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Full Length Research Paper

Assessment of the antiradical and antioxidant actions of leaf, perilla seed and stalk take out

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The antioxidant properties of perilla seed, leaf and stalk extracts were evaluated by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and superoxide radicals scavenging activities, reducing power and metal chelating ability. The DPPH radical scavenging activity of perilla leaf extract was 57.5%, a value which was higher than scavenging activity of other perilla extracts at 15.0 µg/ml. Based on superoxide radical scavenging effect, the perilla leaf extract was the most effective (64.2%) one followed by perilla seed extract (55.1%) and perilla stalk extract (20.1%) at 10.0 µg/ml. Reducing powers of perilla seed, leaf and stalk extracts were excellent. Chelating effects of perilla seed, leaf and stalk extracts were increased with the increasing concentration. The data shows that perilla leaf extract has effective antioxidant functions, especially in DPPH and superoxide radicals scavenging activities and reducing power. The results indicate that the 50% aqueous-methanol extracts of perilla leaf can be used as a new functional food and pharmaceutical agent.

Key words: Perilla, scavenging activity, reducing power, chelating effect, phenolics compound.

INTRODUCTION

Free radicals are a major interest for physiological and biochemical lesions. Although oxygen is essential for aerobic forms of life, oxygen metabolites are highly toxic. As a consequence, reactive oxygen species (ROS) are known to be implicated in many cell disorders and in the development of many diseases including coronary artery disease, aging and cancer (Gülçin 2010). Antioxidants inhibit or prevent oxidation of substrates and evolve to protect biological systems against damage induced by ROS. 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). Therefore, interest in finding natural antioxidants, without undesirable side effects, has increased greatly. The numbers of antioxidant compound by plants play important roles in preventing diseases induced by free radicals (Hirose et al., 1994). Therefore, attention has been directed toward the development of natural antioxidants from plant sources (Chou et al., 2009; Lin et al., 2010b; Lin and Li, 2010).

Perilla (*Perilla frutescens*, Labiatae) is one of popular traditional herbal medicines used in Taiwan and widely distributed in China, Japan and Korea. In general, perilla is used individually in Chinese medicine to treat a variety of diseases (Chinese Pharmacopoeia Commission, 2005). The seeds are employed for dyspnea and cough relief, phlegm elimination and the bowel relaxation. The leaves are said to be helpful for asthma, cold and flu, and to regulate stomach function, while the stalk of the plant is traditionally used as an analgesic and anti-abortive agent (Chinese Pharmacopoeia Commission, 2005). Previously, some studies were focused on antioxidant activity of *Perilla pankinensis* extract (Gülçin et al., 2005)

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and *Perilla frutescens* extract (Chou et al., 2009; Kim et al., 2007; Kim et al., 2008; Lin et al., 2010a). In addition, various medicinal properties of perilla have been ascribed to the antioxidant, anti-inflammatory, anti-allergic and anti-tumor promoting substances (Lin et al., 2007; Makino et al., 2003; Takano et al., 2004; Ueda et al., 2002; Žekonis et al., 2008).

The extraction method was a major factor to determine the composition and their effective in the plant extract. The aims of this work were to estimate the phenolic content and to evaluate the antioxidant and antiradical activities of 50% aqueous-methanol extracts of perilla seed, leaf and stalk. Their antioxidant and antiradical activities were also compared to well known natural antioxidants, such as ascorbic acid and gallic acid.

MATERIALS AND METHODS

Chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH), phenazine methosulfate (PMS), nicotinamide adenine dinucleotide (NADH), nitroblue tetrazolium (NBT), 3-(2-pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine), gallic acid (GA) and ethylenediamine-tetraacetic acid (EDTA) were obtained from Sigma Chemical (St. Louis, MO, U.S.A.). Ascorbic acid (Vit C) was purchased from Fluka (Buchs, Switzerland). All other reagents were of analytical grade.

Extraction procedure

The perilla seed, leaf and stalk were purchased locally (Goangder Tarng Ginseng Co., Taoyuan, Taiwan). The areca flower extracts were prepared according to Köksal and Gülçin (2008) with modification (Chou et al., 2009). The dried of perilla seed, leaf and stalk were ground in a mortar, and extracted twice that combined the same volume of distilled water and methanol under reflux for 4 h at 70°C. The supernatant was separated from the solid residue by paper filtration (No. 1, Advantec, Tokyo, Japan). The 50% aqueous-methanol extracts were combined and evaporated at 60°C under reduced pressure. All dried extracts were stored at 4°C until use.

