Petroleum ether extract of *Chenopodium album* L. prevents cell growth and induces apoptosis of human lung cancer cells

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Abstract. *Chenopodium album* L. is a common edible herb distributed in China that has been used as a traditional Chinese medicine for antiviral, antifungal, anti-inflammatory and cancer treatment. However, to the best of our knowledge no previous reports have investigated its the function of its phytochemical extracts in lung cancer cells. The purpose of the present study was to assess the anticancer activities of the phytochemical extracts of *C. album* L. on human non-small cell lung cancer A549 cells. The present findings demonstrated that the petroleum ether (PE) extract of *C. album* L. exhibited significant growth inhibitory effects on A549 with an IC_{50} value of 33.31±2.79 μg/ml. As determined by MTT and colony formation assays, its growth inhibitory effects were dose- and time-dependent. Furthermore, PE extract-treated A549 cells exhibited dose-dependent cell growth arrest at the G1 phase of the cell cycle and cell apoptosis was induced. These results provide useful data on the anticancer activities of *C. album* L. in human lung cancer and demonstrated the novel possibilities of this plant in developing lung cancer therapies.

Introduction

Lung cancer, as one of the most malignant tumors, has a huge social and economic impact on human health in China and the world (1). According to statistics from the National Office on Tumor Cure and Prevention of China, 700,000 people die of lung cancer annually (2). Despite notable advances in the diagnosis and treatment of lung cancer, many of the chemotherapeutic drugs currently used to treat lung cancer are either not highly effective or may lose their efficacies due to the development of drug resistance (3). Hence, it is important to discover and develop novel drugs for lung cancer treatment.

Natural chemicals have much more chemical diversity than synthetic ones, and have long been recognized as privileged scaffolds to develop drugs due to their evolved biological target specificities, and their proven biological targets are predominantly diverse functional proteins of organisms (4,5). Natural chemical library screenings typically yield higher hit rates of drug-like active compounds than ones that are acquired from synthetic molecule library screenings (6). Previous studies have demonstrated that phytochemical extracts or mixtures from several medicinal herbs exhibit anticancer activities *in vitro* or *in vivo* and are valuable natural sources for drug-like active natural compound screenings (7-10).

Chenopodium album Linne is a fast-growing annual weedy plant, belonging to the *Chenopodium* family, which is widely distributed in hot sub-tropical and tropical climates, as well as temperate regions of the world. Studies on various phytochemical constituents of *C. album* have indicated that the plant contains phytochemicals with various pharmacological effects, including antiviral, antifungal, antioxidant, anti-inflammatory, antiallergic and antiseptic activities (11-13). However, to date little research pertaining to the possible anticancer phytochemical constituents of this plant has been performed. Khoobchandani *et al* (14) reported that the ethyl acetate and methanol extracts of *C. album* prevented the cell growth of human breast cancer MCF-7 cells. Although folk medical usage of *C. album* L. in China has been documented, there are no reports of its phytochemical extracts on the possible activity against lung cancer. The present study used medicinal plant phytochemical extract library screening to identify the petroleum ether (PE) extract of *C. album* L. in order to investigate its effects on the proliferation and cell cycle progression of A549 cells. The present results may provide data to support the use of phytochemicals from *C. album* L. to develop novel cancer therapies.

Materials and methods

Preparation of the extracts of plants. Medicinal plant materials were acquired from the wild in Kunming (Yunnan, China) during the summer of 2014 to prepare a phytochemical extract library, which was identified by Dr. Haizhou Li from

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the Faculty of Life Science and Technology of Kunming University of Science and Technology (Kunming, China). For the preparation of the phytochemical extracts, the plant materials, including branches and leaves, were washed, dried, and finely chopped and grinded. The samples were first extracted with 95% ethanol by an ultrasonic method (15), and were subsequently evaporated using a rotary evaporator (EYELA, Tokyo, Japan). Following this, the dried material was successively extracted using PE, and was subsequently treated with chloroform, ethyl acetate, n-butyl alcohol in a Soxhlet extractor (EYELA). Extracts were filtered and concentrated using a rotary evaporator to evaporate until they were dry. All the dried extracts were weighed and solved with 99.9% (v/v) DMSO (Beyotime Institute of Biotechnology, Haimen, China) to prepare stock solutions at concentration of 100 mg/ml. Subsequently, 100 μl of each phytochemical stock solution was allotted into each well of a 96-well microplate to form a phytochemical extract screening library.

