

Antiproliferative and apoptosis inducing effect of essential oil extracted from *Cyrtomium fortunei* (J.) Smith leaves

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Abstract *Cyrtomium fortunei* (J.) Smith is an endemic species in China, which has been proved to be an important Chinese herbal medicine. However, chemical composition and bioactivity of essential oil (EO) of *C. fortunei* (J.) Smith leaves remain unclear. In present study, we isolated EO from the plant by supercritical carbon dioxide extraction assay (SFE-CO₂), and investigated on cancer cells MGC-803, MCF-7, BGC-823, Bcap-37, A375, and A549 in vitro by MTT assay. 26 compounds were identified by GC–MS analysis, and the EO showed significant antitumor activities against MGC-803, Bcap-37, and A549 cancer cell lines (IC₅₀ values ranging from 0.15 to 0.24 mg/mL), and the activities of its main component were also studied. Subsequent fluorescence staining and flow cytometry analysis indicated that the EO could induce apoptosis in MGC-803, Bcap-37, and A549 cell lines, and the apoptosis ratios

reached 26.44 % after 48 h of treatment at 0.15 mg/mL in MGC-803 cells. Caspase 3 activity in MGC-803 cells was also determined when the cells treated with the oil, and the activity of caspase 3 enzyme was increased compared to the control. This study suggests that the EO isolated from *C. fortunei* (J.) Smith could inhibit the growth of human carcinoma cells, and it could induce apoptosis of cancer cells.

Keywords *Cyrtomium fortunei* (J.) Smith · Essential oil · Antitumor activity · Cell apoptosis · Caspase 3

Introduction

Natural products of various sources, particularly from plants and marines have been regarded as a precious alternative to modern medicine (Lope Pihie *et al.*, 2012). In recent years, a number of natural products isolated from Chinese herbs have been found to inhibit proliferation, induce apoptosis, suppress angiogenesis, retard metastasis, and enhance chemotherapy, exhibiting anticancer potential both in vitro and in vivo (Tan *et al.*, 2011; Hostanska *et al.*, 2007). The essential oils (EOs) extracted from herbs might have more therapeutic or preventive activity than alone, because the different components in herbs may have synergistic activities or buffering toxic effects (Yu *et al.*, 2012). Mostly plant-derived EOs consist of chemical components such as terpenoids including monoterpenes, sesquiterpenes and their oxygenated derivatives. These compounds have the ability to easily diffuse across cell membrane to induce biological reactions (Al Nomaani *et al.*, 2013). Plant EOs and their components have multiple and varied biological activities such as antiparasitic, insecticidal, antimicrobial, antitumor, and antioxidant

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properties (Jeong *et al.*, 2009; Wang *et al.*, 2010; da Silva, 2004; Prabuseenivasan *et al.*, 2006; Namita and Mukesh, 2012). For more than a decade, there has been a considerable interest in screening plant EOs for medical use all over the world (Lin *et al.*, 2012). They have received much attention to preventing plant and animal diseases, as well as to preventing oxidative damage (Rubalya and Neelamegam, 2012; Okigbo *et al.*, 2009).

Earlier studies done revealed that the plants of Dryopteridaceae had many biological properties and pharmacological functions (Herrmann *et al.*, 2011). *Cyrtomium fortunei* (J.) Smith is mainly grown in China, Korea, and Japan and has been used as a medicinal plant for a long time in the Orient (Choi, 2013; Yang *et al.*, 2013a). And it belongs to the Dryopteridaceae family which has 14 genera and 1700 species distributed in the temperate and tropical zones all over the world (Suksathan *et al.*, 2010; Kessler, 2006; Barrington, 2012). Many species of Dryopteridaceae family are rich in fatty acids, phloroglucinols, terpenoids, steroids, and flavonoids, and are well-known medicinal plants (Ito *et al.*, 2000). The roots of *C. fortunei* (J.) Smith are used as folk medicines for treatment of migraine, cancer, acute and chronic pharyngitis, and influenza in China (Kapadia *et al.*, 1996). Also, the herb was used to cure severe acute respiratory syndrome, a life-threatening viral respiratory illness believed to be caused by a coronavirus (Zhao *et al.*, 2007). It was reported that it exhibited a variety of bioactivities such as cytotoxicity (Yang *et al.*, 2013b), antibacterial (Song *et al.*, 2008), antifungal (Jahan *et al.*, 2010), and antioxidant (Yang *et al.*, 2013c) effects. Recently, researchers found that extracts of its roots processed significant antitumor activities via inducing cell apoptosis (Yang *et al.*, 2013a). Moreover, Choi *et al.* reported that methanol extract of its roots appeared to inhibit tyrosinase activity and melanin production in melan-a cells and it could exhibit depigmenting ability on Ultra violet-induced hyper pigmentation in brown guinea pig skin (Choi, 2013). In our previous work, we investigated the chemical constituents of the plant systematically, and tested the biological activities of its extracts and compounds. The results revealed that its major physiologically active ingredients included triterpenoids, flavonoids, and alkaloids (Yang *et al.*, 2013a). However, few studies have analyzed the chemical composition of EO of *C. fortunei* (J.) Smith leaves, and evaluated its biological activity.

