Journal of Medicinal Plants Research Vol. 4(6), pp. 477-483, 18 March, 2010
Available online at http://www.academicjournals.org/JMPR
DOI: 10.5897/JMPR10.035
ISSN 1996-0875© 2010 Academic Journals

Full Length Research Paper

Antioxidant and antiproliferative activities of methanolic extracts of *Perilla frutescens*

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Accepted 16 February, 2010

***Perilla frutescens* is a common traditional Chinese medicinal plant used to treat a variety of diseases. The methanolic extracts from different parts of *P. frutescens* were evaluated for their antioxidant and antiproliferative activities. The antioxidant activities of *P. frutescens* were evaluated by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging methods. Reducing power, metal chelating ability, total phenolic content and total flavonoid content were also detected. In all the tested models, the methanolic extracts of stalk, leaf and seed of *P. frutescens* showed their ability to scavenge the free radicals, reducing power and chelating capacity in a does-dependent manner. The antiproliferative activities of methanolic extracts from different parts of *P. frutescens* were also studied in vitro by using human non-small cell lung A549 cancer cells. The methanolic extract of stalk exhibited moderate antiproliferative activity. The methanolic extract of stalk had higher antioxidant and antiproliferative activities than of leaf and seed. These results indicated that stalk of *P. frutescens* might be used as a potential source of natural antioxidants and as an anti-tumor agent.**

Key words: *Perilla frutescens*, methanolic extract, antioxidant activity, radical scavenging, reducing power, antiproliferative.

INTRODUCTION

Oxidation is essential to many living organisms for the production of energy to fuel biological processes. Free radicals can lead to a variety of physiological and biochemical lesions (Ames, 1998) and induce degenerative diseases such as coronary artery disease, aging and cancer (Gorman et al., 1997). Although almost all organisms possess anti-oxidant defense and repair systems that have evolved to protect them against oxidative damage, these systems are insufficient to prevent the damage entirely. Antioxidants are such substances that can delay or restrict oxidative cellular oxidizable substrates. Interest in finding naturally occurring antioxidants in foods or medicines to replace synthetic antioxidants has increased considerably, given that synthetic antioxidants are being restricted due to their side effects (Zheng and Wang, 2001). The antioxidants in some plants play important roles in preventing diseases induced by free radicals (Hirose et al., 1994). Therefore, attention has been directed toward the development and isolation of natural antioxidants from plant sources.

Perilla frutescens, referred to as “zi-su” in China, belongs to the family Labiatae. *P. frutescens* is an annual herbaceous plant frequently used as one of the most popular garnishes and food colorants in some Asian countries such as China and Japan. In general, the stalks, leaves and seeds of *P. frutescens* are used individually in Chinese medicine to treat a variety of diseases (Chinese Pharmacopoeia Commission, 2005). The stalks of the plant are traditionally used as an analgesic and anti-abortive agent. The leaves are said to be helpful for asthma, colds and flus, and to regulate stomach function, while the seeds are employed for dyspnea and cough relief, phlegm elimination, and the bowel relaxation (Chinese Pharmacopoeia Commission, 2005). Considerable attention has been given to the anti-inflammatory, anti-allergic and anti-tumor promoting substances contained in *P. frutescens* (Lin et al., 2007;

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Makino et al., 2003; Takano et al., 2004; Ueda et al., 2002; Žekonis et al., 2008). Recently, animal studies hint that *P. frutescens* might also be useful for a different type of allergy: the severe, rapid reaction known as anaphylaxis, commonly associated with shellfish, peanut, and bee-sting allergies (Makino et al., 2001, 2003). Although studies were focused mainly on some antioxidant activity of *P. frutescens* extract (Chou et al., 2009; Gu et al., 2009; Jung et al., 2001; Meng et al., 2009; Nakamura et al., 1998), but the antioxidant and antiproliferative activities of methanolic extract of *P. frutescens* has not been reported. In general, the extraction method is another major factor to determine the composition of effective components and their contents in the resulting extract from the plant. Therefore, the objective of this study was to investigate the antioxidant and antiproliferative properties of methanolic extracts of *P. frutescens*.