Cell lines and culture. Human non-small cell lung cancer A549 cell line was purchased from the Kunming Institute of Zoology, Chinese Academy of Sciences (Kunming, China). A549 cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (ScienCell Research Laboratories, Inc., Carlsbad, CA, USA) and 100 U/ml penicillin and streptomycin (Solarbio Science & Technology Co., Ltd., Beijing, China), asnd were incubated at 37˚C in a humidified incubator (Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 5% CO_2 supplementation.

Anticancer phytochemical extract screening and IC_{50}s determination. A549 cells in 100 μl medium were seeded in a 96-well plate at a density of $5x10^3$ cells/well. Following 24 h, the cells were either treated with phytochemical extracts at different concentrations (3.91, 7.81, 15.63, 31.5, 62.5,125, 250 and 500 μg/ml) for 24, 48 and 72 h, respectively, or treated with 0.5% DMSO as controls. Subsequently, 5 mg/ml MTT (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) solution was added into each well and incubated for 4 h. Following this, the supernatant in each well was discarded and 100 μl DMSO was added. Optical density of each culture was measured at 490 nm using a microplate reader (Infinte-M200 Pro; Thermo Fisher Scientific, Inc.). The percentage of cell growth inhibition was calculated using the following formula: Percentage of cell growth inhibition = (C-T) / C x 100, where C denotes absorbance of control cells and T denotes absorbance of treatment cells. Data were presented in percentages of cell inhibition relative to the control. Percentage of cell growth inhibition was used to determine the IC_{50} values of the anticancer activity of phytochemical extracts using Probit analysis with GraphPad Prism 5.0 software (GraphPad Software, San Diego, CA, USA).

Colony formation assay. A549 cells were plated in 6-well plates at a density of 200 cells/well. Each culture was mixed with a PE extract at concentrations of 0, 16.5, 31.5, 62.5, 125 and 250 μg/ml respectively. Following 12 days of incubation, the cell colonies formed in each well were stained with crystal violet (Beyotime Institute of Biotechnology) after fixation with formaldehyde, and the number of colony formed in each well was manually counted.

Morphological observation of A549 cells treated with a PE extract. Morphology of A549 cells treated with a PE extract concentrations of 62.5 or 31.25 μg/ml, or with 0.5% DMSO control for 72 h was observed under a bright field using an inverted fluorescence microscope (Olympus Corp., Tokyo, Japan) at x200 magnification.

Briefly, A549 cells were cultured in 24-well plates at $1x10^4$ cells/well and were analyzed following 24-h treatment with PE extract (31.25 and 62.5 μg/ml, respectively). Treated cells were fixed with cold 4.0% formaldehyde for 10 min, washed with phosphate-buffered saline (PBS), and incubated with 10 μM Hoechst 33342 (Sigma-Aldrich; Merck Millipore) at 37˚C for 15 min. Subsequently, the cells were washed with PBS and the cell nuclei were observed under a fluorescence microscope (Olympus Corp.).

Cell cycle analysis. A549 cells at 50-60% confluence were treated with a phytochemical extract at concentrations of 31.25 and 62.5 μg/ml, respectively, for 24 h. Cells were subsequently harvested by trypsinization and washed twice with PBS. Afterwards, the cells were fixed with cold 70% ethanol for 24 h at 4˚C and centrifuged at 1,000 x *g* for 5 min. Cell pellets were collected and washed with cold PBS. Finally, the cells were suspended in 500 μl staining buffer of 50 μg/ml propidium iodide (PI) with 100 μg/ml RNaseA (Beyotime Institute of Biotechnology), and incubated at 37˚C for 30 min in the dark. Cell cycle progression was then analyzed by a flow cytometer (BD Biosciences, San Jose, CA, USA). A minimum of 10,000 cells were used for each assay and DNA content histograms were further analyzed by FlowJo 7.6 software (Tree Star, Inc., Ashland, OR, USA) for cell cycle analysis.