This study aimed clarifying the chemical composition of the EO of *C. fortunei* (J.) Smith leaves, determining its cytotoxicity in human gastric cancer cell lines (MGC-803, BGC-823), human breast cancer cell lines (MCF-7, Bcap-37), human melanoma cancer cell line (A375), and human lung cancer cell line (A549) and underlying the mechanism of its antitumor action.

Table 1 Chemical components of EO

Compound	RT	Area (%)
Trans-1,4-dimethylcyclohexane	3.19	0.84
Octane	3.58	0.41
5-Nonadecen-1-ol	39.67	8.42
<i>n</i> -Hexadecanoic acid	39.93	8.34
9Z,12Z-Octadecadienoic acid	45.09	4.41
Oleic acid	45.32	6.51
Octadecanoic acid	45.96	1.14
Octadecanal	53.17	0.42
1,21-Docosadiene	58.32	1.04
1-Nonadecene	59.81	1.14
1-Nonadecanol	62.19	0.61
Squalene	62.88	0.58
1,19-Eicosadiene	63.09	1.06
Cyclotetracosane	64.51	7.41
1-Hentetracontanol	66.71	0.44
1,19-Eicosadiene	67.56	1.36
1-Heptacosanol	66.71	0.44
17-Pentatriacontene	68.89	0.80
Campesterol	71.01	4.54
1,30-Triacontanediol	71.76	1.56
7-Oxo-5 α -cholestane-3 β -ol acetate	71.96	0.84
Diploptene	72.46	2.35
22,23-Dihydrostigmasterol	72.89	34.15
β -Amyrin	74.23	1.31
3-Methylheneicosane	74.79	0.69
Stigmast-4-en-3-one	75.74	2.38

Results and discussion

EO was extracted by SFE-CO₂ assay from the fresh aerial parts (leaves) of *C. fortunei* (J.) Smith, and its chemical composition were determined by gas chromatography-mass spectroscopy (GC-MS). 26 compounds, representing 93.19 % of the total oil, were identified by GC-MS analysis for the first time. These compounds identified by GC-MS analysis, their retention time (RT) and relative area percentages are shown in Table 1. As shown in Table 1, the major compounds detected in EO are 22,23-dihydro-stigmasterol (34.15 %), 5-nonadecen-1-ol (8.42 %), *n*-hexadecanoic acid (8.34 %), cyclotetracosane (7.41 %), oleic acid (6.51 %), campesterol (4.54 %), 9Z,12Z-octadecadienoic acid (4.41 %), stigmast-4-en-3-one (2.38 %), and diploptene (2.35 %) comprising the main portion of EO. In addition, β -amyrin, a kind of pentacyclic triterpenoids, was also identified. Steroids and organic acids were the dominant class of compounds, constituting 41.07 and 19.26 % of the total oil, respectively. Pentacyclic triterpenoids constituted 3.66 % of the total oil.

