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Essential Oil from *Siegesbeckia pubescens* Induces Apoptosis through the Mitochondrial Pathway in Human HepG2 Cells*

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Summary: *Siegesbeckia pubescens* (SP) has been used as a traditional medicine for the treatment of and inflammatory diseases. However, the activities of SP against hepatocellular carcinoma and the related mechanisms remain unclear. The present study aimed to examine the effects of the essential oil of SP (SPEO) on the proliferation of hepatocellular carcinoma cells and the possible mechanisms. The growth inhibition of HepG2 cells was analyzed by MTT assay. Hoechst 33258 and fluorescence microscopy were utilized to observe the nuclear morphological changes of apoptotic cells. Flow cytometry was used to detect cell apoptosis and cell cycle. The expressions of the target proteins were detected by Western blotting. The results showed that SPEO obviously inhibited the proliferation of HepG2 cells in a dose-dependent manner. SPEO activated a series of apoptotic proteins in HepG2 cells, increasing expression levels of Bax, caspase-3 and caspase-9, and decreasing the bcl-2 expression level. SPEO displayed promising anti-hepatocellular carcinoma activities *in vitro*, partly by inducing apoptosis in HepG2 cells through activating the mitochondrial pathway.
Key words: *Siegesbeckia pubescens*; essential oil; hepatocellular carcinoma; apoptosis; mitochondrial pathway

Hepatocellular carcinoma (HCC), a common malignant tumor of the liver, has become one of the most common tumors and the third leading cause of cancer deaths in the world[1]. The Global Burden of Cancer 2013 showed that the growth of HCC mortality is faster than any other cancers, with over 662 000 deaths worldwide per year[1]. Nowadays, there have been no satisfactory treatments available for HCC. Liver transplantation is generally regarded as a very effective method for treating HCC, but only a small proportion of patients can benefit from this therapy because of the advanced stage of disease, donor shortage and expensive treatment cost. Most of HCC patients have to rely on systemic chemotherapy. However, resistance to commonly used chemotherapy and radiotherapy is a great challenge for physicians, and effective anti-HCC drugs have always been desirable.

A number of natural products have been developed as anticancer drugs[2]. *Taxol* is one of the best natural anti-cancer drugs, which has been widely used for clinical breast cancer, ovarian cancer, part of head and neck cancers and lung cancer[3]. Another anticancer drug vincristine, which is purified from *Catharanthus roseus* (Madagascar periwinkle), has long been used for the treatment of non-Hodgkin's lymphoma, acute lymphoblastic leukemia and nephroblastoma[4]. The development of natural compounds as chemo-therapeutic agents against cancers has become a very promising area of research.

Siegesbeckia pubescens (Compositae, SP), a kind of commonly used Chinese herbal medicine "Xi-Xian", is an annual herb widely distributed in China. The aerial parts of SP have been used as a traditional medicine for the treatment of allergic diseases, hypertension, malaria, acute hepatitis, and snakebite[5]. Many reports showed that SP has antitumor activities besides its anti-allergic and anti-inflammatory activities[6–8]. However, the effect and mechanism of the essential oil of SP (SPEO) against HCC has not yet been clarified. In the present study, we aimed to examine the chemical profiling of SPEO, validate the potential anti-HCC effects of SPEO *in vitro*, and elucidate its probable anti-HCC mechanisms.

1 MATERIALS AND METHODS

1.1 Materials and Equipment

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazoliun bromide (MTT) and dimethyl sulphoxide (DMSO) were bought from Sigma (USA). The antibodies used for Western blotting was bought from Cell Signaling Technology (USA). DMEM culture medium (high sugar) was purchased from HyClone (USA), annexin Ⅴ-fluorescein

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isothiocyanate (FITC)/propidium iodide (PI) Apoptosis Detection Kit from BD Pharmingen (China), protein assay kits (BCA) from Beyotime Biotechnology (China), the chemical reagents from Sinopharm Chemical Reagent Co., Ltd. (China) and the microplate reader from Bio Rad 3550 (USA).