MATERIALS AND METHODS

Chemicals

Gallic acid, ethylenediaminetetraacetic acid (EDTA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine) were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). Ascorbic acid was purchased from Fluka (Switzerland). 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzen disulfonate (WST-1) was purchased from Roche Molecular Biochemicals (Mannheim, Germany). All other chemicals used were of analytical grade and obtained from either Sigma-Aldrich or Merck.

Preparation of *Perilla frutescens* extracts

The *P. frutescens* stalks, leaves and seeds, which were purchased locally (Goangder Tarng Ginseng Co., Taoyuan, Taiwan). The dried of *P. frutescens* were ground in a mortar, and extracted twice using 50 ml of methanol (60°C) under reflux for 4 h. The supernatant was separated from the solid residue by paper filtration (No. 1, Advantec, Tokyo, Japan). The extracts were combined and evaporated at 60°C under reduced pressure. All dried extracts were stored at 4°C until use.

Antioxidant activity assays

Determination of total phenolic and flavonoid content

Total polyphenols were determined applying the Folin-Ciocalteu method by Chou et al. (2009). Gallic acid was used for constructing the standard curve and the results were expressed as µg of gallic acid equivalents per ml of extract. Flavonoid contents in the extracts were determined by a colorimetric method described by Chou et al. (2009). Rutin was used for constructing the standard curve and the results were expressed as µg of rutin equivalents per ml of extract.

DPPH radical scavenging activity

Scavenging activity on DPPH radical was measured by the method of Chou et al. (2009). DPPH radical scavenging activity was expressed as the inhibition percentage and was calculated as (1 - absorbance of sample/absorbance of control) × 100. The IC_{50} value (µg/ml) is the effective concentration at which the DPPH radicals were scavenged by 50% and was obtained by interpolation from linear regression analysis. Ascorbic acid and gallic acid were used for comparison.

Reducing power

The reducing power was determined according to the method of Chou et al. (2009). A higher absorbance indicated a higher reducing power. The IC_{50} value (µg/ml) is the effective concentration at which the absorbance was 0.5 for the reducing power.

Chelating effect on ferrous ions

The chelating effect of ferrous ions was estimated by the method of Chou et al. (2009). Chelating effect was calculated using the equation: (1 - absorbance of sample/absorbance of control) × 100. The IC_{50} value (µg/ml) is the effective concentration at which ferrous ions were chelated by 50%. EDTA and citric acid were used for comparison.

Cell culture

Human non-small cell lung cancer cell lines A549 (ATCC CCL-85) was obtained from the American Type Cell Culture Collection (ATCC, Manassas, VA). It was maintained in monolayer culture at 37°C and 5% CO_2 in DMEM supplemented with 10% fetal calf serum, 100 U/ml of penicillin G, 100 µg/ml of streptomycin, and 0.25 mg/ml of amphotericin B to culture for anticancer assay.

WST-1 assay for cell proliferation

Inhibition of cell proliferation of methanolic extracts from *P. frutescens* was measured by WST-1 assay. Briefly, cells were plated in 96-well culture plates (1×10^4 cells/well). After 24 h of incubation, the cells were treated with vehicle alone (0.1% DMSO) and various concentrations of the methanolic extracts of *P. frutescens* for 48 h. 5 µl of WST-1 labeling reagent was then added to each well. After 1 h of incubation, the absorbance was measured on an ELISA reader at a test wavelength of 595 nm. The percentage inhibition activity was calculated from $[1–(A_1/A_0)] \times 100$, where A_1 and A_0 indicated the optical density of methanolic extracts and the solvent control, respectively. The concentration of 50% cellular cytotoxicity of cancer cells (IC_{50}) of the methanolic extracts was calculated based on 48 h absorbance values (Chen et al., 2009).

Statistical analysis

All data were presented as the mean ± standard deviation (S.D.) of triplicate parallel measurements. Statistical analysis was performed using student's t- test for paired values.

RESULTS AND DISCUSSION

Amount of total phenolic and flavonoid

Although the different parts of *P. frutescens* are used individually in Chinese medicine to treat a variety of diseases, but there has been little report on the

Table 1. Total phenolic and flavonoid content of methanolic extracts of *P. frutescens*.

