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Article

Antioxidant and Anticancer Activities of Methanol and Water Extracts from Leaves of *Cirsium japonicum*

Yu Yin, Seong-Il Heo, and Myeong-Hyeon Wang*

School of Biotechnology, Kangwon National University, Chuncheon 200-701, Korea

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Potential antioxidant activities of methanol and water extracts of *Cirsium japonicum* var. *ussuriense* (CJ) leaves were examined. The reducing power and hydroxyl raical-scavenging activity assays showed that the methanol extract had a significantly higher activity than the water extract. In addition, the methanol extract showed a concentration-dependent reducing power, ranging from 0.228 to 1.072 (0.1~0.5 mg/mL), as well as a high DPPH free radical-scavenging activity (EC_{50} = 40.25 μg/mL). The total phenolic (as tannic acid) and flavonoid (as quercetin) contents of the extract were 62.41 mg/g and 13.48 mg/g, respectively. The cytotoxic activity indicated that the methanol extract has an inhibition activity in the stomach carcinoma cell (35.40%), suggesting that the methanol extract of CJ leaves could be used as a potential source of pharmaceutical material.

Key words: *anticancer activity, antioxidant activity,* Cirsium japonicum *var.* ussuriense, *methanol and water extracts*

Free radicals are implicated in the etiology of several degenerative diseases, such as coronary artery disease, stroke, rheumatoid arthritis, diabetes, and cancer from both endogenous and exogenous sources [Halliwell *et al.*, 1992]. Attention is being paid to the cytotoxicity of the reactive oxygen as the cause of various pathological conditions. Lipid peroxides produced from unsaturated fatty acids via radicals cause cytotoxicity and promote the formation of additional free radicals through a series of chain reactions [Choi *et al.*, 2006]. Furthermore, the formation of cancer cells can be directly induced by the free radicals [Athukorala *et al.*, 2006]. Malignant tumors are one of the most serious diseases that damage the human health worldwide. However, chemically synthesized medicines are costly and may have side effects. For these reasons, natural medicines for the treatment of cancer, possibly by inhibiting the growth of tumors, are becoming important. Polyphenols belong to a heterogeneous class of compounds with a variety of antioxidant actions. The antioxidative and the radical-scavenging activities of medicinal plants have been studied [Singh *et al.*, 2002; Zhang *et al.*, 2006]. Among the various natural antioxidants, phenolic compounds were found to quench the oxygen-derived free radicals by donating a hydrogen atom or an electron to the free radicals [Wanasundara *et al.*, 1996].

CJ, which belongs to the Compositae family, is a wild perennial herb found in many areas of China, Korea, and Japan. The main compounds of CJ are triterpenoid, steroids, essential oil, and flavones [Zhi *et al.*, 2001]. CJ has been used as a hypertensive, a uretic, and a hepatitis agent in the treatment of tumors such as liver cancer, uterine cancer, and leukemia [Lui *et al.*, 2006].

To determine whether CJ could be a source of natural antioxidants or potential anticancer agents for pharmaceutical application, the present study evaluated the biological activities including free radical-scavenging activity, reducing power, total phenolics content, and flavonoid content of CJ. We evaluated the antiproliferative effect of CJ extracts in the human colon carcinoma cell line (HT-29) and the human stomach carcinoma cell line (NCI-N87), as well as a normal kidney cell line (293) as the control.

Material and Methods

Preparation of extracts. CJ was obtained from

*Corresponding author
Phone: +82-33-250-6486; Fax: +82-33-241-6480
E-mail: mhwang@kangwon.ac.kr

Abbreviations: CJ, *Cirsium japonicum* var. *ussuriense*; DMSO, dimethyl sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; MTT, methyl thiazolyl tetrazolium; OD, optical density; PBS, phosphate buffered saline; Que, quercetin; Tan, tannic acid; TBA, thiobarbituric acid; TCA, trichloroacetic acid

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Table 1. The yield, total phenolic and flavonoid content of methanol and water extracts of CJ leaves

Extracts	Yield (%)	Total phenolic content (mg Tan[a]/g)	Total flavonoid content (mg Que[b]/g)
MeOH ext.	12.36	62.41±0.24	13.48±0.36
Water ext.	24.13	47.27±0.05	0.34±0.04

[a]Tannic acid (Tan) was used as a standard for measuring of the total phenolic content.
[b]Quercetin (Que) was used as a standard for measuring of the total flavonoid content.
Values are expressed as mean±SD (n=3).

