

# ACETOACETATE EXTRACT OF *Pleione bulbocodioides* (FRANCH.) ROLFE INDUCES APOPTOSIS OF HUMAN LEUKEMIA THP-1 CELLS THROUGH A MITOCHONDRIA-REGULATED INTRINSIC APOPTOTIC PATHWAY

## EXTRATO DE ACETOACETATO DE *Pleione bulbocodioides* (FRANCH.) ROLFE INDUZ A APOPTOSE DE CÉLULAS THP-1 DE LEUCEMIA HUMANA ATRAVÉS DE UMA VIA APOPTÓTICA INTRÍNSECA REGULADA POR MITOCÔNDRIAS

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**ABSTRACT:** The tubers of three orchidaceous plants, including *Pleione bulbocodioides* (Franch.) Rolfe, have been used as ‘Shan-Ci-Gu’ in traditional Chinese medicine for the treatment of bacterial infections and cancers for thousands of years. In this study, the effects of an acetoacetate (EtOAc) extract of *P. bulbocodioides* on the cell viability and apoptosis of THP-1 (human acute monocytic leukemia cell line) cells and its interaction with possible apoptotic pathways were investigated. THP-1 cells were treated with the EtOAc extract of *P. bulbocodioides* at different concentrations. The results showed that THP-1 cell viability was significantly inhibited by the EtOAc extract of *P. bulbocodioides* with an  $IC_{50}$  of  $51.37 \pm 2.68 \mu\text{g}/\text{mL}$  at 24 h. The examination of cytotoxic effects on healthy cells showed that the EtOAc extract of *P. bulbocodioides* did not show any effect on healthy Vero cells. Selectivity indexes were greater than 15.57, suggesting that the EtOAc extract of *P. bulbocodioides* had selective toxicity against THP-1 cells. The results of annexin V-FITC/PI and DAPI staining showed that the EtOAc extract of *P. bulbocodioides* induced cell apoptosis in a dose-dependent manner. The apoptotic rate was increased in the treatment groups compared with that in the control group ( $P < 0.05$ ). The distribution of cells in the  $G_2$  phase of the cell cycle increased along with typical cell apoptosis-induced morphological changes. The levels of the pro-apoptotic proteins Bax, cleaved PARP and cleaved caspase-3 increased with increasing concentration of acetoacetate extract of *P. bulbocodioides*, while the anti-apoptosis protein Bcl-2 was downregulated. Cyt c and AIF, which are characteristic proteins of the mitochondria-regulated intrinsic apoptosis pathway, also increased in the cytosol with increasing concentrations of the EtOAc extract of *P. bulbocodioides*. These results showed that the EtOAc extract of *P. bulbocodioides* significantly inhibits cell viability and induces cell apoptosis in the human leukemia cell line THP-1 through a mitochondria-regulated intrinsic apoptotic pathway.

**KEYWORDS:** EtOAc extract of *Pleione bulbocodioides* (Franch.) Rolfe. THP-1 cell line. Cell viability. Cell apoptosis. Mitochondria-regulated intrinsic apoptotic pathway.

## INTRODUCTION

According to worldwide statistics, leukemia accounts for approximately 3% of the total incidence of tumors and is the most common cause of malignant tumors in children and young people. The highest incidence rate of leukemia in the world is in Europe and North America, with a mortality rate of 3.2-7.4 per 10 million people. The lowest incidence rate is in Asia and South America, with a mortality rate of 2.8-4.5 per 10 million people. The mortality rate is highest among children and people under the age of 35 years (CREUTZIG et al., 2018; SANDLER; ROSS, 1997). Acute myeloid leukemia (AML) is the most common malignant myeloid disorder in adults, accounting for the largest number of annual deaths from leukemia (XIAN et al., 2016).

Combined chemotherapy is still a critical therapeutic method for human acute monocytic leukemia. However, resistance and intolerance to molecular targeted therapies are important clinical issues. Therefore, the discovery of highly effective drugs with low toxicity for the treatment of leukemia is still an important and urgent task.

The tubers of three orchidaceous plants, *Pleione bulbocodioides* (Franch.) Rolfe, *Cremastra appendiculata* (D. Don) Makino and *Pleione yunnanensis* Rolfe, have been used as ‘Shan-Ci-Gu’ in traditional Chinese medicine for the treatment of bacterial infections for thousands of years (WANG et al., 2013). Phenanthrene and bibenzyl compounds are the main constituents of *P. bulbocodioides* and *C. appendiculata*, and some of these compounds isolated from *Cremastra appendiculata* (D. Don)

Makino have been reported to possess cytotoxic activities in vitro (LIU et al., 2013, 2016). Currently, traditional Chinese medicinal powders, including *P. bulbocodioides*, are attracting increasing attention for their novel uses in the treatment of various cancers, peptic ulcers and uroschisis (MA, 2012; WANG 2012; TONG, 2010). However, few studies have investigated the bioactivities of *P. bulbocodioides* extract, and the official quality control methods for these herbs are still inadequate. There is no report on the bioactivities of *P. bulbocodioides* for the treatment of leukemia. In this study, the anti-leukemia bioactivity of the EtOAc extract of *P. bulbocodioides* was evaluated in one type of acute myeloid leukemia (AML) with the human acute monocytic leukemia cell line, THP-1.

## MATERIAL AND METHODS

### Plant materials, cell lines and reagents

The tubers of *P. bulbocodioides* were collected in Guizhou Province, People's Republic of China. The plant identity was verified by Professor Weike Jiang (Guiyang University of Traditional Chinese Medicine), and a voucher specimen (No. LXP-064540) was deposited at the Herbarium of Guiyang College of Traditional Chinese Medicine (GCTCM).

