

Polyphenol-rich extract of *Salvia chinensis* exhibits anticancer activity in different cancer cell lines, and induces cell cycle arrest at the G₀/G₁-phase, apoptosis and loss of mitochondrial membrane potential in pancreatic cancer cells

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Abstract. Pancreatic cancer (PC) is one of the most aggressive types of human malignancy, which has an overall 5-year survival rate of <2%. PC is the fourth most common cause of cancer-associated mortality in the western world. At present, there is almost no effective treatment available for the treatment of PC. The aim of the present study was to evaluate the anticancer potential of a polyphenol enriched extract obtained from *Salvia chinensis*, a Chinese medicinal plant. An MTT assay was used to evaluate the cell viability of five cancer cell lines and one normal cell line. In addition, the effects of the extract on apoptotic induction, cell cycle phase distribution, DNA damage and loss of mitochondrial membrane potential ($\Delta\Psi_m$) were evaluated in MiapaCa-2 human PC cells. The effects of the extract on cell cycle phase distribution and $\Delta\Psi_m$ were assessed by flow cytometry, using propidium iodide and rhodamine-123 DNA-binding fluorescent dyes, respectively. Fluorescence microscopy, using 4',6-diamidino-2-phenylindole as a staining agent, was performed in order to detect the morphological changes of the MiapaCa-2 cancer cells and the presence of apoptotic bodies following treatment with the extract. The results of the present study demonstrated that the polyphenol-rich extract from *S. chinensis* induced potent cytotoxicity in the MCF-7 human breast cancer cells, A549 human lung cancer cells, HCT-116 and COLO 205 human colon cancer cells, and MiapaCa-2 human PC cells. The COLO 205 and MCF-7 cancer cell lines were the most susceptible to treatment with the extract, which exhibited increased rate of growth inhibition. Fluorescence microscopy

revealed characteristic morphological features of apoptosis and detected the appearance of apoptotic bodies following treatment with the extract in the PC cells. Flow cytometric analysis demonstrated that the extract induced G₀/G₁ cell cycle arrest in a dose-dependent manner. In addition, treatment with the extract induced a significant and concentration-dependent reduction in the $\Delta\Psi_m$ of the PC cells.

Introduction

Pancreatic cancer (PC) is one of the most aggressive types of human malignancy, exhibiting an overall 5-year survival rate of <2%, and is the fourth most common cause of cancer-associated mortality in the western world (1). PC is characterized by rapid disease development and the absence of specific symptoms, thus limiting early diagnosis and the success of curative treatment (2,3). Surgical resection is currently the only curative treatment for PC; however, due to late diagnosis, the majority of patients are diagnosed with advanced stage PC and only a minority (10-20%) respond well to surgery (4). Owing to the high recurrence rate, patients with PC who have undergone surgery require adjuvant chemotherapy with or without radiotherapy, resulting in 5-year survival rates of between 15 and 25% (5-7). Since the majority of cases of PC are inoperable, the majority of patients rely on palliative treatment using conventional chemotherapy. Gemcitabine and 5-fluorouracil (5-FU) are the standard chemotherapeutic drugs used to treat PC, which offer mild improvement of tumor-associated symptoms and minimal improvements in survival rates. Despite providing improvements in quality of life, current standard treatment with gemcitabine or 5-FU results in a median survival rate of just a few months (8,9). The limitations of conventional chemotherapy are due to the profound resistance of PC cells towards anticancer drugs, which results from efficient protection against chemotherapeutic drugs due to an altered balance of pro- and anti-apoptotic proteins, which results in significantly reduced susceptibility to apoptosis (10,11). Since the majority of established anticancer therapeutic strategies depend on the elimination of tumor cells by apoptosis, the capability of tumor cells to escape apoptosis is a major hurdle in treatment. As with other cancer cells, PC cells have developed resistance mechanisms, which enable them

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to resist chemotherapy (12). Among these mechanisms, protection from apoptosis appears to be the most relevant. With such poor response rates to current chemotherapeutics, there is an immediate requirement to identify novel and effective therapeutic strategies to treat PC (13-15). The present study aimed to determine the cytotoxic potential of the polyphenol-rich extract of *Salvia chinensis*, and to investigate its role in cell cycle arrest, mitochondrial membrane potential loss and apoptosis in pancreatic cancer cells.

Salvia chinensis Benth, also referred to as Shijianchuan (Chinese Sage) is a plant belonging to the Labiatae plant family. *S. chinensis* is an annual plant that is native to several provinces in China, including Hubei, Sichuan, Guangxi, Guangdong and Hunan, and grows in forests and in clusters of grass on hillsides or plains at 100 and 500 m elevation. *S. chinensis* grows on stems, which are erect or prostrate, up to a height of 20-60 cm (16). *S. chinensis* was primarily recorded in the Compendium of Materia Medica (Ming Dynasty, A.D. 1590), in which it was recorded as a treatment for ostealgia and swollen carbuncles (17). In addition, ethnopharmacological investigation revealed that this herbal medicine has been used to treat breast, liver and stomach cancer, and hepatitis (18). Phytochemical investigation of *S. chinensis* has resulted in the detection of >50 chemical constituents, in four classes of compounds: Terpenoids (monoterpenoids, sesquiterpenes and triperpenoids), phenolic acids, flavonoids, and dibenzylcyclooctadiene lignans (19). In addition, boswellic acids, blumenol A, pinafaenoic acid, salvianolic acid B, salvianolic acid D, 5,7,4'-trihydroxydihydroflavonol, protocatechuic acid, 3,5,7-trihydroxychromone and kaempferol have been reported to be present in *S. chinensis* (20-27).