1.2 Preparation of Essential Oil

The whole plants of SP were collected in Aug. 14, 2013 in Wufeng county, Hubei province, China, and identified by Professor Ding-rong WAN of School of Pharmaceutical Sciences, South-Central University for Nationalities (SCUN), Wuhan, China. A voucher specimen (No. SC0723) was deposited in the Herbarium of School of Pharmaceutical Sciences, SCUN. Air-dried whole plants of SP (500 g) were boiled with distilled water (3 ×3500 mL, 3 h each) to yield crude essential oil in a 5-L round-bottom flask fitted with a volatile oil distillation apparatus. The obtained residue was extracted with cyclohexane, then dried with anhydrous sodium sulfate and filtered to yield the SPEO. The resulting SPEO was stored at 4°C prior to further analyses and bioassay.

1.3 Gas Chromatographic-mass Spectrometric (GC-MS) Analysis of SPEO

GC-MS analysis of SPEO was performed on an Agilent 7890N/5973iN GC system equipped with a HP-5MS capillary column (30 m × 0.25 mm id; 0.25 μm) and a HP 5975C mass spectrometer (Agilent Technologies, USA). The ionizing energy was set at 70 eV with a scan range of 500 to 700 m/z. The composition was reported as a relative percentage of the total peak area. The flow rate was set at 1.0 mL/min with helium as the carrier gas[9]. Injector and detector temperatures were set at 250°C and 280°C, respectively. Column temperatures were gradually increased from 50°C to 150°C at 4°C /min, and increased to 250°C at 20°C/min, then held for 10 min. SPEO was dissolved in diethylether, and 0.2 μL of the diluted sample was injected manually. The chemical components were identified based on the comparison of their relative retention time (Rt) and their mass spectra with those in the NIST08 database[10], as well as by comparison of their retention indices (RI) with literature data[11, 12].

1.4 Cell Culture

Hepatocellular carcinoma cell lines (HepG2, Hep3B, Huh7, SMMC-7721) and normal liver cells LO-2 were from the American Type Culture Collection (ATCC, USA). HepG2 cells, a kind of human liver cancer cells, were cultured in DMEM with 10% fetal bovine serum (Sijiqing Co., China), 100 U/mL penicillin and 10 μg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2. Cells were usually used in the logarithmic phase for the experiment[13].

1.5 MTT Assay

HepG2, Hep3B, Huh7, SMMC-7721 and LO-2 cells at the logarithmic phase were seeded in the 96-well culture plates at a concentration of 5 × 10^4/mL (100 μL/well). They were treated with different concentrations (0, 12.5, 25, 50, and 100 μg/mL) of SPEO and 25 μg/mL cis-dichlorodiamineplatinum (CDDP). The final volume was set at 200 μL/well. After 24-h treatment with different concentrations of SPEO in 5% CO_2 incubator, the culture medium in each well was discarded, which was followed by addition with 100 μL (5 μg/mL) MTT solution to continue to develop for 4 h. Finally, the MTT solution was discarded, and 150 μL DMSO was added to cultivate for 10 min at 37°C to dissolve the crystals. Absorbance (A) was recorded at 490 nm (A_{490}) using a microplate reader. Inhibition rate= (1–A_{490} value of drug group/A_{490} value of control group) ×100%[14].

1.6 Hoechst 33258 Staining

We observed the apoptosis of nuclear morphology through staining with Hoechst 33258[15]. HepG2 cells in exponential growth were seeded at a final concentration of 5×10^5 cells per well in a 6-well plate, which was treated with 0, 25, 50, 100 μg/mL SPEO, or 25 μg/mL CDDP for 24 h. Then these cells were washed three times with phosphate buffered saline (PBS), and 1.0 mL/well stationary liquid (methanol : acetic acid=3 : 1) was added. Thirty min later, Hoechst 33258 (5 μg/mL) was added to the 6-well plate. Subsequently, the stained cells were observed under an inverted fluorescent microscope (Olympus, Japan).