Cell apoptosis assays. Prepared A549 cells were cultured in 6-well plates at a density of $5x10^5$ cells/ml and were treated with a PE extract at concentrations of 31.25 and 62.5 μg/ml, respectively, for 24 h. Following treatment, the cells were collected and washed with 1 ml cold PBS, and were resuspended with 250 μl staining buffer (Beyotime Institute of Biotechnology) with Annexin V/fluorescein isothiocyanate (5 μl) and PI (10 μl, 20 μg/ml). Cells were incubated at 37˚C in the dark for 15 min. Finally, the stained cells were analyzed using a flow cytometer. Data were analyzed by FlowJo 7.6 software.

Statistical analysis. All data were presented as the mean ± standard deviation. Student's t-tests were performed to analyze the significant difference between treatment and control data. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Growth inhibitory effects of C. album L. extracts on A549 cells. Cytotoxic activities against A549 cell growth following treatment with phytochemical extracts of PE, chloroform, ethyl acetate, and n-butyl alcohol of *C. album* L. at concentrations of 3.91, 7.81, 15.63, 31.25, 62.5,125, 250 and 500 μg/ml were

Table I. IC_{50} values of extracts of *Chenopodium album L.* and gemcitabine on A549 cell growth.

Samples	IC_{50} values (μg/ml)
Petroleum ether extract	33.31±2.79
Chloroform extract	84.96±5.43
Ethyl acetate extract	304.79±3.92
N-butyl alcohol extract	ND
Gemcitabine	0.45±1.28

IC_{50} values of each extracts of *Chenopodium album L.* or gemcitabine (positive control) were acquired via dose inhibitory assays of A549 cell growth. ND, not determined. Data presented as the mean ± standard deviation of six replicates (n=6). IC_{50}, half maximal inhibitory concentration.

screened and measured respectively for 72 h. Gemcitabine treatment was used as a positive control for cytotoxicity (Table I). The IC_{50} values of these extracts of *C. album* L. toward A549 cell growth were calculated and the PE extract of *C. album* L. exhibited the strongest cell growth inhibitory effect with the lowest IC_{50} value of 33.31±2.79 μg/ml among the extracts screened (Table I). Dose effect assays showed the PE extract of *C. album* L. repressed A549 cell growth in a dose-dependent manner (Fig. 1A). Time effect assays demonstrated that the PE extract of *C. album* L. inhibited A549 cell growth in a time-dependent manner at the various extract concentrations tested (Fig. 1B). These results demonstrated the PE extract of *C. album* L. had a potent and specific growth inhibitory effect on A549 cells.

Inhibitory effects of the PE extract of C. album L. on colony formation in A549 cells. The capability of cell colony formation may represent cell viability after cell inoculation and indicate how cell growth depends on the cell population and the ability of cell propagation. When A549 cells were treated with increasing concentrations of the PE extract of *C. album* L. from 7.81, 15.63, 31.25 and 62.5 to 125 μg/ml, the number of the cell colonies formed was reduced in a dose-dependent manner (Fig. 2). These results demonstrated that the colony formation and cell propagation abilities of A549 cells were sensitive to the treatment of PE extract of *C. album* L.

Morphological changes of A549 cells treated with the PE extract of C. album L. When comparing the morphological properties of control A549 cells treated with 0.5% DMSO and the PE-treated A549 cells, the morphologies of A549 cells treated with the PE extract of *C. album* L. at concentrations of 31.25 and 62.5 μg/ml for 24 h exhibited apoptotic-associated cellular phenotypes, including cell roundness and shrinkage (Fig. 3). PE extract treatment induced the nuclear compaction of A549 cells, whereas the control cells treated with 0.5% DMSO showed normal nuclear morphology (Fig. 4). These cellular phonotypical results indicated that treatment with the PE extract of *C. album* L. may have induced A549 cell apoptosis.

