

# Preliminary evaluation of antitumor effect and induction apoptosis in PC-3 cells of extract from *Patrinia heterophylla*

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Article

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**Abstract:** *Patrinia heterophylla* Bunge, Caprifoliaceae, is a traditional Chinese medicine that has been used for cancer therapy. In our study, a panel of human cancer cells was treated with extract of *Patrinia heterophylla* Bunge. (PHEB), MTT study showed that PC-3 Human prostate adenocarcinoma was the most responsive (IC<sub>50</sub> 9.21±0.32 µg/mL) one to cell growth inhibition, the further study also demonstrated that PHEB could inhibit the proliferation of PC-3 based on a concentration- and time-dependent manner. The transplanted model of sarcoma 180 (S180) and hepatoma 22 (H22) was established in mice, the study demonstrated that *i.p.* administration of 20, 40, 60 mg/kg PHEB exhibited a significant inhibitory effect on the growth of transplantation tumor, with inhibition rate 23.9, 48.4 and 53.6% on S180 and 21.0, 46.3 and 57.2% on H22, respectively. To investigate the molecular mechanism of PHEB in PC-3, the morphological changes of apoptosis were observed by fluorescent microscopy, apoptosis rate was analyzed by flow cytometry (FCM). Morphological characterizations such as apoptotic bodies and membrane blebs were shown by microscopy. The increase of an early apoptotic population was observed in a dose-dependent manner. These results suggest that PHEB has anti-tumor effects and its mechanism is attributed partially to apoptosis induced.

**Keywords:**

antitumor effect  
apoptosis  
MTT  
*Patrinia heterophylla*  
PC-3  
PHEB

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## Introduction

Chinese herbal Mutouhui is the dry root and rhizome of *Patrinia heterophylla* Bunge. which belong to family Caprifoliaceae. As a traditional Chinese medicine, it functions dissipating heat and detoxification, activating blood flow, drying dampness and curing leukorrhagia, and it is usually be used in folk medicine for treating intestinal carbuncle, dysentery, cervical cancer and gastric cancer. Modern pharmacological studies show that plants of *Patrinia* have significant antitumor effects (Chiu et al., 2006; Lu et al., 2009; Zhang et al., 2008a). However, there is still no systematic study on antitumor effect of *P. heterophylla*.

PHEB is a fraction that has been extracted and isolated based on bioactivity-guided fractionation from the root and rhizome of *P. heterophylla* by the authors. The results of Tilden reaction were positive, and it indicated that PHEB is of the unsaturated terpenoids. We have established the preparation method for it, and chemical components studies show that there existed iridoids in PHEB. Seven iridoids have been isolated and identified from PHEB (the chemical components studies will be reported in the future). Iridoid is a

wide class of the main components existed in *Patrinia* (Liu et al., 2006; Yang et al., 2004). Reports showed that iridoids have the effect of sedative and hypnotic (Cometa et al., 2009; Chen et al., 2007). However, there is almost no other report about the antitumor effect of iridoids. In this study, we report the first evaluation in vitro and in vivo of the antitumor effects of PHEB in which the main chemical components are iridoids.

Apoptosis is a form of cell death triggered under a variety of physiological conditions and is tightly regulated by a number of genes that promote or block cell death at different stages. Apoptosis has been suggested as a novel target for cancer chemoprevention and chemotherapy (Makin & Dive, 2001; Sun et al., 2004). In this study, PHEB was also evaluated whether it has the activity of induction apoptosis in PC-3.

## Material and Methods

### *Plant material and PHEB preparation*

*Patrinia heterophylla* Bunge, Caprifoliaceae, was purchased from Hangzhou Chinese medicine herbal tablets factory and authenticated by Prof. RuSong Zhang, College of Pharmaceutical Sciences, Zhejiang

Chinese Medical University. Voucher specimens (No. 080928) were deposited at the Chinese Herb Resources and Engineering Laboratory of the university.

Dried root and rhizome of *P. heterophylla* (5 kg) were smashed into crude powder, then, it was extracted with twenty times 80% ethanol by percolation method at room temperature. The ethanol extract was partitioned between water and ethyl acetate. The extract of ethyl acetate (PHE) was then isolated by silica gel column chromatography, and was gradient eluted by dichloromethane-ethyl acetate. According to the different polarity, PHE was isolated to A, B, C, D four fractions, the fraction B is PHEB.

