±16.16 | 60.38±26.02 | 341.470[a] | 0.000[a] |
| F | 66.156 | 53.733 | 160.083 | 1471.489 | 308.147[a] | F = 7.747[b] | |
| P | 0.000 | 0.000 | 0.000 | 0.000 | 0.000[a] | P = 0.000 | |

[a] meant main effect; [b] meant interaction effect. The values expressed were mean ± standard deviation of triplicate measurements.

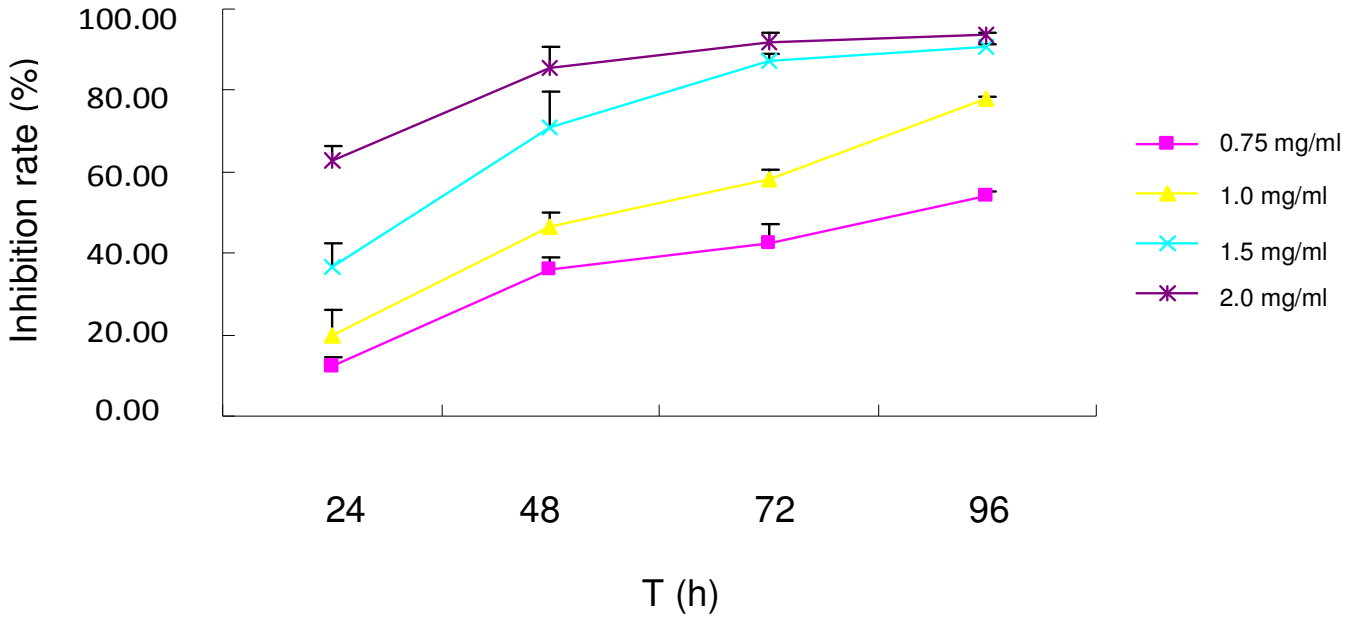


**Figure 1.** Inhibition of CNE-1 cells proliferation by PAESB. Cells were treated with different concentrations of PAESB. The optical density of cells were determined at 24, 48, 72 and 96 h, respectively by MTT assay. Analysis of variance of factorial design showed that the growth of CNE-1 cells was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

greater increases in the proliferation inhibition rate of CNE-1 cells (Table 1). MTT assay showed that PAESB could inhibit growth of CNE-1 cells in a time- and concentration -dependence manner (Figure 1).

## Morphological monitoring

The number of CNE-1 cells was decreased after treatment with PAESB when compared with negative group. The higher the concentration of PAESB used to treat CNE-1 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 after treated with either 0.75 or 1.5 mg/ml PAESB.