Chuncheon, Korea. Leaves of CJ were dried at 60°C and powdered, and 100 g CJ powder was refluxed with methanol or water (w/v = 1:20) for 3 days at room temperature. The mixtures were filtered and evaporated under vacuum. The yield of each extract is shown in Table 1.

Cell lines and culture medium. HT-29 cells (human colon carcinoma cell line), NCI-N87 cells (human stomach carcinoma cell line), and 293 cells (human kidney normal cell line) were purchased from the Korean Cell Line Bank. HT-29 and NCI-N87 cells were cultured in RPMI 1640, supplemented with 10% (v/v) FBS and 100 U/mL penicillin-streptomycin solution. The 293 cells were cultured in Dulbecco's Modified Eagle's Medium, supplemented with 10% (v/v) FBS, 100 U/mL penicillin-streptomycin solution [Lavia *et al.*, 2006].

Measurement of total phenolic and flavonoid contents. One milliliter samples of different concentrations were each mixed with 2 mL Folin-Denis reagent and 2 mL of 35% sodium carbonate. The mixtures were shaken thoroughly and made up to 10 mL with distilled water. The absorbance at 765 nm was determined after incubation at room temperature for 30 min. Phenolic content was determined from a standard curve obtained with Tan [Birt *et al.*, 2001; Lin *et al.*, 2007].

Flavonoid content was determined as follows: One milliliter of the methanol extract (10 mg/mL) was mixed with 1 mL aluminium trichloride in ethanol (20 mg/mL), and the absorbance was measured at 415 nm. Blank samples were prepared from 1 mL plant extract and 1 mL ethanol. The absorption of a standard Que solution (0.5 mg/mL) in ethanol was measured under the same conditions. All determinations were carried out in duplicates. The Que equivalents of the flavonoids in CJ were calculated using the following equation:

$$X = (A \cdot m_0)/(A_0 \cdot m)$$

where X is the flavonoid content (mg/mg) of plant extract, A is the absorption of CJ, A_0 is the absorption of the standard quercetin solution, m is the weight of CJ denoted in g, and m_0 is the weight (mg) of Que in the solution [Lin and Tang, 2007].

DPPH radical-scavenging activity assay. The scavenging activity the DPPH free radical was determined by using the method of Yoshida *et al.* [1989] with a slight modification. Two milliliters of CJ or water as the control was mixed with 2 mL DPPH· solution (0.02 mM in MeOH) and left standing at room temperature for 30 min. The optical density was measured at 517 nm with a UV/VIS spectrophotometer. The experiments were performed in triplicates. Ascorbic acid and α-Tocopherol were used as the positive controls. The DPPH radical scavenging activity was calculated according to the following equation:

$$\text{Scavenging activity (\%)} = [1 - (A_1 - A_2)/A_0] \times 100\%$$

where A_0 is the absorbance of the control (blank), and A_1 is the absorbance in the presence of CJ and A_2 the absorbance without DPPH. EC_{50} value is the concentration of the sample at which 50% of the DPPH free radicals is scavenged.

Reducing power assay. The reducing power of CJ was determined using the method of Oyaizu [1986]. CJ (1 mL) was mixed with 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. After incubation for 20 min at 50, 2.5 mL of 10% TCA was added to the mixture, followed by centrifugation at 3,000 rpm for 15 min. The upper layer solution (2.5 mL) was mixed with an equal volume of water and 0.5 mL of ferric chloride, and the absorbance was measured at 700 nm. The tests were run in triplicates.