The human acute monocytic leukemia cell line THP-1 and the mammalian healthy Vero cells (African green monkey kidney cells) were purchased from the Cell Resource Center of the Shanghai Institutes for Biological Sciences of the Chinese Academy of Sciences. XTT was purchased from Biological industries (Beit Haemek, Israel). DMSO was purchased from Sigma (No. D4540,  $\geq 99.5\%$ ). DAPI and PI were obtained from Solarbio (Beijing, China). Annexin V-FITC was purchased from BD Biosciences (San Jose, CA). Antibodies against cleaved poly (adenosine diphosphate [ADP]-ribose) polymerase (PARP) (Asp 214), cleaved caspase-3 (Asp175) (5A1E), Bax and Bcl-2(124) were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against cytochrome c (A-8) and apoptosis-inducing factor (AIF, E-1) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

### Preparation of extract

The dried and pulverized tubers of *P. bulbocodioides* (350 g) were extracted for 24 h with 90% ethanol (1:10 solid to liquid ratio) and then ultrasonically processed for 2 h. The residues were extracted again, and the filtrates were combined.

These two combined filtrates were concentrated in a rotary evaporator at 65°C until dried, resulting in 56 g of crude extract. The crude extract was suspended in water and then partitioned exhaustively with equal volumes of petroleum ether (PE), acetoacetate (EtOAc) and n-butyl alcohol (n-Bu). The obtained fractions, including PE, EtOAc and n-Bu, were evaporated and stored in dark bottles at 4°C. PE, EtOAc and n-Bu fractions were dissolved in DMSO and adjusted to 400 µg/µl. The treatment concentration was obtained by dilution in RPMI-1640 cell media.

### Cell culture

THP-1 and Vero cells were grown in RPMI-1640 medium (Invitrogen, Guangzhou, China) and Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA), respectively, and supplemented with 10% fetal bovine serum, 100 u/mL penicillin, and 100 µg /mL streptomycin; the cells were kept in 5% CO<sub>2</sub> at 37°C and saturated humidity. The cells were passaged with medium changes every 1-2 days. Vero cells were expanded when the monolayer reached confluence after 3±1 days. After reaching 80% confluence, the cells were digested by using Trypsin/EDTA solution (0.25% trypsin and 1 mmol l<sup>-1</sup> EDTA). Cells in the logarithmic phase whose activity was above 98% were used in all experiments starting with 1.6×10<sup>5</sup>/mL.

### XTT assay for detecting cell viability

Exponentially growing THP-1 and healthy Vero cells (1.6×10<sup>5</sup> cells/mL) were seeded into each well of 96-well plates, and EtOAc extract of *P. bulbocodioides* was added at concentrations of 25-800 µg/mL. Culture media with an equal volume of RPMI-1640 and DMEM medium was used as a blank control. Six duplicates were created for each concentration with a total volume of 100 µL per well. Cells without extract but with the same concentration of DMSO (<0.1%) were used as the control group (0 µg/mL). The cells were incubated for 24-48 h, and then 50 µL XTT was added into each well. After further incubation for 4 h, the optical density (A) at 490 nm was measured with a BioRad M450 microplate reader. Each experiment was repeated three times, and the cellular proliferation inhibition rate (CPIR) was calculated using the following equation: CPIR = (1-mean of experimental group/mean of control group) × 100%. IC<sub>50</sub> was calculated with GraphPad Prism software. Optimal organic extracts and extract concentrations were selected for follow-up experiments.

### Toxicity and selectivity index (SI)

The cytotoxic effect of treatment on nonmalignant cells was evaluated using Vero cells as a model. The cytotoxicity of the EtOAc extract of *P. bulbocodioides* against tumor and nonmalignant cells was compared using the selectivity index (SI), which is the ratio between the 50% inhibitory concentration (IC<sub>50</sub>) of the cell proliferation for nonmalignant and tumor cells, where  $SI = IC_{50} \text{ Vero} / IC_{50} \text{ tumor cell}$ . A high SI value ( $\geq 2$ ) of a compound or plant extract suggests selective toxicity against cancer cells, while a compound with low SI value ( $< 2$ ) is considered to have general toxicity, which can also cause cytotoxicity in normal cells (AWANG et al., 2013).

### Apoptosis measurement

THP-1 cells were cultured in the presence of the indicated concentrations of EtOAc extract of *P. bulbocodioides* for 24 h. In this experiment and hereafter, cells treated with the same concentration of DMSO ( $< 0.1\%$ ) but without extract were used as the control group (0  $\mu\text{g/mL}$ ). Apoptosis was measured by flow cytometry using annexin V/propidium iodide (PI) double staining. The cells were resuspended in 100  $\mu\text{L}$  of 1 $\times$  binding buffer. A volume of 5  $\mu\text{L}$  of annexin V-FITC and 10  $\mu\text{L}$  of PI were added and mixed gently. The cells were incubated in the dark for 15 min at room temperature, and then 500  $\mu\text{L}$  of each of the buffers mentioned above were added. Machine detection was performed within 1 h. Flow cytometry was performed with BD FACS CantoII. Each experiment was repeated three times.

