Food Sci. Biotechnol. Vol. 16, No. 5, pp. 710 ~ 714 (2007)

Food Science and Biotechnology

Antioxidant Activity of *Gomchi* (*Ligularia fischeri*) Leaves

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Abstract *Ligularia fischeri* (LF) has been used to treat jaundice, scarlet-fever, rheumatoidal arthritis, and hepatic diseases. This study was carried out to determine the antioxidant activity of the extract from the leaves of LF by using various established *in vitro* systems with α-tocopherol as positive control. The results showed that the ethanol extract of LF (0.05-0.5 mg/mL) displayed a strong free radical scavenging activity against 1,1-diphenyl-2-picrylhydrazyl, $O_2^{\cdot-}$, and H_2O_2 radicals. It was observed that LF extract (1 mg/mL) significantly decreased the levels of malondialdehyde (MDA) to 69 and 89% of control, measured by ferric thiocyanate (FTC) and thiobarbituric acid (TBA) test, respectively. LF extract (0.5-2 mg/mL) was also found to inhibit significantly the amount of malondialdehyde formed from liver homogenate to 69-56% of control. Similarly, in the high Fe^{2+}/ascorbate induction system LF extract (1-2 mg/mL) inhibited carbonyl formation measured by 2,4-dinitrophenylhydrazine reaction to 89-79% of control. Like antioxidant activity, the reducing power of LF extract was excellent at concentration of 0.5-2 mg/mL. These results indicate that LF could be used as a potential source of natural antioxidant.

Keywords: *Ligularia fischeri*, scavenging effect, antioxidant activity

Introduction

In the past decade natural antioxidants have generated considerable attention in preventive medicine. Oxidation of cellular constituents by free radicals provokes several human diseases such as diabetes, arteriosclerosis, cardiovascular illnesses, cancer, several neurodegenerative disorders, and the aging process (1-3). An imbalance between antioxidants and reactive oxygen species results in oxidative stress, leading to cellular damage. Consequently, much attention has been directed toward the discovery of new natural antioxidants, including herbal products, aimed at quenching biologically harmful radicals. Many herbal plants contain antioxidative compounds and these compounds protect cells against the damaging effects of reactive oxygen species, such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals, and peroxynitrite (4, 5).

Edible plants are also candidates for preventing aging and diseases. *Gomchi* (Korean name, *Ligularia fischeri*; LF) has been used as wild vegetables in Korea. LF is mainly distributed in damp shady regions besides brooks and sloping field in the east part of Korea and has been used as wild vegetables. The leaves of this plant have been used to treat jaundice, scarlet-fever, rheumatoidal arthritis, and hepatic diseases (6). Park *et al.* (7) reported on the isolation of a eudesmane-type sesquiterpene, (+)-intermedeol, and 6-oxoeremophilenolide from the leaves of LF. Intermedeol isolated from the leaves of LF was reported to induce the differentiation of leukemia HL-60 cells (8). Thus, as a part of our continuing studies of the biological activity of natural products, we evaluated the antioxidant activity *in vitro* of the extract from the leaves of LF using different assay systems and well-known antioxidant, α-tocopherol, was used as positive control.

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Received September 12, 2006; accepted April 19, 2007

Materials and Methods

Preparation of extract The dried leaves of *gomchi* [*Ligularia fischeri* (Ledebour) Turcz. var. *spiciformis*; LF] were collected at Gangwon-do, Korea, and identified by Dr. KT Lee, Dept. of Pharmacy, Kyung Hee Univ. (Seoul, Korea). A voucher specimen was deposited at our Institute. The dried leaves were extracted 3 times with 70 % ethanol at room temperatures for 3 days each and the combined extracts were concentrated *in vacuo* and then freeze dried (LF, yield: 21%). α-Tocopherol was used as a positive control.