Previous pharmacological investigations have demonstrated that water extract of *S. chinensis* markedly inhibits the proliferation of CNE human nasopharynx cancer cells and MGC-803 human gastric cancer cells (28). In addition, polysaccharides isolated from *S. chinensis* exhibit marked antitumor activity (29,30), B-lymphocyte stimulation and, at a concentration of 20 mg/l, protection of PC-12 cells against H₂O₂-induced injury (31,32). Furthermore, *S. chinensis* has been reported to protect against CCl₄-induced acute liver injury in mice, possibly due to the antioxidant activity of the phenolic acids present (33).

In view of the reported use of *S. chinensis* in traditional medicine, in combination with reports of its use against various types of cancer, the present study aimed to determine the phytochemical composition and anticancer activity of the polyphenol-rich extract of *S. chinensis*. In addition, the mechanism of action of this extract was evaluated by investigating its effects on cell cycle phase distribution, apoptosis and mitochondrial membrane potential using flow cytometry and fluorescence microscopy.

Materials and methods

Plant material and extraction procedure. *S. chinensis* was collected between June and July 2013 from a local site in Jianguo, China, and the plant material was confirmed by Professor JW Chen (College of Pharmaceutical Science, Nanjing University of Chinese Medicine, Nanjing, China). The aerial parts of *S. chinensis* were washed thoroughly with tap water, air dried and then sectioned into small pieces. Methanol (95%) was used for the hot extraction, which was performed

after 4 h using a Soxhlet extraction apparatus (BSXT-02; Shanghai Bilon Instrument Co., Ltd. Shanghai, China). In this method, the finely ground crude drug is placed in a porous bag made of strong filter paper, which is placed in chamber E of the Soxhlet apparatus. The extract was concentrated under reduced pressure in a rotary evaporator at 45°C, and was maintained at in a refrigerator at 4°C prior to use.

Liquid chromatography-electrospray ionization/multi-stage mass spectrometry (LC-ESI-MSMS)/high performance liquid chromatography (HPLC) analyses. The LC-MS equipment consisted of a chromatographic system (LC-MS Infinity; Agilent Technologies, Inc., Santa Clara, CA, USA) coupled with an Agilent 1100 Series LC system (Agilent Technologies, Inc.), which was equipped with a binary solvent delivery system, auto-sampler, column temperature controller, photo diode array detector and Finnigan LCQ Deca XP Plus ion trap mass spectrometer (Thermo Finnigan; Thermo Fisher Scientific, Waltham, MA, USA) via an ESI interface. MS spectra were obtained using positive and negative modes; nebulizer gas, 45 Psi; capillary voltage, 4,000 V. The operating parameters for MS were as follows: Collision gas, ultrahigh-purity helium (He); nebulizing gas, high purity nitrogen (N₂); ion spray voltage, -5.5 kV; sheath gas (N₂) at a flow rate of 70 arbitrary units; auxiliary gas (N₂) at a flow rate of 30 arbitrary units; capillary temperature, 360°C; capillary voltage, -15 V; and tube lens offset voltage, -30 V. Full scan data acquisition was performed between 80 and 1,800 m/z in MS scan mode.

HPLC analysis was performed on an Agilent 1260 Infinity series (Agilent Technologies, Inc.) using a Chromolith RP-18e column (4.6 mm ID, 60 mm length). The mobile phase consisted of (A) 0.5% aqueous acetic acid and (B) methanol. Mobile phase gradient: 0-10 min, linear gradient between 10 and 20% of B; 10-15 min, isocratic conditions at 25% of B; 15-20 min, linear gradient between 25 and 40% of B; 20-40 min, linear gradient between 40 and 50% of B; 40-50 min; linear gradient between 50 and 100% of B. Flow rate: 1.5 ml/min.

Chemicals and reagents. RPMI-1640 growth medium was purchased from Hangzhou Sijiqing Biological Products Co., Ltd. (Hangzhou, China). Fetal calf serum (Gibco Life Technologies, Carlsbad, CA, USA), trypsin, penicillin, MTT, streptomycin, dimethyl sulfoxide and phosphate-buffered saline (PBS) were used in the present study (all purchased from Sigma-Aldrich, St. Louis, MO, USA). The MTT kit was obtained from Roche Diagnostics (Indianapolis, IN, USA). Camptothecin was used as a positive control for the mitochondrial membrane loss and was purchased from Sigma-Aldrich. An Annexin V-Fluorescein Isothiocyanate (FITC)-Propidium Iodide (PI) Apoptosis Detection kit was purchased from Sigma-Aldrich. All other chemicals and solvents used were of the highest purity grade. Cell culture plasticware was purchased from Falcon® (Corning Life Sciences, Tewkesbury, MA, USA).

Cell lines. MCF-7 human breast cancer cells, A549 human lung cancer cells, HCT-116 human colon cancer cells, COLO-205 human colon cancer cells, MiapaCa-2 human pancreatic cancer cells, and the normal cell line, NIH-3T3 mouse embryonic fibroblasts, were obtained from the Shanghai Institute of Cell Resource Center of Life Science (Shanghai, China). All the cell

lines were cultured in a humidified atmosphere of 5% CO₂ at 37°C in RPMI-1640 medium supplemented with 10% heat-inactivated fetal calf serum, 100 IU/ml penicillin and 100 µg/ml streptomycin.

