

# Chemical constituents of volatile oil from *Pyrolae herba* and antiproliferative activity against SW1353 human chondrosarcoma cells

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**Abstract.** The objective of the present study was to identify chemical constituents of volatile oil from *Pyrolae herba* (PHVO) and evaluate the antiproliferative activity of PHVO against SW1353 human chondrosarcoma cells. The volatile oil from *Pyrolae herba* was prepared by hydrodistillation and characterized by gas chromatography-mass spectroscopy (GC-MS). A total of 12 components in PHVO were identified representing 81.62% of the total integrated chromatographic peaks. The major compounds were found to be n-hexadecanoic acid (29.29%), cedrol (17.08%), 6,10,14-trimethyl-2-pentadecanone (9.59%) and cis-9-octadecadienoic acid (8.23%). The antiproliferative activity of PHVO against SW1353 cells was investigated using MTT assay, flow cytometry and western blot analysis. Our results demonstrated that PHVO inhibited SW1353 cell viability in a dose- and time-dependent manner. Furthermore, PHVO treatment decreased the number of cells entering the S phase and caused a reduction in the expression of cyclin D1, cyclin-dependent kinase (CDK)4 and CDK6, whereas it caused an increase in the expression of p21. PHVO demonstrated potent antitumor activity against SW1353 cells, suggesting its potential use as a therapeutic agent in the treatment of chondrosarcoma.

## Introduction

Chondrosarcoma is the most common primary malignant bone tumor in children and adolescents, comprising almost 60% of all bone sarcomas (1,2). To date, the main treatments for patients with osteosarcoma are surgical resection and chemotherapy (3). However, despite advances in chemotherapy and surgery, the survival rate for osteosarcoma has reached a plateau and 40% of osteosarcoma patients eventually succumb to the disease; thus, there is an urgent need for the development of novel therapeutic agents (4).

Over the past few years, natural products have attracted extensive attention due to their relatively few side-effects and have been used in clinical practice for thousands of years as important alternative remedies for a variety of diseases (5-7). Moreover, natural products are an attractive source of new therapeutic candidate compounds, which make an enormous contribution to drug discovery today (8). An analysis of the number of chemotherapeutic agents and their sources indicates that over 60% of approved drugs are derived from natural compounds (9). Several plant-derived compounds are currently successfully employed in disease treatment. For instance, arteannuin, which was isolated from *Artemisia annua*, has been used for the treatment of malaria and the vinca alkaloid isolated from the periwinkle *Catharanthus roseus* has been used for the treatment of cancer (10,11). Volatile oils are a type of secondary plant metabolite, and have been shown to exhibit antioxidant and antibacterial activities (12,13).

*Pyrolae herba* listed in Chinese Pharmacopoeia is commonly used in traditional Chinese medicine for the treatment of cancer, hypertension, coronary heart disease and various inflammatory diseases, including rheumatoid arthritis and arthralgia. *Pyrolae herba* functions by expelling wind and removing dampness, and promoting blood circulation for the regulation of menstruation and the relief of pain (14,15). In modern pharmacological studies, the active components of *Pyrolae herba* such as chimaphilin, ursolic acid and hyperoside had previously been shown to possess anti-inflammatory, antioxidant and antibacterial properties (16-21). Recent studies have suggested that ursolic acid inhibits cell proliferation and downregulates the expression of anti-apoptotic and metastatic gene products, potentiating antitumor activity (22-25).

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*Abbreviations:* PHVO, volatile oil from *Pyrolae herba*; GC-MS, gas chromatography-mass spectroscopy; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethyl sulfoxide

*Key words:* *Pyrolae herba*, volatile oil, chemical constituent, gas chromatography-mass spectroscopy, antiproliferative activity

However, the chemical constituents and antitumor activity of volatile oil from *Pryrolae herba* have not yet been reported.

In this study, volatile oil from *Pyrolae herba* (PHVO) was obtained by hydrodistillation and identified by gas chromatography-mass spectrometry (GC-MS). To further investigate the antiproliferative activity of PHVO against SW1353 cells, MTT assay was used to evaluate its inhibitory activity. Moreover, cell cycle distribution and protein expression levels of cyclin D1, cyclin-dependent kinase (CDK)4, CDK6 and p21 were determined by flow cytometry and western blot analysis, respectively.