Scavenging effect on DPPH radical The effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was estimated according to the method of Shimada *et al.* (9). Samples (0-1 mg/mL) in 4 mL of methanol were added to a solution of DPPH (10 mM, 1 mL) in methanol. The mixture was shaken and left to stand at room temperature for 10 min; the absorbance of the resulting solution was measured spectrophotometrically at 517 nm. Electron donating ability (EDA) was calculated as follow:

$$\text{EDA}\ (\%) = 100 \times \left(1 - \frac{A_{517nm}\ \text{of sample}}{A_{517nm}\ \text{of control}}\right)$$

Scavenging effect on superoxide radical Inhibition of superoxide anion radical generation was estimated by the xanthine-xanthine oxidase (XOD) system (10). The sample solution (0.1 mL) was added to the mixture (1 mL), consisting of 0.4 mmol/L xanthine and 0.24 mmol/L nitro blue tetrazolium (NBT) in phosphate buffer (pH 8.0). Xanthine oxidase (from butter milk, 0.049 unit/mL) (1.0 mL) diluted in 0.1 mol/L phosphate buffer (pH 8.0) was added, followed by incubation in a shaking water bath at 37°C for 20 min, and then the coloration of NBT was measured at 560 nm. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity.

Scavenging effect on H_2O_2 H_2O_2 was measured by the

formation of a brown color (recorded at 436 nm) in reaction mixtures containing, in a final volume of 1 mL, 0.15 M KH_2PO_4/KOH buffer, pH 7.4, 50 μL of guaiacol solution (100 μL of pure liquid in 50 mL of water) and 10 μL of type IV horseradish peroxidase (5 g/L in the same phosphate buffer). The rate of absorbance change at 436 nm is proportional to the concentration of H_2O_2 added. Samples to be tested for their reaction with H_2O_2 were incubated with 10 mM H_2O_2 for 30 min at 37°C and assayed for remaining H_2O_2 by using the peroxidase system (11).

Determination of antioxidant activity ***Ferric thiocyanate method (FTC):*** The FTC method was adapted from the report of Osawa and Nakimi (12). Samples (4 mL) in 99.5% ethanol were mixed with 2.51% linoleic acid in 99.5% ethanol (4.1 mL), 0.05 M phosphate buffer, pH 7.0 (8 mL), and distilled water (3.9 mL) and kept in screw cap containers under dark conditions at 40°C for 1 day. To 0.1 mL of this solution, 9.7 mL of 75% ethanol and 0.1 mL of 30% ammonium thiocyanate were added. Precisely 3 min after addition of 0.1 mL of 0.02 M ferrous chloride in 3.5% hydrochloric acid to the reaction mixture, the absorbance was measured at 500 nm.

Thiobarbituric acid test (TBA): The test was conducted according to the method of Kikuzaki and Nakatami (13). The same samples as prepared for the FTC method were used. To 1 mL of sample solution, 20% trichloroacetic acid (2 mL) was added. This mixture was then placed in a boiling water bath for 10 min. After cooling, it was centrifuged at 700×g for 20 min. Absorbance of supernatant was measured at 532 nm. In this study, a malondialdehyde (MDA) standard was used to construct a standard curve.

Measurement of lipid peroxides in the mouse liver homogenate The chilled exsanguinated mouse liver was cut into on ice and stored separately at -70°C in plastic vials. Homogenate from liver was prepared after the addition of 1.0 mL phosphate buffer per 100 mg of liver as described previously (14). Protein preparations were measured by the Bio-Rad Bradford protein assay kit. Inhibition of lipid peroxides was estimated by quantitating the amount of TBARS formed from liver homogenate in a Fe^{2+}/ascorbate free-radical-induction system (15) in the presence and absence of extract (mg/mL of the reaction mixture). The reaction mixture contained 0.1 mL of 25% rat liver homogenate (w/v) prepared in 40 mM Tris-HCl buffer (pH 7.0), 30 mM KCl, 0.16 mM ferrous iron, and 0.06 mM ascorbic acid in a final volume of 0.5 mL. The reaction was initiated with the addition of Fe^{2+}. The mixture was incubated for 1 hr at 37°C. The amount of lipid peroxides formed was determined by the TBA reaction method (16). Briefly, the reaction was carried out using 0.4 mL of the above reaction mixture treated with 0.2 mL of 8.1% SDS and 3 mL of TBA reagent (equal volumes of 0.8% TBA and 20% acetic acid pH 3.5). Total volume was made up to 4 mL with distilled water and kept at 95°C for 1 hr in a water bath. Color was extracted with *n*-butanol and pyridine (15:1 v/v), and the absorbance was measured at 530 nm. A MDA standard was used to construct a standard curve.