### DAPI staining for nuclear morphology detection

THP-1 cells were cultured in the presence of the indicated concentrations of EtOAc extract of *P. bulbocodioides* for 24 h, harvested and washed with PBS one time. The cells were collected in 1.5 mL of an EP tube. After centrifugation, the supernatant fluid was removed and fixed with 1 ml 1:1 methanol to acetone solution (including 0.1% Triton x100) for 5 min. After centrifugation and supernatant fluid removal, the cells were stained with 200  $\mu\text{l}$  DAPI for 7 min, followed by two washes with PBS. The cells were suspended in 100  $\mu\text{l}$  PBS, and images were obtained with analytical fluorescence light microscopy.

### Cell cycle analysis

THP-1 cells were cultured in the presence of the indicated concentrations of EtOAc extract of *P. bulbocodioides* for 24 h. Treated and control THP-1

cells were harvested, washed with PBS, and fixed with 70% ethanol overnight. The cells were centrifuged and washed with PBS and then stained with 50  $\mu\text{g/mL}$  propidium iodide (PI) and 2.5  $\mu\text{g/mL}$  RNase in PBS solution for 30 min at room temperature. DNA content was analyzed by flow cytometry at an emission wavelength of 488 nm. Each experiment was repeated three times.

### Western blot analysis for the expression of apoptosis-related protein

Treated and control THP-1 cells were harvested and washed with PBS. Total cell protein was extracted by RIPA lysis buffer (Solarbio, R0010) with PMSF and quantified by Lowry assay (ZHOU et al., 2015). The cytosolic fraction was prepared with digitonin extraction buffer to detect the levels of Cyt c and AIF in the cytosol, as described previously. The gels and samples were prepared according to conventional methods for protein electrophoresis, and the protein was transferred to the membranes (ZHOU et al., 2015). Rabbit anti-human cleavage-PARP, cleavage-caspase-3, Bcl-2, and Bax and mouse anti-human AIF and cytochrome c monoclonal antibodies (1:800) were added, and the membrane was incubated overnight. After rinsing, horseradish peroxidase (HRP)-labeled goat antirabbit IgG (1:2000) was added, and the membrane was incubated on a shaker for 1 h. Finally, electrochemical luminescence reagents were used in X-ray imaging. GADPH was used as an internal control.

### Statistical analysis

The results are expressed as the mean  $\pm$  standard deviation (SD) values. Statistical differences between the samples were evaluated using appropriate statistical tests (one-way ANOVA, repeated measures ANOVA, Student's t-test). A P-value of  $< 0.05$  was considered significant, where probability values were found to be equal to or less than 0.05. SPSS version 16 was used for statistical analysis.

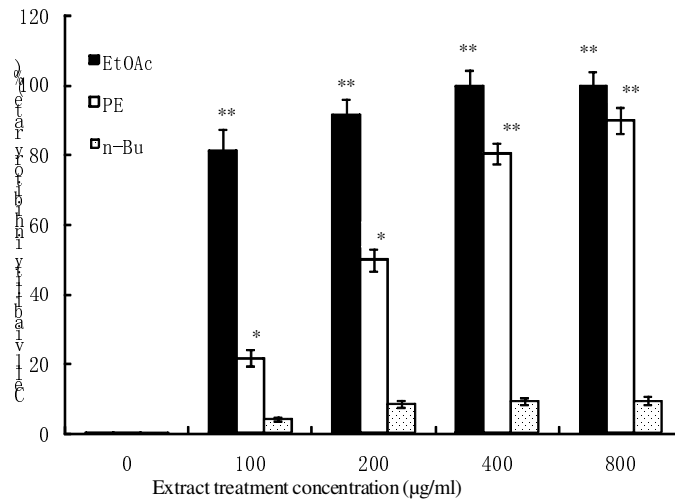
## RESULTS

### Effects of different solvent extracts of *P. bulbocodioides* on THP-1 cell viability

The XTT assay was used to evaluate the cytotoxicity of 800, 400, 200 and 100  $\mu\text{g/mL}$  of PE, EtOAc and n-Bu extracts of *P. bulbocodioides* on the THP-1 cell line. The results showed that the EtOAc extract caused significant decreases in THP-1 cell viability ( $P < 0.01$ ). PE showed weak

cytotoxicity on THP-1 cells ( $P < 0.05$ ), while no significant inhibition of cell viability was detected below a concentration of 800  $\mu\text{g/mL}$  n-Bu extract treatment ( $P > 0.05$ ). Treatment with several concentrations of EtOAc (100, 200, 400  $\mu\text{g/mL}$ ) resulted in a significant increase in the inhibition of

cell viability of THP-1 cells by 83.13%, 94.34% and 100%, respectively ( $P < 0.01$ ) (Figure 1). Therefore, concentrations of 200, 100, 50 and 25  $\mu\text{g/mL}$  of the EtOAc extract treatments were selected for the following experiments.

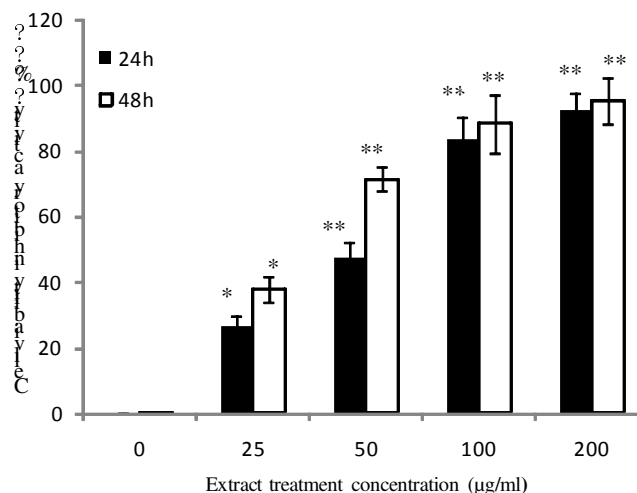


**Figure 1.** The effects of treatment with *P. bulbocodioides* extracts prepared with 3 solvents on the viability of THP-1 cells. Bars represent mean  $\pm$  SD.  $n=6$ . \*  $P < 0.05$  and \*\*  $P < 0.01$  vs 0  $\mu\text{g/mL}$  extract group.