## Materials and methods

**Materials and reagents.** *Pyrolae herba* was purchased from Jiangxi Herb Co., Ltd., Nanchang, Jiangxi, China (Chinese FDA approval no. 1202001). Fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and trypsin-EDTA were purchased from HyClone (Logan, UT, USA). MTT was obtained from Sigma-Aldrich (St. Louis, MO, USA). The cycle test kit was purchased from Becton-Dickinson (San Jose, CA, USA). The total protein extraction kit was purchased from Nanjing KeyGen Biotech (Nanjing, China). Polyvinylidene fluoride (PVDF) membrane was purchased from Millipore (Lincoln Park, NJ, USA). Rabbit anti-rat cyclin D1, CDK4, CDK6 and p21, and  $\beta$ -actin HRP secondary goat anti-rabbit antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

**Extraction of essential oil.** A total of 300 g of *Pyrolae herba* was placed in a 5-l round-bottom flask with 3 l deionized water. The material was soaked for approximately 2 h before it was subjected to hydrodistillation, then *Pyrolae herba* were distilled for 6 h to collect volatile oil, according to the method recommended by Chinese Pharmacopoeia (26). The essential oil was dried over anhydrous sodium sulfate and stored at  $-20^{\circ}\text{C}$  in the dark.

**GC-MS analysis.** GC-MS analysis was performed on an Agilent 6890N Network GC System, fitted with a HP-5MS capillary column [30 m x 0.25 mm inner diameter (i.d.) x 0.25  $\mu\text{m}$  film thickness; maximum temperature,  $350^{\circ}\text{C}$ ], coupled to an Agilent 5975 inert XL Mass Selective Detector. Helium was used as the carrier gas with a flow rate of 1.0 ml/min and the injector was set at  $250^{\circ}\text{C}$ . The GC oven initial temperature was  $50^{\circ}\text{C}$  for 3 min, then programmed to  $160^{\circ}\text{C}$  at  $1.5^{\circ}\text{C}/\text{min}$  and increased to 280 at  $3^{\circ}\text{C}/\text{min}$ , finally holding at  $280^{\circ}\text{C}$  for 40 min. The ion source temperature was  $230^{\circ}\text{C}$ . MS scanning was carried out at 70 eV over a mass range of 30-500 amu. Samples were injected (1  $\mu\text{l}$ ) with a split mode ratio of 50:1. Compounds were identified by comparing their spectral data and retention time with data from NIST Mass Spectral Library.

**Cell culture.** The SW1353 human chondrosarcoma cell line was obtained from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). SW1353 cells were grown in DMEM containing 10% (v/v) FBS, and 100 U/ml penicillin and 100  $\mu\text{g}/\text{ml}$  streptomycin. Cells were grown in a  $37^{\circ}\text{C}$  incubator supplied with 5%  $\text{CO}_2$ .

**Measurement of cell viability.** Cell viability was determined by MTT assay. SW1353 cells were seeded in 96-well plates at a density of  $1 \times 10^4$  cells/well in 0.1 ml medium. The cells were treated with various concentrations of PHVO for 48 h or 100  $\mu\text{g}/\text{ml}$  of PHVO for different periods of time. The medium was then removed and 20  $\mu\text{l}$  of 0.5% MTT solution were added to each well followed by incubation for 4 h at  $37^{\circ}\text{C}$ . The purple-blue MTT formazan precipitate was dissolved in 100  $\mu\text{l}$  DMSO. The absorbance of each well was determined at 490 nm using an ELISA reader (BioTek, Model ELX800, Winnoski, VT, USA).

**Flow cytometry detection of cell cycle.** To determine the effect of PHVO on the cell cycle distribution, SW1353 cells were treated with various concentrations (0, 50, 100, 150  $\mu\text{g}/\text{ml}$ ) of PHVO for 48 h. The cells were harvested and washed with PBS. The cell concentration was adjusted to  $1 \times 10^6$  cells/ml following centrifugation. Solutions A, B and C were added according to the manufacturer's instructions. DNA content of the cells was measured by FACSCalibur (Becton-Dickinson) flow cytometry system and analyzed using Modfit LT version 3.0 (Verity Software House, Topsham, ME, USA).