Hydroxyl radical scavenging. Hydroxyl radical scavenging was carried out according to the method of Chung *et al.* [1997]. The Fenton reaction mixture consisted of 200 μL of each $FeSO_4$ (10 mM), EDTA (10 mM), and 2-deoxyribose (10 mM). Two hundred microliters of the sample and 1 mL of 0.1 M phosphate buffer (pH 7.4) were added to a total volume of 1.8 mL. Subsequently, 200 μL of 10 mM H_2O_2 was added, and the reaction mixture was incubated for 4 h at 37°C. After incubation, 1 mL of 2.8% TCA and 1 mL of 1% TBA were added, and the mixture was placed in a boiling water bath for 10 min. The mixture was then centrifuged (5 min, 300 rpm), and the absorbance was measured at 532 nm. The hydroxyl radical-scavenging activity was calculated according to the following equation:

Scavenging activity (%) = $[1 - (A_1 - A_2)/A_0] \times 100\%$

where A_0 is the absorbance of the control (blank), A_1 the absorbance in the presence of CJ, and A_2 the absorbance without 2-deoxyribose.

Antiproliferative assay. Cell growth inhibition activity was measured by using the MTT assay [Athukorala *et al.*, 2006; Wang *et al.*, 2006]. The cells were seeded in a 96-well plate at 2×10^5 cells/mL. After 4 h incubation at 37°C under a humidified atmosphere of 5% CO_2, the extracts were added to the final concentration of 300 μg/mL. The cells were further incubated for an additional 72 h at 37°C. MTT solution (50 μL, 2 mg/mL in PBS) was added to each well for a total reaction volume of 250 μL. After further incubation for 4 h, the supernatants of each well were spilt out. The formazan crystals in each well were dissolved in 150 μL DMSO. After 15-20 min, the amount of the purple formazan was determined by measuring the absorbance at 540 nm. For the treated cells, the viability was expressed as a percentage of the control cells. All determinations were carried out in triplicates.

Result and Discussion

Phenolic compounds are known to possess strong antioxidant activity [Rice-Evans *et al.*, 1997; Zhao *et al.*, 2006]. We, therefore, measured the total phenolic and flavonoid contents of the CJ leaves. The phenolic contents in methanol and water extracts were 62.41 and 47.27 mg Tan/g, respectively (Table 1). Flavonoids, such as flavones, isoflavones, flavonones, and anthocyanins, and catechins are natural phenolics [Wang *et al.*, 2005]. These compounds possess a broad spectrum of chemical and biological activities including radical-scavenging and strong antioxidant capacities. The flavonoid contents of methanol and water extracts respectively were 13.48 and 0.34 mg Que/g (Table 1). These results show the methanol extracts have higher phenolic and flavonoid contents than those of the water extracts.

DPPH· is used to evaluate the free radical-scavenging activity of natural compounds [Halliwell, 1995]. Table 2 shows EC_{50} of the scavenging activity measured with different extracts; the methanol extracts showed higher DPPH· (EC_{50} = 40.25 μg/mL) than the water extracts (EC_{50} = 222.71 μg/mL). Under the same conditions, EC_{50} of ascorbic acid and α-Tocopherol were 2.72 and 7.60 μg/mL, respectively.

Table 2. Antioxidant activities of methanol and water extracts of CJ leaves evaluated by DPPH radical-scavenging ability

Extracts	EC_{50}: (μg/mL)
MeOH ext.	40.25
Water ext.	222.71
Positive Control	
Ascorbic acid	2.72
α-Tocopherol	7.60

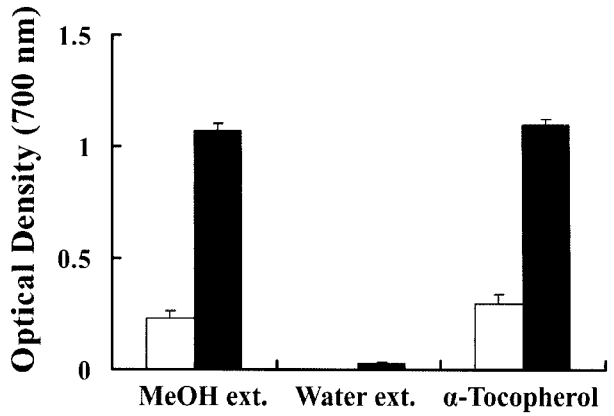

Fig. 1. Reducing power of methanol and water extracts from leaves of CJ. α-Tocopherol was used as a positive control (□: 100 μg/mL; ■: 500 μg/mL). Values are expressed as mean±SD (n=3).

