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Swertia mussotii extracts induce mitochondria-dependent apoptosis in gastric cancer cells

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ABSTRACT

Swertia mussotii (Gentianaceae) is a traditional Chinese medicinal plant grown in the Qinghai-Tibet Plateau. Three fractions from *S. mussotii* extract, named SWF50, SWF 70 and SWF100, were screened for in vitro anti-proliferative activity on two gastric cancer cell lines, MGC-803 and BGC-823 cells using MTT assay. Our results demonstrated that SMF70 showed an anti-proliferative effect in MGC-803 cells and SMF100 showed an anti-proliferative effect in BGC-823 cells in vitro. Moreover, both two fractions induced apoptosis via depolymerization of cytoskeletal filaments, increased cytoplasmic levels of ROS and Ca^{2+} and disrupted mitochondrial transmembrane potential. In addition, flow cytometry analysis indicated that both two fractions could induce cell apoptosis and arrest the cell cycle at S phase. Our results indicate that SMF70 induces apoptosis of MGC-803 cells and SMF100 induces apoptosis of BGC-823 cells via a mitochondrial-dependent pathway. Meanwhile, we also investigated antitumor effect of SMF70 in vivo, and exhibited effective tumor growth inhibition. Our findings demonstrate that *S. mussotii* extracts could be a potential new alternative therapeutic agent gastric cancer.

1. Introduction

Despite the advancement in earlier diagnosis and clinical intervention, cancer remains a severe disease and a leading cause of death worldwide and the prevalence of cancer continues to rise [1]. According to the American Cancer Society, gastric cancer (GC) is the second-leading cause of death due to cancer worldwide [2]. However, many drawbacks exist in the current cancer treatment, including low selectivity, resistance and high toxic [3]. Natural products, which have rich resources and fewer side effects, provide a good source of candidates for anticancer drug's discovery. In fact, many different sources of natural products have been reported to exert anticancer activities both in vitro and in vivo [4,5] and over 60% of anticancer agents are directly or indirectly originated from natural sources [6]. Thus, we selected the traditional Chinese medicinal materials *Swertia mussotii* Franch. that has anti-proliferation activity of gastric cancer cells MGC-803 and BGC-823, demonstrated in our previous study, as the research object to carry out the research on antitumor activity.

The genus *Swertia*, which belongs to the Gentianaceae family, comprises about 170 species recorded throughout the world and about 79 species present in China [7]. Together with 11 species of *Halenia*, 40 species of *Swertia* were used as traditional Tibetan folk medicinal species called "Zangyinchen" that have long been used to treat febrile diseases of the liver and gallbladder, such as hepatitis, cholecystitis and fatty liver [8]. *Swertia mussotii* Franch., the most commonly used in "Zangyinchen", grows mainly in the high alpine lands of the Qinghai-Tibet Plateau at altitudes between 3200 and 4200 m [9]. Previous phytochemical studies found that *S. mussotii* contains xanthones, flavonoids, iridoids and steroids and that xanthone derivatives are its major components [10–13]. Modern pharmacological investigations have shown that *S. mussotii* extract can depress the hyperlipidemia on rats with experimental hyperlipemia, repair the fibrillation of the liver and alleviate the damage of immunological liver injury [14–16]. Anti-proliferative activity studies conducted on some "Zangyinchen" species in recent years have found that extracts of these species have significant anti-tumour activity, such as *Swertia davidii* and *Gentianopsis paludosa* [17,18]. As regards *S. mussotii* that mian contents are xanthones, except for our previous study on anti-proliferation of MGC-803 and BGC-823

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cells, there is no report on any anticancer activity of this species available to date [19].

Therefore, in the present study, we aimed to investigate the cytotoxic effects of different fractions from *S. mussotii* extract on the viability and apoptosis of two gastric cancer cell lines, MGC-803 and BGC-823 cells. We also investigated the anticancer effect in vivo. In vivo experiments also were performed to validate the in vitro anticancer activity of the promising fraction.

2. Materials and methods

2.1. Plant material

The plants of *S. mussotii* were collected from Qinghai Province, China, in October 2015, and authenticated by Professor Mei Lijuan (Northwest Institute of Plateau Biology, Chinese Academy of Sciences, Xining, China). A voucher specimen (2014080101) was deposited in the herbarium of Northwest Institute of Plateau Biology, Xining, China.

2.2. Chemicals

Dimethyl sulfoxide (DMSO) and ethanol were purchased from Sigma Aldrich, MO, USA. RNase purchased from Thermoscientific, Waltham, MA; and the cell culture media RPMI-1640, DMEM, fetal bovine serum, and phosphate buffer solution (PBS) were obtained from Hyclone, UT, USA. DAPI, PI, JC-1, DCFH-DA, Fluo-3/AM and Hoechst 33,258 were purchased from Sigma (St. Louis, MO, USA). AnnexinV-fluorescein isothiocyanate (FITC) and PI apoptosis detection kit were obtained from BD Pharmingen (SanDiego, CA, USA). FITC-phalloidin, was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). All other chemicals were used analytical grade and purchased from Tianjing Baishi company (Tianjing, China).