#### Reagents

RPMI-1640 and DMEM medium was purchased from Invitrogen Life Technologies Corporation (USA). Bovine serum was purchased from Hangzhou Sijiqing Biotechnology Co. (Hangzhou, China). Trypsin, Acridine Orange (AO) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was obtained from Boehringer Mannheim (Mannheim, Germany). Penicillin/streptomycin solution was purchased from Sigma (St. Louis, MO). Cyclophosphamide was obtained from Jiangsu Hengrui Medicine Co., LTD.

#### Cell cultures and animals

PC-3 Human prostate adenocarcinoma, COLO-205 colorectal carcinoma, MCF-7 breast carcinoma, gastric SGC-7901 cancer, NB4 leukemia cells, HepG2 hepatocellular carcinoma were provided by the Cell Bank of Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). MCF-7, SGC-7901, NB4 and HepG2 were cultured in RPMI 1640 medium, PC-3 and COLO-205 in DMEM medium, all supplemented with 0.25% sodium bicarbonate, 10% heat-inactivated fetal bovine serum (FBS), and 2% penicillin-streptomycin. All the cell cultures were incubated at 95% relative humidity, 5% CO<sub>2</sub>, and 37 °C, and passaged two times a week. Ascitic sarcoma 180 and ascitic hepacarcinoma 22 tumor cells were provided by Zhejiang Academy of Medical Science.

Healthy male ICR mice which weigh 20±2 g were provided by Animal Experimental Center of Zhejiang Chinese Medical University. Certificate of the mice is syxk (Zhe) 2008-003. All experiments involving mice were approved by the Institutional Animal Care and Use Committee.

#### Antiproliferative effect of PHEB on different cancer cells

Antiproliferative effect was measured by

microculture tetrazolium (MTT) assay (Mosmann, 1983). Six cancer cells, namely PC-3 human prostate adenocarcinoma, COLO-205 colorectal carcinoma, MCF-7 breast carcinoma, gastric SGC-7901 cancer, NB4 leukemia cells, HepG2 hepatocellular carcinoma, were seeded in 96-well flat-bottom plates at cell density of  $2.0 \times 10^3$  per well for 24 h. PHEB with various concentrations, 2.5-100 µg/mL were then added, for 72 h. After incubation, followed by adding 20 µL MTT solution (5 mg/mL, w/v, in phosphate-buffered saline (PBS) pH 7.4) in each well and being left for 3 h. The blue formazan precipitate was dissolved using 150 µL DMSO, absorbance was read on a Microplate Reader (Eix800, Bio-TEK instruments, Inc, USA) at 570 nm after shaking plates for 5 min.

#### Concentration- and time-dependent effects of PHEB on the viability of PC-3 cells

Above results of MTT study showed that PC-3 was the most responsive one to cell growth inhibition. The relations of PC-3 cell growth inhibition and PHEB concentration, exposure time was then be studied by MTT assay. Briefly, PC-3 cells were seeded within 96-well culture plates ( $1.5 \times 10^3$  cells/well). After overnight adherence, the cells were treated with PHEB 5, 10, 20 µg/mL or without PHEB (normal control, 0.1% DMSO) respectively for 0, 24, 48, 72, 96 h, respectively. Each concentration group included four wells. The medium was replaced at two-day intervals. At the end of treatment, the medium was discarded and the cells were washed twice with PBS. 20 µL MTT at a concentration of 5 mg/mL in PBS was added to each well. The cells were incubated for another 4 h and then 150 µL DMSO was added to each well. Absorbance at a test wavelength of 570 nm was measured using a Microplate Reader. All experiments were conducted three times.

#### Tumor transplantation in vivo

According to protocols of mouse tumor transplants models (Wand, 1997). Under sterile circumstance, ascites of bearing sarcoma 180 mice and hepatoma 22 mice were drawn and diluted with normal saline to final concentrations of  $1 \times 10^7$  cells/mL. ICR mice were inoculated with S180 or H22 cells subcutaneously into right axillary fossa, each mouse in a volume of 0.2 mL. After 24 h, mice were randomly divided into five experimental groups (n=10 mice for each group): Control, cyclophosphamide (CP) group and PHEB groups, and were treated intraperitoneally (*i.p.*) with 0.9% NaCl, CP 25 mg/kg and PHEB 20, 40, 60 mg/kg daily, respectively. The tumor bearing mice implanted with S180 or H22 were sacrificed ten days after inoculation and the tumors were removed and

immediately weighed.