MTT cell viability assay. The inhibition of cell proliferation following treatment with the extract was measured using an MTT assay. Briefly, the MCF-7, A-549, HCT-116, COLO-205, MiapaCa-2 and NIH-3T3 cells were plated in separate 96-well culture plates (1x10⁵ cells/well). Following 24 h incubation at 37°C, the cells were treated with the polyphenol-rich extract (10, 20, 40, 60, 80 and 100 µg/ml; eight wells per concentration) for 12, 24 or 48 h. MTT solution (5 mg/ml) was subsequently added to each well. Following incubation for 4 h, the formazan precipitate was dissolved with 100 µl dimethyl sulfoxide, and the absorbance was measured using an ELISA reader (SpectraMax Plus 384 microplate reader; Molecular Devices, LLC, Sunnyvale, CA, USA) at a wavelength of 570 nm. The cell viability ratio was calculated using the following formula: Inhibitory ratio (%) = [(OD_{control} - OD_{treated}) / (OD_{control})] x 100%; OD, optical density. Cytotoxicity was expressed as the half maximal inhibitory concentration.

Investigation of apoptosis using fluorescence microscopy. Fluorescence microscopy was performed to evaluate morphological alterations in the MiapaCa-2 cancer cells, following treatment with the extract. The cells (1x10⁶ cells/ml) were seeded in 6-well plates and treated with the extract (20, 40, 60 and 80 µg/ml concentrations). Following 24 h incubation at 37°C, the cells were centrifuged at 112 x g for 5 min at 4°C. The resuspended pellet was then dissolved in PBS. The air-dried smears were then fixed in methanol at -20°C, stained with 4',6-diamidino-2-phenylindole (DAPI; 2 µg/ml) and incubated at 37°C for 20 min. The culture plates were subsequently observed under an inverted light microscope (Eclipse Ti-E; Nikon Corporation, Tokyo, Japan) for morphological analysis.

Annexin V binding assay and quantification of apoptotic cell death. To establish and confirm that the cells were undergoing apoptosis, an annexin V binding assay was performed using flow cytometry. Briefly, the MiapaCa-2 pancreatic cancer cells (2x10⁶ cells/ml) were treated with the polyphenol-rich extract at 20, 40, 60 and 80 µg/ml for 24 h at 37°C. Subsequently, the treated and untreated cells were harvested by trypsinization. The harvested cells were then incubated with annexin V-FITC (80 ng/ml) and PI (50 µg/ml), at room temperature in the dark for 20 min, and analyzed using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). A minimum of 2x10⁴ cells were measured in each sample.

Agarose gel electrophoresis for the detection of DNA fragmentation. For the detection of DNA fragmentation, the cells were lysed in a solution containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA and 0.5% Triton X-100 at room temperature for 30 min. The lysates were then vortexed and cleared by centrifugation at 15,000 rpm for 15 min. DNA was extracted from the supernatant using a 20:20:10 (v/v/v) equal volume of neutral phenol:chloroform:isoamyl alcohol. The DNA was then separated by electrophoresis on 1.0% agarose gels containing 0.1 µg/ml ethidium bromide (Sigma-Aldrich),

and DNA fragmentation was detected under UV illumination.

Cell cycle analysis. MiapaCa-2 cells (5x10⁶) were seeded into 60 mm dishes and subjected to various concentrations (20, 40, 60 and 80 µg/ml) of polyphenol-rich extract for 48 h at 37°C. The floating and adherent cells were collected by trypsinization and washed twice with PBS. The remaining cells were then incubated in 70% ethanol at -20°C overnight, treated with 10 µg/ml RNase A, and stained with 2.0 µg/ml PI. The stained cells were subsequently analyzed using flow cytometry at a wavelength of 488 nm (FACSCalibur; BD Biosciences), equipped with CellQuest 3.3 software.

Measurement of mitochondrial membrane potential ($\Delta\Psi_m$) loss. Mitochondrial membrane potential ($\Delta\Psi_m$) was measured using 1 mM Rhodamine-123 (Rh-123) dye. Rhodamine fluorescence can be used as a measure of membrane polarization in live cell assays within mitochondria. Briefly, 5x10⁵ MiapaCa-2 cells were treated with different concentrations (20, 40, 60 and 80 µg/ml) of the polyphenol-rich extract for 48 h at 37°C. Subsequently, $\Delta\Psi_m$ was measured using flow cytometry (FACSCalibur; BD Biosciences). Rh-123 (2 mM) was added 1.5 h prior to termination of the experiment. The cells were then collected, washed in PBS and incubated with PI (10 µg/ml) for 15 min at room temperature. The reduction in fluorescence intensity, due to loss of $\Delta\Psi_m$, was analyzed using flow cytometry. The mean fluorescence intensity was detected using the FL1 channel of the FACSCalibur.

Statistical analysis. All the data were analyzed using a one-way analysis of variance, followed by Dunnett's test for pair wise comparison with GraphPad Prism 4.0 (GraphPad Software, Inc., La Jolla, CA, USA). Values are presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

Results

Evaluation of the antitumor activity of the polyphenol-rich extract using cytotoxicity assays. The polyphenol-rich extract was evaluated for antiproliferative activity using an MTT assay. The MCF-7 human breast cancer cells, A549 human lung cancer cells, HCT-116 human colon cancer cells, COLO-205 human colon cancer cells, MiapaCa-2 human pancreatic cancer cells and NIH-3T3 mouse embryonic fibroblast normal cell line were treated with the extract for 24 h (Fig. 1). The extract exhibited potent dose-dependent cytotoxic activity against the different cancer cell lines. The COLO-205 and MCF-7 cancer cells were the most susceptible to treatment with the extract, and exhibited increased growth inhibition. The HCT-116 and MiapaCa-2 cells exhibited higher levels of growth inhibition only following treatment with higher concentrations of the extract. In order to examine the toxic effects of the extract on normal cells, the cytotoxic effects of the extract were assessed against the NIH-3T3 mouse embryonic fibroblast cell line. The extract demonstrated reduced cytotoxicity towards the normal cell line, compared with the cancer cell lines, suggesting that its effects are specific to cancer cells. The effect of the polyphenol-rich extract on the growth of MiapaCa-2 pancreatic cancer cells was