#### Effects of EtOAc extract of *P. bulbocodioides* on THP-1 cell viability and on cytotoxic activity in healthy Vero cells

One objective of this study was to develop a new drug that could be used effectively in the treatment of acute myeloid leukemia. To address this objective, the cytotoxic effects of the EtOAc extract of *P. bulbocodioides* against human acute myeloid leukemia THP-1 cells and healthy Vero cells were investigated. As shown in Figure 2, after treatment with 200, 100, 50 and 25  $\mu\text{g/mL}$  of EtOAc extract for 24 and 48 h, the inhibition of

cell viability in all treatment groups of THP-1 cells was greater than that in the control group. The cell viability inhibitory rate increased significantly ( $P < 0.05$ ) with increasing EtOAc extract concentration and exposure time in a time- and dose-dependent manner. The inhibition rates of THP-1 cell viability were 47.54% and 71.31% after 50  $\mu\text{g/mL}$  EtOAc extract treatment for 24 and 48 h, respectively. The half maximal cell viability inhibitory concentration ( $\text{IC}_{50}$ ) of THP-1 cells treated for 24 h was  $51.37 \pm 2.68$   $\mu\text{g/mL}$ .



**Figure 2.** The effects of the EtOAc extract of *P. bulbocodioides* on the viability of THP-1 cells. Mean  $\pm$  SD,  $n=6$ . \*  $P < 0.05$  and \*\*  $P < 0.01$  vs 0  $\mu\text{g/mL}$  EtOAc extract group.

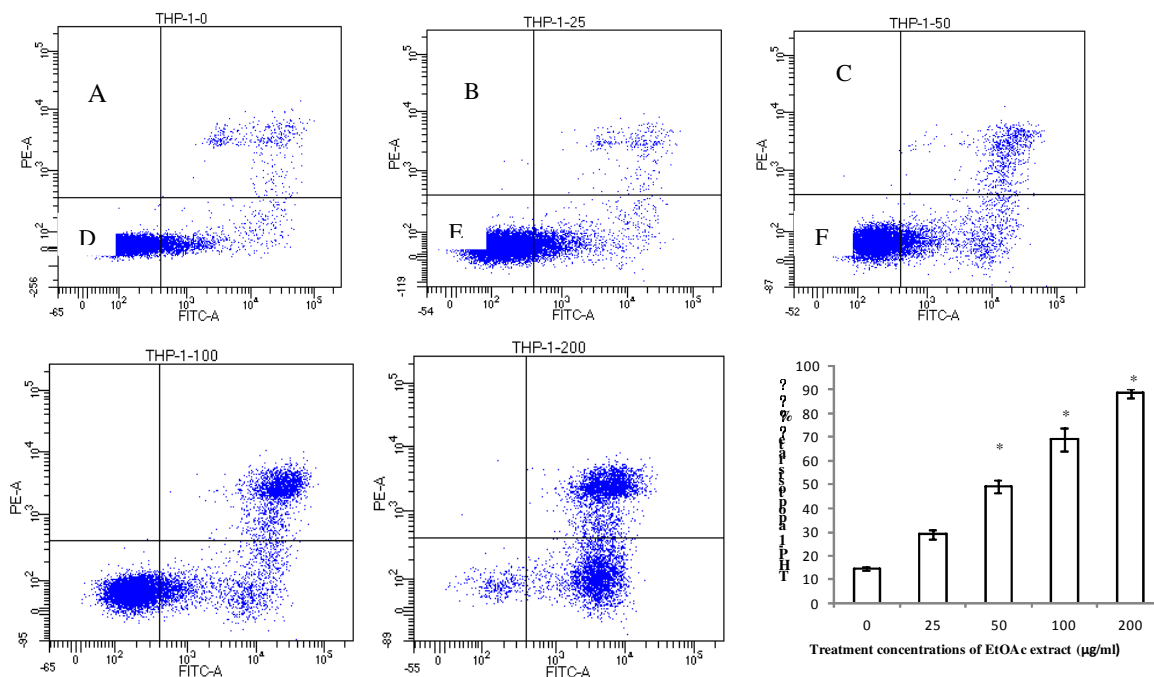
After treatment with 25-800  $\mu\text{g/mL}$  of EtOAc extract for 24 and 48 h, the inhibition of cell viability in healthy Vero cells of all groups was similar to that in the control group (0  $\mu\text{g/mL}$ ) ( $P > 0.05$ ) (data not shown). There was no significant change in cell viability below a concentration of 800  $\mu\text{g/mL}$  EtOAc extract treatment for 48 h ( $P > 0.05$ ). These results indicated that no significant inhibition of cell viability was detected in healthy Vero cells. The half maximal cell viability inhibitory concentration ( $\text{IC}_{50}$ ) of healthy Vero cells was greater than 800  $\mu\text{g/mL}$ . From these results, we know that the EtOAc extract was much more cytotoxic against THP-1 cells because the  $\text{IC}_{50}$  of the healthy Vero cells was approximately 15 times higher than the  $\text{IC}_{50}$  for THP-1 cells. Selectivity indexes ( $\text{SI} = \text{IC}_{50} \text{ Vero} / \text{IC}_{50} \text{ THP-1 cells}$ ) was greater than 15.57. These results suggest that the EtOAc extract of *P. bulbocodioides* did not show cytotoxic effects on healthy Vero cells and had selective toxicity against THP-1 cells.