1.7 Annexin Ⅴ Staining for Apoptosis

HepG2 cells were incubated at 1×10^6 cells/well in 6-well plates containing DMEM medium with 10% FBS. After 24 h, different concentrations of SPEO were added in the plates. The cold PBS was used to wash the cells twice and centrifuged. Then 300 μL 1× binding buffer was added to re-suspended cells, and the cells were incubated with 5 μL of annexin Ⅴ-FITC at room temperature for 15 min in the dark[16]. Then, 5 μL of PI was added to the above suspended cells and continued to incubate for 1 h. Before measurement, another 200 μL 1× binding buffer was added. Flow cytometric analysis was used with a BD Pharmingen (Becton, Dickinson and Company, USA) for detection of apoptosis.

1.8 Western Blotting Assay

Western blotting was used to analyze the expression of proteins in cells. Firstly, cells were collected after lysis with lysis buffer. Cell lysates were put in the fridge at 4°C for 15 min, and centrifuged at 10 000× g for 15 min at 4°C[17]. The supernatant was boiled in loading buffer and separated by 10%–15% SDS-PAGE, and then transferred to a nitrocellulose (NC) membrane, which was incubated in the skim milk for 2 h. Then the NC membrane was immuneblotted with primary antibodies including anti-caspase-3, caspase-9, Bcl-2, Bax and β-actin antibodies (at a dilution of 1 : 1000 to 1 : 4000) at 4°C overnight. After washing three times with TBST, the protein bands were incubated with anti-rabbit (or anti-mouse) antibody for 2 h at 4°C[18]. Finally, the quantitative analysis of membranes was determined by enhanced chemiluminescence (ECL).

1.9 Statistical Analysis

Data were analyzed by using GraphPad Prism 5.0 software. Student's t-test was used to evaluate statistical significance. A P value less than 0.05 was considered to be significant.

2 RESULTS

2.1 Chemical Characterization of SPEO

SPEO was obtained by hydro-distillation with a yield of 0.58% (w/w) according to the dry weight of SP. GC-MS analysis (fig. 1 and table 1) showed that there were 10 different compounds in SP, representing 98.27% of the total oil. SPEO was dominantly comprised of

2-ethyl hexanol (38.84%) and dibutyl phthalate (20.76%), followed by heptacosane (11.04%). Among other compounds, a variety of compounds were found at considerable amounts, including cholesta-5,7,9 (11)-trien-3-ol acetate (6.69%), phthalic acid dodecyl octyl ester (4.58%), phytol (4.08%), 6,10,14-trimethyl-2-pentadecanone (3.95%), 3,5-dehydro-6-malateethoxy cholest-22-ene-21-ol pivalate (3.57%), 2,6,10,15-tetramethyl heptadecane (2.50%), and phthalic acid isobutyl pentadecyl ester (2.26%).

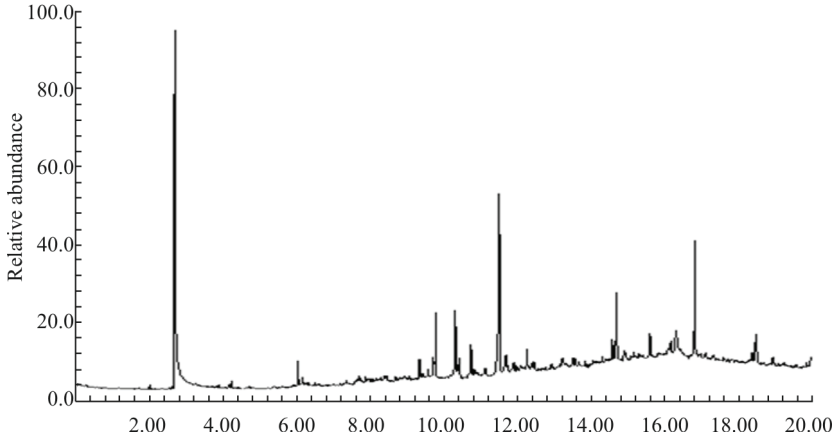

Fig. 1 The GC-MS analysis of SPEO

Table 1 Main components (%) of SPEO

No.	Rt (min)	Compounds	Percentage (%)
1	2.65	2-ethyl hexanol	38.84
2	6.03	2,6,10,15-tetramethyl heptadecane	2.5
3	9.61	6,10,14-trimethyl-2-pentadecanone	3.95
4	10.15	Phytol	4.08
5	10.57	Phthalic acid isobutyl pentadecyl ester	2.26
6	11.32	Dibutyl phthalate	20.76
7	14.43	Phthalic acid dodecyl octyl ester	4.58
8	16.05	Cholesta-5,7,9(11)-trien-3-ol acetate	6.69
9	16.54	Heptacosane	13.04
10	18.15	3,5-dehydro-6-malateethoxy cholest-22-ene-21-ol pivalate	5.57