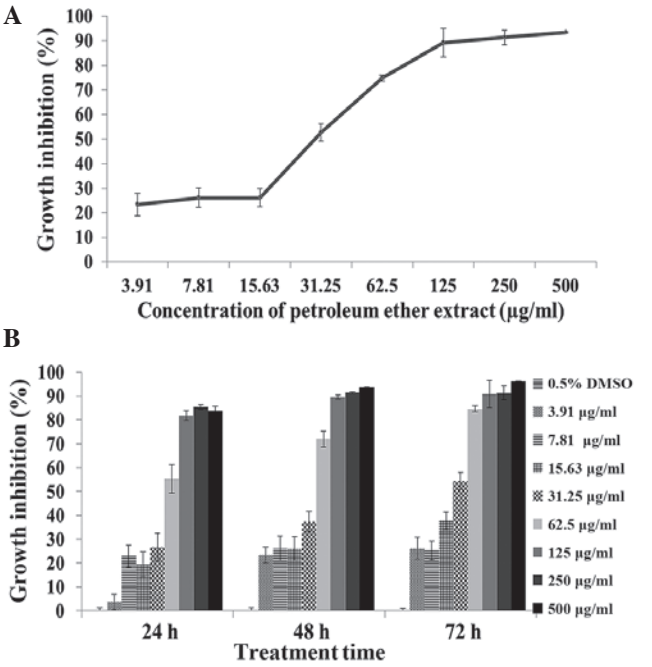

Figure 1. Growth inhibitory effects of the PE extract of *Chenopodium album L.* on A549 cells. (A) Growth inhibition percentages of A549 cells treated with the PE extract at 7.81, 15.63, 31.25, 62.5, 125, 250 and 500 μg/ml, respectively, for 72 h. (B) Growth inhibition percentages of A549 cells treated with the PE extract at 3.91, 7.81, 15.63, 31.25, 62.5, 125, 250 and 500 μg/ml, respectively, for 24, 48 and 72 h. Data presented as the mean ± standard deviation of at least three experiments. PE, petroleum ether.

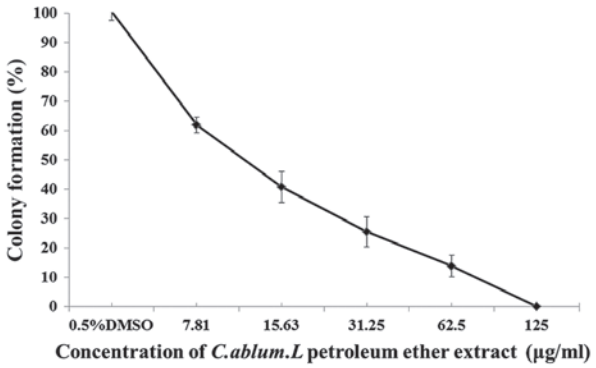

Figure 2. Inhibitory effects of the PE extract of *Chenopodium album* L on the colony formation of A549 cell. PE extract of *Chenopodium album* L. was administered at concentrations of 7.81, 15.63, 31.25 and 62.5 to 125 μg/ml and their cell colony formations were visualized by crystal violet staining and formaldehyde fixing after culturing for 12 days. 0.5% DMSO treatment was the. Data presented as the mean ± standard deviation of at least three experiments. PE, petroleum ether.

A549 cells exhibited G1 phase arrest and apoptosis after treatment with the PE extract of C. album L. To investigate the mechanism of the cell growth inhibitory effect induced by the PE extract on A549, the cell cycle of A549 cells was assessed following treatment with 31.25 and 62.5 μg/ml PE extract for 24 h. The results showed that these phytochemical treatments significantly increased the ratio of the G1 population of the cells (Fig. 5) in a concentration-dependent manner (untreated, 59.37%; 31.25 μg/ml, 69.74%, $P<0.01$; 62.5 μg/ml, 76.93%, $P<0.001$).

