Antitumor Activity of Dichloromethane Extract from *Salvia plebeia* and Induction of Apoptosis on K562 Cells

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Abstract: **Objective** To study the antitumor activity of extract from *Salvia plebeia* and investigate whether the extract induce apoptosis of K562 cells. **Methods** The aqueous, petroleum ether, dichloromethane (CH₂Cl₂), ethyl acetate, and butanol extracts were prepared from the aerial parts of *S. plebeia*. Taking fluorouracil as reference, the cytotoxic activities of these extracts on HeLa, A549, SGC-7901, HCT-116, K562, LoVo, DU-145, and HepG2 cells were evaluated. To clarify the apoptosis of K562 cells induced by CH₂Cl₂ extract, the methods of Hoechst 33258 staining, flow cytometry assay, and DNA ladder assay were investigated. **Results** The CH₂Cl₂ extract showed the most potent cytotoxic effect against K562 cells, with an IC₅₀ < 15 μg/mL for 3 d treatment. The characteristic apoptotic symptoms such as DNA fragmentation and chromatin condensation were also observed in the K562 cells. **Conclusion** The CH₂Cl₂ extract from *S. plebeia* may inhibit the cancer cell proliferation by inducing cell apoptosis.

**Key words:** antitumor activity; apoptosis; dichloromethane extract; K562 cells; *Salvia plebeia*

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Introduction

*Salvia plebeia* R. Brown is an annual or biennial grass, widely distributed in many countries, especially in China and India. In traditional Chinese medicine, it is named as *Lizhicao* (Jiang, Luo, and Zheng, 1987). Previous phytochemical studies of this plant reported that it contained flavones (Gupta, Ayengar, and Rangaswami, 1975), lignans (Plattner and Powell, 1978; Richard and Ronald, 1976), and diterpenoids (Su, Toshihiko, and Mitsuo, 1986). It has been used as a folk medicine for the treatment of hepatitis (Qu et al, 2009), cough, inflammation (Jung et al, 2009), and haemorrhoids. It was also identified to be a potent anti-oxidant plant (Weng and Wang, 2000). 6-Methoxy-luteolin-7-glucoside, β-sitosterol, and 2'-hydroxy-5'-methoxybiochanin, isolated from *S. plebeian*, had strong anti-oxidant activities (Weng and Wang, 2000). However, whether it has antitumor activity is not yet confirmed.

Apoptosis or programmed cell death is a normal physiological process serving to eliminate unwanted cells and maintain homeostasis in healthy tissues. Tumor growth is regulated by the balance between cell proliferation and apoptosis. Deregulated cell proliferation and suppressed cell death together provide the underlying platform for neoplastic progression (Evan and Vousden, 2001; Kaufmann and Earnshaw, 2000). One essential strategy for cancer therapy is to target the lesions that suppress apoptosis in the tumor cells. It has been found that most cancer chemotherapy drugs exert cytotoxic effects on malignant cells by inducing apoptosis (Kaufmann and Earnshaw, 2000). In search for new cancer therapeutics, the herbs used in traditional medicines for cancer treatment are promising candidates. This paper shows the five different extracts prepared from *S. plebeian* had antitumor activities and our preliminary study found that the dichloromethane (CH₂Cl₂) extract exhibited significant antitumor activities. Hence, the CH₂Cl₂ extract has been further analyzed by testing its apoptotic induction on K562.

Materials and methods

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Reagents
The eight kinds of cells were obtained from Shanghai Jiao Tong University, Shanghai, China. The aerial parts of *Salvia plebeia* R. Brown were collected from Changzhou, Jiangsu, China. MTT [3-(4,5-dime-thylthiazol-2-yl)-2,5-diphenytetrazoliumbromide] was obtained from Biosharp and dissolved in 0.01 mol/L PBS. Annexin V-FITC Apoptosis Detection Kit was obtained from Ruibang Xingye Science Co., Ltd., Beijing, China. All chemical reagents were purchased from Changzhou Runyou Chemical Reagent Co., Ltd., Jiangsu, China.

Preparation of extract
*S. plebeia* was dried at room temperature. Totally, 1.5 kg crude herb was minced and extracted in boiling water for three times, 2, 1.5, and 1 h, respectively. The extracts were combined by filtering with gauze and concentrated to yield 132.0 g water extracts which were extracted in an equal volume of petroleum ether (PE). The insoluble residue was re-extracted in the same volumes of PE for three times. The residue left was extracted by equal total volumes of CH2Cl2, ethyl acetate (EtOAc), and butanol (BuOH), consecutively, in a similar manner as for PE. The specific process was illustrated by Fig. 1. All chemicals and solvents used were of analytical grade. The liquid phase was separated from the solid by filtration and concentrated by a rotary evaporator to dryness, and then redissolved in DMSO at 300 mg/mL for the bioassays.