#### Morphological study of apoptosis by acridine orange staining assay

Acridine orange staining assay (Renvoize et al., 1998) was used to observe the morphological changes of apoptotic cells treated by PHEB. PC-3 human prostate adenocarcinoma cells were seeded within 6-well culture plates ( $1 \times 10^5$  cells/well). After incubation for 24 h, PHEB of different concentrations (final dose is 5, 10, 20  $\mu\text{g/mL}$ ), DMSO (normal control, 0.1%) was added to the wells. Having been treated with PHEB for 48 h, the medium were discarded and the cells were washed twice with D-Hanks, it was then stained with 5  $\mu\text{L}$  acridine orange for 10 min at room temperature in the dark. Processed cells were observed with a fluorescence microscope (Olympus, Japan).

#### Cell apoptosis analysis by flow cytometry

AnnexinV-FITC/PI staining assay was used to analysis cell apoptosis (Cao et al., 2006). PC-3 human prostate adenocarcinoma cells were seeded in T25 culture flask for 24 h. The cells were then treated with 5, 10, 20  $\mu\text{g/mL}$  PHEB, DMSO (0.1%) for 24 h, respectively. After incubation, the cells were trypsinized, washed with PBS, and fixed overnight in ice-cold 70% ethanol. After fixation,  $1 \times 10^6$  cells were washed twice with PBS, resuspended in 1 mL of DNA binding buffer solution, then, 10  $\mu\text{L}$  propidium iodide (PI) and 5  $\mu\text{L}$  AnnexinV-FITC was added, and analyzed with FACSCalibur flow cytometer (BD Biosciences, USA). Proportion of apoptotic cells was measured using the control software of the flow cytometer.

#### Statistics

All data are expressed as mean $\pm$ SD. The divergence between the treated and the controlled was analyzed by t-test. A probability of  $p < 0.05$  was considered significant.

## Results

#### Antiproliferative effects of PHEB on different cancer cells

The PC-3 human prostate adenocarcinoma, COLO-205 colorectal carcinoma, MCF-7 breast carcinoma, gastric SGC-7901 cancer, NB4 leukemia cells, HepG2 hepatocellular carcinoma cells were treated respectively with various concentrations of PHEB, for 72 h. Among those cancer cells tested, HepG2 was the most resistant ( $\text{IC}_{50}$  24.7 $\pm$ 0.60  $\mu\text{g/mL}$ )

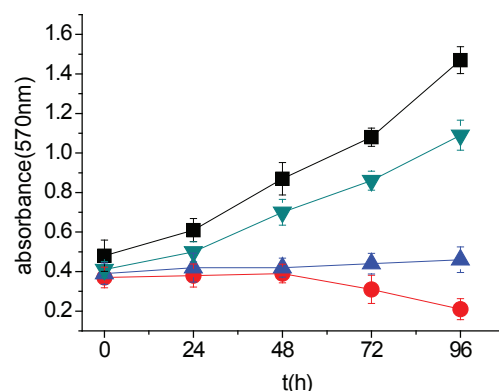
while PC-3 was the most sensitive ( $\text{IC}_{50}$  9.21 $\pm$ 0.32  $\mu\text{g/mL}$ ) cancer cell to the PHEB-induced growth inhibition (Table 1). Therefore, mechanistic actions on the growth inhibition of PHEB were further investigated in the PC-3 human prostate adenocarcinoma cells.

**Table 1.** Values of  $\text{IC}_{50}$  for PHEB on growth of various cancer cells (mean $\pm$ SD, n=3).

Cancer cell line	Cell type	Value of $\text{IC}_{50}$ ( $\mu\text{g/mL}$ )
PC-3	Prostate	9.21 $\pm$ 0.32
COLO-205	Colon	12.3 $\pm$ 0.59
MCF-7	Breast	13.47 $\pm$ 0.53
SGC-7901	Stomach	13.90 $\pm$ 0.34
NB-4	Leukemia	18.40 $\pm$ 0.56
HepG2	Liver	24.7 $\pm$ 0.60

#### Concentration- and time-dependent effects of PHEB on the viability of PC-3 cells

Using MTT assay, the growth inhibitory effect of different concentrations of PHEB on PC-3 cells is shown in Figure 1. The data indicated that growth inhibitory effect of PHEB on the tested cell line increased in a concentration- and time-dependent manner, particularly between 48 and 96 h of treatment. The graph clearly demonstrates that the  $\text{IC}_{50}$  of PHEB on PC-3 is about 10  $\mu\text{g/mL}$  at 72 h.