2.3. Preparation of S. mussotii extracts

The air-dried and finely ground plant of *S. mussotii* (1 kg) were exhaustively extracted with 90% ethanol under reflux three times (65 °C, 3 × 10 L). The combined ethanol extracts were concentrated in vacuum at 40 °C to yield a dark brown crude extract (150 g). The residue was dissolved in a small amount of EtOH, and then applied to column chromatography on polyamide column with a gradient of water-EtOH (100:0; 50:50; 30:70; 0:100, v/v, 3 volumes). Three fractions were subsequently obtained, including 50% EtOH fraction (named SMF50), 70% EtOH fraction (named SMF70) and 100% EtOH fraction (named SMF100).

2.4. Cell culture

The human MGC-803 cell lines were obtained from the Cell Bank of the Chinese Academy of Sciences Shanghai Institute of Cell Biology (Shanghai, China). MGC-803 cells were cultured in DMEM containing 10% FBS and maintained at 5% CO_2 and 37 °C for 24 *h*.

2.5. MTT assay

Cytotoxicity was determined using an MTT assay [20]. The MGC-803 and BGC-823 cell lines were plated in 96-well plates at density of 1×10^4 cells/well and incubated at 37 °C for 12 *h*. After attachment, and then the cells were treated with various concentrations of the fractions (100–1200 μg/mL) for 24 *h*. All tests included a vehicle control with an equivalent concentration of DMSO (0.2%). At the end of each incubation period, 20 μL of MTT (final concentration 0.5 μg/mL) was added to each well and the plates were further incubated for 4 *h* at 37 °C. The supernatants were then replaced with 100 μL DMSO. The absorbance was detected with a microplate reader at 570 nm. The results were expressed in terms of cell viability (%) and half maximal inhibitory concentration (IC_{50}, μg/ml). All experiments were carried out in triplicate.

2.6. Immunofluorescence assay

Microfilament visualization was conducted using FITC-phalloidin according to the procedure previously described [21]. MGC-803 cells and BGC-823 cells were grown on glass coverslips at a density of 2×10^5 cells/mL and treated with different concentrations of fractions (0, 300, 600 and 900 μg/mL) for 24 *h*. The cells were washed twice with PBS, fixed for 10 min in.4% formalin at room temperature and again washed twice with PBS, and permeabilized for 5 min in 0.1% Triton X-100. After washed with PBS, microfilaments were stained using Actin-Tracker Green working solution and cells nuclei were stained by DAPI solution. The cells were set aside for 15 min and then washed with PBS prior to observation using CLSM (Confocal Laser Scanning Microscopy) (FV500, Olympus, Japan).

2.7. Flow cytometric analysis of apoptosis with annexin-v FITC/PI

The apoptotic effect of fractions from *S. mussotii* on MGC-803 cells and BGC-823 cells were evaluated by Annexin V-FITC and PI double staining [22]. The cells were seeded to approximately 1×10^6 cells/well in 12-well plates. After 12 *h* of attachment, cells were treated with 0, 300, 600 or 900 μg/mL SMF70 or SMF100 for 24 *h*. Then, the cells were harvested and washed with cold PBS and incubated with Annexin V-FITC (5 μL) and PI solution (20 μg/mL, 10 μL) for 15 min at room temperature in the dark. The labeled cells were then analyzed using flow cytometry (Becton Dickinson, USA). The percentages of early and late apoptotic and live cells in three independent experiments were statistically analyzed using GRAPHPAD PRISM 6 software.

2.8. Flow cytometric analysis of cell cycle

MGC-803 cells and BGC-823 cells were seeded to approximately 1×10^6 cells/well in 12-well plates. After 12 *h* of attachment, cells were treated with 0, 300, 600 or 900 μg/mL SMF70 or SMF100 for 24 *h*. Next, the cells were harvested and fixed with 70% ice-cold ethanol at −20 °C for 2 *h*. The fixed cells were washed with cold PBS, incubated with 0.1% Triton X-100 for 10 min. Before the flow cytometry analysis, the cells were stained with 0.5 mL staining solution containing 40 μg/mL propidium iodide and 0.1 mg/mL RNase, and incubated in the dark at room temperature for 30 min.

2.9. Measurement of mitochondrial membrane potential, reactive oxygen species and intracellular free calcium concentration

MGC-803 cells and BGC-823 cells were seeded to approximately 2×10^6 cells/well in 6-well plates. After 12 *h* of attachment, cells were treated with 0, 300, 600 or 900 μg/mL SMF70 or SMF100 for 18 *h*. The cells were harvested and washed twice with PBS, and then re-suspended in JC-1 solution (4 μM) for mitochondrial membrane potential determination, re-suspended in Fluo-3 AM solution (10 μM) for the level of intracellular free calcium and in DCFH-DA solution (10 μM) for the level ROS determination, according to the manufacturer's protocols. Samples were incubated with the specific fluorescent markers at 37 °C for 30 min in the dark room and washed twice with PBS to remove the spare dye subsequently. Stained cells were then analyzed immediately by flow cytometry.

2.10. Acute toxicity study

Given SMF70 have better anti-proliferation activity on MGC803 cells, we choose SMF70 as the research object of subsequent animals experiments. BalB/c female mice (15–20 g; six-week old; obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd., Beijing,