**Western blot analysis.** SW1353 cells were seeded in culture flasks and treated with or without PHVO for 24 h at  $37^{\circ}\text{C}$ . Cells were scraped from the culture, washed twice with PBS and then suspended in 30  $\mu\text{l}$  western blot lysis buffer. The protein concentration was then determined using the bicinchoninic acid (BCA) protein assay (Beyotime Biotechnology, Haimen, China). Samples were loaded with 20  $\mu\text{g}$  of protein and separated by electrophoresis on 12% SDS-polyacrylamide gels. Following electrophoresis, the protein blots were transferred onto a PVDF membrane. The membrane was blocked with 5% skimmed milk in TBST solution and incubated overnight with primary antibodies at  $4^{\circ}\text{C}$ . The membranes were then washed in TBST and exposed to secondary antibodies. Finally, the bands were quantified by scanning densitometry (170-8070; Molecular Imager ChemiDoc XRS System, Bio-Rad, Hercules, CA, USA).

**Statistical analysis.** All data are the means of three determinations and the data were analyzed using the SPSS 16.0 package. Student's t-tests and one-way ANOVA test were used to compare the differences among groups and P-values  $< 0.05$  were considered to indicate statistically significant differences.

## Results

**GC-MS analysis of the oil samples.** The results of GC-MS analyses are listed in Table I. GC-MS analysis of the oil samples revealed the presence of 16 different components, 12 of which were listed (Table I), representing 81.62% of the total oil composition from *Pyrolae herba*. n-Hexadecanoic acid (29.29%) was the main component of the oil, followed by cedrol (17.08%), 6,10,14-trimethyl-2-pentadecanone (9.59%) and cis-9-octadecadienoic acid (8.23%).

**PHVO inhibits the proliferation of SW1353 cells.** In order to investigate the effect of PHVO on SW1353 cells, we first observed the cell density by phase-contrast microscopy. As

Table I. Characteristics of the components of volatile oil from *Pyrolae herba* as shown by GC-MS analysis.

Peak no.	Component	RT (min)	RI	Peak area (%)
1	4-Hexyl-2,5-dihydro-2,5-dioxo-3-furanacetic acid	23.684	1490	1.26
2	Cedrol	27.133	1580	17.08
3	1,6-Dimethyl-4-isopropyl-naphthalene	29.187	1622	1.47
4	Tetradecanoic acid	31.439	1712	2.35
5	6,10,14-Trimethyl-2-pentadecanone	33.67	1781	9.59
6	Di-iso-butyl phthalate	34.308	1807	2.25
7	Isophytol	36.595	1843	1.27
8	n-Hexadecanoic acid	37.049	1862	29.79
9	Hexadecanoic acid ethyl ester	38.047	1889	1.90
10	Phytol	41.865	1914	4.72
11	Cis-9-octadecadienoic acid	42.715	1927	8.23
12	11-Decyl-tricosane	55.584	2101	1.71

RT, retention time; RI, retention index.