**Figure 1.** Concentration- and time-dependent effects of PHEB on the growth inhibition of PC-3 cells. ■, DMSO 0.1%; ▼, PHEB 5  $\mu\text{g/mL}$ ; ▲, PHEB 10  $\mu\text{g/mL}$ ; ●, PHEB 20  $\mu\text{g/mL}$ .

#### Tumor transplantation in vivo

The anti-tumor effect of PHEB *in vivo* was evaluated by the inhibition rate of tumor mass. Data (Table 2 and 3) showed that *i.p.* PHEB 40 and 60 mg/kg had a significant antitumor effect on the growth of S180 with inhibition rate 48.4% and 53.6%, and 46.3% and 57.2% on H22, respectively.

**Table 2.** Inhibitory effects of PHEB on the growth of S180 in tumor-bearing mice (mean±SD, n=3).

Groups	Dose (mg/kg)	n	Tumor weight (g)	weight gain/g		Inhibitory rate (%)
				Beginning	End	
Control	-	10/10	2.453±0.421	20.6±1.34	28.7±1.47	-
	20	10/10	1.867±0.485	20.3±1.28	27.4±1.41	23.9
PHEB	40	10/10	1.266±0.575**	20.3±1.39	22.1±1.37	48.4
	60	10/10	1.138±0.493**	20.4±1.27	21.3±1.29	53.6
CP	25	10/10	1.065±0.312**	20.2±1.43	23.9±1.52	56.6

**Table 3.** Inhibitory effects of PHEB on the growth of H22 in tumor-bearing mice (mean±SD, n=3).

Groups	Dose (mg/kg)	n	Tumor weight (g)	weight gain/g		Inhibitory rate (%)
				Beginning	End	
Control	-	10/10	2.659±0.364	20.4±1.18	29.4±1.51	-
	20	10/10	2.100±0.392	20.3±1.30	26.3±1.46	21.0
PHEB	40	10/10	1.427±0.416**	20.5±1.28	21.5±1.34	46.3
	60	10/10	1.138±0.295**	20.2±1.25	20.4±1.38	57.2
CP	25	10/10	1.254±0.254**	20.3±1.42	23.6±1.19	52.8

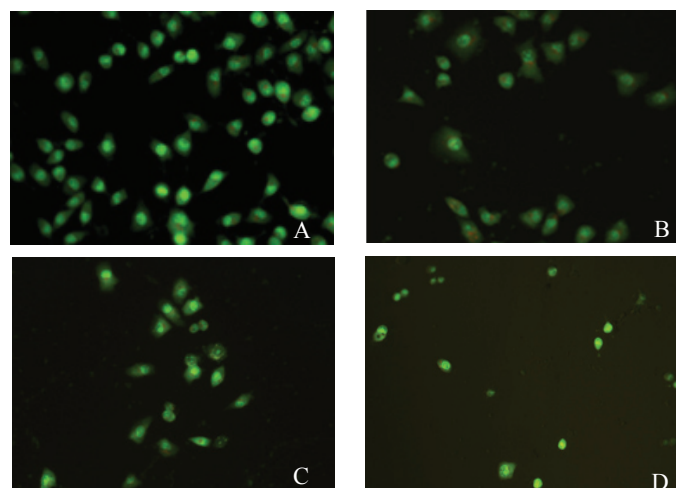
Note: Control: 0.9% NaCl, CP: cyclophosphamide; Inhibition rate (IR %) =  $(1 - \text{Tumor weight}_{\text{Treated}} / \text{Tumor weight}_{\text{Controlled}}) \times 100\%$ . \*  $p < 0.05$ , \*\*  $p < 0.01$  vs. control.

