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 (CH_2Cl_2), 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 CH_2Cl_2 extract, the methods of Hoechst 33258 staining, flow cytometry assay, and DNA ladder assay were investigated. **Results** The CH_2Cl_2 extract showed the most potent cytotoxic effect against K562 cells, with an $IC_{50} < 15$ 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 CH_2Cl_2 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 (CH_2Cl_2) extract exhibited significant antitumor activities. Hence, the CH_2Cl_2 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 CH_2Cl_2, 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.

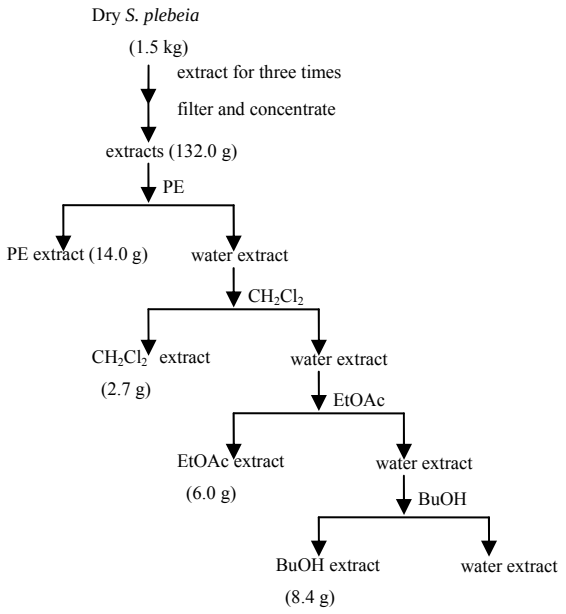

Fig. 1 Flow chart of the procedure for preparation of various extracts from *S. plebeia*

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 ℃ in humidified atmosphere with 5% CO_2. 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 3×10^4 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 CO_2 incubator with 5% CO_2 at 37 ℃. 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 IC_{50} 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 10^5 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 FACScan (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.

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 3×10^4 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 CH_2Cl_2 extract tested was added, and maintained in an incubator with 5% CO_2 at 37 ℃. The concentrations of the CH_2Cl_2 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 1.5×10^5 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 CH_2Cl_2 extract tested was added, and maintained in a CO_2 incubator with 5% CO_2 at 37 ℃. The concentrations of the CH_2Cl_2 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 ℃. 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 IC_{50} for different extracts was calculated using the MTT assay (Table 1). EtOAc and CH_2Cl_2 extracts exhibited significant inhibition of cell growth, while the H_2O and PE extracts did not show a consistent effect on cell growth. CH_2Cl_2 extract even showed better effect than 5-fluorouracil (5-Fu) on K562, DU-145, HepG2, and LoVo cells. CH_2Cl_2 extract, whose IC_{50} value was relatively the lowest on K562, was chosen for all the subsequent experiments.

K562 cells were treated with 0, 2.5, 5, 10, 20, and 40 μg/mL of CH_2Cl_2 extract for 24, 48, and 72 h and cell proliferation was observed. Mean proliferation inhibitory rate was calculated as follows: 1 − (mean optical density of experimental group / mean optical density of control) × 100%. This inhibition occurred in a dose- and time-dependent manner (Fig. 2).

Dose-dependent apoptosis induced by CH_2Cl_2 extract

K562 cells were treated with various doses (0, 15, and 30 μg/mL) of CH_2Cl_2 extract for 24 h. The cells were harvested as described in "Materials and methods" and apoptosis was examined by flow cytometry. Apoptotic induction was 2.1-fold higher after treating with 30 μg/mL CH_2Cl_2 extract and this effect was dose-dependent (Fig. 3).

Table 1 Inhibitory effects of five extracts and 5-Fu by MTT assay on different cells

Extracts	IC_{50} / (μg·mL^{-1})							
	HeLa	A549	SGC-7901	HCT-116	K562	DU-145	HepG2	LoVo
CH_2Cl_2	28	24	13	20	13	12	27	19
EtOAc	> 100	> 100	15	72	38	26	> 100	30
BuOH	> 100	> 100	43	> 100	64	> 100	> 100	37
PE	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
water	> 100	> 100	> 100	> 100	> 100	> 100	> 100	> 100
5-Fu	23	23	2	2	62	31	> 100	49

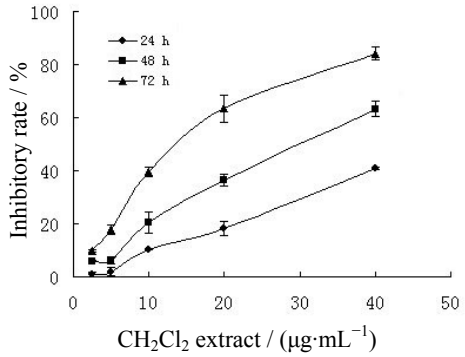

Fig. 2 K562 cells treated with different concentrations of CH_2Cl_2 extract for 24, 48, and 72 h ($\bar{X} \pm s$, $n = 3$)