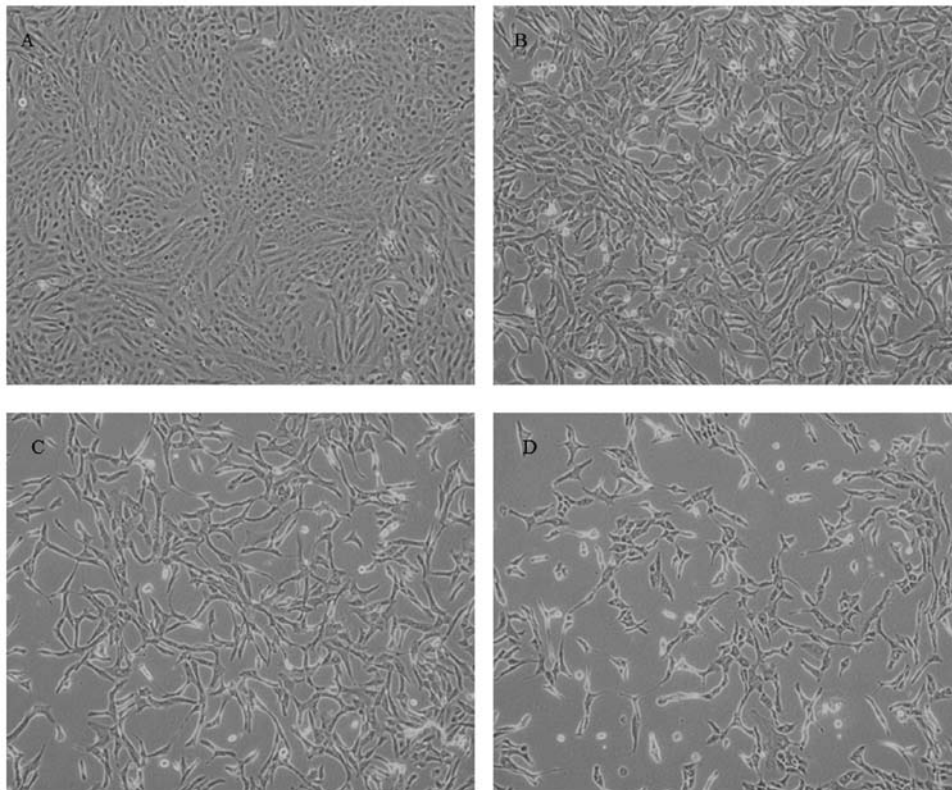


Figure 1. Effect of PHVO on the morphology of SW1353 cells. (A) Untreated control SW1353 cells. (B-D) SW1353 cells were treated with 50, 100, 150  $\mu\text{g/ml}$  PHVO for 48 h. Morphological changes of SW1353 cells were observed under a phase-contrast microscope. Images were taken at a magnification of  $\times 100$ .

shown in Fig. 1, PHVO treatment significantly reduced the cell density compared with the untreated control cells. The effect of PHVO on the viability of SW1353 cells was determined by MTT assay. As shown in Fig. 2, PHVO treatment decreased the cell viability in a dose- and time-dependent manner compared to the untreated control cells ( $P < 0.05$ ). Taken together, these data demonstrate that PHVO inhibits the proliferation of SW1353 cells.

PHVO blocks the progression of SW1353 cells from the G1 to the S phase. In order to examine the effect of PHVO treatment on the progression of SW1353 cells from the G1 to the S phase, we performed flow cytometric analysis of the SW1353 cells treated with 50, 100 and 150  $\mu\text{g/ml}$  PHVO for 48 h. As shown in Fig. 3, the treatment of SW1353 cells with PHVO led to a decrease in the number of cells in the S phase, associated with a concomitant increase in the number of cells

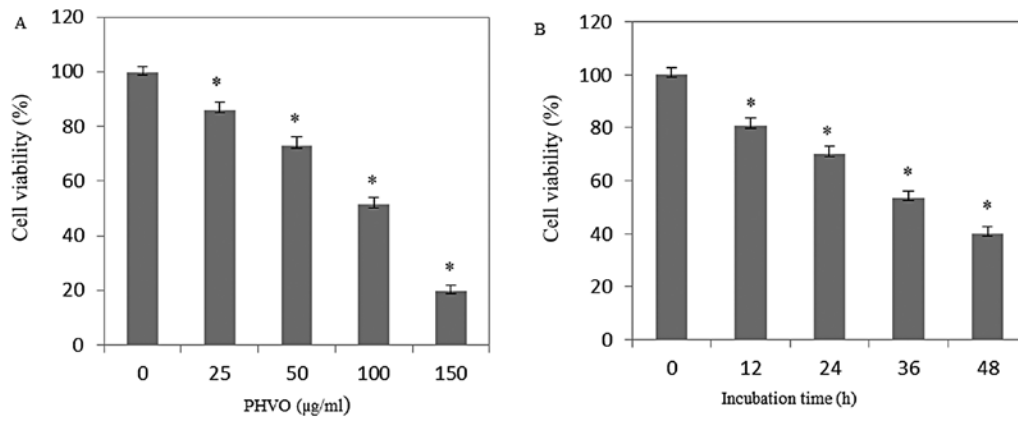


Figure 2. Effect of PHVO on SW1353 cell viability (A) SW1353 cells were treated with the indicated concentrations of PHVO for 48 h. (B) SW1353 cells were treated with 100 µg/ml of PHVO for 12, 24, 36 and 48 h. SW1353 cell viability was determined by MTT assay. The data were normalized to the viability of the untreated cells (100%). Data are the means ± SD (error bars). \*P<0.05, statistically significant vs. untreated cells.