## Effect of PAESB on CNE-1 cells apoptosis

To further confirm that PAESB induced cell apoptosis, CNE-1 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

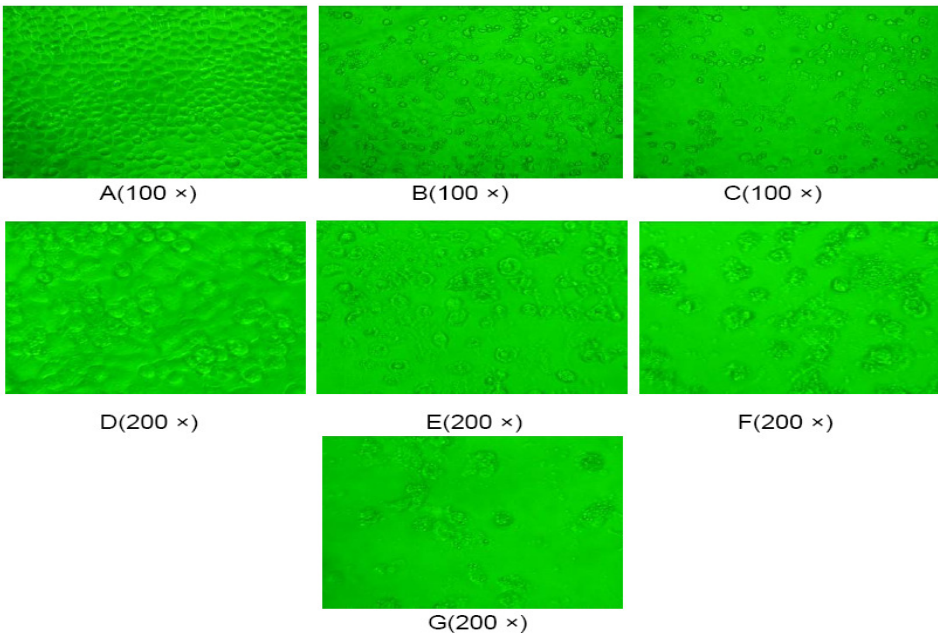


**Figure 2.** Morphological changes of CNE-1 cells after treatment with PAESB. (A) cells untreated for 24 h; (B) cells treated with 1.5 mg/ml PAESB for 24 h; (C) cells treated with 250 µg/ml 5-FU for 24 h; (D) cells untreated for 48 h; (E) cells treated with 0.75 mg/ml PAESB for 48 h (F) cells treated with 1.5 mg/ml PAESB for 48 h (G) cells treated with 250 µg/ml 5-FU for 48 h. Cell photos were observed by an inverted microscope.

**Table 2.** Apoptosis rate of CNE-1 cells treated with PAESB for 48 h.

| Groups | Apoptosis rate (%) |
|---|---|
| Negative | 1.42±0.26 |
| Positive | 8.58±1.56[a] |
| Test | 5.13±0.70[a] |
| F | 38.573 |
| P | 0.000 |

[a]P< 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 expressed were mean ± standard deviation of triplicate measurements.

PAESB treating CNE-1 cells for 48 h, the cells apoptosis rate (5.13 ± 0.70%) had statistical significance as compared with the negative group (1.42 ± 0.26%) (P = 0.004). These results suggested that PAESB (0.75 mg/ml) had positive effects on apoptosis of CNE-1 cells (Figure 3).

### Effect of PAESB on cell cycle distributions of CNE-1 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 S phase occurred while the percentage of cells at $G_0/G_1$ and $G_2/M$ phases were decreased by comparison with the corresponding values of negative group ($P < 0.05$). These results showed that 0.75 mg/ml PAESB could arrest cell cycle progression in S phase (Figure 4 A-C ).

## DISCUSSION

The incidence and mortality rates of nasopharyngeal carcinoma still rank high in the worldwide population. Currently, chemotherapy is still the main treatment method, but only a few cancer patients can be survival. Because more cancer cells can resist to current