Total phenolic determination

Total phenolic contents were quantified according to the method of Chou et al. (2009). Basically, aqueous-methanol extract solution (50 µl) was mixed with 50 µl of Folin-Ciocalteu's phenol reagent. Then, 500 µl of a 15% sodium carbonate solution was added to the mixture and it was adjusted to 400 µl with distilled water. The mixture was allowed to stand for 10 min with intermittent shaking, after which the absorbance was read at 725 nm (Ultrospec 2100 pro spectrophotometer, Amersham Pharmacia Biotech, UK). Results were expressed as gallic acid equivalent. All experiments were done in triplicate.

DPPH radical scavenging activity

The DPPH radical scavenging activity was measured according to the method of Chou et al. (2009). A stock solution of DPPH radical (1 mM) in methanol was prepared. An aliquot (100 µl) of 1 mM methanol solution of DPPH was mixed with varying concentrations of the perilla extract. After a 30 min incubation period at room temperature, the absorbance of the resulting solution was read at 517 nm. For each sample a methanol blank was also measured. 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.

Superoxide radical scavenging effect

Scavenging activity on superoxide radical was evaluated according to the method of Chou et al. (2009). The reaction mixture contained the same volume of 120 µM PMS, 936 µM NADH, perilla extract, and 300 µM NBT in a total volume of 1 ml of phosphate buffer (100 mM, pH 7.4). After 5 min of incubation at ambient temperature, the absorbance of the resulting solution was measured at 560 nm. The superoxide radical activity was calculated as scavenging effect (%) = (1 – absorbance of sample/absorbance of control) × 100. The IC_{50} value (µg/ml) is the effective concentration at which the superoxide radical were scavenged by 50%. 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). The perilla extract (250 µl) was mixed with 250 µl of 200 mM sodium phosphate buffer (pH 6.6) and 250 µl of 1% potassium ferricyanide, and the mixture was incubated at 50°C for 20 min. Later, 250 µl of 10% trichloroacetic acid was added to the mixture to stop the reaction. The mixture was centrifuged at 3000 rpm for 10 min. The supernatant (500 µl) was mixed with 400 µl of deionized water and 100 µl of 0.1% ferric chloride solution, allowed to stand for 10 min, and the absorbance was measured at 700 nm. A higher absorbance indicated a higher reducing power. The IC_{50} value (µg/ml) is the effective concentration at which the absorbance was 1.0 for the reducing power. Ascorbic acid and gallic acid were used for comparison.

Chelating effect on ferrous ions

The chelating effect of ferrous ions was estimated according to the method of Chou et al. (2009). The perilla extract was added to a solution of 2 mM $FeCl_2$ (20 µl). The reaction was initiated by the addition of 5 mM ferrozine (40 µl) and the mixture was shaken vigorously and left standing at room temperature for 10 min. The absorbance of the mixture was measured at 562 nm. 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. The data were presented as the means ± standard deviation (SD) of triplicate parallel measurements. Statistical analysis was performed using Student's t-test and $p < 0.05$ was regarded as significant.

RESULTS

We measured the total phenolic content to investigate if they may contribute to the antioxidant activity of the perilla extracts. As shown in Table 1, the total phenolic contents in perilla seed, leaf and stalk extracts were 424.86 ± 4.68, 549.00 ± 9.97 and 586.69 ± 2.74 µg/ml

Table 1. Total phenolic content of perilla seed, leaf and stalk extracts.

Extract	Seed	Leaf	Stalk
Total phenolic content[a] (μg/ml)	424.86±4.68	549.00±9.97	586.69±2.74

[a]Values represented mean ± SD of three parallel measurements. Total phenolic content was expressed as μg gallic acid equivalents/ml extract.

Table 2. IC_{50} values of perilla seed, leaf and stalk extracts and standard in antioxidant properties.

Parameter	Samples (μg/ml)					
	Seed	Leaf	Stalk	Vit. C	GA	EDTA
DPPH radical scavenging activity	16.52	10.89	11.04	10.79	3.59	nd[a]
Superoxide radical scavenging effect	5.96	6.93	16.11	8.39	10.48	nd[a]
Reducing power	21.46	16.38	15.80	12.27	12.32	nd[a]
Chelating ability on ferrous ions	62.78	71.27	72.39	nd[a]	nd[a]	8.82

[a]nd: not detected.

