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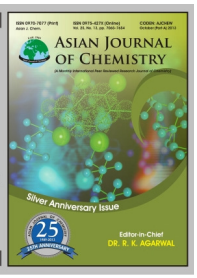

Antitumor and Antioxidant Activities of Extracts from *Cyrtomium fortumei* (J.) Smith

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The *in vitro* antitumor activities of crude petroleum ether, ethyl acetate and *n*-BuOH extracts of *Cyrtomium fortumei* (J.) Smith were studied on the A375, Bcap-37, BGC-823 and PC3 cell lines. The antioxidant activities of these crude extracts were also tested by DPPH (2,2-diphenlyl-1-picrylhydrazyl) radical scavenging assay. The results relvealed that ethyl acetate and *n*-BuOH extracts showed significant antitumor activities and exhibited high DPPH free radical scavenging effects. In addition, Fr.5 isolated from ethyl acetate extract had good antitumor activities and the inhibitory ratios of Fr.5 at 100 µg/mL were 82.4, 81.2, 75.1 and 80.9 % against the four cell lines respectively.

Key Words: *Cyrtomium fortumei*, Ethyl acetate extract, Isolate, Antitumor, Antioxidant.

INTRODUCTION

In recent years, malignant neoplasm is the major cause of human deaths worldwide, due to its high incidence and mortality. Thousands of people die of cancer every year, despite aggressive treatment regimens including surgery, chemotherapy, radiation therapy and palliative care. Because of the infiltrative nature and the rapid recurrence of the malignant tumor, complete surgical resection of these tumors is typically not achieved[1,2] and the conventional radiation and chemotherapy are often intolerable due to the strong systemic toxicity and local irritation[3-5]. These factors highlight the urgent need for new therapies of therapeutic combinations to improve the survival and quality of life of cancer patients. Oxidative stress has been suggested to be a contributory factor in development and complication of diabetes[6]. Antioxidants are substances that may protect cells from the damage caused by unstable molecules known as free radicals[7]. Free radical damage may lead to cancer[8]. Antioxidants interact with and stabilize free radicals and may prevent some of the damage free radicals might otherwise cause[9]. Some research have shown that natural antioxidants, such as tea and some Chinese herbs, have scavenging activity and also decrease in lipid peroxidation activity[10,11].

In recent years, interest in phytochemicals from traditional Chinese medicinal herbs has been growing rapidly because of their ability to inhibit the growth of cancer cells and exhibit scavenging activity. *Cyrtomium fortumei* (J.) Smith, one of the popular Chinese herbal medicines, belongs to the Dryopteridaceae family which comprises approximately 14 genera and 1700 species throughout the world. In China, it is mainly distributed in Guizhou, Yunnan, Guangxi and Sichuan provinces. It is a traditional Chinese herbal medicine used in the treatment of several viral diseases listed in the Chinese Pharmacopoeia (2005 edition). Powdered rhizomes and extracts from the plant have been used in the past as vermifuges for human[12]. It is widely used to cure many diseases such as influenza, acute and chronic pharyngitis, cancer, migraine[13]. Also, the herb was used to cure severe acute respiratory syndrome (SARS), a life-threatening viral respiratory illness believed to be caused by a coronavirus[14]. Many species of the Dryopteridaceae family contain phoroglucinols and favonoids in their rhizome and scales. Flavonoids are known to display a wide array of pharmacological and biochemical actions. Flavonoids and other phenolic compounds act as antioxidants by the free radical scavenging properties of their hydroxyl groups and are also effective metal chelators[15]. The extensive conjugation across the flavonoid molecule and numerous hydroxyl groups enhances their antioxidant propertis[16].

As part of our ongoing research on the plant, the rhizomes of *Cyrtomium fortumei* (J.) Smith were extracted using different solvents and the crude extracts were tested the antitumor activities against the PC3, BGC-823, Bcap-37 and A375 cell lines as well as DPPH (2,2-diphenlyl-1-picrylhydrazyl) free radical scavenging activities. The results showed that the ethyl acetate and *n*-BuOH extracts had great antitumor activities and high DPPH free radical scavenging effect among the three extracts. Furthermore, some fractions isolated from the two extracts had good antitumor activities and strong antioxidant activities.