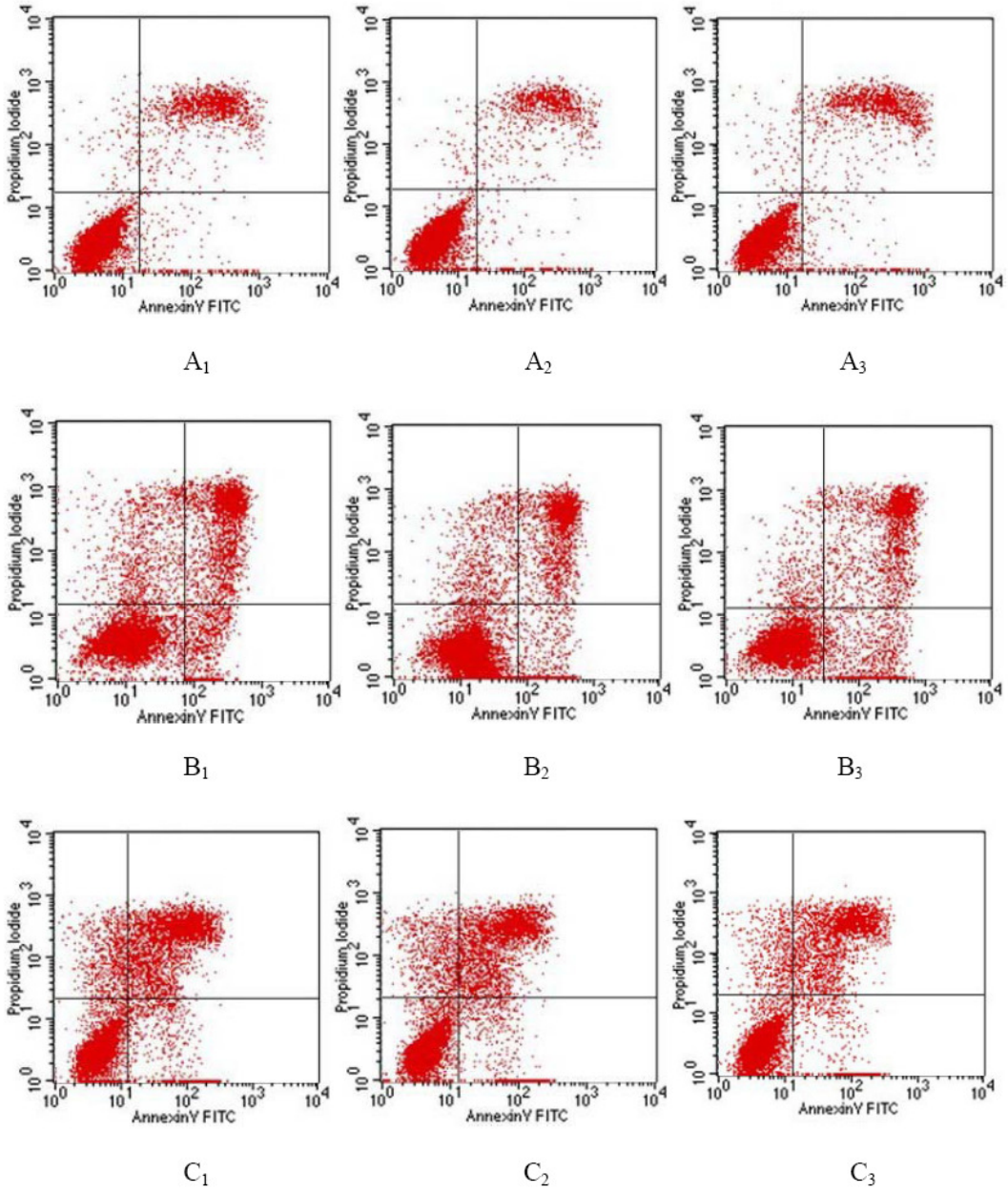


**Figure 3.** The apoptosis of CNE-1 cells after treated with PAESB for 48 h. (A) negative; (B) positive; (C) cells treated with 0.75 mg/ml PAESB.

**Table 3.** The cycle distributions of CNE-1 cells after treated with PAESB for 48 h.

| Groups | Cell cycle distribution (%) | | |
|---|---|---|---|
| | $G_0/G_1$ | S | $G_2/M$ |
| Negative | 61.05±1.57 | 27.77±2.25 | 11.18±0.79 |
| Positive | 63.15±0.92 | 36.85±0.92[a] | 0.00±0.00[a] |
| Test | 57.7±1.22[ab] | 37.30±1.87[a] | 5.01±1.23[ab] |
| F | 14.175 | 27.588 | 131.834 |
| P | 0.005 | 0.001 | 0.000 |