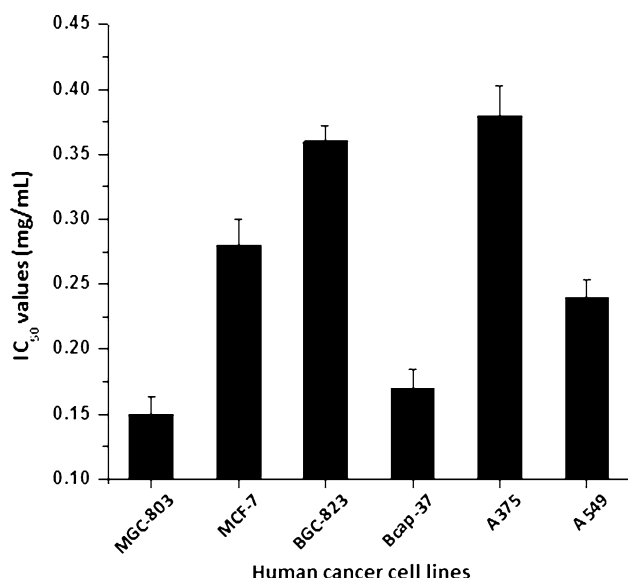


Fig. 1 Antitumor activities of EO from *Cyrtomium fortunei* (J.) Smith leaves. Values are mean of three experiments

The *in vitro* antitumor activity was evaluated for EO from *C. fortunei* (J.) Smith leaves against MGC-803, MCF-7, BGC-823, Bcap-37, A375, and A549 cell lines. EO was dissolved in DMSO, and then diluted with culture medium containing 0.1 % DMSO. The control cells were treated with culture medium containing 0.1 % DMSO. Adriamycin (ADM) was used as a positive control. The inhibitory ratios of ADM at 0.1 mg/mL against the six cell lines were 99.1, 98.3, 99.4, 97.6, 95.4, and 99.7 %, respectively. The IC₅₀ values of EO against the six cells are shown in Fig. 1. Furthermore, EO showed dose dependent antitumor activities against the two cell lines, and summarized in Fig. 2.

As shown in Fig. 1, EO had potential antitumor activities against MGC-803, Bcap-37, and A549 cell lines, and IC₅₀ values were 0.15, 0.17, and 0.24 mg/mL, respectively. It can be seen from Fig. 2, the proliferation of these three cancer cells were significantly inhibited by EO in a concentration-dependent manner. Interestingly, when the concentration reached at 0.3 mg/mL, the cell growth inhibition did not depend upon the concentration.

Steroids and pentacyclic triterpenoids are the most important classes of natural products occurring widely in the plant kingdom (Janeczko, 2012; Nicholas, 1962). And they have been shown to possess several medicinal properties including anticancer and anti-HIV activities. A number of studies have indicated that 22,23-dihydrostigmast-4-en-3-one may have anticancer properties (Patocka, 2003), and it was found to reduce growth of human prostate and colon cancer cells, and displayed antitumor activity against lymphocytic leukemia (Awad *et al.*, 2000; Park *et al.*,

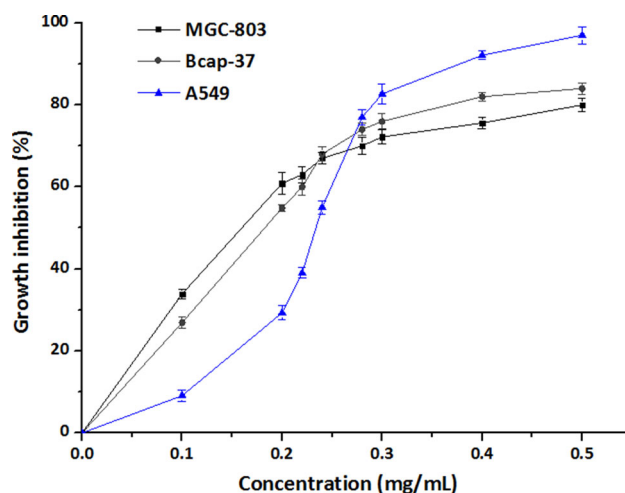


Fig. 2 Effect of EO on proliferation of tumor cells. Values are mean of three experiments

Table 2 Effect of steroids and triterpenoids from EO against cell viability of different cancer cells

Compound	IC ₅₀ (μM) ^a		
	MGC-803	Bcap-37	A549
22,23-Dihydrostigmast-4-en-3-one	58.2 ± 1.21	74.3 ± 0.81	92.4 ± 0.67
β-Amyrin	49.3 ± 0.94	83.4 ± 1.02	56.0 ± 0.78
Stigmast-4-en-3-one	60.5 ± 0.43	73.6 ± 0.27	>100 ^b
ADM ^c	0.73 ± 0.11	1.23 ± 0.16	0.94 ± 0.13

^a IC₅₀ means the concentration required to inhibit cell growth by 50 % as determined from the dose–response curve

^b When 50 % inhibition could not be reached at the highest concentration, then >100 μM was given

^c Adriamycin, positive control

2008). El-Alfy *et al.* found that β-amyrin showed significant cytotoxicity against MCF-7 and colon HCT-116 cells (El-Alfy *et al.*, 2011). Also, some studies have shown that stigmast-4-en-3-one has marked antitumor effects and cytotoxic activity toward various types of cancer cell lines (Habsah *et al.*, 2005). Therefore, the antitumor activities of these compounds were also evaluated against MGC-803, Bcap-37, and A549 cell lines, respectively. The results are shown in Table 2.