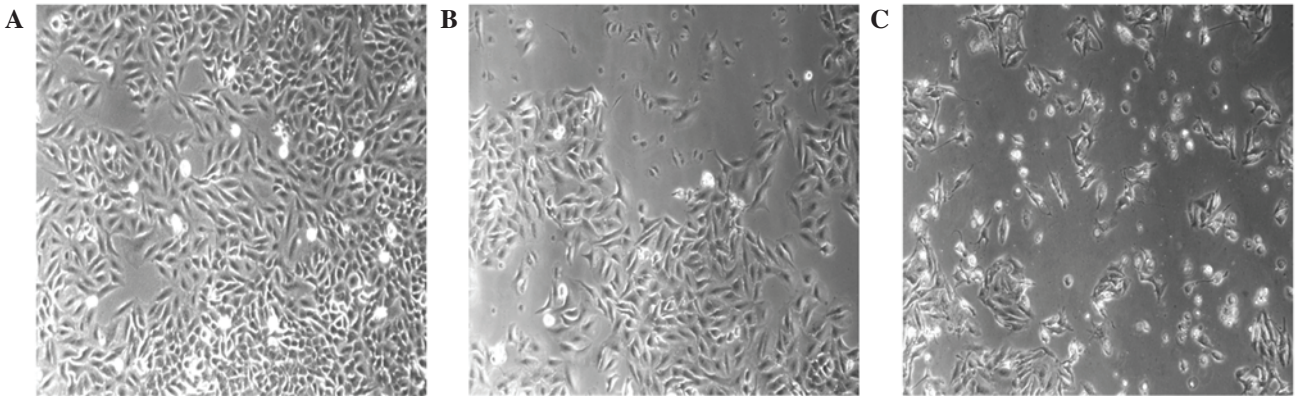

Figure 3. Morphological changes of A549 cells treated with the PE extract of *Chenopodium album* L or (A) 0.5% DMSO as the control. A549 cells grew with the PE extract of *Chenopodium album* L. at concentrations of (B) 31.25 and (C) 62.5 μg/ml for 24 h and were visualized under a bright field using an inverted fluorescence microscope (magnification, x200). PE, petroleum ether.

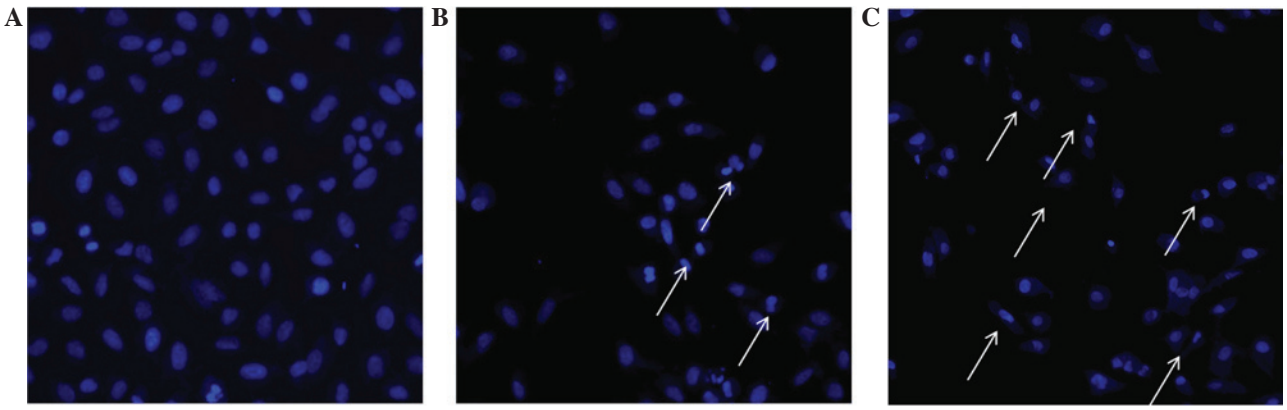

Figure 4. Morphological changes of the nucleus of A549 cells treated by the PE extract of *Chenopodium album* L or (A) 0.5% DMSO as the control. Nuclear morphological changes of A549 cells treated with the PE extract at concentrations of (B) 31.25 and (C) 62.5 μg/ml were visualized under an inverted fluorescence microscope (magnification, x200) for 24 h. Arrows indicate nuclear compaction. PE, petroleum ether.

In addition, the effect of PE extract of *C. album* L. on cell apoptosis was assessed by measuring the ratio of apoptotic cells in the cell population following different PE extract treatments. PE extract-treated A549 cells were subjected to cell apoptosis analysis using a flow cytometer after the cells were stained by Annexin V-FICT/PI. The results showed that A549 cells treated with either 31.25 or 62.5 μg/ml of the PE extract for 24 h exhibited significant increases in the ratio of apoptotic cells in the cell population (untreated, 0.775%; 31.25 μg/ml, 11.9%, P<0.001; 62.5 μg/ml, 22.3%, P<0.001; Fig. 6). These findings indicated that A549 cell growth inhibition following treatment with the PE extract of *C. album* L. may be associated with the induction of cell cycle G1 phase arrest and apoptosis.

Discussion

Chinese medicinal herbs have been widely been used as a folk medicine for centuries in China and southeast Asia (16-18). However, empirical studies related to the action mechanisms of the phytochemicals from these widely used Chinese medicinal herbs remain insufficient. Therefore, the present pilot study was initiated by building a small phytochemical extract library from >50 Chinese medicinal herbs, which was subsequently used as a platform to screen plant constituents of possible novel anticancer activities on an array of *in vitro* human cancer cell lines. This study specifically focused on the phytochemical extracts from *C. album* L. and explored their possible anticancer activities against human non-small cell lung cancer A549 cells. The present findings demonstrated for the first time that the PE extract of *C. album* L. significantly inhibited A549 cell growth in a time- and dose-dependent manner, as determined via MTT and colony formation assays.