*Morphological study of apoptosis by acridine orange staining assay*

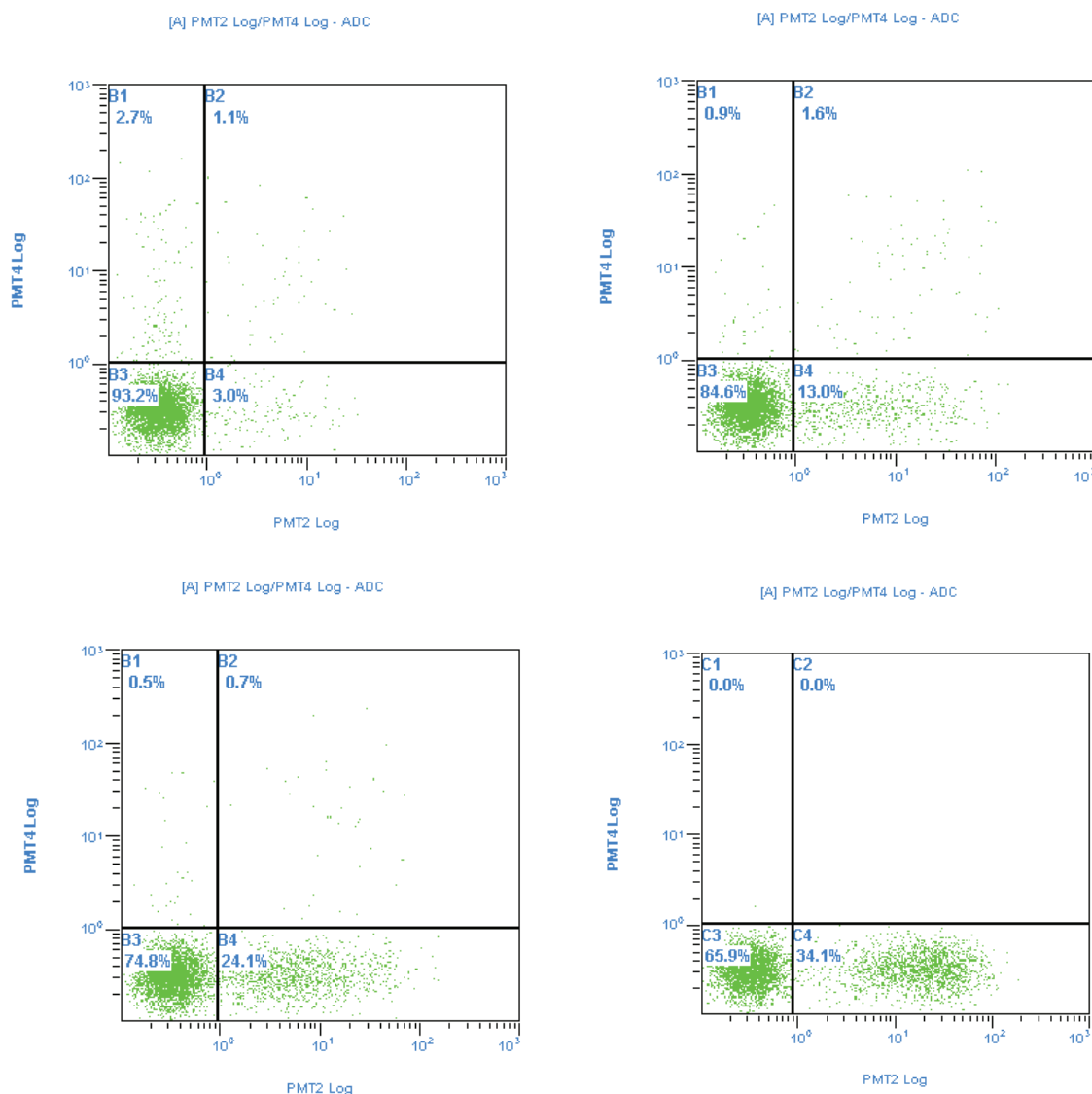
After treatment of PC-3 cells with PHEB at different concentrations for 48 h, compared with vehicle treated control, marked morphological changes of cell apoptosis such as fragmentation and condensation of chromatin, nuclear fragmentations, membrane blebs and apoptotic bodies were found clearly in the cells treated with 10, 20 µg/mL PHEB, using acridine orange (AO) staining (Figure 2). These suggest that apoptosis occurred in these cells, and these effects were increased by increasing in concentration of PHEB.