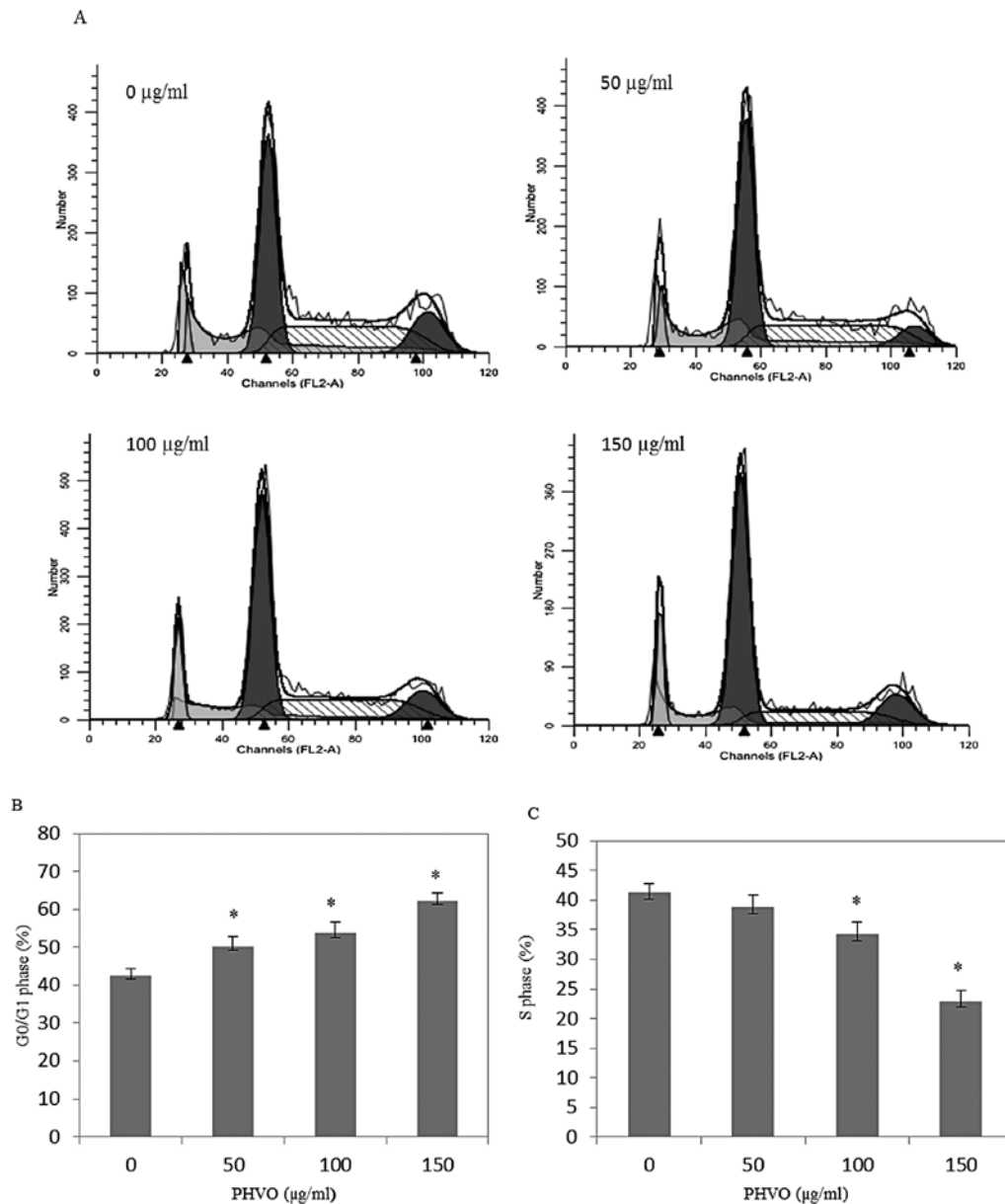


Figure 3. Effect of PHVO on the cell cycle progression of SW1353 cells. (A) SW1353 cells were treated with the indicated concentrations of PHVO for 48 h, stained with PI and analyzed by FACS. (B) Percentage of SW1353 cells in the G0/G1 phase after treatment with PHVO. (C) Percentage of SW1353 cells in S phase after treatment with PHVO. The data shown are the means ± SD (error bars) from three independent experiments. \*P<0.05 vs. control cells.