EXPERIMENTAL

Column chromatography was performed using silica gel (200-300 meshes) (Qingdao Marine Chemistry Co., Qingdao, China) and silica gel H (Qingdao Marine Chemistry Co., Qingdao, China), Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden), HP-20 (Mitsubishi Chemical Corp., Toukyu Met, Japan) and MCI-gel CHP 20P (Mitsubishi Chemical Corp., Tokyo Met, Japan). All other chemicals were of analytical reagent grade and used without further purification. Sodium dodecyl sulfate (SDS) were purchased from Beijing Dingguo Co. Ltd.; 2,2-diphenlyl-1-picrylhydrazyl (DPPH) and Vitamin C (Vc) were purchased from Aladdin Reagent Inc; 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and DMSO was purchased from Roche molecular biochemicals (1465-007); adriamycin (ADM) was purchased from Zhejiang Hisun Pharmaceutical Co. Ltd.; All the other chemicals were of analytical reagent grade and used without further purification.

Fresh sample of *Cyrtomium fortumei* (J.) Smith was collected in Longli, Guizhou Province, China, on July, 2011. The plant material was identified by Prof. Qing-De Long, Department of Medicine, Guiyang Medical University. A voucher specimen is deposited at Guiyang Medical University, Guiyang, China.

Extraction and isolation: Dried rhizomes of *Cyrtomium fortumei* (J.) Smith (10 kg) were cut into pieces and extracted with 80 % EtOH (3 × 40 L) under reflux, 6 h for the first time, 3 h for the second time and 1 h for the last time. The combined EtOH extracts were evaporated to dryness to yield a dried EtOH extract (424 g). The extract was suspended in water and then extracted with petroleum ether (10 L × 3 times), ethyl acetate (10 L × 3 times) and *n*-BuOH (10 L × 3 times), respectively. Then petroleum ether extract (46 g), ethyl acetate extract (95 g) and *n*-BuOH extract (110 g) were obtained, respectively. The antitumor and antioxidant activities of these extracts were tested before further isolation process and it was found that ethyl acetate and *n*-BuOH extracts exhibited best inhibitory activities against *in vitro* proliferation of PC3, BGC-823, Bcap-37 and A375 cell lines and had high antioxidant activities. Thus, only ethyl acetate and *n*-BuOH extracts were isolated. The organic ethyl acetate extract (95 g) was subjected to CC on silica gel (200-300 mesh) eluted with a gradient of petroleum ether-EtOAc (20/1, 10/1, 5/1, 2/1 and 1/1, v/v) and finally a mixture of chloroform and methanol (10/1, 5/1 and 1/1, v/v, respectively) to get 10 fractions, namely, fractions 1-10. The fractions were monitored by TLC. The *n*-BuOH extract (110 g) was suspended in H_2O, adsorbed on an MCI-gel column (500 g) and eluted using mixtures of H_2O and MeOH starting with 20 % MeOH (v/v) to 100 % MeOH in 10 % increments (2.5 L each fraction) to afford a total of 6 fractions, namely fraction 11-16. The fractions were also monitored by RP-TLC. All spots on TLC were visualized by heating silica gel plates sprayed with 10 % Phosphomolybdic acid hydrate in EtOH and 1 % $FeCl_3$ in EtOH.

Cell culture: Human malignant melanoma cell line A375, breast cancer cell line Bcap-37, gastric cancer cell line BGC-823, prostate cancer cell line PC3 and mouse fibroblast cell line NIH3T3 were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The entire cancer cell lines were maintained in the RPMI 1640 medium and NIH3T3 was maintained in the DMEM medium. They were supplemented with 10 % heat-inactivated fetal bovine serum (FBS). All cell lines were maintained at 37 ºC in a humidified 5 % carbon dioxide and 95 % air incubator.

MTT (thiazolyl blue tetrazolium bromide) assay: All tested extracts were dissolved in DMSO and subsequently diluted in the culture medium before treatment of the cultured cells. 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 (2×10^3/well) were plated in 100 µL of medium/well in 96-well plate. After incubations overnight, the cells were treated with different concentrations of extracts in RPMI 1640 with 10% FBS for 72 h. In parallel, the cells treated with 0.1 % DMSO served as negative control and adriamycin as positive control. Finally, 100 µL of MTT was added and the cells were incubated for 4 h. The MTT-formazan formed by metabolically viable cells was dissolved in 100 µL of SDS for 12 h. The absorbance was then measured at 595 nm with a microplate reader (BIO-RAD, model 680), which is directly proportional to the number of living cells in culture[17]. The percentage cytotoxicity was calculated using the formula.