As shown in Table 2, 22,23-dihydrostigmast-4-en-3-one and β-amyrin suppressed proliferation of the three cell lines in different extents (IC₅₀ values: 49.3–92.4 μM). Although stigmast-4-en-3-one did not show significant activity against A549 cells, it possessed potent activities against MGC-803 and Bcap-37 cells, with IC₅₀ values of 60.5 and 73.6 μM, respectively. The results revealed that 22,23-dihydrostigmast-4-en-3-one, β-amyrin, and stigmast-4-en-3-one may be responsible for the antitumor activity of EO.

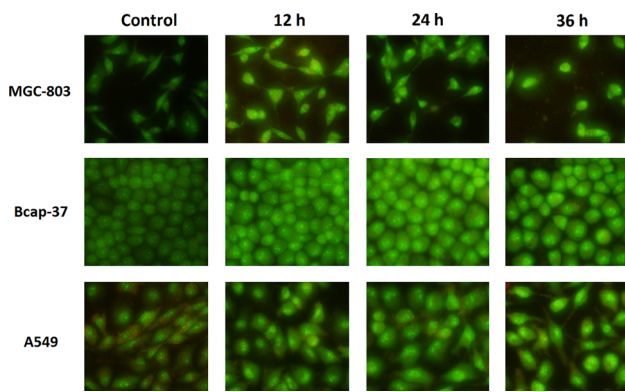


Fig. 3 Nuclei morphological changes during EO-induced apoptosis in MGC-803, Bcap-37, and A549 cell lines detected by AO/EB staining

Apoptosis, a highly structured and orchestrated process, performs a significant role in regulating cell number for the growth and homeostasis of tissues by eliminating aged, damaged, and unwanted cells (Lope Pihie *et al.*, 2012), and it is an important phenomenon in cytotoxicity induced by anticancer drugs (Kim *et al.*, 2002). The execution of apoptosis, or programmed cell death, is associated with characteristic morphological. Thus, the antiproliferative activity of EO was further investigated by apoptosis assay to determine the mode of cell death involved. For this purpose, treated and untreated cells were subjected to acridine orange/ethidium bromide (AO/EB) double-staining, and the induction of apoptosis was further confirmed by Hoechst 33258 staining.

AO is taken up by both viable and nonviable cells and emits green fluorescence if intercalated into double stranded nucleic acid (DNA) or red fluorescence if bound to single stranded nucleic acid (RNA), whereas EB is taken up only by nonviable cells and emits red fluorescence by intercalation into DNA. Thus the stained cells revealed four different types under a fluorescence microscope: (1) Viable cells have uniform bright green nuclei with organized structure; (2) Early apoptotic cells have green nuclei, but perinuclear chromatin condensation is visible as bright green patches or fragments; (3) Late apoptotic cells have orange to red nuclei with condensed or fragmented chromatin; (4) Necrotic cells have a uniformly orange to red nuclei with organized structure (Attaril *et al.*, 2009). MGC-803, Bcap-37, and A549 cells were treated with EO at 0.15 mg/mL for 12, 24, and 36 h. The cells treated with 1 % DMSO were used as negative control. The results are present in Fig. 3. Finding from the microscopic examination showed that live cells in the negative group displayed normal green nuclei, while the nuclei of the cells treated with EO were found disintegrated and fragmented into distinct spherical fragments with highly dense chromatin. These phenomena are associated with cell apoptosis.