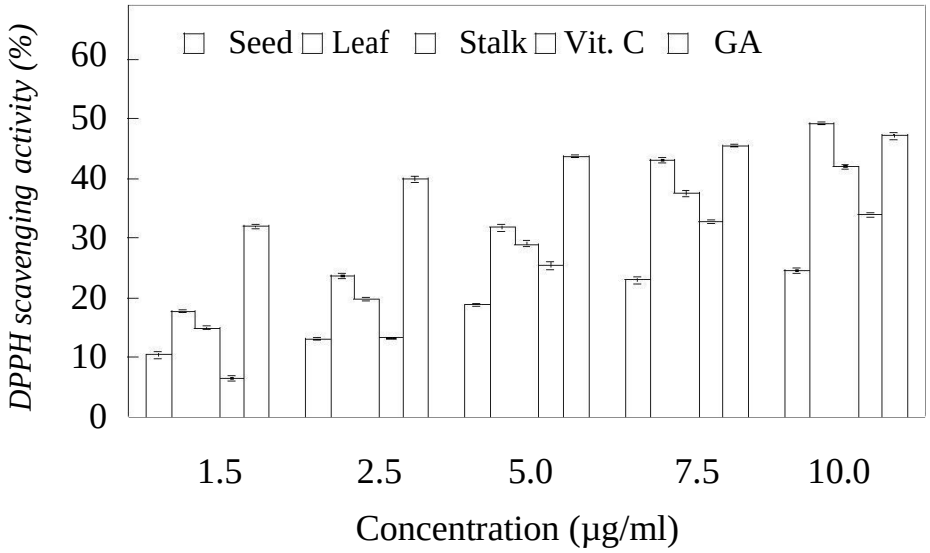

Figure 1. DPPH radical scavenging activity on of perilla seed, leaf and stalk extracts. Ascorbic acid and gallic acid were taken as the standards. Data expressed as mean ± SD (*n*=3).

extract, respectively. Obviously, the phenolic content of perilla stalk extract was higher than those of perilla leaf and perilla seed extracts. The results are expressed as gallic acid equivalents. The free radical scavenging activities of perilla extracts, measured by the DPPH method, are shown in Figure 1. The extracts of perilla leaf and perilla stalk had better DPPH radical scavenging activity than perilla seed extract at the same concentration. The perilla extract concentrations were expressed as gallic acid equivalent/ml extract. The scavenging effects of perilla extracts and standards on the DPPH radical were in the following order: gallic acid > perilla leaf > perilla stalk > ascorbic acid = perilla seed (39.9, 23.8, 19.9, 13.3 and 13.3%, respectively), at the same concentration (2.5 μg/ml). The extracts of perilla leaf and perilla stalk showed significant dose-dependent scavenging activity, especially, and it reached up to 49.2 and 42.0%, respectively, at the concentration of 10.0 μg/ml. As shown in Table 2, the IC_{50} value for perilla seed, perilla leaf and perilla stalk extracts in this assay were 16.52, 10.89 and 11.04 μg/ml, respectively. On the other hand, IC_{50} values of ascorbic acid and gallic acid were found to be 10.79 and 3.59 μg/ml, respectively.