$$\text{Cytotoxicity \%} = \left(\frac{(\text{Control abs} - \text{Blank abs}) - (\text{Test abs} - \text{Blank abs})}{(\text{Control abs} - \text{Blank abs})} \right) \times 100$$

Flow cytometry analysis: Cells in different states were observed with an annexin V-EGFP/PI double staining. A375 cells were treated with 50 µg/mL of Fr. 5 for 72 h. And cells (1×10^6/mL) were washed twice with cold PBS and then re-suspended gently in 500 µL binding buffer. Thereafter, cells were stained in 5 µL Annexin V-FITC and shaked well. Finally, 5 µL propidium iodide (PI) was added to these cells and incubated for 15 min in the dark, analyzed by FACS Calibur, Becton Dickinson[18]. All experiments were performed three times.

DPPH free radical scavenging assay: The DPPH free radical has been widely used to evaluate the antioxidant capacity of fractions and pure compounds, which is stable due to its resonance stability and special blockade of benzene rings. The purple chromogen radical DPPH is reduced by antioxidant compounds to the corresponding pale yellow hydrazine[19]. The antioxidant activity of plant extracts and antioxidant standard were assessed on the basis of radical scavenging effect of the stable DPPH free radical[20]. In its radical form, DPPH has a characteristic absorption at 515 nm in ethanol, which disappears with acceptance of an electron from the antioxidant sample. All tested extracts were dissolved in ethanol. 100 mL of DPPH in ethanol was added in a 96-well plate and was mixed with the test samples (100 mL) at different concentrations. After shaken for 60 s in microplate reader (BIO-RAD, model 680), it was left in the dark at 37 ºC for 0.5 h. The absorbance was then measured at 515 nm with a microplate reader. All experiments were carried out in triplicate. Ethanol was used as the blank control and vitamin C (Vc) served as positive control. The DPPH radical scavenging activity of the extracts and fractions were calculated according to the following formula.

$$\%\,\text{DPPH scavenging activity} = \left(\frac{OD_{blank} - OD_{sample}}{OD_{blank}} \right) \times 100$$

TABLE-1
ANTITUMOR ACTIVITIES OF DIFFERENT EXTRACTS OF *Cyrtomium fortumei* (J.) Smith

Extracts	Conc. (μg/mL)	Inhibition rate for different cancer cell lines (%, mean ± SD)[a]			
		A375	Bcap-37	BGC-823	PC3
Petroleum ether	50	27.7 ± 3.1	13.5 ± 2.2	19.8 ± 2.9	35.5 ± 1.1
	200	60.5 ± 3.2	65.0 ± 0.6	69.9 ± 3.8	68.0 ± 1.9
Ethyl acetate	50	69.8 ± 2.1	59.6 ± 3.3	65.7 ± 1.7	73.1 ± 2.2
	200	88.7 ± 3.3	86.1 ± 3.1	86.6 ± 2.1	89.6 ± 1.5
n-BuOH	50	35.9 ± 2.0	41.3 ± 2.7	33.6 ± 3.5	37.6 ± 2.1
	200	85.2 ± 3.7	82.1 ± 1.2	84.1 ± 1.6	80.2 ± 2.4
Adriamycin	50	94.1 ± 2.2	97.5 ± 2.6	95.6 ± 3.1	95.4 ± 1.9

Note: [a] Inhibitory percentage of cells of each fraction for 72 h and SD = standard deviation