### Effects of EtOAc extract of *P. bulbocodioides* on THP-1 apoptosis

The annexin-V/PI double staining assay quantitatively detected the effect of EtOAc extract on apoptosis of THP-1 cells. The results suggested that the proportion of apoptotic THP-1 cells gradually increased with the increase in the concentration of EtOAc extract. After treatment with 25, 50, 100 and 200  $\mu\text{g/mL}$  of EtOAc extract

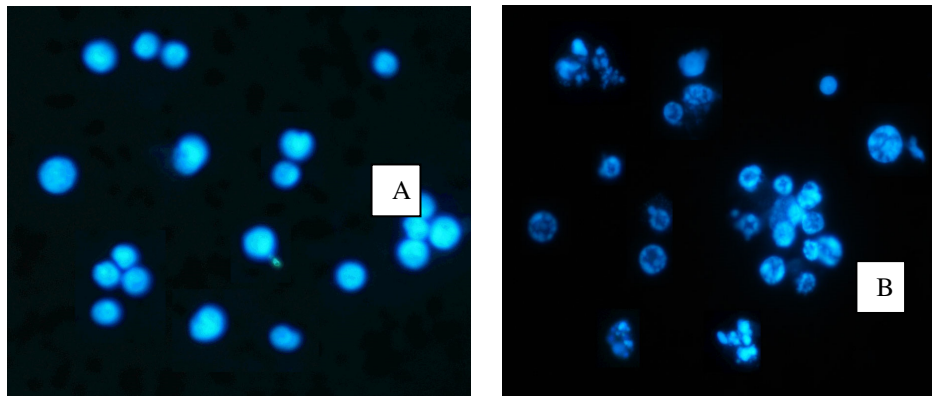
for 24 h, the percentage of apoptotic THP-1 cells was  $(29.1 \pm 2.09)\%$ ,  $(48.9 \pm 3.14)\%$ ,  $(69.3 \pm 3.69)\%$  and  $(88.7 \pm 2.73)\%$ , respectively; there were significant differences compared with the  $(14.7 \pm 1.29)\%$  apoptotic cells in the control group ( $P < 0.05$ ) (Figure 3). These results also indicated that THP-1 cells are sensitive to EtOAc extract treatment.

To further evaluate EtOAc extract-induced apoptosis in THP-1 cells, nuclear morphology was imaged after DAPI staining. Figure 4 shows that cells of the control group had normal nuclear morphology under a fluorescence microscope after DAPI staining, indicating that the chromatin was equivalently distributed in the nucleus. After treatment with different concentrations of EtOAc extract for 24 h, the test group was marked with nuclear fragmentation, condensation of chromatin and the following morphological characteristics of apoptosis: the disappearance of microvilli on the cell surface; blebbing on the cell surface; increased cytoplasmic density; condensed and marginalized chromosomes; condensed nuclei; and the formation of apoptotic bodies. These results indicated that the EtOAc extract of *P. bulbocodioides* is capable of inducing apoptosis in THP-1 cells in a dose-dependent manner. Taken together, these results confirmed the proapoptotic effect of the EtOAc extract of *P. bulbocodioides* on THP-1 cells.



**Figure 3.** EtOAc extract of *P. bulbocodioides* induces apoptosis in THP-1 cells. Cells were exposed to the EtOAc extract of *P. bulbocodioides* at concentrations ranging from 0 to 200  $\mu\text{g/mL}$  for 24 h. Then, the cells underwent annexin V/PI double staining for the apoptotic cell death assay. Panels show (A) control, (B) 25  $\mu\text{g/mL}$ ,

(C) 50  $\mu\text{g}/\text{mL}$ , (D) 100  $\mu\text{g}/\text{mL}$ , (E) and 200  $\mu\text{g}/\text{mL}$ , (F) groups. Bars represent mean $\pm$ SD.  $n=3$ . \*  $P<0.05$  vs 0  $\mu\text{g}/\text{mL}$  EtOAc extract group.



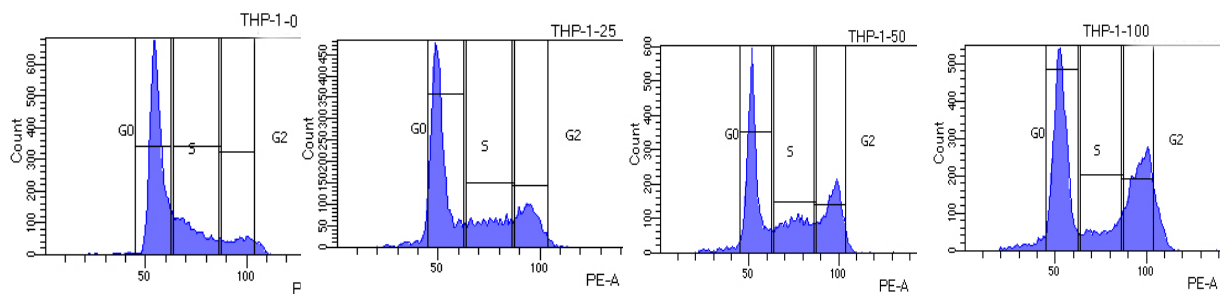
**Figure 4.** The effects of EtOAc extract on apoptosis-induced morphological changes in THP-1 cells (DAPI staining,  $\times 400$ ).