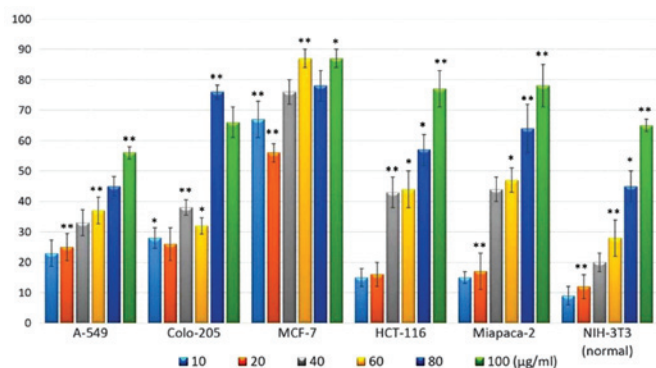


Figure 1. Cytotoxic effect of the *Salvia chinensis* polyphenol-rich extract on different cancer cells, determined using an MTT assay. The cells were treated with different concentrations of the extract (10, 20, 40, 60, 80 and 100 $\mu\text{g/ml}$) for 24 h. Data are expressed as the mean + standard deviation of three independent experiments. * $P < 0.05$, ** $P < 0.01$, vs 0 $\mu\text{g/ml}$ (control).

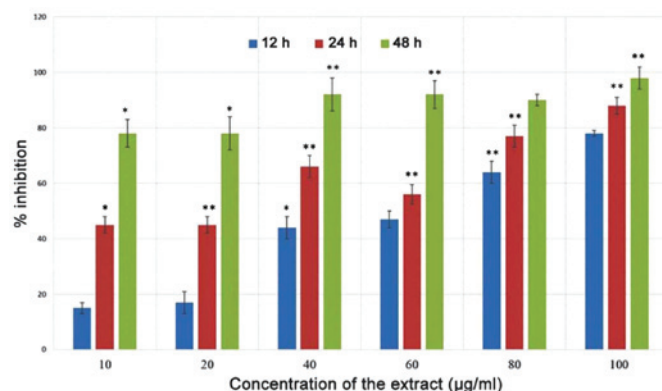


Figure 2. Cytotoxic effect of *Salvia chinensis* extract on Miapaca-2 human pancreatic cancer cells at different durations of treatment (12, 24, and 48 h). Data are expressed as the mean + standard deviation of three independent experiments. * $P < 0.05$, ** $P < 0.01$, vs 0 $\mu\text{g/ml}$ (control).

evaluated using an MTT assay at three different time intervals (12, 24 and 48 h). The cytotoxic effect of the extract on the cells was dose- and time-dependent. At increased time intervals, high levels of growth inhibition were observed (Fig. 2).

Evaluation of apoptotic morphological changes in Miapaca-2 cells. In order to establish whether the polyphenol-rich extract of *S. chinensis* induced apoptosis in Miapaca-2 cells, the cells were treated with various concentrations of the extract (0, 20, 40, 60 and 80 $\mu\text{g/ml}$) for 48 h. Subsequently, the representative morphological features of apoptosis were examined under an inverted light fluorescence microscope, using DAPI as a staining agent. As shown in Fig. 3, compared with the untreated viable cells, treatment with the extract resulted in the appearance of cell contraction and membrane blebbing, both of which are distinguishing features of apoptosis. When treated with a higher concentration of the extract (100 $\mu\text{g/ml}$), the majority of the cancer cells had shrunk considerably, and no cells exhibiting normal morphological features were detected.

Quantification of apoptotic cell death using an annexin V binding assay. The translocation of phosphatidylserine to the exterior surfaces of the plasma membrane is a distinguishing

feature of early apoptosis, which can be identified and detected by annexin V-FITC binding. If cell death occurs, fragmented and damaged DNA becomes permeable for binding with PI (34). Following staining of cells with annexin V in combination with PI, this reagent enters the cells only when the plasma cell membrane has deteriorated. In the present study, flow cytometry revealed that, in the extract-treated cells, a higher number of annexin V-positive cells were detected, compared with the untreated control cells (Fig. 4A and B). The percentage of apoptotic cells was low following treatment with lower concentrations of the extract. However, at higher extract concentrations (60 and 80 $\mu\text{g/ml}$), the total number of apoptotic cells increased considerably. This assay provided a quantitative estimation of the rate of apoptotic cell death following drug exposure.

Effects of the polyphenol-rich extract on cell cycle distribution. Apoptosis and cell cycle dysfunction are closely associated biochemical processes, and any disturbance in cell cycle progression may lead to apoptotic cell death (35). In order to determine the mechanism underlying the growth inhibitory effect of the extract on Miapaca-2 cancer cells, flow cytometric analysis was performed to detect whether the extract induced cell cycle arrest. Treatment with different concentrations of the extract for 48 h induced G_0/G_1 -phase growth arrest in the Miapaca-2 cells. As shown in Fig. 5, following treatment of the Miapaca-2 cells with different concentrations of the extract (20, 40, 60 and 80 $\mu\text{g/ml}$), considerable G_0/G_1 cell cycle growth arrest was observed. The apoptotic cells were observed as shrunken cells with degraded chromatin, increased side scatter and decreased forward scatter properties. The increase in the sub- G_1 cell population (hypodiploid DNA content) may be due to DNA fragmentation, which eventually results in apoptotic cell death. Inhibition of cell cycle progression may be one of the molecular events associated with the cytotoxic activities of the extract. Following treatment of the cells with low concentrations of the extract, no significant differences were observed in the levels of apoptosis; however, following treatment with extract concentrations of 60 and 80 $\mu\text{g/ml}$, the percentage of cells undergoing apoptosis (G_0/G_1 arrest) increased significantly.