2.2 Inhibition of Cell Viability by SPEO

As shown in fig. 2A, after treatment for 24 h, SPEO significantly inhibited the proliferation of HCC cells (HepG2, Hep3B, Huh7, SMMC-7721) in a dose-dependent manner. The 50% proliferation inhibition concentrations (IC_{50}) of SPEO were 42.0–95.2 μg/mL against the four HCC cell lines, in which HepG2 cells exhibited better sensitivity to SPEO with the IC_{50} value being 42.0 μg/mL. However, the IC_{50} value of SPEO in L-O2 cell line was higher than 100 μg/mL (fig. 2A). Furthermore, it was found that SPEO could time- and dose-dependently inhibit the proliferation of HepG2 cells (fig. 2B).

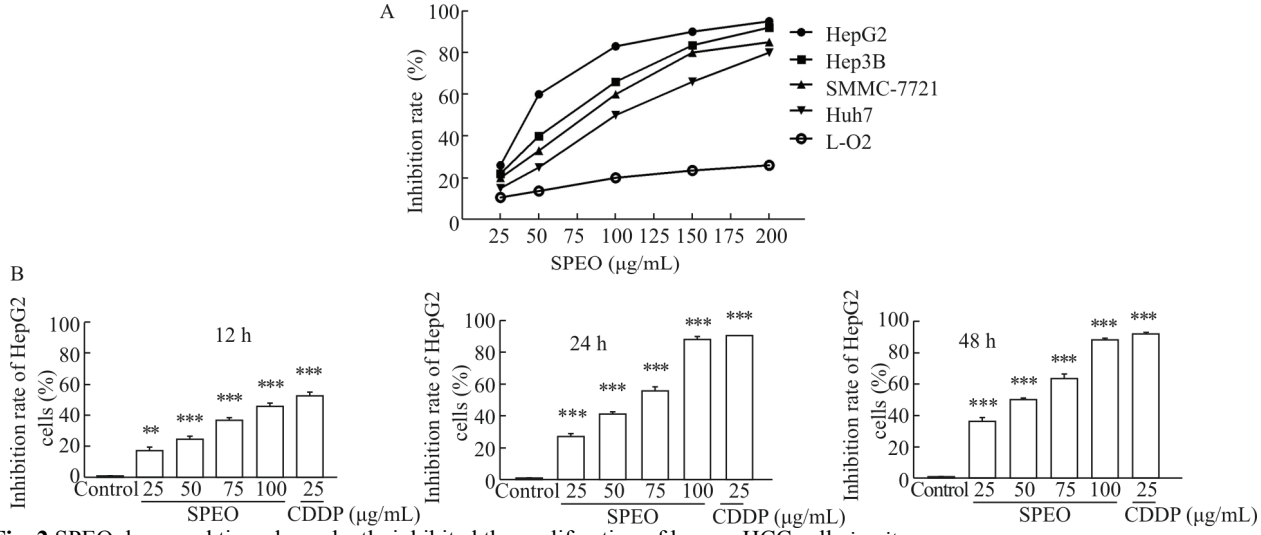

Fig. 2 SPEO dose- and time-dependently inhibited the proliferation of human HCC cells *in vitro*

A: MTT analysis of the inhibitory rate of different hepatic cells treated by SPEO at different concentrations; B: MTT analysis of the inhibitory rate of HepG2 cells treated with 25–100 μg/mL SPEO or 25 μg/mL CDDP for 12, 24 and 48 h. $^{**}P<0.01$ and $^{***}P<0.001$ *vs.* control group