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

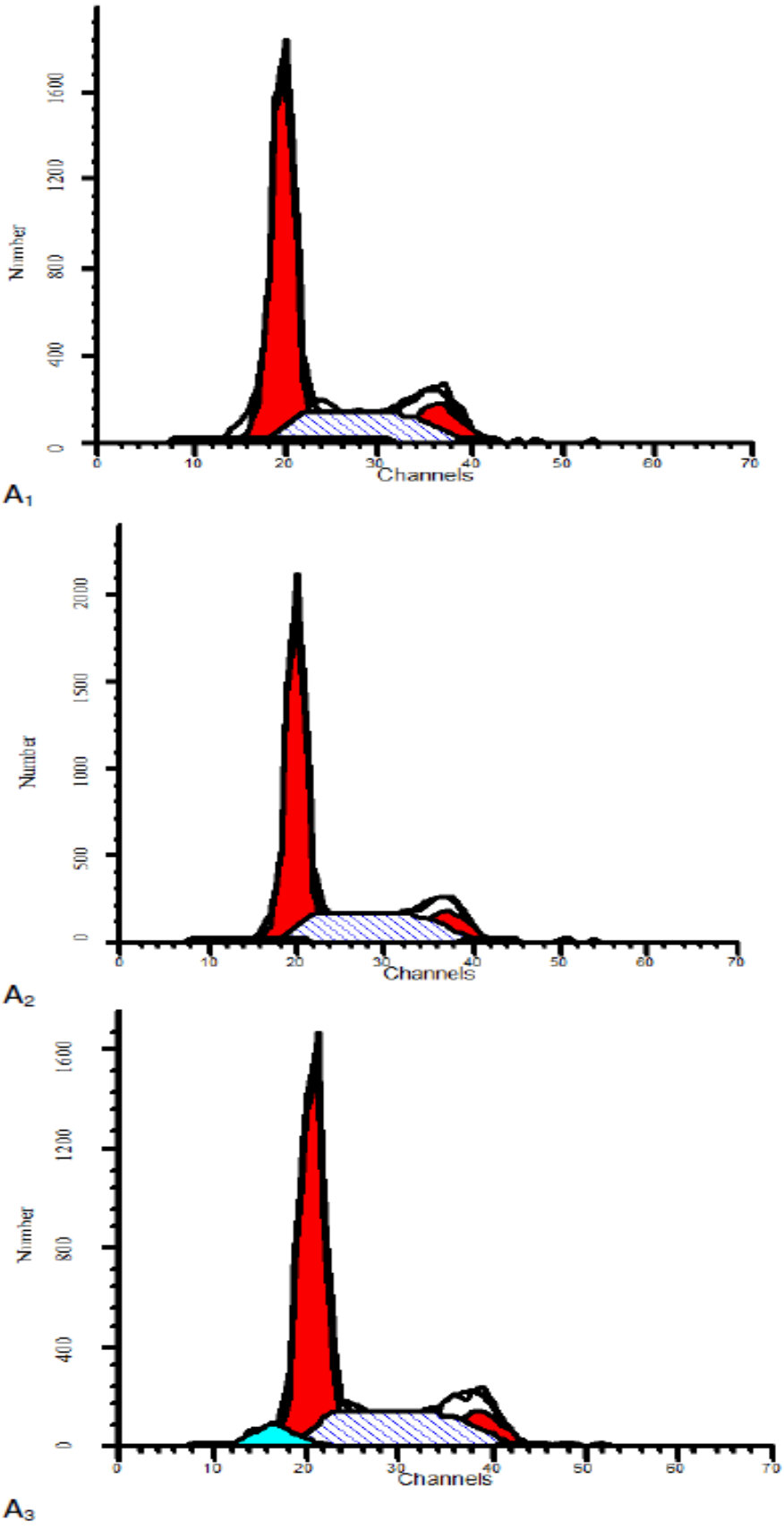


**Figure 4.** Flow cytometric analysis of CNE-1 cells cycle distribution after treated with PAESB for 48 h. (A) negative; (B) positive; (C) cells treated with 0.75 mg/ml PAESB.