Cancer cells generally evade the programmed cell death regulatory pathways of normal tissues to support their malignant growth (19,20) and uncontrolled proliferation, thus the suppression of apoptosis has a key role in cancer development (21,22). To date, various anti-cancer drugs targeting cancer cell apoptosis have been developed from natural chemicals (23,16). The present study demonstrated that the PE extract of *C. album* L. affected the cellular morphology of human non-small cell lung cancer A549 cells, and their proliferative abilities. The present findings also showed that the phytochemical extract induced cellular apoptosis and G1 cell cycle arrest, which may provide important information to develop novel cancer therapies. To evaluate how the PE extract induced A549 cell apoptosis, the nuclear morphology of A549 cells treated with the extract was analyzed using Hoechst 33342 immunofluorescent staining, and the externalization of

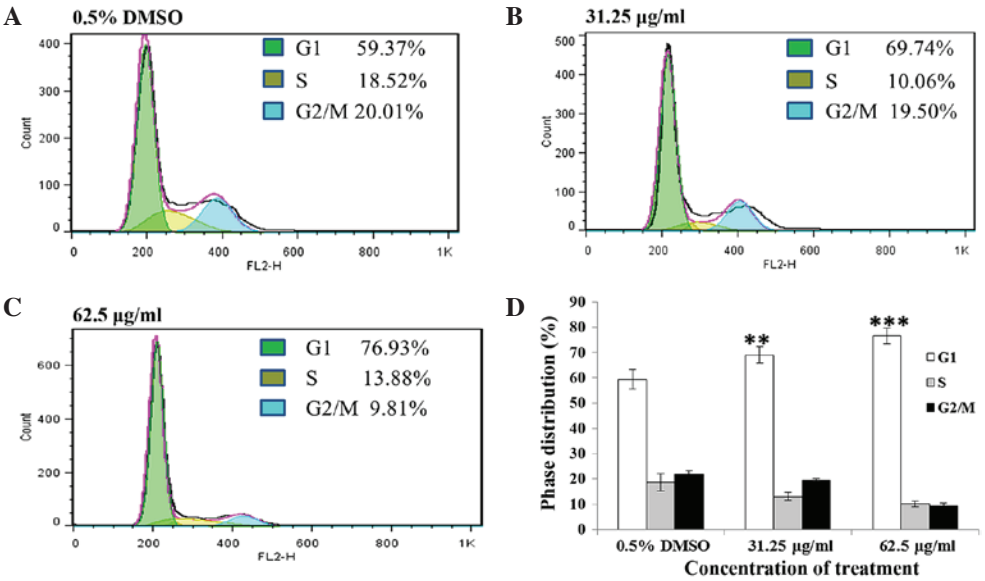

Figure 5. Cell cycle analysis of A549 cells cultured with (A) 0.5% DMSO (control), and (B) 31.25 μg/ml and (C) 62.5 μg/ml of petroleum ether extract of *Chenopodium album* L. for 24 h using flow cytometry after the cells were stained by propidium iodide. (D) Histograms present the percentage of each cell populations at the different cell cycle stage following treatment. Data presented as the mean ± standard deviation of at least three experiments. **P<0.01 and ***P<0.001 vs. the controls.