2.3 SPEO-induced Apoptosis of HepG2 Cells *In Vitro*

Human HepG2 cells were treated with 0, 25, 50 and 100 μg/mL SPEO or 25 μg/mL CDDP. After 24 h, cells were directly observed under a phase contrast microscope or a fluorescence microscope after staining with Hoechst 33258. Phase-contrast micrographs revealed that SPEO induced typical morphological characteristics of apoptosis, including cell shrinkage, apoptotic vacuoles, membrane blebbing and formation of floating cells, in a dose-dependent manner (fig. 3A). Fluorescence micrographs showed that more cells significantly shrunk and separated from the culture plate, and apoptotic bodies were increased with the SPEO concentration increasing. Flow cytometry analysis (FACS) was used to examine the cell cycle distribution after treatment with different SPEO concentrations for 24 h. In fig. 3B, the number of HepG2 cells in the G_2/M phase increased significantly, and that in the G_0/G_1 phase decreased with the SPEO concentration increasing during 24 h treatment, suggesting that SPEO arrested HepG2 cells at G_2/M phase. To analyze apoptotic rate of HepG2 cells incubated with different concentrations of SPEO at 24 h, FACS was also performed with Annexin V-FITC apoptosis detection kits. As shown in fig. 3C, SPEO obviously induced the apoptosis of HepG2 cells and cells progressed gradually from the early apoptotic stage to the late apoptotic stage.