Panels show (A) untreated THP-1 cells and (B) THP-1 cells treated with 50  $\mu\text{g}/\text{mL}$  EtOAc extract

#### Effect of EtOAc extract of *P. bulbocodiioides* on the cell cycle of THP-1 cells

After exposing THP-1 cells to various concentrations of EtOAc extract for 24 h, cell cycle analysis was conducted by using flow cytometry with PI staining. After treatment with different concentrations of EtOAc extract for 24 h, the distribution of the cell cycle changed subsequently.

The proportion of THP-1 cells in the  $G_2$  phase increased gradually with increasing concentrations of EtOAc extract, from 11.4% to 34.5%, while cells in the  $G_1$  phase decreased in a dose-dependent manner from 59.1% to 31.8% ( $P<0.05$ ). The effect on cells in S phase was not obvious (Table 1, Figure 5). The results suggested that the EtOAc extract arrested the THP-1 cell cycle primarily at  $G_2$  phase.



**Figure 5** The effects of EtOAc extract of *P. bulbocodiioides* on the cell cycle distribution of THP-1 cells.

**Table 1.** EtOAc extract of *P. bulbocodiioides* induced cell cycle arrest at  $G_2$  phase in THP-1 cells (Mean $\pm$ SD,  $n=3$ )

EtOAc extract ( $\mu\text{g}/\text{mL}$ )	$G_1$ phase/%	$G_2$ phase/%
0	59.1 $\pm$ 2.89	11.4 $\pm$ 1.58
25	50.2 $\pm$ 3.12	19.1 $\pm$ 2.12
50	40.4 $\pm$ 3.46*	26.4 $\pm$ 3.21*
100	31.8 $\pm$ 2.57**	34.5 $\pm$ 2.31**

\*  $P<0.05$  and \*\*  $P<0.01$  vs 0  $\mu\text{g}/\text{mL}$  EtOAc extract group

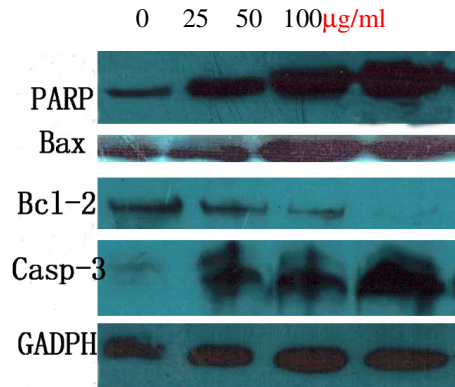
#### Western blot results showed that the EtOAc extract of *P. bulbocodiioides* induced apoptosis through the mitochondrial pathway

The EtOAc extract of *P. bulbocodiioides* treatment was observed to modulate the expression of apoptosis-related proteins in THP-1 cells. Western blot analysis revealed that all

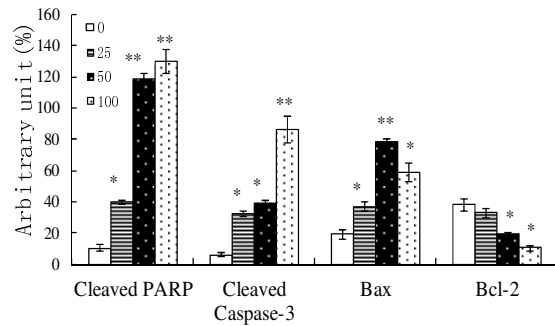
concentrations of EtOAc extract (25, 50, 100, and 200  $\mu\text{g}/\text{mL}$ ) resulted in a significant increase in cleaved caspase-3 and cleaved PARP, which are hallmarks of apoptosis (Figure 6). Mitochondrial dysfunction is regulated by Bcl-2 family proteins; thus, the Bcl-2 family proteins were examined in this study. As shown in Figure 6, the expression of

Bcl-2, one of the antiapoptotic members of the Bcl-2 family, was significantly suppressed with increasing EtOAc extract concentrations, whereas the expression of Bax was significantly increased.

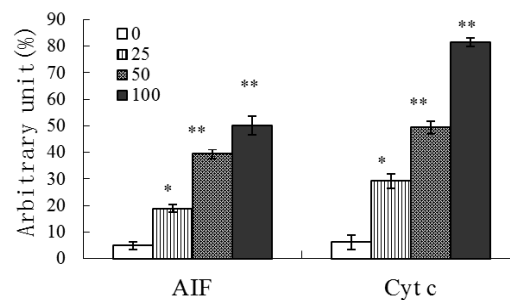
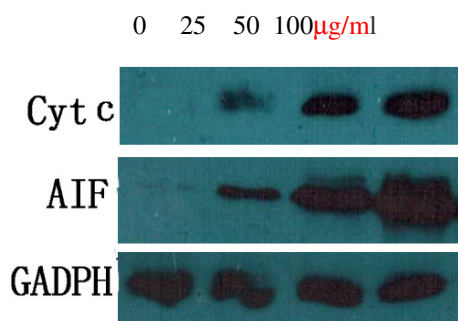
Most chemotherapeutic agents induce apoptosis by triggering the release of Cyt c and AIF from mitochondria into the cytosol. To evaluate the apoptosis pathway that was activated by EtOAc extract treatment, THP-1 cells were exposed to



different concentrations of EtOAc extract, and then Cyt c and AIF levels in the cytosolic fraction were examined by Western blotting. Cyt c and AIF were undetectable in the cytosol of control cells but were released from the mitochondria into the cytosol after EtOAc extract treatment (Figure 7). The results implied that the EtOAc extract could induce apoptosis in THP-1 cells through the mitochondria-regulated intrinsic apoptotic pathway.