TABLE-2
ANTITUMOR ACTIVITIES OF 16 FRACTIONS

Fractions	Inhibition rate for different cell lines (%, mean ± SD)[a]				
	A375	Bcap-37	BGC-823	PC3	NIH3T3
Fr.1	25.8 ± 2.5	24.3 ± 3.6	30.1 ± 0.8	23.3 ± 3.1	10.3 ± 4.4
Fr.2	56.6 ± 2.6	59.9 ± 1.4	65.0 ± 0.7	67.7 ± 0.9	13.0 ± 2.2
Fr.3	83.5 ± 4.2	72.3 ± 4.2	77.1 ± 5.7	80.1 ± 3.0	43.6 ± 1.9
Fr.4	64.1 ± 2.8	66.4 ± 4.4	67.2 ± 3.6	63.7 ± 3.4	25.6 ± 1.5
Fr.5	82.4 ± 5.1	81.2 ± 3.8	75.1 ± 3.2	80.9 ± 3.6	19.4 ± 2.6
Fr.6	80.3 ± 2.6	72.5 ± 4.3	72.2 ± 2.5	70.2 ± 0.5	36.1 ± 0.8
Fr.7	32.5 ± 2.0	31.1 ± 1.7	23.8 ± 1.4	34.5 ± 2.3	5.7 ± 3.5
Fr.8	51.3 ± 1.1	52.3 ± 1.5	45.6 ± 3.8	50.5 ± 2.4	8.2 ± 1.2
Fr.9	60.1 ± 2.3	64.9 ± 6.1	66.7 ± 5.4	61.2 ± 4.4	13.1 ± 2.0
Fr.10	40.4 ± 2.7	45.6 ± 2.5	47.8 ± 2.3	44.4 ± 3.3	21.7 ± 3.6
Fr.11	38.4 ± 2.6	40.3 ± 3.2	34.4 ± 4.0	30.3 ± 4.2	15.0 ± 3.9
Fr.12	83.1 ± 5.0	81.0 ± 2.3	83.3 ± 3.7	85.4 ± 4.9	45.8 ± 2.3
Fr.13	69.2 ± 2.4	76.6 ± 4.8	61.1 ± 2.7	67.9 ± 3.3	22.2 ± 5.1
Fr.14	75.1 ± 2.0	73.0 ± 1.4	78.8 ± 3.4	65.2 ± 5.5	39.9 ± 1.0
Fr.15	23.4 ± 0.8	20.3 ± 1.4	15.6 ± 4.0	10.3 ± 2.5	13.2 ± 3.3
Fr.16	34.6 ± 4.9	37.9 ± 1.6	32.2 ± 2.3	40.8 ± 3.6	12.9 ± 2.7
Adriamycin	96.8 ± 3.2	95.3 ± 1.1	94.5 ± 2.4	98.0 ± 2.8	95.9 ± 0.5

Note: [a] Inhibitory percentage of cells treated with 100 μg/mL of each fraction for 72 h

RESULTS AND DISCUSSION

Antitumor activities of the plant extracts: The potential effect of the extracts from *Cyrtomium fortumei* was investigated on the viability of A375, Bcap-37, BGC-823 and PC3 cell lines using MTT assay, with adriamycin being used as the positive control and culture medium containing 0.1 % DMSO used as the negative control. The antitumor activities of different extracts are presented in Table-1. Table-1 showed the antitumor activities of different extracts (petroleum ether, ethyl acetate and *n*-BuOH extracts). The ethyl acetate and *n*-BuOH extracts had significant antiproliferative activities against the four cell lines at 200 μg/mL. Thus, the ethyl acetate and *n*-BuOH extracts were further fractioned to yield 16 fractions. The antitumor activities of the 16 fractions were listed in Table-2. Table-2 showed that some fractions also had moderate activities. Moreover Fr.3, Fr.5, Fr.6 and Fr.12 showed the best *in vitro* antiproliferation activities at 100 μg/mL. At the same time, the 16 fractions were tested on normal cell line NIH3T3. It can be seen from Table- 2 that most of the fractions were less toxic on normal cell line than on the investigated tumor cell lines and were selective to cancer cells. Furthermore, the inhibitory ratios of Fr.5 at 72 h after treatment were 82.4, 81.2, 75.1, 80.9 and 19.4 % against A375, Bcap-37, BGC-823, PC3 and NIH3T3 cell lines. Therefore, the results indicated Fr.5 had great antitumor activity and more selective to cancer cells than other fractions. In addition, Fr.5 was selected to analyze the mechanism by flow cytometry analysis (FCM). As show Fig. 1, the upper left quadrant of apoptosis diagrams demonstrated that mechanically injured cells; the upper right quadrant demonstrated that the late apoptotic cells; the lower left quadrant demonstrated that the normal cells; the lower right quadrant demonstrated that the early apoptotic cells. It is evident from Fig. 1B that the second and forth quadrant of Fr.5 presented positive results. These observations demonstrated that Fr.5 group exhibited simultaneously early and late apoptotic cells against A375 cells and the apoptosis ratios reached 34.63% after 72 h at 50 μg/mL.