The superoxide radical scavenging activities of perilla

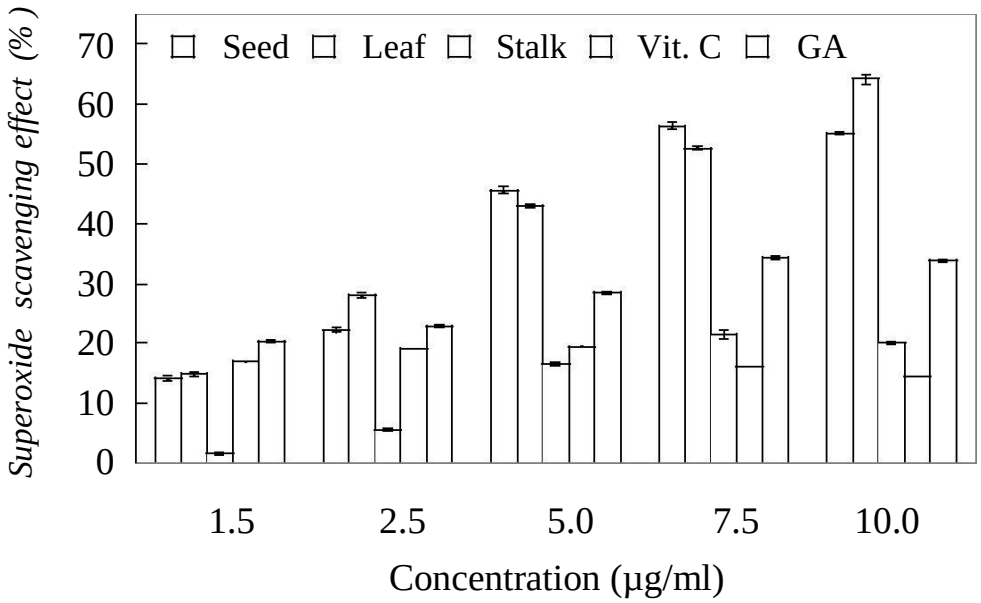

Figure 2. Superoxide radical scavenging effect of perilla seed, leaf and stalk extracts. Ascorbic acid and gallic acid were taken as the standards. Data expressed as mean ± SD (*n*=3).

extracts were increased with increasing sample concentrations (Figure 2). The extracts of perilla leaf and perilla seed showed significant dose-dependent scavenging activity on superoxide radical. Scavenging abilities of perilla leaf and perilla seed extracts were similar and better than that of perilla stalk; these activities were more than that of the standards at the concentration of 2.5 to 10.0 µg/ml. The extracts of perilla seed and perilla leaf exhibited good superoxide radical scavenging activity (IC_{50} = 5.96 and 6.93 µg/ml, respectively) that were comparable with standards (8.39 µg/ml for ascorbic acid and 10.48 µg/ml for gallic acid). The extract of perilla stalk had shown a very weak activity, IC_{50} = 16.11 µg/ml. All results are shown in Table 2.

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 3, the reducing powers of perilla extracts were increased with increasing amount of sample, but values remained lower than that for ascorbic acid and gallic acid, at the concentration of 2.5 to 25.0 µg/ml. However, the reducing powers of perilla were similar in the following order: perilla stalk (2.02) > perilla leaf (1.99) > perilla seed (1.78) at the concentration of 37.5 µg/ml (ascorbic acid and gallic acid were 1.97 and 1.96, respectively). In addition, reducing powers of perilla extracts and standards were found similar statistically at 50.0 µg/ml (data not shown). The perilla leaf and perilla stalk extracts had shown higher reducing power with IC_{50} = 16.38 and 15.80 µg/ml, respectively. The IC_{50} for perilla seed was 21.46 µg/ml (Table 2). However, these values of ascorbic acid and gallic acid were found to be 12.27 and 12.32 µg/ml, respectively.

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 (Gülçin et al., 2009). Figure 4 shows that the absorbance of Fe^{2+}-ferrozine complex was decreased dose-dependently from 12.5 to 100 µg/ml for all perilla extracts. The iron chelating abilities of all perilla extracts showed moderate were in the range of 4.9 to 9.3% to 62.6 to 67.4%. Obviously, all perilla extracts had a steady increase on metal chelating activity with increasing concentrations. IC_{50} values of perilla seed extract was 62.78 µg/ml whereas that of perilla leaf and perilla stalk extracts were 71.27 and 72.39 µg/ml, respectively (Table 2). However, EDTA showed very strong chelating ability of 94.2% at 12.5 µg/ml (IC_{50} = 8.82 µg/ml) and citric acid had no iron ion chelating.

DISCUSSION

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. The radical scavenging activity values were expressed as the ratio percentage of sample absorbance decrease and the absorbance of DPPH solution in the absence of extract at 517 nm (Gülçin 2009). The decrease in absorption is taken as a measure of the extent of radical scavenging. From the analysis of Figure 1, the scavenging activities on DPPH radical of all perilla extracts increased with the concentration increase and were excellent, especially for perilla leaf extract. The DPPH radical scavenging activity