Measurement of protein oxidation in the mouse liver homogenate The reaction was carried out according to Gassen *et al.* (17). One mL of the reaction mixture contained liver homogenate with protein concentration of 1 mg incubated at 37°C for 1 hr in the presence of 15 mM ascorbic acid and 250 mM $FeSO_4$ in 100 mM Tris-HCl (pH 7.4). The reaction was initiated by the addition of Fe^{2+}. Liver homogenate was prepared as described by Cao and Cutler (18). The assay was performed immediately, or the extracted protein was stored in liquid nitrogen until used. In our assay, we used a homogenate with the ratio of A_{280}/A_{260} higher than or equal to 1.1. The amount of protein carbonyls formed in the presence and absence of extract (mg/mL of the reaction mixture) was measured using 2,4-dinitrophenylhydrazine (19). The values were calculated as nmol carbonyl/mg protein, and percentage inhibition was calculated by comparing both the experimental and control (without extract), and expressed as percent control.

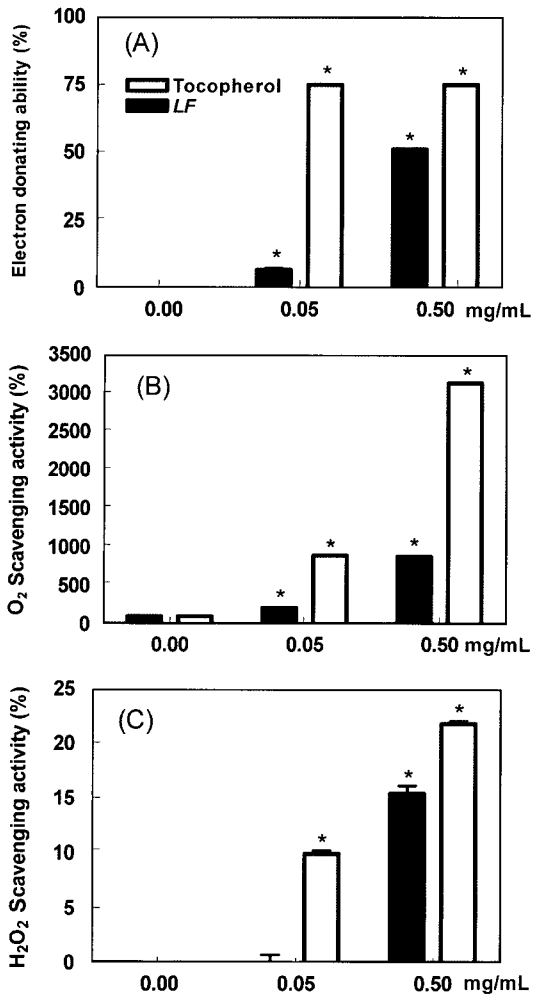
Determination of the reducing power ***Reducing power:*** The reducing power of samples was determined according to the method of Oyaizu (20). Sample in 1 mL methanol was mixed with phosphate buffer (5 mL, 0.2 M, pH 6.6) and potassium ferricyanide (5 mL, 1%), and the mixture was incubated at 50°C for 20 min. Five mL of trichloroacetic acid (10%) were added to the reaction mixture, which was then centrifuged at 3,000×g for 10 min. The upper layer of the solution (5 mL) was mixed with distilled water (5 mL) and ferric chloride (1 mL, 1%), and the absorbance was measured at 700 nm. Increased absorbance indicated increased reducing power.