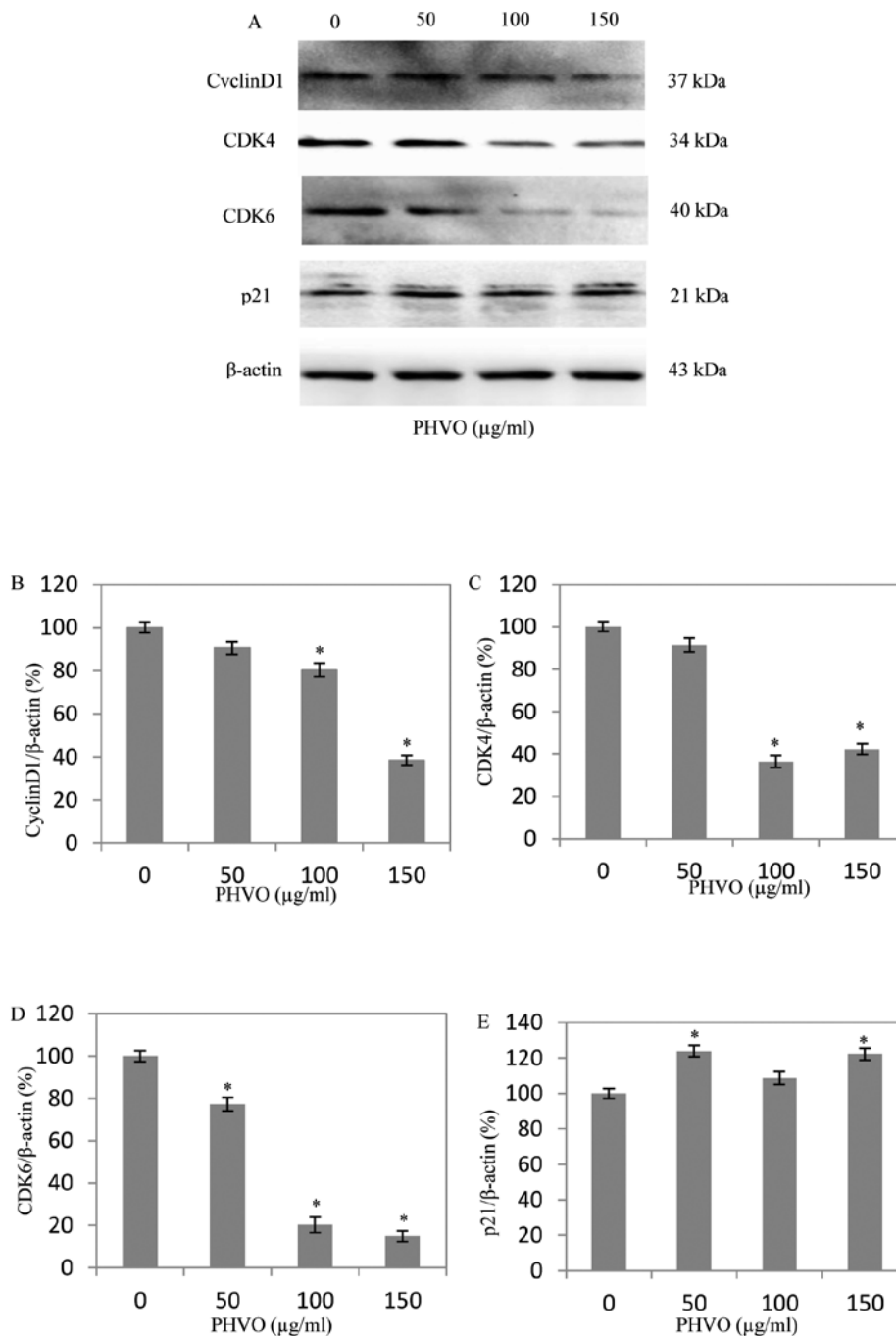


Figure 4. Effect of PHVO treatment on the protein expression levels of cyclin D1, CDK4, CDK6 and p21 in SW1353 cells. (A) SW1353 cells were treated with the indicated concentrations of PHVO for 48 h. The protein expression levels of cyclin D1, CDK4, CDK6 and p21 were determined by western blot analysis.  $\beta$ -actin was used as the internal control. (B-E) Densitometric analysis. The data were normalized to the mean protein expression of the untreated controls (100%). \* $P < 0.05$  vs. controls.

in the G1 phase compared to the untreated cells ( $P < 0.05$ ). These results suggest that PHVO exerts growth inhibitory effects on SW1353 cells by leading to cell cycle arrest at the G1 phase, and thus not allowing the cells to progress further in the cell cycle and proliferate.