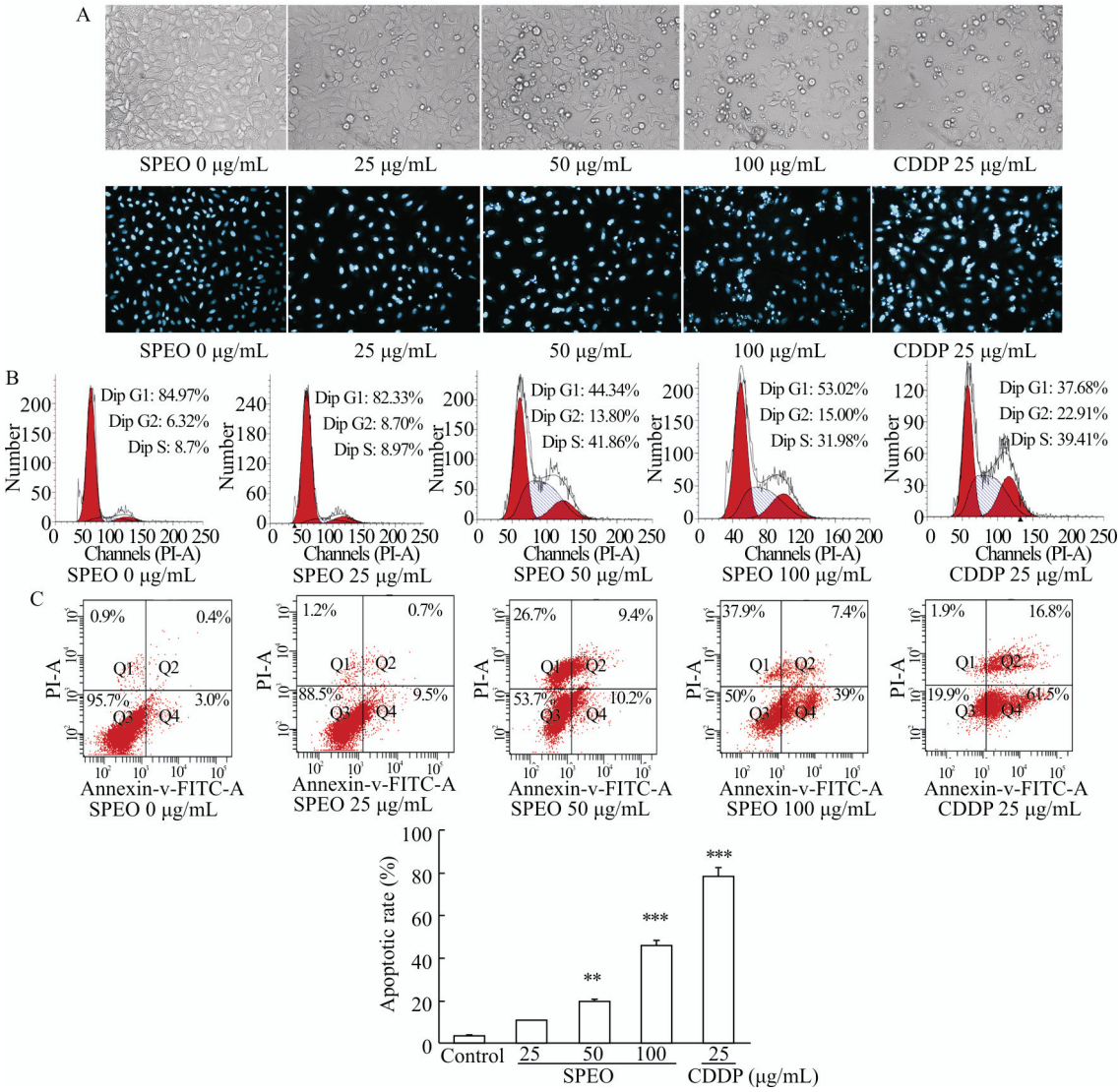

Fig. 3 SPEO dose-dependently induced human HepG2 cell apoptosis *in vitro*.
In the above picture, those left to right represent negative control group, SPEO groups (25, 50, 100 μg/mL), and CDDP group (25 μg/mL), respectively. A: HepG2 cells were observed under a phase contrast microscope (×200, upper row) or an inverted fluorescence microscope (×200, lower row) with Hoechst 33258 staining. B: FACS of cell cycle distribution of HepG2 cells treated with different concentrations of SPEO for 24 h; C: FACS of cell apoptosis after 24-h treatment with SPEO (25–100 μg/mL). Upper and lower quadrants on the right part of the picture represent the apoptosis area. Data were presented as $\bar{x}\pm s$ from three independent experiments, $^{**}P<0.01$, $^{***}P<0.001$ *vs*. control group

2.4 Inhibition of Mitochondrial Pathway by SPEO Treatment

Western blotting (fig. 4) showed that cleaved Bcl-2 expression levels were significantly decreased in SPEO-treated cells. Moreover, the high expression of Bax and low expression of Bcl-2 increased the Bax/Bcl-2 ratio, leading to activation of caspase-9 and caspase-3 through mitochondria. Thus, SPEO induced apoptosis in

HepG2 cells at least partly through mitochondria-dependent pathway.

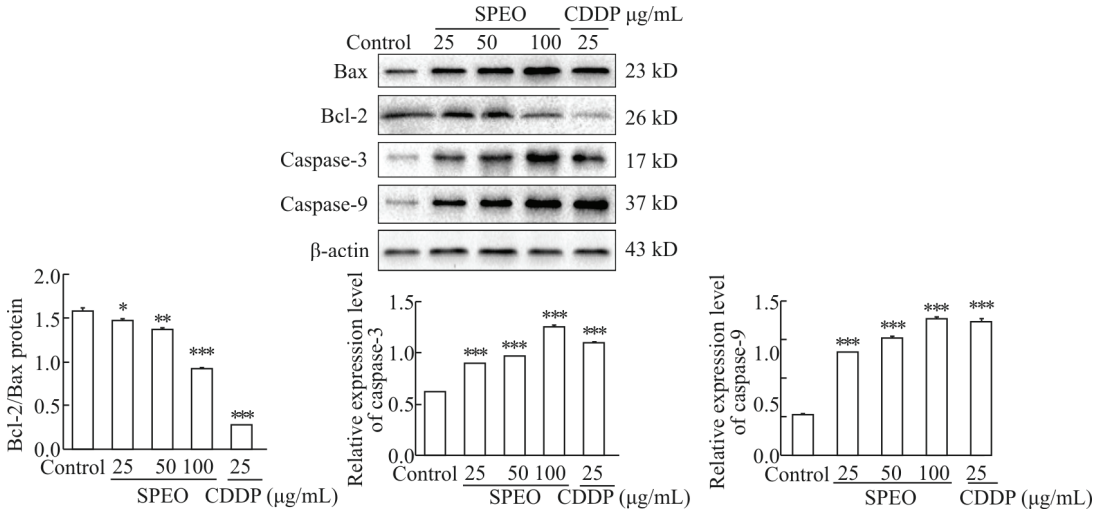

Fig. 4 Western blotting analysis of the caspase-3, caspase-9, Bax and Bcl-2 protein levels in HepG2 cells
β-actin was used as an internal control. The data were shown as $x\pm s$ of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs*. control group