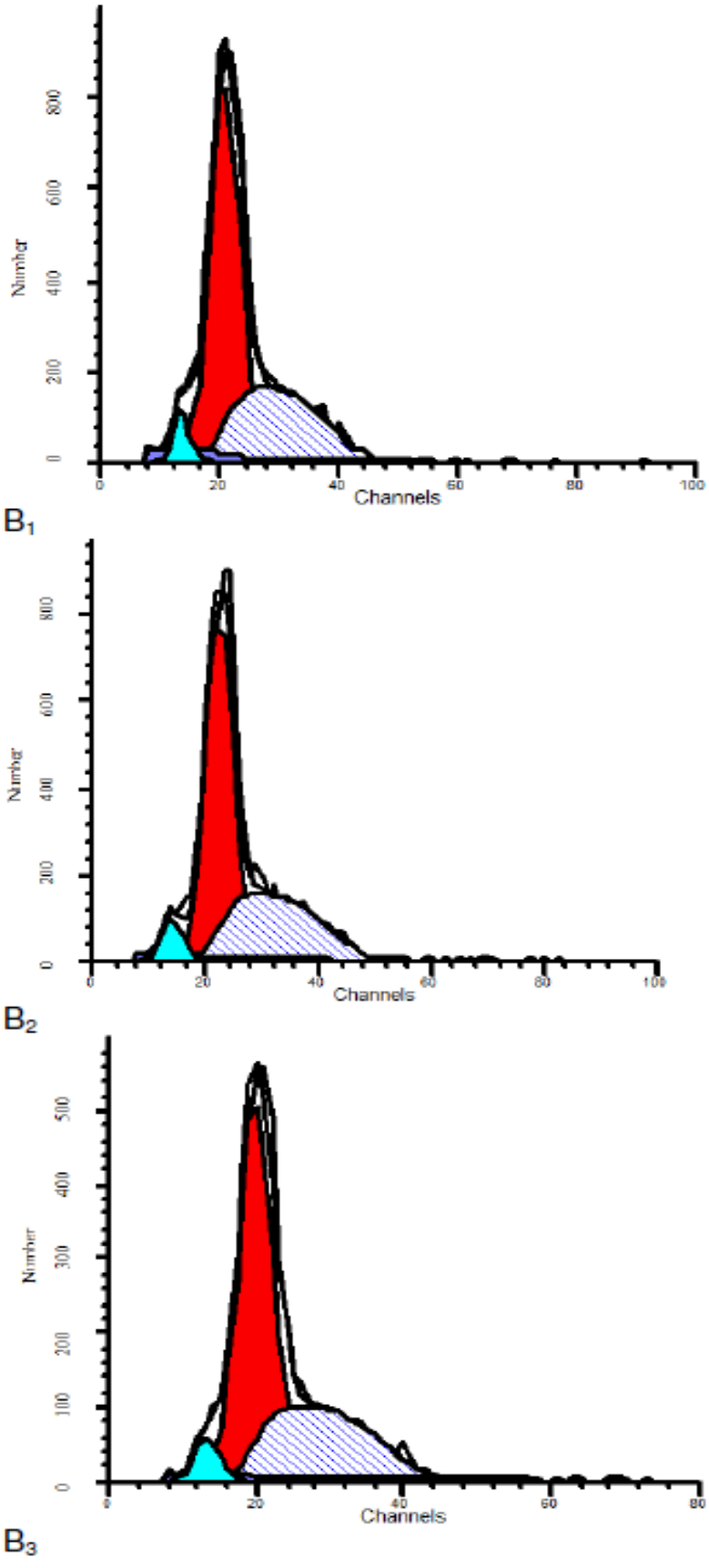


**Figure 4 B.** Contd.

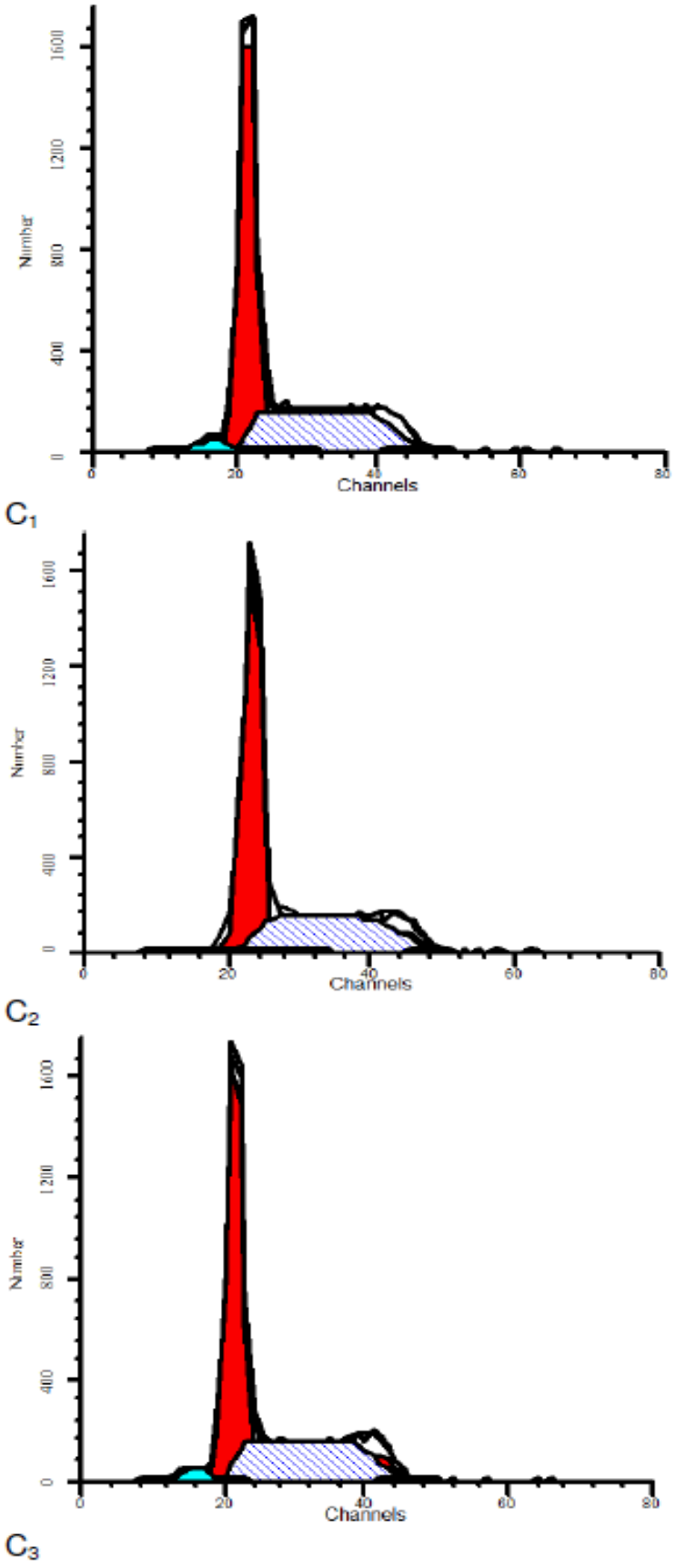


**Figure 4 B.** Contd.

chemotherapy and the chemotherapy will eventually dominate the normal cell population, resulting in much higher mortality (Kang et al., 2005). So, the development of new anticancer drugs has been a key issue in cancer chemotherapy. Recently, there is a growing interest in the use of plant materials for the treatment of various human diseases including cancer (Schwartsmann et al., 2002). More and more people have begun to seach for new antitumor agents from natural products. According to recent reports, uses of plant extracts have effective in anticancer therapy.

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, and known in traditional Chinese medicine as “Ban-Zhi-Lian”. 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 gynecological tumor cells, leukemia cells, colon cancer cells, hepatoma cells, lung cancer cells, skin cancer cells and so on. Different cancer cell lines may have a different sensitivity to the extract. Thus, the present study investigated the effects of PAESB on the proliferation, apoptosis or cell cycle of cultured nasopharyngeal carcinoma cells. The MTT assay showed that PAESB could inhibit the proliferation of CNE-1 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 CNE-1 cells.