*PHVO regulates protein expression of cyclin D1, CDK4, CDK6 and p21.* To further investigate the mechanism behind the anti-proliferative activities of PHVO, we performed western blot analysis to detect the protein expression of cyclin D1, CDK4,

CDK6 and p21 in the PHVO-treated SW1353 cells. The results showed that PHVO treatment decreased the expression of cyclin D1, CDK4 and CDK6 in dose-dependent manner, but increased that of p21 in the SW1353 cells (Fig. 4).

#### Discussion

Chondrosarcoma is the most common primary bone tumor and the second highest cause of cancer-related mortality in the pediatric age group. To date, surgery and adjuvant chemo-

therapy are the main therapeutic approaches for patients with osteosarcoma, leading to extensive side-effects and multidrug resistance. Thus, the prognosis of osteosarcoma after surgery is poor (27-29). Therefore, the ultimate goal remains to discover effective agents, which are affordable and have minimal toxicity. In this study, the volatile components from *Pyrolae herba* were extracted by hydrodistillation and analyzed by GC-MS. The results showed the presence of a wide range of compounds in the volatile oil of *Pyrolae herba*, including terpenoids, aromatics, long-chain hydrocarbons, alcohols, ketones, acids and esters. Twelve components were identified representing 81.62% of the total integrated chromatographic peaks. Terpenoids and acids constitute more than 58% of the *Pyrolae herba* volatile oil, with n-hexadecanoic acid (29.29%) and cedrol (17.08%) as the major components.

In the present study, we investigated the antitumor activity of PHVO. MTT assay was used to evaluate the effects of PHVO on SW1353 cell viability. The results showed that PHVO was effective in inhibiting the proliferation of SW1353 cells.

Cell proliferation is primarily governed by the cell cycle (30). The G1/S transition is one of the two main checkpoints of the cell cycle (31), which is responsible for initiation and completion of DNA replication. In addition, cell cycle progression is also a tightly controlled event regulated positively by main cyclin-dependent kinases (CDK4/CDK6) and their cyclin-regulatory subunits (cyclin D1). Cyclin D1 is a positive regulator which binds to CDK4 or CDK6 to control cell cycle progression from the G1 to the S phase (32). An unchecked or hyperactivated cyclin D1/CDK4 complex often leads to uncontrolled cell division and malignancy (33-35). p21, the first identified inhibitor of cyclin/CDK complexes, was independently isolated as a CDK-binding protein (36). The main role of p21 in the cell cycle regulation is performed by inhibiting the activity of cyclin-CDK complexes (37,38). G1 phase lengthening and S phase shortening could lead to cell cycle arrest and eventually to the inhibition of cell proliferation. In this study, cell cycle distribution was examined by flow cytometry. The results demonstrated that PHVO significantly increased the cell population in the G0/G1 phase and reduced that in the S phase. Furthermore, the expression of cell cycle regulating proteins (cyclin D1, CDK4, CDK6 and p21) was detected by western blot analysis. The results demonstrated that PHVO treatment led to the downregulation of cyclin D1, CDK4 and CDK6 and the upregulation of p21. It was suggested that the molecular mechanism behind the PHVO inhibition of SW1353 cell proliferation was cell cycle arrest at the G1/S phase.

The results of this study demonstrate that the volatile oil of *Pyrolae herba* significantly inhibits the growth of SW1353 cells. Nevertheless, volatile oil is a complex combination of a variety of chemical components, any of which may play a role in inhibiting cell growth. It is not clear as to which of the components contribute most significantly to antitumor activity. Therefore, components of volatile oil from *Pyrolae herba* should be further investigated in order to elucidate the individual antitumor activity of each component. The antitumor activity of the mixture was then compared with the expected antitumor activity calculated by the sum of efficiencies of each compound separately, relative to their proportions in the mixture. If it was higher, a synergy was pointed out, whereas a lower value was representative of an antagonism. In so

doing, it may be helpful to discover more effective antitumor compounds from the volatile oil of *Pyrolae herba*.

In conclusion, the direct application of volatile oil in the treatment of chondrosarcoma has thus far not been considered. However, the presence of potential antitumor compounds in the volatile oil from *Pyrolae herba*, as well as the low toxicity of the oil at rather low doses may provide possibilities for further investigations in the field of antitumor compounds.

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