Extract	Total phenolic content (µg/ml)	Total flavonoid content (µg/ml)
Stalk	137.40±1.82	205.75± 5.11
Leaf	116.08±1.58	158.55±11.14
Seed	139.25±0.60	182.15±14.22

Values represented mean ± S.D. of three parallel measurements. Total phenolic content was expressed as µg gallic acid equivalents/ml extract. Total flavonoid content was expressed as µg rutin equivalent/ml extract.

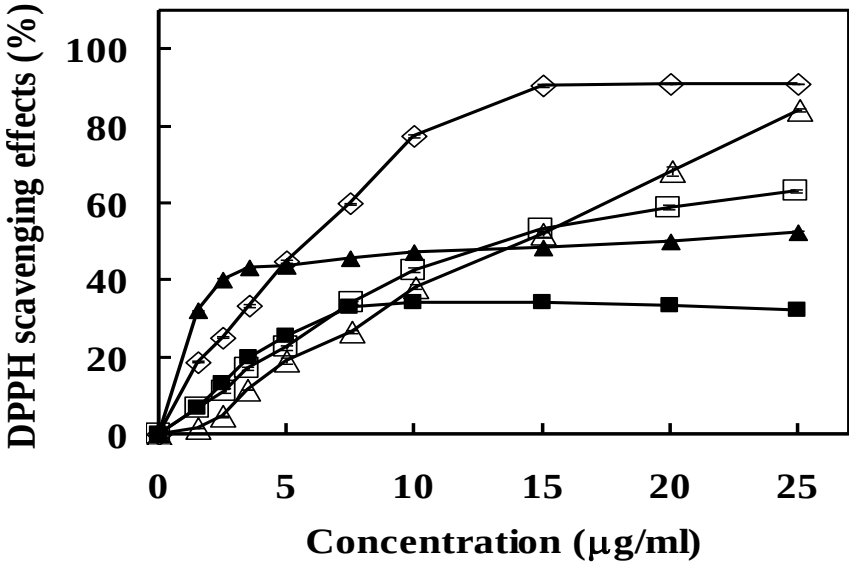

Figure 1. DPPH radical scavenging activity of methanolic extracts of *P. frutescens*. Absorbance values represent means of triplicates of different samples analysed. ◇ Stalk, □ Leaf, △ Seed, ▲ ascorbic acid, and ■ gallic acid.

quantitative presence of polyphenols in *P. frutescens* (Meng et al., 2009; Peng et al., 2005). Table 1 shows the methanolic extracts of seed and stalk were found to have the highest phenolic content (139.25 ± 0.60 µg/ml and 137.40 ± 1.82 µg/ml, respectively) among the *P. frutescens*. On the other hand, total flavonoid content of stalk was also found to be superior to the other *P. frutescens* extracts (205.75 ± 5.11 µg/ml). The lowest flavonoid content was exhibited of leaf (158.55 ± 11.14 µg/ml). Polyphenols display important role in stabilizing lipid oxidation that associated with its antioxidant activity (Osakabe et al., 2002; Gülçin et al., 2003a). The main phenolic compounds have been proven to be rosmarinic acid, and there are small amounts of flavonoids and phenolic acids such as catechin, apigenin, luteolin, caffeic acid, and ferulic acid found in the leaves and seeds of *P. frutescens* (Aritomi et al., 1985; Ishikura, 1981; Masahiro et al., 1996). Flavonoids have been proven to display a wide range of pharmacological and biochemical actions, such as antimicrobial, antithrombotic, antimutagenic and anticarcinogenic activities (Benavente-Garcia and Castillo, 2008; Hoensch and Kirch, 2005).