**Figure 6.** The effects of EtOAc extract of *P. bulbocodioides* on the protein levels of cleaved PARP, cleaved caspase-3, Bax and Bcl-2. Mean±SD.  $n=3$ . \*  $P<0.05$  and \*\*  $P<0.01$  vs 0 µg/mL EtOAc extract group.



**Figure 7.** The effects of EtOAc extract of *P. bulbocodioides* on the protein expression of AIF and Cyt c in the cytosol. Mean±SD.  $n=3$ . \*  $P<0.05$  and \*\*  $P<0.01$  vs 0 µg/mL EtOAc extract group.

## DISCUSSION

In this study, we described for the first time the detailed pro-apoptotic activity and mechanism of action of *P. bulbocodioides* on the human acute monocytic leukemia cell line THP-1. The results showed that treatment with the n-Bu extract had no significant inhibition of cell viability in THP-1 cells ( $P>0.05$ ), while treatment with the PE extract showed weak inhibition of cell viability, and the EtOAc extract caused significant decreases in cell viability ( $P<0.05$ ). These results indicated that the EtOAc extract of *P. bulbocodioides* has anti-leukemia bioactivity. Some studies have indicated that phenanthrene and bibenzyl compounds are the

main constituents of *P. bulbocodioides* and *C. appendiculata* (LIU et al., 2013, 2016). Therefore, we speculate that the main anti-leukemia active ingredients of *P. bulbocodioides* may be phenanthrenes and bibenzyl compounds. In addition, our study also showed that the EtOAc extract of *P. bulbocodioides* did not show obvious cytotoxic effects on healthy Vero cells. The result of selectivity indexes suggested that the EtOAc extract of *P. bulbocodioides* had selective toxicity against THP-1 cells.

Additionally, the EtOAc extract of *P. bulbocodioides* inhibited the viability of THP-1 cells, and the inhibition was positively correlated with exposure time and concentration. The  $IC_{50}$  of

THP-1 cells treated for 24 h was  $51.37 \pm 2.68 \mu\text{g}/\text{mL}$ . At the same time, EtOAc extract had a strong apoptosis-inducing effect. After being treated with 25-200  $\mu\text{g}/\text{mL}$  EtOAc extract for 24 h, the apoptosis rate of THP-1 cells increased significantly, and typical apoptotic morphological changes were observed. Wang et al also reported that one bibenzyl compound of *Cremastra appendiculata* showed moderate cytotoxic activity against the A549 cell line (WANG et al., 2013).

The apoptosis-inducing effect of EtOAc extract may be closely related to its role in cell cycle arrest. Along with increasing concentrations of EtOAc extract, the proportions of cells in G<sub>2</sub> phase increased gradually, and the proportion of cells in G<sub>1</sub> phase correspondingly decreased gradually. However, the EtOAc extract had little effect on the proportion of cells in S phase. This result indicates that EtOAc extract induces apoptosis primarily through blocking THP-1 cells at G<sub>2</sub> phase. The G<sub>2</sub>/M checkpoint is an important checkpoint of the cell cycle. Exposure to many agents that damage DNA (irradiation or chemical reagents) not only result in the arrest of the cell cycle in G<sub>1</sub> but also at the G<sub>2</sub>/M checkpoint (RUBIN et al., 1993). Moreover, G<sub>2</sub>/M arrest in the cell cycle was more common, indicating its significance in tumorigenesis. G<sub>2</sub>/M arrest may be related to DNA repair. Cells with DNA that has not been repaired exit the cell cycle and enter an apoptotic pathway (HILARY et al., 2017). Heinrich et al reported that the DNA cross-linker-induced G<sub>2</sub>/M arrest in group C Fanconi Anemia Lymphoblasts reflects normal checkpoint function (HEINRICH et al., 1998). Some studies have also shown that chronic myelocytic leukemia (CML) cells transiently arrest in G<sub>2</sub> following X-ray radiation but rapidly progress to apoptosis (BEDI et al., 1995; NISHII et al., 1996). Therefore, we hypothesize that the reversal of G<sub>2</sub> arrest by some agents could abolish the protective effect of BCR-ABL kinase and induce leukemia cell entrance into apoptosis, which is important for the treatment of leukemia.

There are two main apoptotic pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (ELMORE et al., 2007). However, there is now evidence that the two pathways are linked and that molecules in one pathway can influence the other (IGNEY et al., 2002). Activation of the mitochondria-mediated intrinsic apoptotic pathway is governed by Bcl-2 family proteins and is one of the key mechanisms involved in the function of anti-tumor drugs (ZHANG et al., 2015). Bcl-2 is an upstream effector molecule in the apoptotic pathway and has been