Phosphomolybdenum method: The reducing power of sample having antioxidant capacity was also evaluated by the method of Prieto *et al.* (21). An aliquot of 0.1 mL of sample solution was combined with 1 mL phosphate and 4 mM ammonium molybdate. The tubes were capped and incubated in a boiling water bath at 95°C for 90 min. After the samples had been cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm.

Statistical analysis Statistical significance was determined by analysis of variance and subsequent Dunnett's *t* test ($p<0.05$). The analysis was performed using SAS statistical software.

Results and Discussion

Radical scavenging effect The radical scavenging activity, using a DPPH radical, was tested with extract of LF, along with α-tocopherol. As shown in Fig. 1A, LF showed a 50% scavenging capacity for DPPH at 0.5 mg/mL. These results reveal that LF extract contain powerful inhibitor compounds, which may act as primary antioxidants that react with free radicals. Figure 1B shows the superoxide radical scavenging activity of the LF extract. In the xanthine-xanthine oxidase system, superoxide anion derived oxygen by xanthine-xanthine oxidase coupling reaction reduces NBT. The decreased of absorbance at 560 nm with antioxidants thus indicates the consumption of superoxide anion in the reaction mixture. As shown in Fig.



Fig, 1. Scavenging effect of *Ligularia fischeri* extract on DPPH/ superoxide/hydroperoxide radicals. (A) Scavenging effect on DPPH radical, (B) Scavenging effect on superoxide radical, (C) Scavenging effect on H_2O_2. Results are expressed as mean±SEM of 4 experimental determinations. *p<0.05 vs. control.

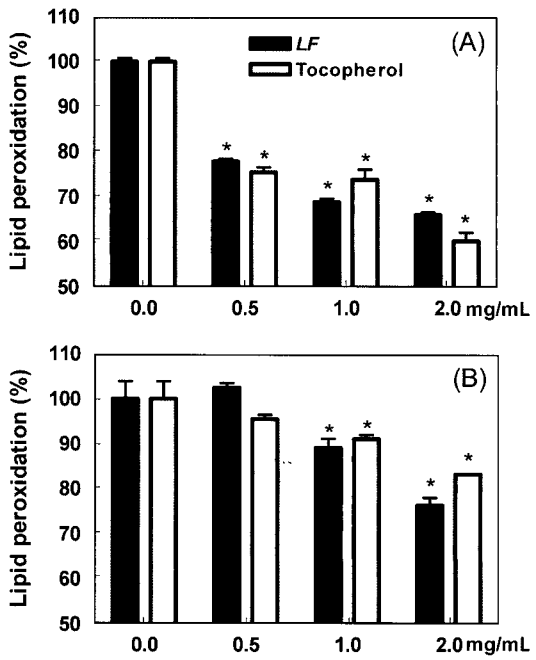


Fig. 2. Antioxidant activity of *Ligularia fischeri* extract in the linoleic acid peroxidation system. The values obtained without antioxidants were used for 100% lipid peroxidation: (A) Ferric thiocyanate method; (B) TBA method. Results are expressed as mean±SEM of 4 experimental determinations. *p<0.05 vs. control.

1C, incubation of 0.5 mg/mL LF extract with 10 mM-H_2O_2 for 30 min at 37°C caused significant loss of H_2O_2, as measured by a peroxidase/guaiacol assay. It thus seems likely that LF could be effective radical scavenger under physiological conditions.

The elevated radical scavenging ability of LF extract might be due to the presence of flavonoids. Moreover, it has also been reported that, in addition to tannins, a free 3-hydroxyl substitution would confer potent antisuperoxidative properties on the compounds (22). Similarly, Amarowicz *et al.* (23) reported that the tannins extracted from canola and rapeseed hulls exhibited a high scavenging efficiency toward DPPH radicals. On the other hand, the presence of the 4'hydroxy configuration in the main compound of stem bark, flavan-3,4-diol (fistucacidin), could also be responsible for the hydrogen donating capacity (24). The peroxyl radicals is a key step in lipid peroxidation (25) and scavenging of non-lipid radicals is of important for protection against early events in oxidative damage, i.e., formation of first radicals by metal catalysis or light exposure (26). Our experiments show that LF is a good scavenger of DPPH, O_2^-, and H_2O_2 *in vitro*, which results demonstrate that LF contains scavengers that may contribute to decompose peroxyls produced during inflammatory states (27); hence LF consumption may afford cytoprotective effects.