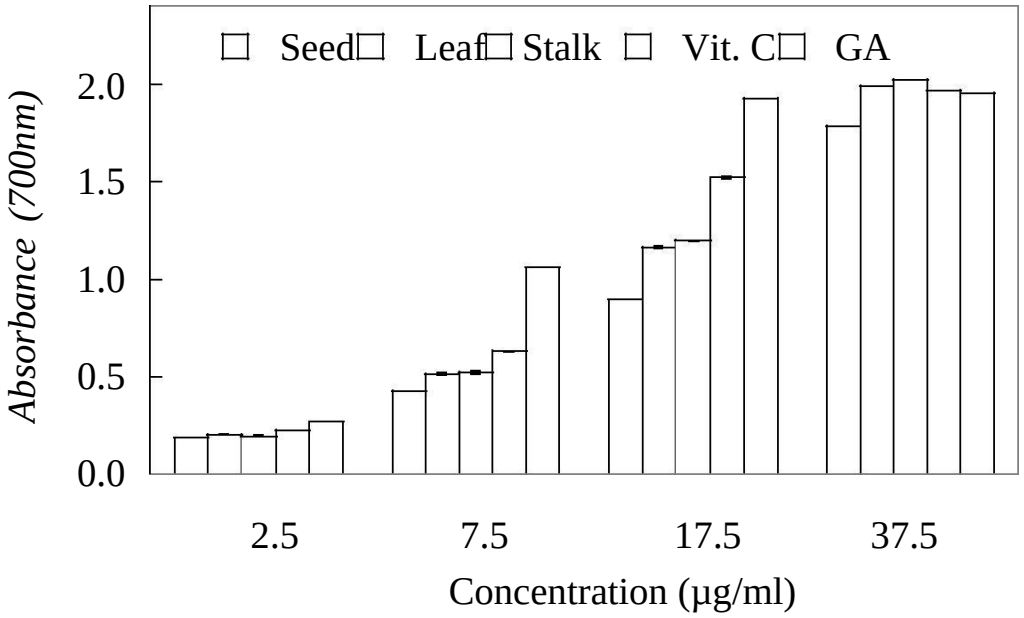

Figure 3. Reducing power of perilla seed, leaf and stalk extracts. Ascorbic acid and gallic acid were taken as the standards. Data expressed as mean ± SD (*n*=3).

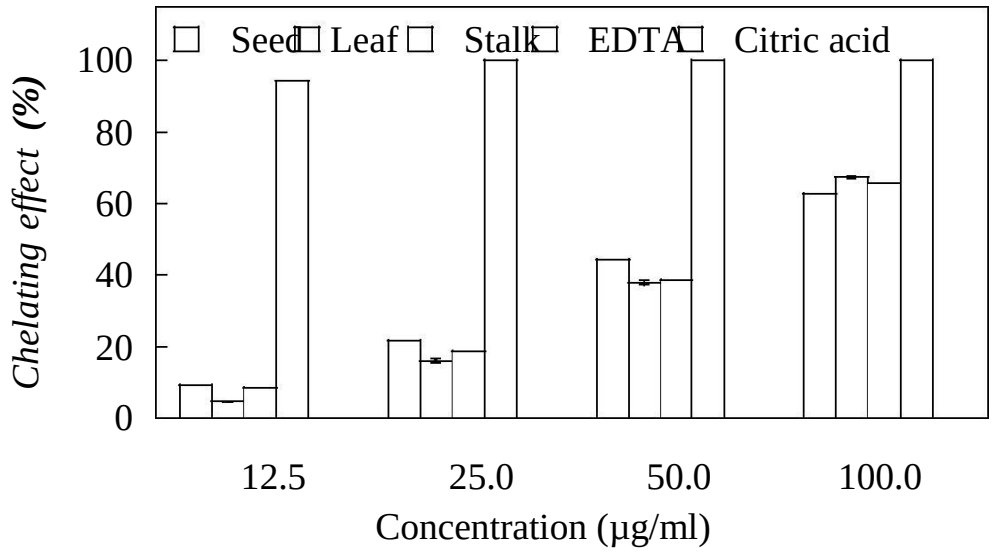

Figure 4. Chelating effect of perilla seed, leaf and stalk extracts on ferrous ions. EDTA and citric acid were taken as the standards. Data expressed as mean ± SD (*n*=3).

was also good for perilla stalk extract, but perilla seed extract revealed a very low value.