DPPH radical scavenging activity

Antioxidant activity assays employed the inhibition of free radical DPPH test/method which is one of the oldest and most frequently used methods for total antioxidant potential/capacity of food and biological extracts. It is based on the ability of an antioxidant to give hydrogen radical to synthetic long-lived nitrogen radical compound DPPH. A blue-violet color changes gradually to green and yellow (absorption maximum at 405 nm), and a decrease in absorbance at 517 nm is monitored. At 1.5 - 25 µg/ml, the scavenging abilities of the methanolic extracts of stalk, leaf and seed of *P. frutescens* on DPPH radicals were in the range of 18.7-91.0%, 6.7-63.1% and 1.6-84.3%, respectively (Figure 1). Obviously, these methanolic extracts of *P. frutescens* showed excellent activities at the

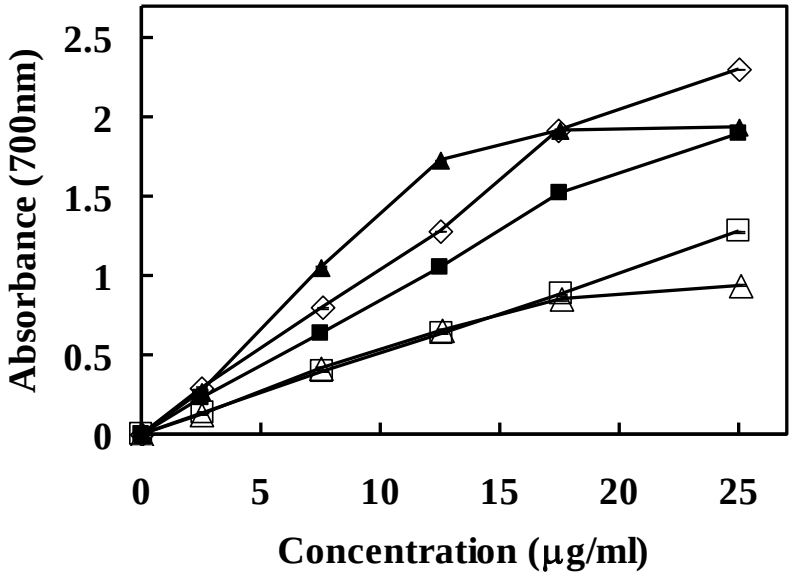

Figure 2. Reducing power of methanolic extracts of *P. frutescens*. Absorbance values represent means of triplicates of different samples analysed. ◇ Stalk, □ Leaf, △ Seed, ▲ ascorbic acid, and ■ gallic acid.

concentrations tested. At 25 µg/ml, ascorbic acid and gallic acid showed excellent scavenging abilities of 32.2 and 52.3%, respectively. The hot water extract of stalk showed moderate DPPH radical scavenging abilities of 54.8% at 10 µg/ml, whereas those of leaf and seed scavenged DPPH radicals by 5.5 and 6.7% at 10 µg/ml, respectively (Chou et al., 2009). It seemed that the methanolic extracts from stalk, leaf and seed were high effective in scavenging activities than hot water extracts.

Reducing power

The reducing power indicates compounds that are electron donors, which can act as primary and secondary antioxidants (Yen and Chen, 1995). As seen in Figure 2, reducing powers of methanolic extracts from different parts of *P. frutescens* were enhanced by increasing concentration of samples. Reducing powers of methanolic extracts of stalk of *P. frutescens* was 2.30, whereas those of leaf and seed were 1.28 and 0.94 at 25 µg/ml, respectively. However, at 2.5 - 25 µg/ml, ascorbic acid and gallic acid showed increase in reducing powers from 0.23 to 1.90 and 0.27 to 1.94, respectively. Therefore, the methanolic extracts of *P. frutescens* showed significant effects on the reducing capacity at all amount. Chou et al. (2009) mentioned that reducing powers of hot water extracts at 25 µg/ml were 1.53, 1.53 and 1.40 for stalk, leaf and seed, respectively. It seemed that with regard to reducing power, it can be concluded that the methanolic extracts of stalk of *P. frutescens* showed higher absorbance at low concentrations. Reducing power of a compound may serve as a significant indicator of its potential antioxidant activity (Gülçin et al., 2003b).