Antioxidant activity in the linoleic acid peroxidation system The antioxidative activities of the LF extract were measured using ferric thiocyanate (FTC) and thiobarbituric acid (TBA) method. As shown in Fig. 2A measured by FTC method, LF extract (0.5-2 mg/mL) had significant antioxidative activities, comparable to α-tocopherol. The extent of lipid peroxidation can be estimated by measurement of thiobarbituric reactive substances (TBARS). TBARS can be determined by reaction of malondialdehyde a secondary breakdown product of lipid hydroperoxides, with thiobarbituric acid (TBA) method. Using the TBA method, the LF extract (1-2 mg/mL) showed a significant inhibition of lipid peroxidation (Fig. 2B). Based on the results obtained, it is highly possible that several compounds may contribute to the antioxidative activity of LF. Part of the antioxidative activity may be due to flavonoids. In addition, antioxidative activities observed in these plants could be the synergistic effect of more than two compounds that may present in the plant. It has been reported that most natural antioxidative compounds often work synergistically with each other to produce a broad spectrum of antioxidative activities that creates an effective defense system against free radical attack (28).

Inhibition of lipid peroxides and protein oxidation in the mouse liver homogenate Virtually all cellular components appear to be sensitive to oxidative damage. Lipids, proteins, nucleic acids, and carbohydrates are all known to undergo oxidative modification (29). As shown in Fig. 3A, the production of lipid peroxides by ferrous/ascorbate systems in liver homogenates was significantly inhibited by the LF extract (0.5-2 mg/mL). Lipid peroxidation is the process that involves the chain reaction of free radicals with polyunsaturated fatty acids. These reactions lead to rearrangements of double bonds in conjugated diens, hydroperoxide generation, and lipid breakdown into lower molecular weight fragments such as ketones, alcohol, hydrocarbons, acids, and epoxides. The process of lipid peroxidation is initiated by the abstraction of a hydrogen atom in an unsaturated fatty acyl chain and propagated as a chain reaction (30). Therefore, inhibition of lipid peroxidation is of great importance in the disease processes involving free radicals.

Free-radical-mediated oxidation of some amino acid residues of proteins such as lysine, arginine, and proline leads to the formation of carbonyl derivatives. Other oxidative mechanisms are also involved in the formation of carbonyl derivatives such as glycation and glycoxida-tion reactions (31). In any case, the presence of carbonyl group has become a widely accepted measure of oxidative damage of proteins under conditions of oxidative stress, which react with DNPH to form stable hydrazone derivatives (32). Figure 3B shows that LF extract (1-2 mg/mL) could inhibit protein carbonyl formation in a dose-dependent manner. The antioxidant activity of LF may be attributed to various mechanisms, among which are prevention of chain reaction, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, and radical scavenging (33, 34).

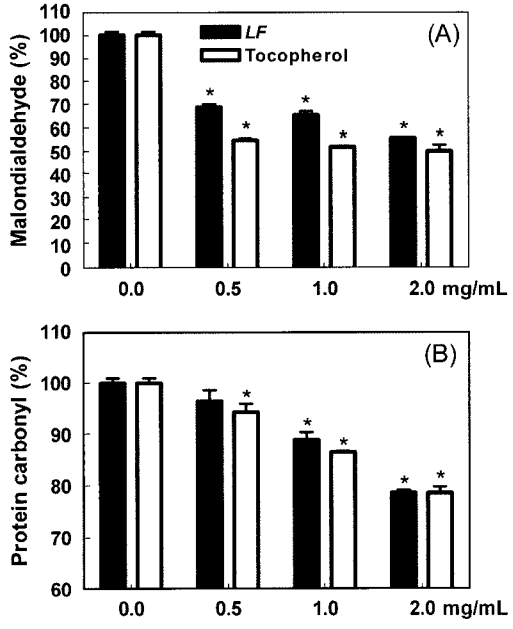


Fig. 3. Inhibition of lipid peroxides and protein oxidation in the liver homogenate. (A) Lipid peroxidation, (B) Protein oxidation. Results are mean±SEM (n=7). *p<0.05 vs. control.