Superoxide anion is a reduced form of molecular oxygen and has been implicated in the initiating oxidation reactions associated with ageing (Cotelle et al., 1996). Superoxide anions play an important role in formation of other ROS such as singlet oxygen, hydrogen peroxide and hydroxyl radical which induce oxidative damage in DNA, lipids and proteins (Pietta, 2000). We investigated the superoxide radical scavenging activity of perilla extracts using the nitroblue tetrazolium chloride-NADH-phenazine methosulphate (NBT-NADH-PMS) system. Decrease of optical density values against control is the indication of the presence of bioactive compounds possessing superoxide radical scavenging activity. The scavenging abilities on superoxide radical were good for perilla leaf and perilla seed extracts, but perilla stalk extract revealed a very low value (Figure 2).

Nakamura et al. (1998) previously reported that a high concentration of rosmarinic acid in *P. frutescens* leaf was found to have elevated superoxide radical scavenging activity. In addition, Gülçin et al. (2005) reported that the anthocyanins from *P. pankinensis* exhibited a high scavenging efficiency toward superoxide radicals. Therefore, phenolic compounds effectively exhibited antioxidative activity in the biological systems through the scavenging of O_2^-.

The reducing properties of natural components may have a reciprocal correlation with the antioxidant activities (Gülçin et al., 2003b). For the measurements of the reductive ability, the Fe^{3+}–Fe^{2+} transformation was investigated of perilla extract using the potassium ferricyanide reduction method. In this assay, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of each antioxidant sample. Reducing power of a compound may serve as a significant indicator of its potential antioxidant activity. Reducing powers of perilla extracts had high reducing activity in dose-dependent manner (Figure 3). The higher phenolic content in perilla leaf and perilla stalk extracts might account for the better results found in their reducing power and DPPH radical scavenging activity. Different studies have indicated that the antioxidant effect is related to the development of reductones (Shon et al., 2003). Furthermore, reductones such as ascorbic acid can react directly with peroxides and also with certain precursors and thereby, prevent peroxide formation. The reducing ability of various extracts might be due to its hydrogen-donating capacity, as described by Shimada et al. (1992). Therefore, the perilla extracts might contain reductones which could react with free radicals to stabilize and terminate free radical chain reactions.

Chelating agents were reported to be effective as secondary antioxidants because they can reduce the redox potential, thereby stabilizing the oxidized form of the metal ion (Gülçin et al., 2007). Measurement of the rate of color reduction allows estimation of the chelating activity of the coexisting chelator (Yamaguchi et al., 2000). Both perilla extracts and standard EDTA interfered with the formation of ferrous and ferrozine complex, suggesting that they have chelating activity and capture ferrous ion before ferrozine. The perilla extracts showed significant iron binding capacity at all amounts (Figure 4). The results demonstrated moderate iron binding capacity, suggesting that the antioxidant activity may be related to the capacity for iron binding. The difference among all perilla extracts were significantly different ($p < 0.05$).

Chelating effect on ferrous ions of an antioxidant molecule prevents oxyradical generation and the consequent oxidative damage. Chelating ability was significant as they reduced the concentration of the catalyzing transition metal in lipid peroxidation (Duh et al.,

1999). Since ferrous ions were the most effective pro-oxidants in food system (Yamaguchi et al., 1988), the moderate to high ferrous-ion chelating abilities of the various perilla extracts would be somewhat beneficial. These assays were used for establishing the abilities of perilla extracts to chelate and had important applications for the pharmaceutical and food industries.

Although the biological activity of perilla as well as its superior safety was well documented, but there has been little report on the quantitative presence of phenolic compounds in perilla (Peng et al., 2005; Meng et al., 2009). Polyphenolics display important role in stabilizing lipid oxidation associated with its antioxidant activity (Osakabe et al., 2002; Gülçin et al., 2003a). The main polyphenolic compounds has 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 leaf and seed of *P. frutescens* (Ishikura, 1981; Aritomi et al., 1985; Masahiro et al., 1996). Polyphenolic compounds are a variety of antioxidant compounds that have been used as dietary supplements for the prevention of pathological diseases and for the improvement of human health conditions (Zhang et al., 2006). The bioactivity of phenolics may be related to their ability to scavenge free radicals, chelate metals and inhibit lipoxygenase (Decker, 1997).

Conclusion

The results demonstrated that the 50% aqueous–methanol extracts of perilla seed, leaf and stalk may have a significant effect on antioxidant and antiradical activities; especially perilla leaf has effective DPPH and superoxide radicals scavenging activities and reducing power. Hence, perilla leaf can be used as an easy accessible source of natural antioxidants, as a food supplement or pharmaceutical agent.

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