Chelating effect on ferrous ions

Measurement of the rate of color reduction allows estimation of the chelating activity of the coexisting chelator (Yamaguchi et al., 2000). Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted with the result that the red color of the complexes decreases. Figure 3 shows the chelating effects of the methanolic extracts of *P. frutescens* on ferrous ions. Chelating abilities of methanolic extracts from different parts of *P. frutescens* on ferrous ions showed a slight increase as the concentration increased. The methanolic extracts of stalk, leaf and seed of *P. frutescens* chelated 27.7%, 4.6 and 8.4% of ferrous ions at 12.5 µg/ml and 63.2, 49.8 and 56.5% at 75 µg/ml, respectively. However, EDTA showed an excellent chelating ability of 94.2% at 12.5 µg/ml, and remained the level of 94.8 - 94.9% to 25 µg/ml. Citric acid was not a good chelating agent for ferrous ions. The chelating abilities of the hot water extracts of stalk, leaf and seed were 7.7, 8.5 and 14.9% at 12.5 µg/ml, and 53.7, 46.6 and 82.2% at 75 µg/ml, respectively (Chou et al., 2009). With regard to reducing power, the methanolic extracts and hot water extracts were comparable except for the seed. The difference among *P. frutescens* extracts concentrations and the control values were statistically significant ($p < 0.01$). Yamaguchi et al. (1988) reported that ferrous ions

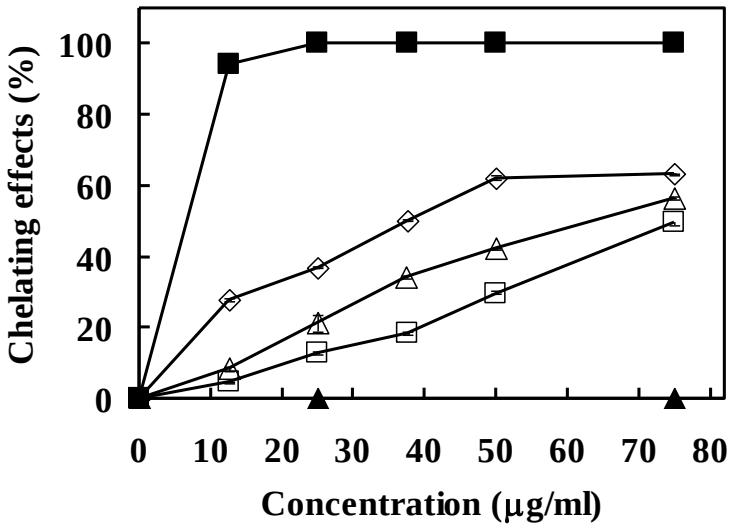

Figure 3. Chelating effect on ferrous ions of methanolic extracts of *P. frutescens* on ferrous ion. Absorbance values represent means of triplicates of different samples analysed. ◇ Stalk, □ Leaf, △ Seed, ■ EDTA, and ▲ citric acid.

Table 2. IC_{50} values of methanolic extracts of *P. frutescens* in antioxidant and antiproliferative activity.

	Extracts (µg/ml)		
	Stalk	**Leaf**	**Seed**
Scavenging ability on DPPH radicals	5.92	7.97	12.34
Reducing power	11.36	13.71	10.89
Chelating ability on ferrous ions	21.78	30.70	46.76
Antiproliferative activity	12.42	10.16	8.75

Values are means of triplicate analyses.

were the most effective pro-oxidants in food system. This result indicates that the low to moderate ferrousion chelating abilities of methanolic extracts of *P. frutescens* would be somewhat beneficial.

IC_{50}

The antioxidant properties assayed herein were summarized in Table 2 and the results were normalised and expressed as IC_{50} values for comparison. The phenolic compounds concentrations equivalents were used for the measurements of methanolic extract concentrations. Effectiveness in antioxidant properties inversely correlated with IC_{50} value. Stalk extracts from *P. frutescens* showed good scavenging ability on DPPH radical as evidenced by their particularly low IC_{50} values. In addition, with regard to effectiveness in reducing power, IC_{50} values of stalk, leaf and seed were similar. All extracted of *P. frutescens* showed chelating ability on ferrous ions but IC_{50} values were higher than 20 µg/ml. Hence, *P. frutescens* can be used as an easy accessible source of natural antioxidants, as a food supplement, or in the pharmaceutical industries.