Cell culture
HeLa, A549, HepG2, SGC-7901, HCT-116, K562, LoVo, and DU-145 cell lines were used in this study. The cell culture was maintained in RPMI-1640 medium except A549 and HepG2 (in DMEM medium) supplemented with 10% newborn bovine serum, 50 μg/mL Penicillin and Streptomycin at 37 °C in humidified atmosphere with 5% CO2. All cells to be tested in the following assays had a passage number of 3.

MTT assay
The eight kinds of cells in exponential growth stage were harvested from culture by centrifuging at 1500 r/min for 3 min, and resuspended in fresh medium at a cell density of 3 × 10⁶ cells/mL. The cell suspension was dispensed into a 96-well microplate at 90 μL/well, incubated for 16 h, to which 10 μL/well of complete medium containing the extracts tested was added and maintained in a CO₂ incubator with 5% CO₂ at 37 °C. After 3 d treatment, 10 μL of PBS solution containing 5 mg/mL MTT was added to each well and further incubated for 4 h (Mosmann, 1983). The cell (except K562) supernatant was then inhaled by aspirator and the cells from each well were dissolved with 100 μL DMSO for optical density reading at 570 and 630 nm, while K562 cells were added with 100 μL 10% SDS and incubated over night. The IC₅₀ of the extracts on cells was calculated by the Logit method (Wang *et al.*, 2007; Fazal *et al.*, 2005).

Measurement of apoptosis by flow cytometry
Apoptosis was determined by an apoptosis detection kit (Annexin V—FITC Apoptosis Detection Kit). Briefly, after drug treatment, K562 cells were collected and washed twice with PBS and once with a binding buffer (HEPES-buffered saline solution supplemented with 2.5 mmol/L calcium chloride). The cells were dissolved in the binding buffer at a concentration of 10⁵ cells/mL. PI and FITC-labeled annexin V were added to the solution, and the samples were incubated for 15 min before being analyzed within 1 h on an FACSscan (Becton-Dickinson). The left lower section of fluorocytogram (An⁻, PI⁻) represents the normal cells; Right lower section of fluorocytogram (An⁻, PI⁺) represents early and median apoptosis cells, Right upper section of fluorocytogram (An⁺, PI⁻) represents late apoptosis cells (Jalving *et al.*, 2006; Liu *et al.*, 2007). Statistical significance was accepted for *P* < 0.05 by One-way ANOVA vs the control.

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**Fig. 1** Flow chart of the procedure for preparation of various extracts from *S. plebeia*
Immunofluorescent staining

K562 cells in exponential growth stage were harvested from culture by centrifuging at 1500 r/min for 3 min, and resuspended in fresh medium at a cell density of $3 \times 10^4$ cells/mL. Cover glasses were placed in a 24-well plate and the cell suspension was dispensed on the cover glasses at 900 μL/well, incubated for 16 h, to which 100 μL/well of complete medium containing the CH2Cl2 extract tested was added, and maintained in an incubator with 5% CO2 at 37°C. The concentrations of the CH2Cl2 extract were 15 and 30 μg/mL.

After treatment for 72 h, cells were harvested and transferred into 1.5 mL centrifuge tubes. The supernatant was dislodged and the cells were fixed with 4% formaldehyde for 30 min. Then the cells were washed with PBS again and incubated with Hoechst 33258 solution (0.5 μg/mL in PBS) for 15 min at RT in dark condition. The nucleus was stained with Hoechst 33258 solution. The cells were washed with PBS for three times and moved to the glass slides with coverslips on them, then the cells were observed through fluorescence microscope (UV). Images were obtained using confocal microscopy (Nikon, Japan).

DNA fragmentation analysis

K562 cells in exponential growth stage were harvested from culture by centrifuging at 1500 r/min for 3 min, and resuspended in fresh medium at a cell density of $1.5 \times 10^5$ cells/mL. The cell suspension was dispensed into a 6-well microplate at 2.7 mL/well, incubated for 16 h, to which 300 μL/well of complete medium containing the CH2Cl2 extract tested was added, and maintained in a CO2 incubator with 5% CO2 at 37°C. The concentrations of the CH2Cl2 extract were 15 and 30 μg/mL.

After treatment for 72 h, cells were harvested and lysed in a buffer containing 20 mmol/L Tris (pH 8.0), 10 mmol/L EDTA, and 0.5% Triton X-100 for 30 min on ice. Cell lysates were then shaked and cleared by centrifugation. DNA in the supernatant was extracted and precipitated with an equal volume of isopropanol with 0.5 mol/L sodium chloride over night at 4°C. After washing with 70% ethanol for three times, the DNA pellets were dissolved in a buffer containing 10 mmol/L Tris (pH 8.0) and 1 mmol/L EDTA (pH 8.0). Fragmented DNA was electrophoresed in 1.5% agarose gels dissolved in 0.5 μg/mL ethidium bromide for 2 h at 60 V.