Polyphenol-rich extract induces $\Delta\Psi_m$ loss. Depolarization of the mitochondrial membrane and subsequent seepage of the outer membrane is a key step in the intrinsic apoptotic pathway. This is usually followed by the release of cytochrome *c* and pro-apoptotic molecules (36). The present study used the fluorescent probe, Rh-123, to detect the $\Delta\Psi_m$ in living cells. Treatment with the extract induced a substantial reduction in the number of cells with intact membrane potential, and increased the number of cells with a low $\Delta\Psi_m$ after 48 h (Fig. 6). Loss of $\Delta\Psi_m$ is a crucial event in the mitochondrial apoptotic pathway. The loss of $\Delta\Psi_m$ was found to exhibit dose-dependence, and the number of cells with reduced $\Delta\Psi_m$ increased with increasing concentrations of the extract.

DNA fragmentation is induced by treatment with polyphenol-rich extract. A DNA fragmentation assay also revealed that treatment with the extract resulted in DNA laddering, which is indicative of apoptosis (Fig. 7A). DNA fragmentation in the polyphenol-rich extract-treated cells was confirmed using agarose gel electrophoresis, which detected the presence of

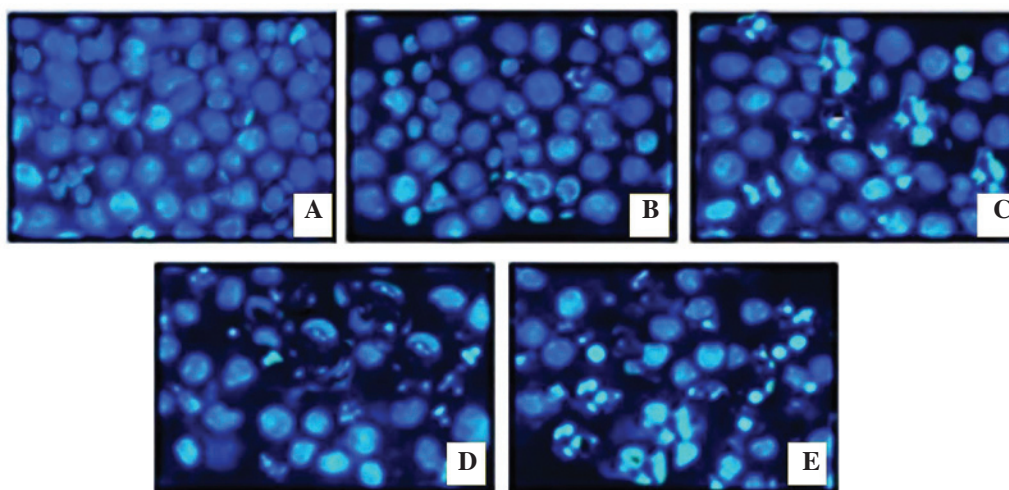


Figure 3. Morphological analyses using 4',6-diamidino-2-phenylindole nuclear staining (magnification, x400). MiapaCa-2 human pancreatic cancer cells were treated (A) without and with polyphenol-rich extract of *Salvia chinensis* at concentrations of (B) 20 µg/ml, (C) 40 µg/ml, (D) 60 µg/ml and (E) 80 µg/ml, for 48 h. Changes in nuclear morphology can be observed with increasing concentration.