3 DISCUSSION

Many promising advances have been made in the development of novel therapeutic strategies against HCC[19]. However, the prognosis of most HCC patients remains poor. CDDP is a traditional chemotherapy drug with high activity of inhibiting HCC. Nevertheless, it has shown severe hepatotoxicity and nephrotoxicity to patients, leading to poor prognosis, and even tumor growth and metastasis[20]. Natural products (NPs) have played a very important role in fighting cancers, including vincristine[21], taxol[22], camptothecin[23], maytansine[24], cephalotaxine[25] and matrine[26], etc. With few side effects, NPs have attracted more and more attention for their use against cancers.

SP, a Chinese traditional medicine, has been used to treat arthritis for centuries[27]. Previous researches reported that SP contains sesquiterpenes and diterpenes as wells as bioflavonoids[28]. However, the anti-HCC activities and mechanisms of SPEO are still unknown. In the present study, the SPEO was prepared from the whole plants of SP, and the main chemical constituents of SPEO were determined by GC-MS. Four human HCC cell lines (HepG2, Hep3B, SMMC-7721, Huh7) were used to evaluate the cytotoxic activity of SPEO. The cytotoxic results showed that SPEO displayed strong cytotoxic activity on four HCC cell lines with low toxic effects on the normal hepatic cell line LO-2, and HepG2 cells displayed stronger sensitivity to SPEO than other HCC cell lines with the IC_{50} value of SPEO being 42.0 μg/mL. The phase contrast microscopy and fluorescence microscopy showed that SPEO treatment dose- and time-dependently increased the apoptosis rates of HepG2 cells. Furthermore, FACS showed that SPEO induced G_2/M cell cycle arrest in HepG2 cells and increased the total apoptotic rate in a dose-dependent manner.

The mitochondria-mediated apoptosis pathway is also referred to as the intrinsic apoptosis pathway, and it plays a vital role in apoptosis induced by the caspase-dependent pathway[29]. Bcl-2 family members are major apoptosis regulators of the mitochondria. In viable cells, Bcl-2 is one of the major anti-apoptotic proteins integrated within mitochondrial membrane, and Bax is present as an inactive protein in the cytosol or loosely attached to intracellular membranes as a monomer[30]. In apoptotic cells, activated Bax is translocated and integrated into mitochondrial membranes[31]. The high expression of Bax and low expression of Bcl-2 eventually leads to caspasse-3 activation through either homologous dimerization or the promotion of mPTP formation in the inner and outer membranes[32]. In our study, the key apoptotic proteins caspase-3, caspase-9, Bcl-2 and Bax were detected by Western blotting. Results showed that the expression levels of cleaved fragments of caspase-3, caspase-9 and Bax were increased, and cleaved Bcl-2 expression level was significantly decreased in a dose-dependent manner after SPEO treatment for 24 h. Moreover, the high expression of Bax and low expression of Bcl-2 resulted in the increased Bax/Bcl-2 levels, and the activation of caspase-9 and caspase-3 through mitochondria. Thus, it was suggested that SPEO induced HepG2 cells apoptosis at least partly through a mitochondria-dependent pathway.

In conclusion, our study provided some evidence that SPEO had obvious anti-HCC activities. SPEO dose-dependently inhibited the proliferation of HepG2 cells, induced typical morphological characteristics of apoptosis of HepG2 cells, arrested HepG2 cells at G_2/M phase, induced apoptosis of HepG2 cells via mitochondria-dependent pathways. These experimental findings suggest that SPEO is a potential source for treatment of HCC.

Conflict of Interest Statement

The authors declare no competing interests.

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