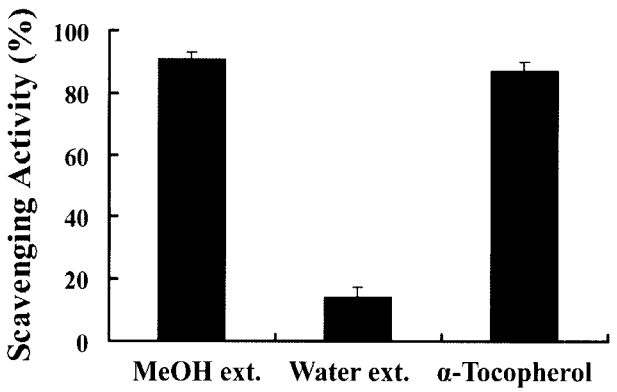

Fig. 2. Hydroxyl radical-scavenging activities of methanol and water extracts from leaves of CJ. α-Tocopherol was used as a positive control. The concentration of the extracts and α-Tocopherol was 100 μg/mL. Values are expressed as mean±SD (n=3).

The reducing activity of the CJ extracts increased with the increasing sample concentration (Fig. 1). Methanol extracts have strong reducing power, higher than that of the water extracts. Reducing power is associated with the presence of reductones, which have been shown to exert antioxidant effect by donating a hydrogen atom and breaking the free radical chain [Duh, 1998]. Reductones react with certain peroxide precursors, which prevent the formation of peroxides [Gordon, 1990]. Methanol extracts showed higher reducing power, suggesting that the methanol extracts contribute significantly to the antioxidant effect (Fig. 2). Scavenging activity of the methanol extracts

Table 3. Growth inhibitory activities of methanol and water extracts of CJ leaves on cell lines

Sample		Growth inhibition (%)		
		HT-29	NCI-N87	293
CJ	MeOH ext.	8.04±23.07	35.40±6.01	10.30±13.64
	Water ext.	-19.67±11.20	13.91±19.77	-18.15±21.13
Control	Paclitaxel	68.02±17.53	67.14±2.26	/
	10% DMSO	/	/	71.55±17.91

Paclitaxel was a positive control in cancer cells, and 10% DMSO was a positive control in normal cells.
Values are expressed as mean±SD (n=3).

(90.67%) was higher than that of α-tocopherol (87.42%,) suggesting that the hydroxyl radical-scavenging activity is dependent on the levels of the phenolic compounds. Phenolic compounds are found in both edible and inedible plants and have been reported to have multiple biological effects [Osman *et al.*, 2008]. The antioxidative activity of phenolic compounds is mainly due to their redox properties, which play an important role in absorbing and neutralizing free radicals [Siriwardhana *et al.*, 2002].

The MTT assay is a method of metabolically quantifying the viable cells through their ability to reduce a soluble yellow tetrazolium salt into the blue-purple formazan crystals [Mossman, 1983]. The absorbance of the solution varies depending on the number of live cells. The methanol extracts have higher cytotoxicity than the water extracts in HT-29 (8.04%) and NCI-N87 cells (35.40%) (Table 3). However, cytotoxicity of the methanol extracts on NCI-N87 cells was higher than 293 cells, suggesting the potential of methanol extracts exerting anticancer activity on the stomach cancer in the NCI-N87 cells. The anticancer activity of the CJ methanol extracts may be induced by phenolics and flavonoids. Triterpenoid [Chan, 2007] and steroids [Malíková *et al.*, 2008] showed cytotoxicity on several types of cancer cells.

In conclusion, CJ could be considered as an ingredient of functional foods as well as for pharmaceutical purposes. Furthermore, studies on the identification of the biologically active compounds in CJ containing antioxidant properties *in vivo* is necessary in the near future.

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