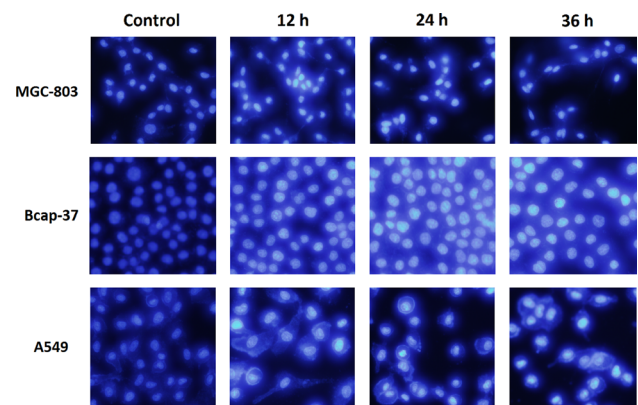


Fig. 4 Nuclei morphological changes during EO induced apoptosis in MGC-803, Bcap-37, and A549 cell lines detected by Hoechst 33258 staining

The inhibitory effect of EO on cancer cells via induction of apoptosis was further confirmed by Hoechst 33258 staining. Live cells with homogeneous light blue nuclei could be observed under fluorescence microscopy after staining with Hoechst 33258, and the apoptotic cells displayed bright blue due to karyopyknosis and chromatin condensation after treating with Hoechst 33258, and the nuclei of dead cells could not be stained (Roy *et al.*, 2013). To determine the mode of cell death induced by EO, the MGC-803, Bcap-37, and A549 cells were treated with EO at 0.15 mg/mL in different time intervals (12, 24, and 36 h). The cells treated with 1 % DMSO were used as negative control. The results are given in Fig. 4. Under fluorescence microscopy, the cells in the negative group displayed normally blue, while EO-treated cells showed obvious morphological changes (bright blue), including cell shrinkage and reduced in number. These phenomena also revealed that EO inhibited the growth of cancer cells involved with apoptosis.

Also, TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling) is a popular method for detecting DNA fragmentation that results from apoptotic signaling cascades. Apoptotic cells with brown color could be observed under a light microscope after staining with TUNEL (Liu *et al.*, 2013a). Thus, the induction of apoptosis could be further confirmed by TUNEL assay again. In this study, the cancer cells (MGC-803, Bcap-37, A549) were treated with EO at 0.15 mg/mL from 12 to 36 h, and the cells treated with 1 % DMSO were used as negative control. The TUNEL stained-cells were observed under a light microscope. The results are illustrated in Fig. 5. Microscopically, the cells in the negative group did not appear as brown precipitates, whereas the cells treated with EO in different time intervals appeared as brown precipitate. In addition, EO-treated cells also displayed other obvious morphological changes (cell shrinkage and

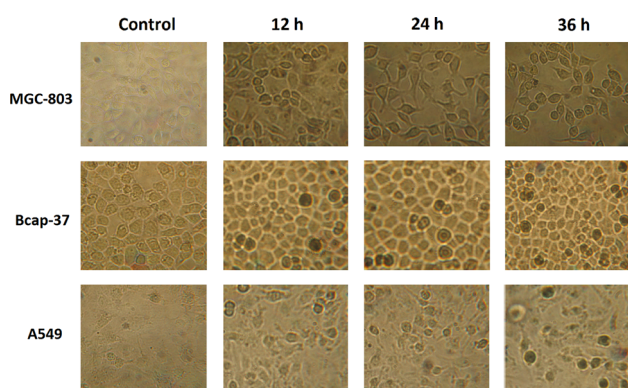


Fig. 5 Nuclei morphological changes during EO induced apoptosis in MGC-803, Bcap-37, and A549 cell lines detected by TUNEL assay

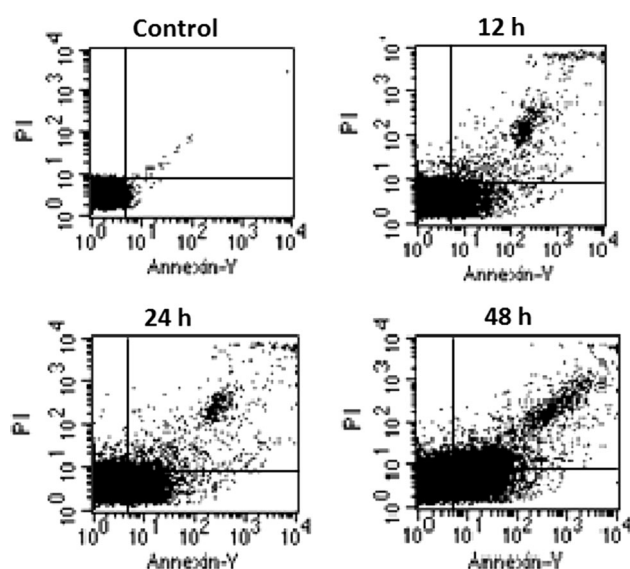


Fig. 6 Annexin V/PI dual staining of MGC-803 cell line

reduced in number). Therefore, we further concluded that EO-induced apoptosis against cancer cells.