Chromatin condensation in K562 cells

The change of nuclear morphology in the K562 cells after treatment with 15 and 30 μg/mL CH_2Cl_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

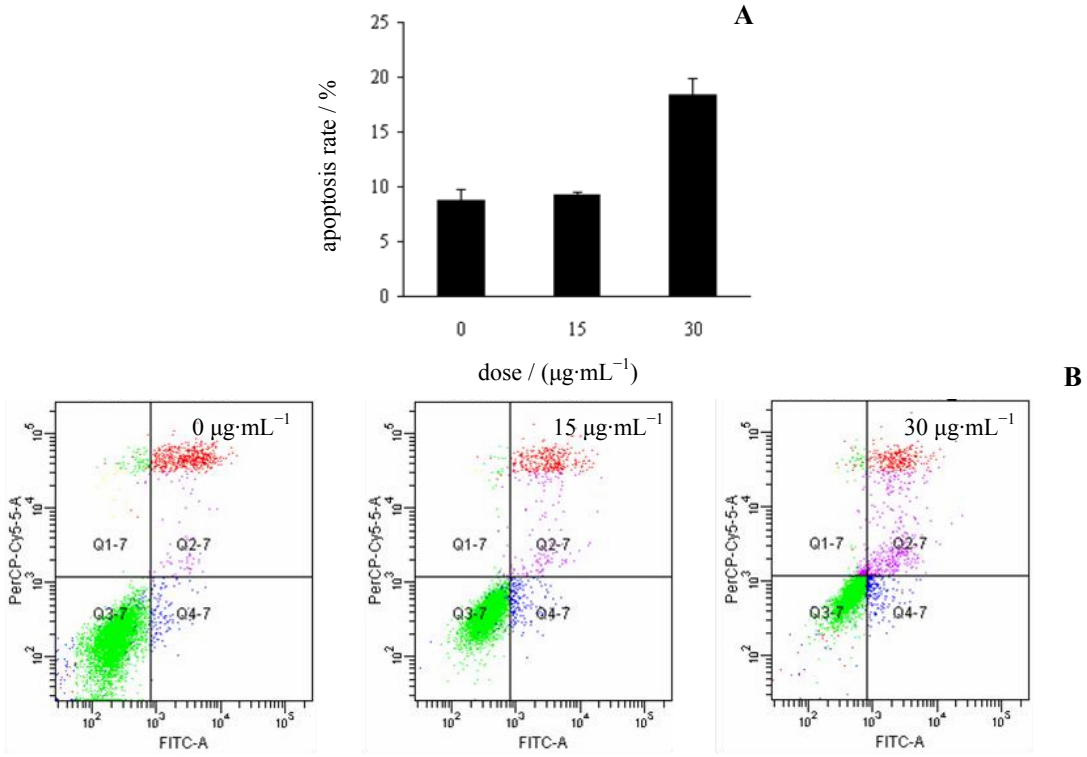

Fig. 3 Dose-dependent apoptosis of K562 cells by CH_2Cl_2 extract

K562 cells were incubated with various doses of CH_2Cl_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_2Cl_2 extract and then increases in a dose-dependent manner. B: Dot plots of apoptosis

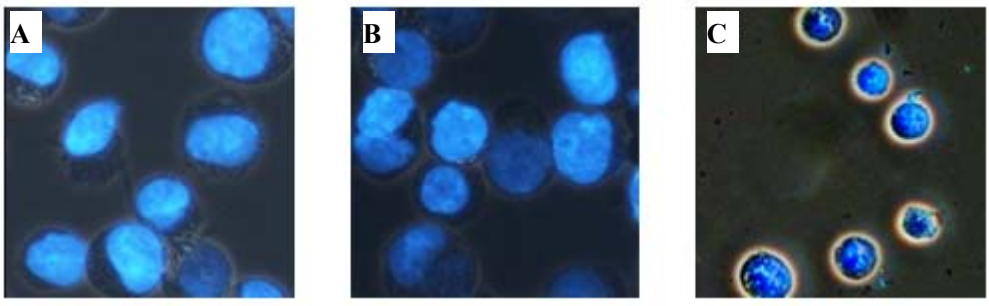

Fig. 4 Nuclear morphology of K562 cells in control culture (A), culture treated with 15 (B), and 30 (C) μg·mL^{-1} CH_2Cl_2 extract for 72 h

Cells were stained with Hoechest 33258 and observed under fluorescence microscopy

in the K562 cells within 72 h after treatment with CH_2Cl_2 extract at 15 and 30 μg/mL (Fig. 5). Compared with the control (B), the genomic DNA of CH_2Cl_2 extract-treated cells at 30 and 15 μg/mL (C and D) shows a disperse pattern and has a dose-effect relationship.