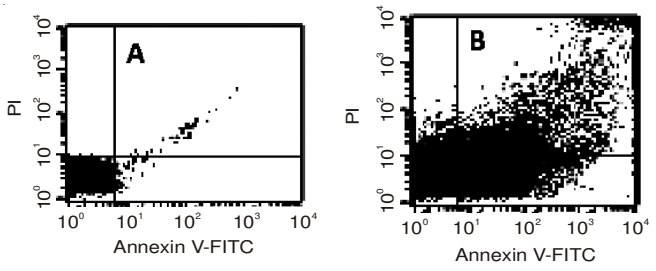

Fig. 1. Scatter diagram of FCM tested apoptosis on A375 cell lines. (A) negative control; (B) cells treated with Fr.5 (50 μg/mL) for 72 h

TABLE-4
ANTIOXIDANT ACTIVITIES OF 16 FRACTIONS

Frs	Scavenging rate (%, mean ± SD)		Frs	Scavenging rate (%, mean ± SD)	
	25 μg/mL	100 μg/mL		25 μg/mL	100 μg/mL
Fr.1	6.2 ± 3.4	9.2 ± 0.9	Fr.9	66.7 ± 1.0	77.1 ± 0.5
Fr.2	51.1 ± 0.5	78.9 ± 2.3	Fr.10	81.9 ± 1.7	85.2 ± 0.9
Fr.3	75.8 ± 3.6	92.9 ± 0.9	Fr.11	26.6 ± 3.9	59.1 ± 1.3
Fr.4	48.4 ± 2.4	81.4 ± 0.7	Fr.12	77.4 ± 2.1	86.1 ± 0.7
Fr.5	85.9 ± 0.6	86.6 ± 0.8	Fr.13	80.2 ± 0.6	82.9 ± 0.5
Fr.6	80.6 ± 2.4	89.7 ± 1.7	Fr.14	21.2 ± 1.0	52.8 ± 0.7
Fr.7	30.8 ± 2.3	50.9 ± 3.2	Fr.15	65.4 ± 2.3	85.0 ± 1.2
Fr.8	17.6 ± 1.8	30.4 ± 2.1	Fr.16	35.9 ± 1.4	67.7 ± 1.1
Vitamin C	96.7 ± 2.1	98.6 ± 1.4	–	–	–

Antioxidant activities of the plant extracts: The antioxidant activities of different extracts are presented in Table-3, which reveals that ethyl acetate and *n*-BuOH extracts exhibited strong DPPH free radical scavenging activities. However, it can be seen from Table-3 that petroleum ether did not have significant activity. At same time, the 16 fractions isolated from ethyl acetate and *n*-BuOH extracts were tested their antioxidant activities and the results were listed in Table-4. As shown in Table-4, Fr.3, Fr.5, Fr.6 and Fr.12 also exhibited high DPPH free radical scavenging activities. In addition, the antioxidant activity likely derived from the flavone ingredients contained in the ethyl acetate and *n*-BuOH extracts.

TABLE-3
ANTIOXIDANT ACTIVITIES OF DIFFERENT EXTRACTS OF *Cyrtomium fortumei* (J.) Smith

Extracts	Conc. (μg/mL)	Scavenging rate (%, mean ± SD)[a]
Petroleum ether	25	24.9 ± 3.1
	100	55.7 ± 0.9
Ethyl acetate	25	85.4 ± 2.4
	100	96.7 ± 3.5
n-BuOH	25	88.8 ± 1.4
	100	97.3 ± 2.2
Vitamin C[b]	25	96.9 ± 3.2

Note: [a] Scavenging rate of each fraction and SD = standard deviation; [b] The standard compound used for comparison of activities

Conclusion

The ethyl acetate extract, *n*-BuOH extract and four fractions (Fr.3, Fr.5, Fr.6 and Fr.12) of *Cyrtomium fortumei* exhibited significant antiproliferative activities against the A375, Bcap-37, BGC-823 and PC3 cell lines. Fr.5, in particular, displayed inhibition rates of 82.4, 81.2, 75.1 and 80.9 % at 100 μg/mL against the four cancer cell lines and less toxic on normal cell line than on cancer cell lines, and the flow cytometry analysis indicated that Fr.5 could induce apoptosis in A375 cells. Also, the ethyl acetate and *n*-BuOH extracts exhibited strong DPPH free radical scavenging activities and the some fractions also had high antioxidant activities.

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