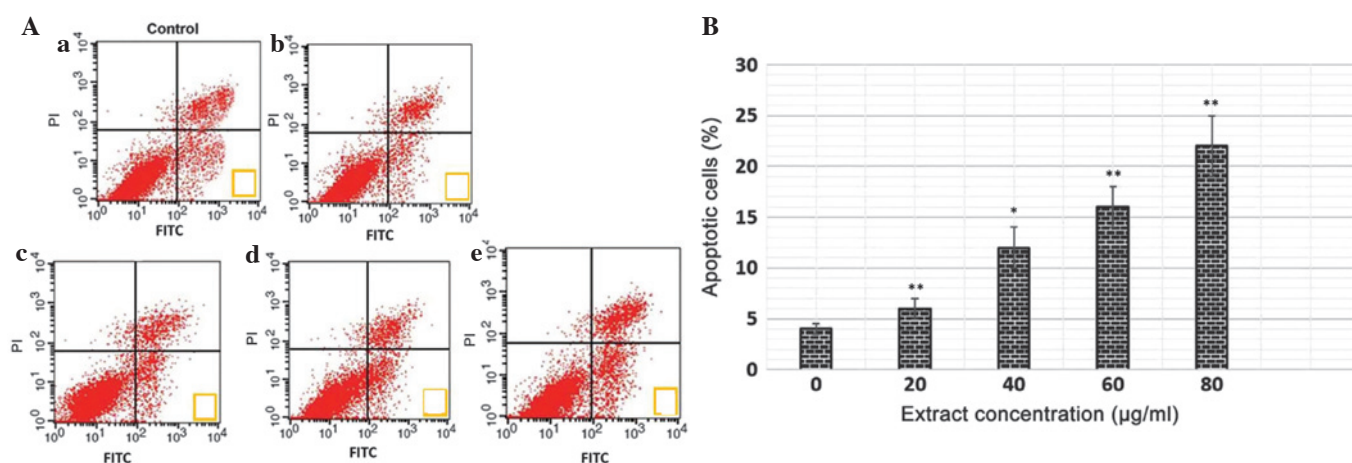


Figure 4. (A) Polyphenol-rich extract of *Salvia chinensis* stimulates apoptosis in MiapaCa-2 human pancreatic cancer cells. (A) Apoptotic cells were evaluated using flow cytometry following annexin V-FITC and PI staining. (a) Control, (b) 20 µg/ml, (c) 40 µg/ml, (d) 60 µg/ml and (e) 80 µg/ml concentration of extract. Cells in the lower left quadrant (annexin V-FITC-/PI-) are viable, those in the lower right quadrant are early apoptotic and those in the upper right quadrant are late apoptotic or necrotic. (B) Percentages of apoptotic cells following treatment with various concentrations of the extract. Data are expressed as the mean + standard deviation of three independent experiments. *P<0.05, **P<0.01, vs 0 µg/ml (control). FITC, fluorescein isothiocyanate; PI, propidium iodide.

DNA laddering, a marker of apoptosis, in the extract-treated MiapaCa-2 cells. By contrast, the untreated control cells demonstrated no evidence of DNA laddering. As shown in Fig. 7B, the number of cells exhibiting degraded DNA (sub-G₁ DNA content) increased in a dose-dependent manner.

LC-ESI-MSMS/HPLC analysis. In the present study, analysis of the *S. chinensis* methanol extract was performed using LC-ESI-MS as well as HPLC techniques. The extract was run under positive and negative ESI-MS conditions, and demonstrated several major and minor peaks. The total ion MS chromatogram, and the structure of the identified molecules are shown in Figs. 8 and 9, respectively. Fragmentation of the major peaks was used for identification of the compounds. Identification of the chemical compounds was also achieved by comparing the molecular ion peaks and MS fragmentation patterns with those described in the literature. The six chemical constituents identified in the extract were as follows: Protocatechuic acid,

salvianolic acid B, salvianolic acid D, xeractinol, kaempferol and apigenin. All these compounds belong to the polyphenol class of natural products.

Discussion

Dysregulation of cell division and apoptosis are associated with the development of the majority of types of cancer. Therefore, the ability of cancer cells to induce apoptosis has been recognized as one of the major mechanisms that may assist in the development of novel anticancer treatment strategies. Of the two apoptotic pathways, the intrinsic pathway is primarily controlled by members of the B-cell lymphoma (Bcl-2) protein family (35,37). The anti-apoptotic Bcl-2 proteins, including Bcl-2 and Bcl-extra large, stimulate cell survival by inhibiting mitochondrial permeability and the release of cytochrome *c*, therefore, effectively inhibiting apoptosis. Pro-apoptotic proteins, including Bcl-2-associated X protein

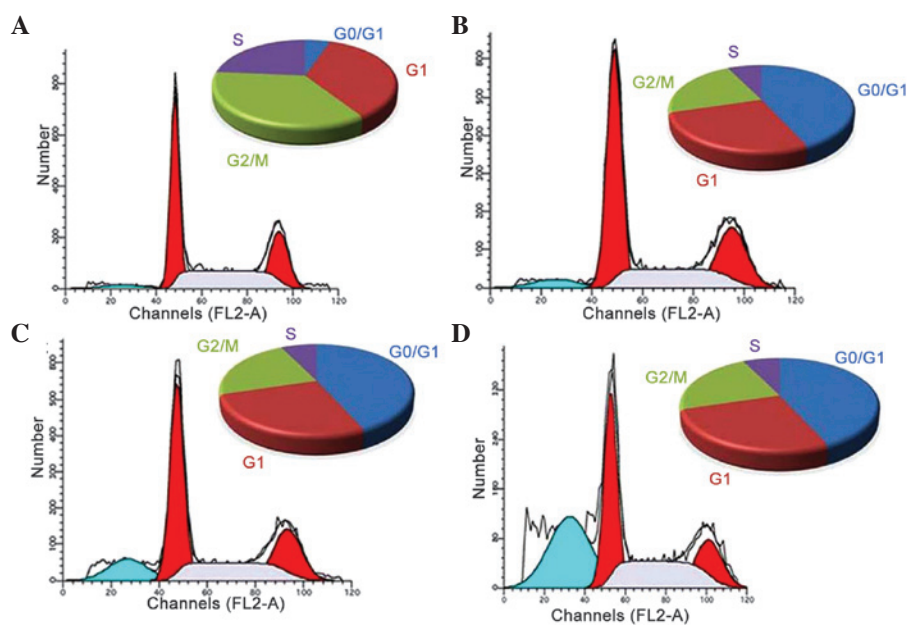


Figure 5. Effects of different concentrations of polyphenol-rich extract of *Salvia chinensis* on the cell cycle distribution of MiapaCa-2 human pancreatic cancer cells, determined using flow cytometry. The effects of (A) 20, (B) 40, (C) 60 and (D) 80 $\mu\text{g/ml}$ of the extract on the cells are shown. The corresponding pie charts indicate the percentage increase in the G_0/G_1 cell populations with increasing extract concentration.