Tumor growth is regulated by the balance between cell proliferation and apoptosis. Cancer is characterised by proliferation disorders and apoptosis obstacles. Inhibiting cell proliferation and increasing apoptosis in tumors are effective tactics for preventing tumor growth. So, the therapeutic application of apoptosis had been regarded as a model for developing anti-tumour drugs (Hong et al., 2003). AnnexinV-FITC and PI staining showed that after 0.75 mg/ml PAESB treating for 48 h, the CNE-1 cells apoptosis rate (5.13 ± 0.70%) had statistical significance as compared with the negative group (1.42 ± 0.26%). This finding suggested that, it may have potential as a cancer-prevention agent. What’s more, many anti-cancer agents arrest the cell cycle at the $G_0/G_1$, S or $G_2$/M phase and then induce apoptotic cell death (Tian et al., 2006).

In the present study, we found that significant increase in the percentage of S phase occurred after 0.75 mg/ml PAESB treatment for 48 h while the percentage of cells at $G_2$/M and $G_0/G_1$ phases were decreased by comparison with the corresponding values for CNE-1 cells without PAESB. This suggested that the anti-proliferative effect of PAESB on CNE-1 cells was related to arrest at S phase of the cell cycle. The present study did not identify or characterize the active chemical components existing in PAESB. Therefore, we have 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 CNE-1 cells. In conclusion, we had demonstrated that PAESB could inhibit the growth of the CNE-1 cells, induce apoptosis and cell cycle arrest at S phase. These results suggested that, the PAESB had anticancer activity *in vitro*. It could be considered as valuable sources 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.

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