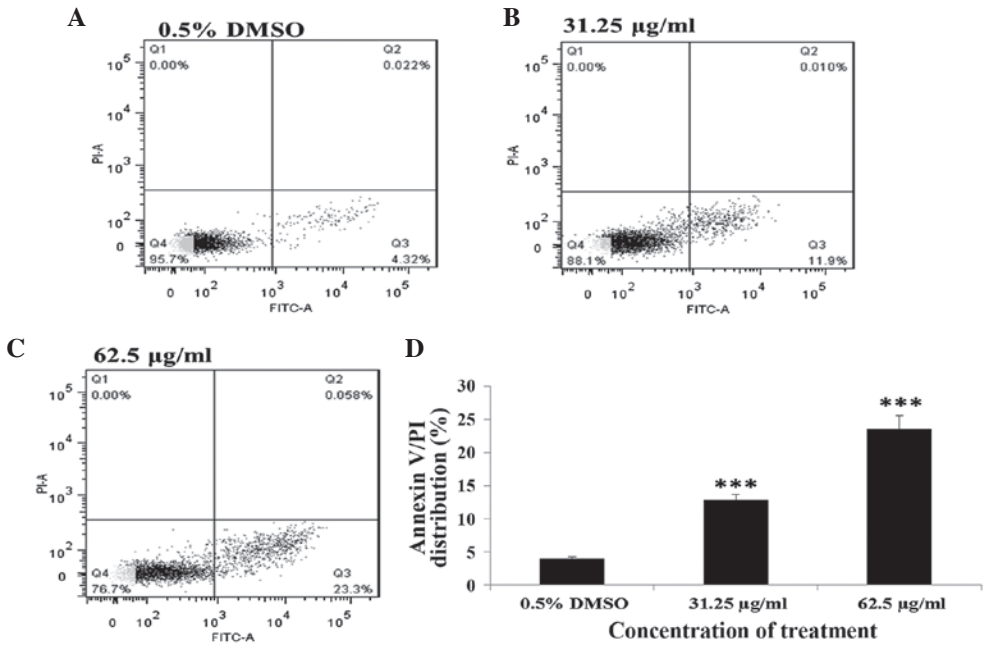

Figure 6. Apoptosis analysis of A549 cells treated with (A) 0.5% DMSO (as control), and (B) 31.25 μg/ml and (C) 62.5 μg/ml petroleum ether extract from *Chenopodium album* L. for 24 h by fluorescence-activated sorting using flow cytometry. (D) Percentages of apoptotic cells in A549 treated with petroleum ether extract at 31.25 μg/ml and 62.5 μg/ml. Data presented as the mean ± standard deviation of at least three experiments. ***P<0.001 vs. the control. FITC, fluorescein isothiocyanate.

phosphatidylserine (PS) of A549 cells treated with the extract using the binding assay of Annexin V to PS followed by cell sorting with a flow cytometer (24,25). The results indicated that the PE extract caused A549 cells to undergo chromatin condensation and externalization of PS, which are typical apoptotic phenotypes.

Cell cycle progress is crucial for cell proliferation (26,27). G1 phase arrest of the cell cycle provides an opportunity for cells to either undergo repair or follow an apoptotic pathway (28,29). Many chemicals developed as anti-tumor agents were designed to target cellular components involved in promoting G1/S transition (30,31). The present results have shown that the PE extract of *C. ambrosioides* L. significantly induced G1 phase arrest of A549 cells, which may be one of the mechanisms to trigger the cell apoptosis. This provides an important base and opportunity to further characterize the natural molecule(s) associated with this significant biological activity in future research.

At present, herbal medicines have been shown to be a promising approach for curing lung cancer (32,33). Since our crude extract is an unfractionated plant extraction, it is possible that the components mediating cell death of different tumor cell are not identical. For a specific plant, different extraction processes may produce a variety of compounds with different concentrations and various bioactivities (34). The present results suggested that there may be valuable active compound(s) against human non-small cell lung cancer A549 cell in the PE extract of *C. album* L. At this stage, it is not possible to elucidate whether these effects on A549 cell growth are induced by specific compounds or are the result of the combined action of multiple compounds in the extract. As an edible Chinese medicinal herb, *C. album* L. has no toxicity and few side effects. *C. album* L is a wild neglected herb which has various pharmacological properties, such as antiviral, antifungal, anti-inflammatory, antiallergic, antiseptic and immunomodulating activities. However it has some side effects since it contains porphyrin. Any plant that contains such a substance belongs to the ling sensitivity plants; after people eat it and are then exposed to sunlight, they are prone to developing a skin disease called phytophotodermatitis (35). therefore, the plant can be consumed as a human food and is expected to benefit individuals with lung cancer (36).

In conclusion, the present study, for the first time, screened different phytochemical extracts from *C. album* L. against non-small cell lung cancer A549 cell to explore their anticancer activities, and demonstrated that the PE extract of *C. album* L. specifically inhibited A549 cell growth by inducing cell cycle G1 phase arrest and cell apoptosis. These results may provide valuable data for assessing the possible usage of phytochemicals from *C. album* L. in exploring and developing novel cancer therapies and healthcare products.

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