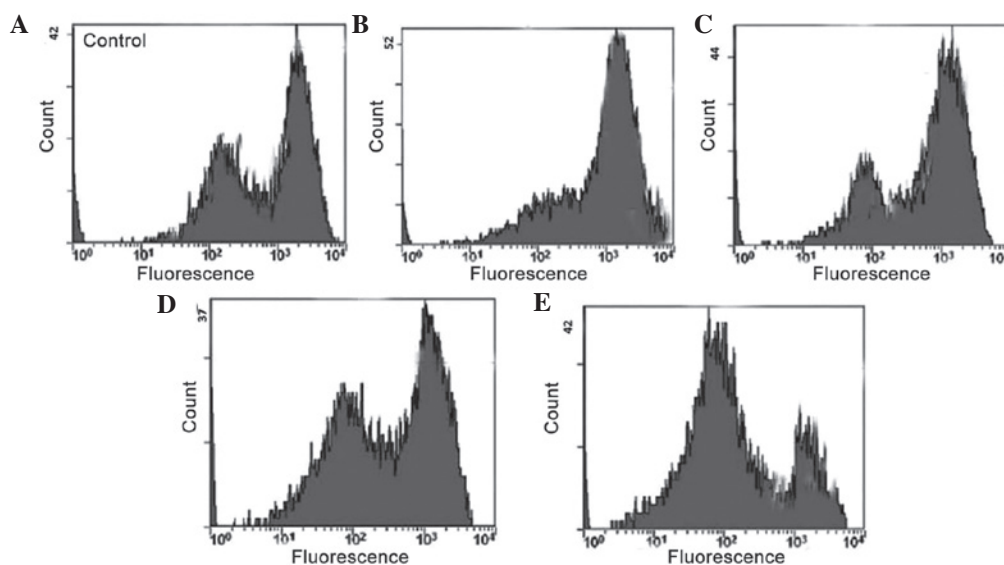


Figure 6. Effects of different concentrations of the polyphenol-rich extract of *Salvia chinensis* on the loss of mitochondrial membrane potential in MiapaCa-2 human pancreatic cancer cells. (A) Camptothecin-treated cells (positive control) and cells treated with (B) 20, (C) 40, (D) 60 and (E) 80 $\mu\text{g/ml}$ of the extract.

and Bcl-2-associated death promoter, stimulate cell death through a reduction in $\Delta\Psi_m$ (38,39). Therefore, the proportion of pro-apoptotic to anti-apoptotic molecules is considered to be a determining factor for mitochondria-associated apoptosis.

In the intrinsic apoptotic pathway, mitochondria have a vital role (36). Disruption of the $\Delta\Psi_m$ results in the release of cytochrome *c* into the cytosol. Release of cytochrome *c*, along with apoptotic protease-activating factor-1, allows formation of the apoptosome complex, which activates caspase-9 (40,41). Activated caspase-9 then cleaves and activates effector caspases, including caspase-3, which results in the apoptotic process. Release of cytochrome *c* from the mitochondria into the cytosol is regulated by pro- and anti-apoptotic Bcl-2 family proteins,

which regulate mitochondrial membrane permeability and polarization. In the present study, the results of the flow cytometric analyses demonstrated that the mitochondrial membranes were depolarized following treatment with the extract, particularly following exposure to higher concentrations.

Although a number of studies have reported the anticancer activity of *S. chinensis* against certain malignancies (28-32), the mechanism of action has not been investigated in detail. The aim of the present study was to evaluate the anticancer effects of the polyphenol-rich extract of *S. chinensis* in various human cancer cell lines, and to determine the mechanism underlying the anticancer action against MiapaCa-2 pancreatic cancer cells by evaluating its effects on cell viability, cell cycle phase distri-

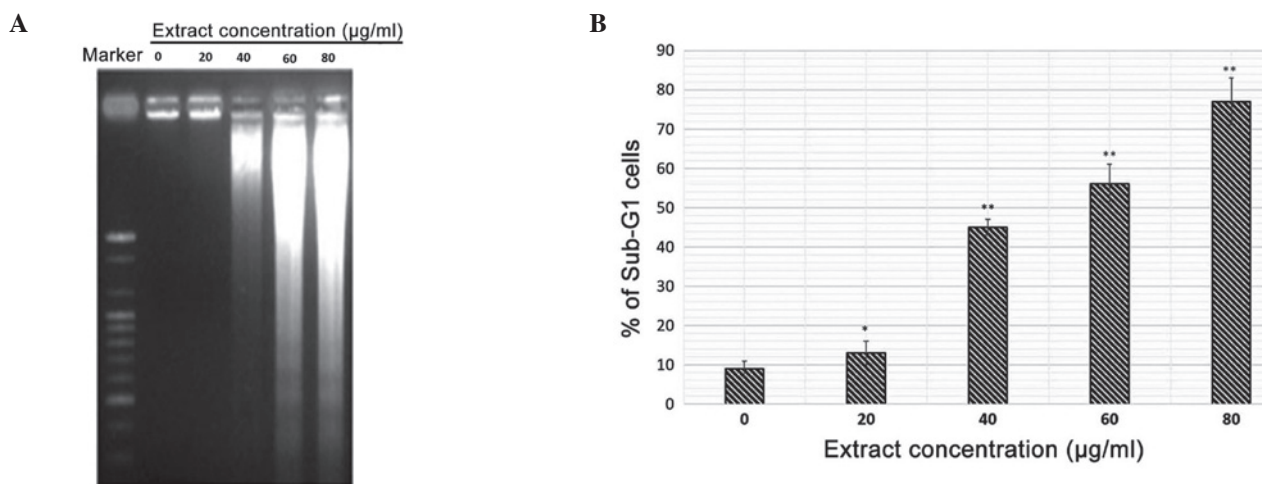


Figure 7. (A) DNA fragmentation was examined by 1.0% agarose gel electrophoresis of genomic DNA, followed by ethidium bromide staining. (B) MiapaCa-2 human pancreatic cancer cells were evaluated using flow cytometry to determine the sub-G₁ DNA content, which is indicative of apoptotic cell death. Data are expressed as the mean + standard deviation of three independent experiments. *P<0.05, **P<0.01, vs 0 µg/ml (control).