Measurement of cell proliferation

Antiproliferative activities of the different extracts of *P. frutescens* on the growth of the human non-small cell lung cancer A549 cell line in vitro were summarized in Figure 4. Cell proliferation was analyzed at 48 h after A549 cells had been cultured with an extract of 0, 5, 10, 20, 40, 80 or 120 µg/ml in the media using the WST-1 assay. The methanolic extract of *P. frutescens* had moderate antiproliferative activities under the experimental conditions. A549 cell proliferation was inhibited in a dose-dependent manner after exposure to the different

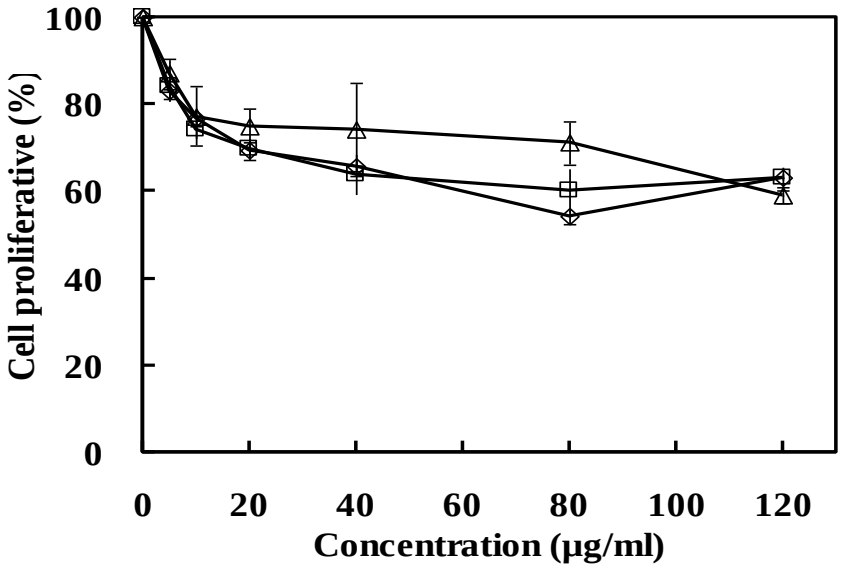

Figure 4. Percent inhibition of A549 human non-small cell lung cancer cell proliferation of methanolic extracts of *P. frutescens*. Absorbance values represent means of triplicates of different samples analysed. ◇ Stalk, □ Leaf, △ Seed.

extracts. The IC_{50} values for methanolic extracted of *P. frutescens* in human lung cancer cell lines was also given in Table 2. The methanolic extract of seed had the lowest IC_{50} of 8.75 µg/ml, followed by leaf (10.16 µg/ml), and stalk (12.42 µg/ml). The antioxidant activities of the different methanolic extracts were directly correlated to the total amount of phenolic and flavonoid found in these extracts, but there were no relationship between antioxidant and antiproliferative activities. This experiment suggests that the inhibition of tumor cell proliferation in vitro by the methanolic extract of *P. frutescens* can not be solely explained by the concentration of phenolic /flavonoid compounds. The inhibition of cancer cell proliferation may be attributed to some unknown compound(s) present in the *P. frutescens* extracts. Other phytochemicals may play a major role in the antiproliferative activity.

In conclusion, the results demonstrated that the methanolic extracts in *P. frutescens* may have a significant effect on antioxidant and antiproliferative activities. Additionally, the antioxidant activity was directly related to the total amount of phenolic and flavonoid found in *P. frutescens* extracts. The additive roles of methanolic extracts may contribute significantly to the potent antioxidant activity and the ability to inhibit tumor cell proliferation in vitro. Overall, *P. frutescens* in the form of stalk, leaf and seed and their methanolic extracts could be used as food or a food ingredient. Therefore, in addition to their therapeutic effects, *P. frutescens* in human dietary supplements might serve as possible protective agents to help human reduce oxidative damage. Further work should be performed to isolate and identify the antioxidative or antiproliferative components.

ACKNOWLEDGMENTS

We are grateful to Miss Ching-Ting Yang, Mr. Chia-Ching, Li and Dr. Yu-Chi, Chen, Vanung University, for providing necessary facilities to carry out this research work. This study was supported by a research grant (NSC 96-2622-E-438-001-CC3) from the National Science Council of Taiwan, R.O.C.

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