Results

Inhibition of cell growth by various S. plebeia extracts

When cells were treated with various concentrations of different extracts from S. plebeia for 72 h, we found that cell survival rates were decreased compared with the control. The IC50 for different extracts was calculated using the MTT assay (Table 1). EtOAc and CH2Cl2 extracts exhibited significant inhibition of cell growth, while the H2O and PE extracts did not show a consistent effect on cell growth. CH2Cl2 extract even showed better effect than 5-fluorouracil (5-Fu) on K562, DU-145, HepG2, and LoVo cells. CH2Cl2 extract, whose IC50 value was relatively the lowest on K562, was chosen for all the subsequent experiments.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>IC50 / (μg·mL⁻¹)</th>
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<tbody>
<tr>
<td>CH2Cl2</td>
<td>28, 24, 13</td>
</tr>
<tr>
<td>EtOAc</td>
<td>&gt; 100, &gt; 100, 15</td>
</tr>
<tr>
<td>BuOH</td>
<td>&gt; 100, &gt; 100, 43</td>
</tr>
<tr>
<td>PE</td>
<td>&gt; 100, &gt; 100, &gt; 100</td>
</tr>
<tr>
<td>water</td>
<td>&gt; 100, &gt; 100, &gt; 100</td>
</tr>
<tr>
<td>5-Fu</td>
<td>23, 23, 2</td>
</tr>
</tbody>
</table>

Table 1 Inhibitory effects of five extracts and 5-Fu by MTT assay on different cells
Fig. 2  K562 cells treated with different concentrations of CH$_2$Cl$_2$ extract for 24, 48, and 72 h (X ± s, n = 3)

Chromatin condensation in K562 cells
The change of nuclear morphology in the K562 cells after treatment with 15 and 30 μg/mL CH$_2$Cl$_2$ extract for 72 h was observed under fluorescence microscopy (Fig. 4). Chromatin condensation, a specific and distinct feature of apoptotic cells was found in the majority of treated cells.

DNA fragmentation in K562 cells
Another characteristic event of cell apoptosis is the fragmentation of genomic DNA into integer multiples of 180—200 bp units producing a characteristic ladder on agarose gel electrophoresis. This event was observed.

Fig. 3  Dose-dependent apoptosis of K562 cells by CH$_2$Cl$_2$ extract
K562 cells were incubated with various doses of CH$_2$Cl$_2$ extract for 24 h and flow cytometry was performed using Annexin V-FITC. Apoptosis is represented by the apoptosis rate. A: Compared with the control, apoptosis is observed from 15 μg/mL of CH$_2$Cl$_2$ extract and then increases in a dose-dependent manner. B: Dot plots of apoptosis

Fig. 4  Nuclear morphology of K562 cells in control culture (A), culture treated with 15 (B), and 30 (C) μg·mL$^{-1}$ CH$_2$Cl$_2$ extract for 72 h
Cells were stained with Hoechst 33258 and observed under fluorescence microscopy
in the K562 cells within 72 h after treatment with CH2Cl2 extract at 15 and 30 μg/mL (Fig. 5). Compared with the control (B), the genomic DNA of CH2Cl2 extract-treated cells at 30 and 15 μg/mL (C and D) shows a disperse pattern and has a dose-effect relationship.

**Fig. 5 Induction of DNA fragmentation in K562 cells by CH2Cl2 extract**

Lane A: Marker; Lane B: control; Lane C: treated by 30 μg·mL⁻¹ CH2Cl2 extract; Lane D: treated by 15 μg·mL⁻¹ CH2Cl2 extract

**Discussion**

In this research, we demonstrated that the CH2Cl2 extract of *Salvia plebeia* exhibited potent cytotoxicity on K562, SGC-7901, and DU-145 cells with an IC50 < 15 μg/mL. Compared with 5-Fu, the IC50 of K562 cells was relatively the lowest. Based on the above data, we next focused our effort on investigating the cell apoptosis of K562 cells by CH2Cl2 extract.

Apoptosis is a form of programmed cell death that is characterized by a variety of morphological features, including changes in the plasma membrane such as loss of membrane asymmetry and attachment, cell shrinkage, chromatin condensation and chromosomal DNA fragmentation (Reed, 1997; 2000; Finkel, 1999; Sun, Hail, and Lotan, 2004). Annexin V-PI double staining was used to detect apoptosis of K562 cells treated by CH2Cl2 extract. And the number of apoptotic cells increased significantly. Data from cell morphological assessment and flow cytometry showed that, CH2Cl2 extract could induce K562 cell shrinkage, chromatin condensation, and loss of membrane phospholipid asymmetry, with translocation of phosphatidylserine from the inner leaflet of the phospholipid bilayer to the cell surface. These results indicated that CH2Cl2 extract may induce apoptosis in K562 cells.

Based on all those results, we come to a conclusion that CH2Cl2 extract may be a novel inducer of apoptosis in K562 cells and a tumor suppressor *in vitro*. However, the active ingredients responsible for the observed effect and the specific molecular signaling pathways for induction of apoptosis remain to be identified.

**References**