The apoptosis ratios induced by EO in MGC-803 cell line were quantitatively assessed by flow cytometry (FCM). MGC-803 cells were double-labeled by the annexin V/PI after they were treated with EO at 0.15 mg/mL from 12 to 48 h. The cells treated with 1 % DMSO were used as negative control. The results are representative of three independent experiments. Four quadrant images were observed by flow cytometric analysis (Fig. 6): necrotic cells (the upper left quadrant), late apoptotic cells (the upper right quadrant), intact cells (the lower left quadrant) or early apoptotic cells (the lower right quadrant) (Liu *et al.*, 2012). As shown in Fig. 7, results from the study showed that the percentage of apoptotic MGC-803 cells that have been treated with 0.15 mg/mL of EO, was increased in a time-dependent manner with 13.56 % at 12 h, 19.22 % at 24 h, and 26.44 % at 48 h. This

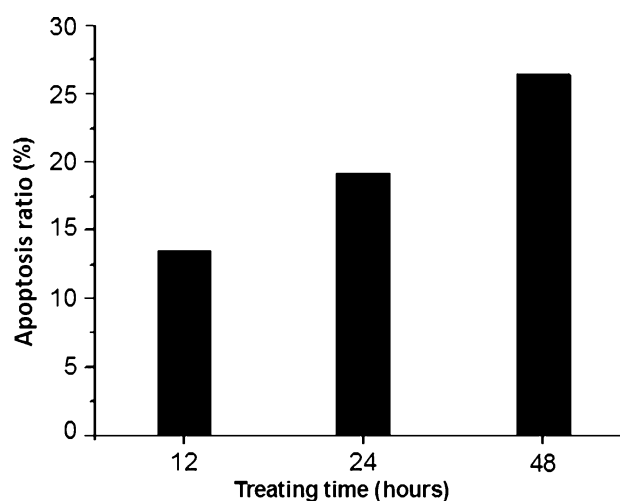


Fig. 7 The apoptosis ratios of MGC-803 cells treated with EO assessed by flow cytometry

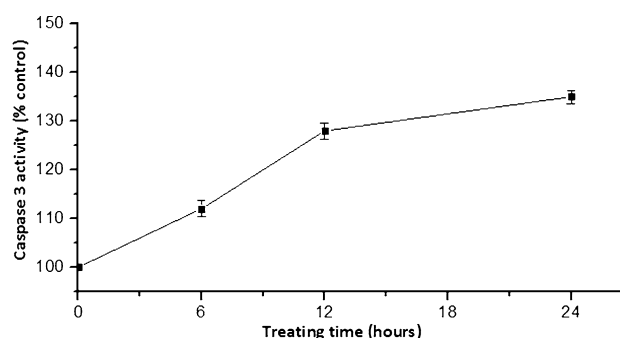


Fig. 8 Activation of caspase 3 on MGC-803 cell line treated with EO. Values are mean of three experiments

observation indicated that longer the exposure time to EO, the higher the percentage of apoptotic MGC-803 cells.

The occurrence of the apoptotic process was confirmed by the presence of caspase 3 (which plays a crucial role and is a key element in the execution phase of apoptosis) (Marchal *et al.*, 2004). We have chosen gastric cancer cell line MGC-803 to investigate general activation of caspases by spectrophotometry, as shown in Fig. 8. After incubation with EO from 6 to 24 h, the activity of caspase 3 increased compared to the control. It can be included that EO seemed to induce apoptosis with the involvement of caspases on gastric cancer cell line.