identified as a potent suppressor of apoptosis (HOCKENBERY et al., 1993), and most cancers generally overexpress Bcl-2 (REED et al., 1995), thereby escaping apoptosis and undermining therapy. We observed that EtOAc extract significantly downregulated Bcl-2 protein in THP-1 cells (Figure 6). Bax/Bcl-2 regulates the release of Cyt c from mitochondria into the cytosol, and cytochrome c in the cytosol initiates caspases cascades (such as caspase-3/9), which leads to cell apoptosis (CHIPUK et al., 2006). Caspase-9 is activated by cytochrome c released from the mitochondria, which in turn leads to the activation of effector caspases, such as caspase-3, -6 and -7 (CAI et al., 1998). Activated caspase-3 induces cleavage of its substrate PARP, which is a DNA repair enzyme, and ultimately apoptosis (GREEN et al., 2000). Our results revealed that EtOAc extract significantly inhibited Bcl-2 expression and promoted Bax and cleaved caspase-3 expression in a concentration-dependent manner accompanied by an increase in Cyt c and apoptosis inducing factor (AIF) in the cytosol (Figures 6 and 7). Maioral et al. also reported that the compound 1-(3,4,5-trihydroxyphenyl)-dodecylbenzoate strongly increased the expression of AIF in both K562 and Jurkat cells (MAIORAL et al., 2000). Thus, through the interaction with apoptotic protease activating factors (Apaf), Cyt c initiated the activation cascade of caspase-3 once it was released into the cytosol under treatment with EtOAc extract in THP-1 cells (Figures 6 and 7) (CAI et al., 1998). The EtOAc extract also increased levels of cleaved PARP in THP-1 cells in a dose-dependent manner (Figure 6). These events caused the cleavage of 35 kD caspase-3 to generate a 17 kD fragment (Figure 6). When apoptosis starts, 116 kD of PARP in the Asp216-Gly217 between caspase-3 is cut into two fragments (31 kD and 85 kD), and then PARP in conjunction with two zinc finger DNA structures and the carboxy-terminal catalytic domain of separation no longer functions properly (EUSTERMANN et al., 2011). PARP is a family of proteins involved in a number of cellular processes, such as DNA repair, genomic stability, and programmed cell death (NORBURY et al., 2004). PARP inactivation induced by caspase-3 could cause increased DNA damage and nuclease activation, resulting in DNA degradation and cell apoptosis under treatment with EtOAc extract in THP-1 cells (Figure 6) (JAVLE et al., 2011). These results indicated that THP-1 cells treated with EtOAc extract were subjected to apoptosis through the mitochondria-regulated intrinsic apoptotic pathway.



## CONCLUSIONS

In conclusion, cell viability was significantly inhibited by the EtOAc extract of *P. bulbocodioides* in THP-1 cells after treatment for 24 h. The EtOAc extract induced cell apoptosis in a dose-dependent manner.

The distribution of cells in the G2 phase of the cell cycle increased along with typical cell apoptosis-induced morphological changes. In addition, the THP-1 cells treated with the EtOAc extract exhibited an increase in cleaved PARP and cleaved caspase-3 expression, while anti-apoptosis protein Bcl-2 was downregulated.

The increased expression of Bax by EtOAc extract resulted in a loss of mitochondrial membrane potential, which allowed the release of AIF by

mitochondria and promoted the induction of apoptosis.

These results showed that EtOAc extract of *P. bulbocodioides* significantly inhibits cell proliferation and induces cell apoptosis in the human leukemia cell line THP-1 through the mitochondria-regulated intrinsic apoptotic pathway.

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**RESUMO:** Os tubérculos de três plantas orquídeas, incluindo *Pleione bulbocodioides* (Franch.) Rolfe, têm sido usados como "Shan-Ci-Gu" na medicina tradicional chinesa para o tratamento de infecções bacterianas e cânceres por milhares de anos. Neste estudo, os efeitos de um extrato de acetoacetato (EtOAc) de *P. bulbocodioides* na viabilidade celular e apoptose de células THP-1 (linhagem celular de leucemia monocítica aguda humana) e sua interação com possíveis vias apoptóticas foram investigados. As células THP-1 foram tratadas com o extrato EtOAc de *P. bulbocodioides* em diferentes concentrações. Os resultados mostraram que a viabilidade das células THP-1 foi significativamente inibida pelo extrato EtOAc de *P. bulbocodioides* com IC50 de  $51,37 \pm 2,68 \mu\text{g/mL}$  às 24 h. O exame dos efeitos citotóxicos em células saudáveis mostrou que o extrato de EtOAc de *P. bulbocodioides* não mostrou nenhum efeito sobre células Vero saudáveis. Os índices de seletividade foram maiores que 15,57, sugerindo que o extrato de EtOAc de *P. bulbocodioides* teve toxicidade seletiva contra as células THP-1. Os resultados da coloração da anexina V-FITC/PI e DAPI mostraram que o extrato de EtOAc de *P. bulbocodioides* induziu a apoptose celular de maneira dose-dependente. A taxa de apoptose foi aumentada nos grupos de tratamento em comparação com o grupo controle ( $P < 0,05$ ). A distribuição de células na fase G2 do ciclo celular aumentou juntamente com alterações morfológicas típicas induzidas pela apoptose celular. Os níveis das proteínas pró-apoptóticas Bax, PARP clivada e caspase-3 clivada aumentaram com o aumento da concentração do extrato acetoacetato de *P. bulbocodioides*, enquanto a proteína anti-apoptose Bcl-2 foi regulada negativamente. Cyt c e AIF, que são proteínas características da via de apoptose intrínseca regulada por mitocôndrias, também aumentaram no citosol com concentrações crescentes do extrato de EtOAc de *P. bulbocodioides*. Estes resultados mostraram que o extrato de EtOAc de *P. bulbocodioides* inibe significativamente a viabilidade celular e induz a apoptose na linha celular de leucemia humana THP-1 através de uma via apoptótica intrínseca regulada por mitocôndrias.

**PALAVRAS-CHAVE:** Extrato EtOAc de *Pleione bulbocodioides* (Franch.) Rolfe. Linha celular THP-1. Viabilidade celular. Apoptose Celular. Via apoptótica intrínseca regulada por mitocôndrias.

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