*Cell apoptosis analysis by flow cytometry*

In view of the morphological changes of cell apoptosis, we were interested in determining whether PHEB also induced apoptosis in PC-3 in the early stage of the treatment, we harvest cells at 24 h, rather than 48 h as many other studies did. The cells were treated with 0.1% DMSO alone or 5, 10, 20 µg/mL PHEB for 24 h, representative results for the PC-3 cells were shown in Figure 3. In PC-3 cells, the percentage of apoptotic cells was 3.0, 13.0, 24.1, and 34.1% respectively. Thus, PHEB has significant apoptosis-inducing effect on PC-3 cells *in vitro*.



**Figure 2.** Morphology studies of PHEB-treated PC-3 cells for 48 h with fluorescent staining AO. The cells were treated with 0.1% DMSO, various concentrations of PHEB, for 48 h. Fixed cells were stained with acridine orange (5 µL) and examined by fluorescence microscopy (A: DMSO 0.1%, B: PHEB 5 µg/mL, C: PHEB 10 µg/mL, D: PHEB 20 µg/mL). The arrow indicates the apoptotic cell.



**Figure 3.** The apoptosis-inducing effect of PHEB on PC-3 cells. PC-3 cells were treated with 0.1% DMSO, various concentrations of PHEB for 24 h, respectively. (A: DMSO 0.1%, B: PHEB 5 µg/mL, C: PHEB 10 µg/mL, D: PHEB 20 µg/mL).

## Discussion

*Patrinia* exhibits diverse pharmacological effects. Volatiles from *Patrinia villosa* (Thunb.) Juss. have the antioxidant activity (Xie et al., 2008). Pretreatment with *Patrinia scabiosifolia* has an anti-inflammatory effect on cholecystokinin (CCK) octapeptide-induced acute pancreatitis (AP) (Seo et al., 2006). Chemical constituents extracted from *P. villosa* has the effect of anti-inflammation (Li et al., 2008). *P. heterophylla* has been used in traditionally Chinese medicine for treating intestinal carbuncle, dysentery; it has also been suggested for treating gastric cancer and cervical cancer. The main components contained in *Patrinia* are triterpenoid saponin, flavonoid, volatile oil etc. There are some iridoids reported recent years. So far studies

have indicated that the material bases of *Patrinia* antitumor effect are triterpenoid saponin (Zhang et al., 2008b; Jung et al., 2004). Our studies clearly indicated that PHEB in which the main components are iridoids has significant antiproliferative effect on a panel of human cancer cells *in vitro*, it could effectively inhibit the growth of sarcoma 180 mice and hepatoma 22 mice in a concentration-dependent manner, simultaneously. This will supply the scientific evidences to reasonably use *P. heterophylla*.

PHEB also induced typical apoptotic features. It is known that apoptosis is important cellular events that can account for the cancer preventive effects of selenium (Kamesaki, 1998). Apoptosis is considered as an active suicidal response to various physiological or pathological stimuli including anticancer agents. It is



not only a genetically controlled mechanism essential for development, maintenance of tissue homeostasis and elimination of unwanted or damaged cells such as tumor cells (Chiu et al., 2006), but also a commonly accepted mechanism of anti-tumor effect of chemotherapeutic drugs. Ethyl acetate extract of *Patrinia scabiosifolia* was found to induce apoptosis in MCF-7 cells, involving downregulated Bcl-2/Bcl-XL expression. Interestingly, the ethyl acetate extract of *Patrinia scabiosifolia* induced apoptosis could not be inhibited by the caspase-9 inhibitor (Chiu et al., 2006). PHEB was isolated from ethyl acetate extract of *P. heterophylla*. The components of PHEB may have some similarity with ethyl acetate extract of *Patrinia scabiosifolia*. Furthermore, the antiproliferative effect of PHEB is better than EAE-PS. So, the mechanism of PHEB-induced apoptosis in PC-3 should be studied in the future.

In our study, PHEB could effectively inhibit the growth of S180 mice and H22 mice in a concentration-dependent manner. Antitumor activity of PHEB *in vivo* is almost the same as cyclophosphamide, this is pleasantly surprised us. For PHEB is still crude extract, after isolation and purification, it may exhibit stronger antitumor effect *in vivo*. Sarcoma 180 and hepatoma 22 are tumor in mice, in further study, a human prostate adenocarcinoma cancer implant model using nude mice will be established to evaluate the antitumor effect *in vivo*.

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