Conclusions

In our study, EO isolated from *C. fortunei* (J.) Smith leaves have been analyzed by GC-MS and its antitumor activities were investigated on MGC-803, MCF-7, BGC-823, Bcap-37, A375, and A549 cell lines. 26 compounds, representing

93.19 % of the total oil, were identified for the first time, and EO exhibited significant antitumor activities against MCG-803, Bcap-37, and A549 cell lines, with IC₅₀ values of 0.15, 0.17, and 0.24 mg/mL, respectively. We also found that 22,23-dihydrostigmasterol, β -amyrin, and stigmast-4-en-3-one, the main components, were responsible for its antitumor activities. In addition, the apoptosis inducing activities of EO on the three cancer cells were investigated through AO/EB staining, Hoechst 33258 staining, and TUNEL assay. At the same time, the apoptosis ratios were detected by FCM, with the apoptosis ratio of 26.44 % after 48 h of treatment at the concentration of 0.15 mg/mL on MGC-803 cells. The results revealed that *C. fortunei* (J.) Smith as a kind of herb medicines had potential to be employed in adjuvant therapy for treating for human gastric tumors. Further studies of the specific mechanisms of EO on human malignant tumors are currently underway.

Materials and methods

General procedures and reagents

The melting points of the products were determined using an XT-4 binocular microscope (Beijing Tech Instrument Co. Ltd., Beijing, China). Infrared spectra were recorded on a Bruker VECTOR22 spectrometer in KBr disks. ¹H-NMR and ¹³C-NMR were recorded using a JEOL-ECX500 spectrometer at 22 °C, with tetramethylsilane as the internal standard and CDCl₃ as the solvent. Column chromatography was performed using silica gel (200–300 meshes) (Qingdao Marine Chemistry Co., Qingdao, China). Sodium dodecyl sulfate (SDS) was purchased from Beijing Dingguo CO., Ltd; 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and DMSO were purchased from Roche Molecular Biochemicals (1465-007); Adriamycin (ADM) was purchased from Zhejiang Hisun Pharmaceutical Co., Ltd; β -Amyrin and stigmast-4-en-3-one had been prepared in previous work (Yang *et al.*, 2013b, 2013c). 22,23-Dihydrostigmasterol was isolated from EO and its purification process and NMR data are presented in supplementary material. All the other chemicals were of analytical reagent grade and used without further purification.

Plant materials

The fresh leaves of *C. fortunei* (J.) Smith (10 kg) were harvested at July 2012 from Longli, Guizhou province in China. The plant was identified by Prof. Qingde Long (Department of medicine, Guiyang Medical University, Guiyang, China) and a voucher specimen (CF2012XH0782) was deposited in the Museum of Guiyang Medical University. The sample was air-dried and ground to a powder.

Extraction of essential oil

About 250 g of dried leaves of *C. fortunei* (J.) Smith was subjected to extraction. A CO₂ flow rate of 30 L/h and an extraction period of 90 min were used. The extraction was performed under a pressure of 33 MPa and at a temperature of 42 °C. EO obtained by supercritical carbon dioxide extraction assay was yellow viscous oil (yield 1.9 %). The oil was dried over anhydrous Na₂SO₄ and placed at a low temperature in the refrigerator until analysis.

Gas chromatography-mass spectroscopy (GC–MS) analysis

A gas chromatographic-mass spectral analysis was performed on EO of *C. fortunei* (J.) Smith leaves using an Agilent 6890 GC with Agilent 5973 mass selective detector (EI-MS, electron energy = 70 eV, scan range = 10–550 amu), and a fused silica capillary column (HP-5 ms, 30 m × 0.25 mm) coated with 5 % phenyl methyl siloxane (0.25 μ m phase thickness). The carrier gas was helium (99.999 %) with a flow rate of 1.0 mL/min. The injector temperature was 250 °C, and the oven temperature was programmed to initially hold for 2 min at 50 °C, then ramp to 290 °C at 5 °C/min for 2 min. The interface temperature was 280 °C. A 1 % (w/v) solution of each sample in dichloromethane CH₂Cl₂ was prepared, and 1 μ L was injected using a split injection technique with split ratio 20:1. The components were identified by comparison of their mass spectra with those of the NIST 5 mass spectra library.

Cell lines and culture

Human gastric cancer cells (MGC-803 and BGC-823), human breast cancer cells (MCF-7 and Bcap-37), human melanoma cancer cells (A375), and human lung cancer cells (A549) from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China), were cultured in RPMI 1640 medium (Gibco BRL, USA) supplemented with 10 % (v/v) heat-inactivated fetal bovine serum, 100 IU/ml penicillin, and 100 μ g/ml streptomycin. The cells were maintained in 5 % CO₂, at 37 °C until reaching approximately 50–70 % confluence and were then treated with different amounts of chemicals as indicated. DMSO alone was used as the vehicle control.