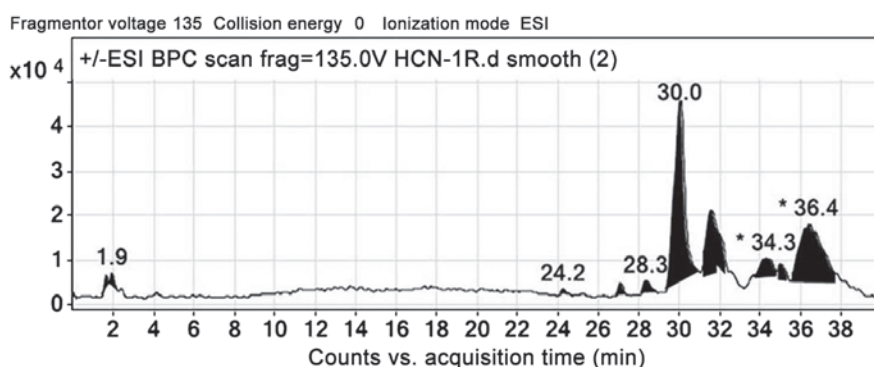


Figure 8. Total ion chromatogram of the methanol extract of *Salvia chinensis*.

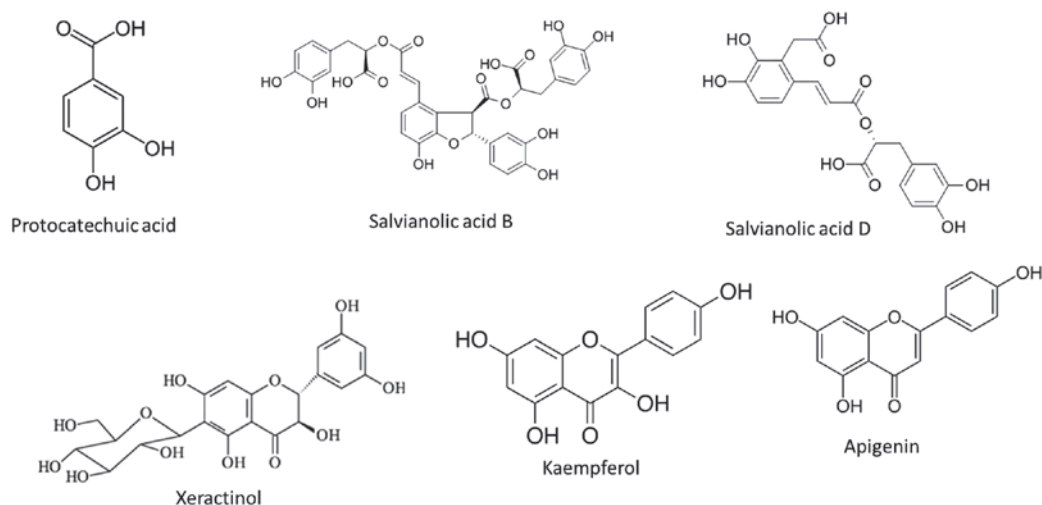


Figure 9. Molecular structures of the compounds in the methanol extract of *Salvia chinensis*, identified using liquid chromatography-mass spectrometry.

bution, apoptosis, DNA fragmentation, and $\Delta\Psi_m$. This is the first study, to the best of our knowledge, to confirm the reported benefits of this plant in Chinese medicine folklore.

The results of the present study demonstrated that the extract from *S. chinensis* exhibited potent cytotoxic effects

against cancer cell lines, including MCF-7 human breast cancer cells, A549 human lung cancer cells, HCT-116 and COLO-205 human colon cancer cells, MiapaCa-2 human pancreatic cancer cells and NIH-3T3 mouse embryonic fibroblast normal cells, following exposure for 24 h. Notably, the extract exhibited

lower cytotoxicity towards the normal cell line. These results are encouraging, since cancer cell specificity is important for the production of novel anticancer agents. In addition, in order to identify the anticancer action of the extract, its effects on the apoptosis of pancreatic cancer cells were evaluated using DAPI staining, flow cytometry and gel electrophoresis. The results demonstrated that the extract induced apoptosis by inducing DNA damage and cell cycle arrest at G₀/G₁ phase. The annexin V binding assay revealed the extent of the apoptosis induced by the extract. The present study also evaluated the role of the extract in disrupting the mitochondrial membrane in pancreatic cancer cells using flow cytometry. The results demonstrated that treatment with the extract induced potent ΔΨ_m loss in the cells.

In conclusion, polypheno-rich extract from *S. chinensis* was found to induce significant growth inhibition of MCF-7 human breast cancer cells, A549 human lung cancer cells, HCT-116 and COLO-205 human colon cancer cells, and MiapaCa-2 human pancreatic cancer cells. The extract was demonstrated to be selective, as it exhibited lower cytotoxicity towards the NIH-3T3 normal cell line, compared with the cancer cell lines. The extract induced apoptosis in the pancreatic cancer cells, determined using flow cytometry, fluorescence microscopy and agarose gel electrophoresis. In addition, the extract induced G₀/G₁ cell cycle arrest and loss of ΔΨ_m. This requires further elucidation of the exact mechanism of action in order to render it an effective therapeutic strategy against different types of cancer.

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