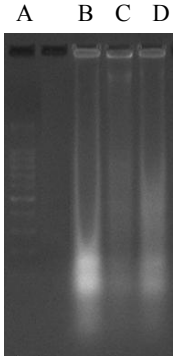

Fig. 5 Induction of DNA fragmentation in K562 cells by CH_2Cl_2 extract

Lane A: Marker; Lane B: control; Lane C: treated by 30 μg·mL^{-1} CH_2Cl_2 extract; Lane D: treated by 15 μg·mL^{-1} CH_2Cl_2 extract

Discussion

In this research, we demonstrated that the CH_2Cl_2 extract of *S. plebeia* exhibited potent cytotoxicity on K562, SGC-7901, and DU-145 cells with an IC_{50} < 15 μg/mL. Compared with 5-Fu, the IC_{50} 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 CH_2Cl_2 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 CH_2Cl_2 extract. And the number of apoptotic cells increased significantly. Data from cell morphological assessment and flow cytometer showed that, CH_2Cl_2 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 CH_2Cl_2 extract may induce apoptosis in K562 cells.

Based on all those results, we come to a conclusion that CH_2Cl_2 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

Evan GI, Vousden KH, 2001. Proliferation, cell cycle and apoptosis in cancer. *Nature* 411: 342-348.

Fazal F, Gu L, Ihnatovych I, Han Y, Hu W, Antic N, Carreira F, Blomquist JF, Hope TJ, Ucker DS, Lanerolle P de, 2005. Inhibiting myosin light chain kinase induces apoptosis *in vitro* and *in vivo*. *Mol Cell Biol* 25: 6259-6266.

Finkel E, 1999. Does cancer therapy trigger cell suicide. *Science* 286: 2256-2258.

Gupta HC, Ayengar KN, Rangaswami S, 1975. Structure and synthesis of salvition, a new flavonid isolated from *Salvia plebeian* India. *J Chem* 13(5): 215-217.

Jalving M, Jong S de, Koornstra JJ, Boersma-van Ek W, Zwart N, Wesseling J, Vries EG de, Kleibeuker JH, 2006. TRAIL induces apoptosis in human colorectal adenoma cell lines and human colorectal adenomas. *Clin Cancer Res* 12: 4350-4356.

Jiang Y, Luo SQ, Zheng MS, 1987. Studies on active principles of *Salvia plebeia*. *J Pharm Indust* 18(8): 349-351.

Jung HJ, Song YS, Lim CJ, Park EH, 2009. Anti-inflammatory, anti-angiogenic and anti-nociceptive activities of an ethanol extract of *Salvia plebeia* R. Brown. *J Ethnopharmacol* 126(2): 355-360.

Kaufmann SH, Earnshaw WC, 2000. Induction of apoptosis by cancer chemotherapy. *Exp Cell Res* 256: 42-49.

Liu T, Hannafon B, Gill L, Kelly W, Benbrook D, 2007. Flex-Hets differentially induce apoptosis in cancer over normal cells by directly targeting mitochondria. *Mol Cancer Ther* 6: 1814-1822.

Mosmann T, 1983. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* 16: 55-63.

Plattner RD, Powell RG, 1978. A secoisolariciresinol branched fatty diester from *Salvia plebeia* seed. *Phytochemistry* 17: 149-150.

Qu XJ, Xia X, Wang YS, Song MJ, Liu LL, Xie YY, Cheng YN, Liu XJ, Qiu LL, Xiang L, Gao JJ, Zhang XF, Cui SX, 2009. Protective effects of *Salvia plebeia* compound homoplantaginin on hepatocyte injury. *Food and Chem Toxicol* 47(7): 1710-1715.

Reed JC, 1997. Bcl-2 family proteins: Regulators of apoptosis and chemoresistance in hematologic malignancies, Semin. *Hematol J* 34: 9-19.

Reed JC, 2000. Mechanisms of apoptosis. *Am J Pathol* 157: 1415-1430.

Richard GP, Ronald DP, 1976. Structure of a secoisolariciresinol diester from *Salvia plebeia* seed. *Phytochemistry* 15: 1963-1965.

Studzinski GP, 1999. *An Overview of Apoptosis.* In: Studzinski GP, editor. *Apoptosis: A Practical Approach.* Oxford University Press: New York, 1-16.

Su JD, Toshihiko O, Mitsuo N, 1986. Screening for antioxidative activity of crude drugs. *Agric Biol Chem* 50(1): 199-203.

Sun SY, Hail NJ, Lotan R, 2004. Apoptosis as a novel target for cancer chemoprevention. *J Natl Cancer Inst* 96: 662-672.

Wang M, Zhang L, Han X, Yang J, Qian J, Hong S, Samaniego F, Romaguera J, Yi Q, 2007. Atiprimod inhibits the growth of mantle cell lymphoma *in vitro* and *in vivo* and induces apoptosis *via* activating the mitochondrial pathways. *Blood* 109: 5455-5462.

Weng XC, Wang W, 2000. Anti-oxidant activity of compounds isolated from *Salvia plebeian*. *Food Chem* 71: 489-493.