MTT assays

In vitro, the cytotoxic activity of EO was determined by the MTT cytotoxicity assay. When the cells were 80–90 % confluent, they were harvested by treatment with a solution

containing 0.25 % trypsin, thoroughly washed and resuspended in supplemented growth medium. Cells were inoculated into 96-well plates in 100 μ L at plating densities ranging 6×10^4 cells/mL depending on the doubling time of individual cell lines. After 24 h, different concentrations of EO or ADM (Positive control) were added to each well. The medium was removed after cells were treated with drug for 72 h, then replaced with 100 μ L of MTT. After 4 h, 100 μ L of SDS was added to dissolve the MTT crystals for 12 h. The optical density was determined using a microplate reader (BIO-RAD, model 680) at a test wavelength of 595 nm (Boncler *et al.*, 2014; Bhavsar *et al.*, 2011). The inhibitory rate and IC₅₀ (concentration of drug that inhibits cell growth by 50 %) were then calculated.

AO/EB staining

Cancer cells in the logarithmic growth phase were seeded at a density of 3×10^5 cells/mL in six-well plates and incubated for 24 h. Afterward, the medium was removed and replaced with fresh medium plus 10 % FBS and then supplemented with EO for a certain range of treatment time. After the treatment period, the cover slip with monolayer cells was inverted on the glass slide with 25 μ L of AO/EB stain (100 μ g/mL) (Liu *et al.*, 2013b). The fluorescence was read using an IX71SIF-3 fluorescence microscope (OLYMPUS Co., Japan).

Hoechst 33258 staining

Cancer cells in the logarithmic growth phase were seeded at a density of 3×10^5 cells/mL in six-well plates and incubated for 24 h. Afterward, the medium was removed and replaced with fresh medium plus 10 % FBS and then supplemented with EO for a certain range of treatment time. Then, the cells were collected and fixed with 4 % paraformaldehyde in PBS for 10 min, followed by staining with Hoechst 33258 staining (Beyotime Institute of Biotechnology, Jiangsu, China) at room temperature for 5 min. Finally, after the cells were washed twice with PBS, morphological changes of the cells were observed under a fluorescence microscope at 350 nm excitation and 460 nm emissions (Jia *et al.*, 2013).

TUNEL assay

Cancer cells in the logarithmic growth phase were seeded at a density of 3×10^5 cells/mL in six-well plates and incubated for 24 h. Afterward, the cells were treated EO for a certain range of treatment time. Afterward, the medium was removed and replaced with fresh medium plus 10 % FBS and then supplemented with EO for a certain range of treatment time. After the treatment period, the assay was performed using a colorimetric TUNEL apoptosis assay kit (Beyotime

Institute of Biotechnology, Jiangsu, China) according to the manufacturer's instructions. At last, the cells were rewashed twice with PBS, and were consequently imaged under an XDS-1B inverted biological microscope (Xu *et al.*, 2011).

Flow cytometry analysis

To determine the apoptosis induced by EO in MGC-803 cells, the annexin V-FITC apoptosis detection kit (Beyotime Institute of Biotechnology, Jiangsu, China) was used following the protocol described in the previous study (Liu, *et al.*, 2012). After exposure to EO at indicated concentrations for a certain range of treatment time, cells were collected, washed twice with PBS, gently resuspended in annexin V binding buffer and incubated with annexin V-FITC/PI in dark for 15 min and analyzed by flow cytometry (Di Rosso *et al.*, 2013). All experiments were performed three times.

Caspase 3 enzyme assay

The enzymatic assay of caspase induced by EO was measured using the manufacture's protocol (Beyotime Institute of Biotechnology, Jiangsu, China). Briefly, cells were lysed in a lysis buffer by freeze and thawing. The lysed cells were centrifuged at 18,000 g for 15 min. 80 μ L of reaction buffer and 10 μ L of Ac-DEVED-pNA were added to 10 μ L of supernatant liquid. After incubating at 37 °C for 3 h, the optical density of the reaction mixture was quantitated spectrophotometrically at a wavelength of 405 nm using 96-well plate reader (Bai *et al.*, 2013).

Statistical analysis

All statistical analyses were performed using SPSS 10.0, and the data were expressed as the mean \pm standard deviation (SD) of the values obtained from at least three replicates. Using analysis of variance (ANOVA), statistical significance was determined. Mean values with probability values of $P < 0.05$ were taken as statistically significant.

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Conflict of interest None.

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