Reducing power Figure 4 shows the reductive capability of LF extract. For the measurements of the reductive activity, we investigated the Fe^{3+}-Fe^{2+} transformation in the presence of LF extract. In our study LF extract (0.5-2 mg/mL) showed high reducing power (Fig. 4A). The phosphomolybdenum method is based on the reduction of Mo (VI) to Mo (V) by the antioxidant components and the formation of a green Mo (V) complex with a maximal absorption at 695 nm. The reducing capacity by phosphomolybdenum method was also enhanced by LF extract (Fig. 4B). The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity (35). It has been cited in the reports that the reducing power was associated with the antioxidant activity and this relationship was also established with the compounds of some anthraquinones (36). Accordingly, LF extract might contain a higher amount of reductone, which could react with free radicals to stabilize and terminate radical chain reactions.

The potent antioxidative activities of the vegetables were observed *in vivo* and *in vitro*. Jeong *et al.* (37) reported that the TBA value of *Aster scaber* was 80% of the value of BHT, a strong synthetic antioxidant, while the value of spinach was only 40% of BHT. The strong antioxidative potency of the wild vegetables may be due to either antioxidant vitamin, phenolic compounds including flavonoid, or the combined effect of all of the above. Flavonoids inhibit both lipid peroxidation and the formation of lipid peroxides (38). The hypocholesterolemic effect has also been reported in other polyphenol-containing foods (39). Dietary flavonoids are an important category of antioxidants that have a sparing effect on vitamin E and β-carotene (40). This suggests that the

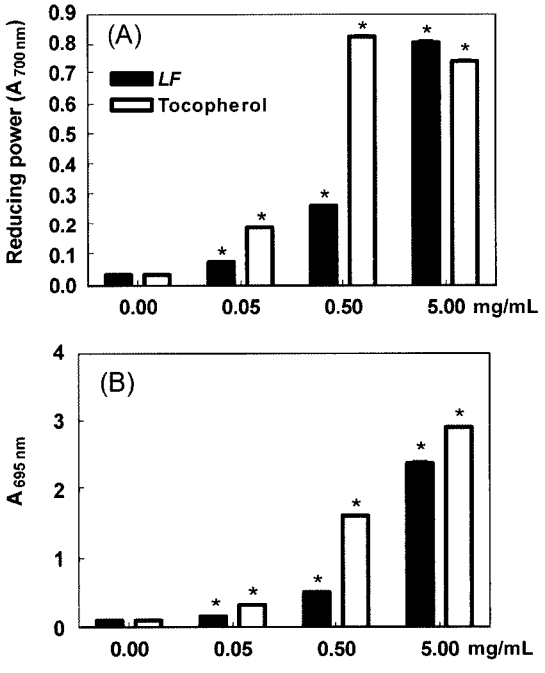


Fig. 4. Reducing power of *Ligularia fischeri* extract. Results are expressed as mean±SEM of 4 experimental determinations. (A) Reducing power by the method of Oyaizu (17); (B) Reducing power by phosphomolybdenum method. *p<0.05 vs. control.

flavonoid and other dietary phenolic compounds, which are known to have potent free-radical scavenging properties (41), are the major contributors to the antioxidative potential of LF. Based on the active profile exposed through various assays, it can be concluded that LF shows strong antioxidant activity, reducing power, free radical scavenging activities. The results of this study show that LF can be of use as an easily accessible source of natural antioxidants and as a possible food supplement or in pharmaceutical industry.

Acknowledgments

This work was supported by the Korea Research Foundation grant funded by Korean Government (MOEHRD) (KRF-2004-206-E00013).

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