Prev. Nutr. Food Sci. 2014;19(1):34-39
http://dx.doi.org/10.3746/pnf.2014.19.1.034
pISSN 2287-1098 · eISSN 2287-8602

Evaluation of the Antioxidant Activity of Cooked *Gomchwi* (*Ligularia fischeri*) Using the Myoglobin Methods

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ABSTRACT: This study evaluated the influence of cooking methods (blanching, pan-frying, and microwaving) on the antioxidant activity of *Ligularia fischeri* (LF) using myoglobin methods against five different reactive oxygen species (ROS) (i.e., 2,2-diphenyl-1-picrylhydrazyl (DPPH), hypochlorite ions, hydroxyl radicals, peroxyl radicals, and peroxynitrite ions). With respect to DPPH scavenging activity, the antioxidant activities of blanched LF and pan-fried LF were significantly higher ($P<0.05$) than that of fresh LF for all cooking times. Pan-fried LF and microwaved LF showed higher antioxidant activities against hydroxyl radicals and peroxyl radicals than uncooked LF, while the protective effect of blanched LF was low, except for the at the 3 min cooking time point. Microwaved LF showed high antioxidant activity against all ROS at the 2 min cooking time point. Interestingly, LF that had been microwaved for 2 min and 3 min had high antioxidant values, whereas blanched LF and pan-fried LF had low antioxidant values. The activities of cooked LF against five different ROS were characterized comprehensively by 5-axe cobweb charts.

Keywords: *Ligularia fischeri*, *gomchwi*, cooking methods, antioxidant activity, myoglobin protective activity

INTRODUCTION

Ligularia fischeri (*L. fischeri*, LF), also called *gomchwi*, is a perennial vegetable plant found mainly in damp shady regions that has long been cultivated for food in Korea (1). In order to reduce its bitter taste, LF is generally consumed salted and roasted after being blanched a state known as *chwinamul*. The leaves of LF are an important herbal medicine traditionally used to treat jaundice, scarlet-fever, rheumatoid arthritis, and hepatic diseases (2). Recently, the chemical constituents of LF leaf tea were analyzed and LF was recognized as a value-added functional food (3). A number of biological activities, including antimutagenic activities and antigenotoxic activities, and cancer prevention activities have been reported for LF (4-6). Many epidemiological studies have verified a relationship between the consumption of fruits, vegetables, and beverages of plant origin (e.g., herbal teas), and a decreased risk of medical disorders caused by the presence of free radicals in the body. There have been many reports about the antioxidant activities of LF extract (1,2,7,8).

In recent years, there has been an increased interest in new developments in disease prevention. The role of free radicals and antioxidants in disease prevention has been of particular interest (9). The role of oxygen free radicals, more generally known as "reactive oxygen species" (ROS) and "reactive nitrogen species" (RNS) in experimental and clinical medicine, has attracted increasing interest. Oxidative stress, which is defined as an imbalance between oxidant formation and the antioxidant-repair capacity of a cell, can generate ROS such as superoxide (O_2^-), hydroxyl (·OH), HO_2 (hydroperoxyl), ROO· (peroxyl), RO· (alkoxyl) as free radicals, and H_2O_2 as a non-radical. Nitrogen-derived oxidant species are mainly NO (nitric oxide), ONOO (peroxynitrate), NO_2 (nitrogen dioxide), and N_2O_3 (dinitrogen trioxide) (10). Antioxidants have been found in various foods, including vegetables, fruits, and grains (11-13). Although various antioxidants have been found in foods, their antioxidant activities have been evaluated mostly by ambiguous methods that give a qualitative or relative value compared to an antioxidant reagent such as ascorbic acid or Ttrolox (14,15). Among those, the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method is a convenient assay that provides quantitative data from a simple experiment (16). While the DPPH method is convenient, the physiological meaning of the activity evaluated by this method is unclear because DPPH is not found in living systems (16). Quantitative measure-

Received 30 December 2013; Accepted 12 March 2014

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ment of physiologically important ROS and free radicals, such as hydroxyl radicals (OH) and hypochlorite radicals, is possible but expensive. The antioxidant activities of physiologically relevant ROS (e.g., hypochlorite ions (CLO) and OH) can be evaluated quickly and simply by measuring absorbance changes of myoglobin. In this study, antioxidants activities against different radicals were evaluated based on myoglobin structural changes and comprehensively displayed using cobweb charts.

MATERIALS AND METHODS

Materials

DPPH, myoglobin (equine skeletal muscle, 95~100 unit), hydrogen peroxide solution (30% (w/w), 2,2'-azobis (2-methylpropionamidine) dihydrochloride, sodium hypochlorite ferrous sulfate, and hydrogen peroxide were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA).

Sample preparation

LF, a number of the *Compositae* family, was collected at Inje-gun, Gangwon-do, Korea. LF was cleaned, washed with water, cut into small pieces, and cooked in a laboratory. The LF was divided into 4 treatment groups (fresh, blanched, pan-fried, and microwaved) and subjected to one of the following methods:

(1) Fresh: LF (200 g) was washed, but not cooked.

(2) Blanched: washed LF (200 g) was added to water (2 L) and blanched in a stainless steel vessel for 1, 3, and 5 min at 95°C.

(3) Pan-fried: washed LF (200 g) was placed in a frying pan with 20 mL of oil, and stirred for 1, 3, and 5 min.

(4) Microwaved: washed LF (200 g) was placed in a glass dish without additional water, and cooked in a microwave oven at 800 W for 0.5, 1, and 2 min.

All of the processed samples were extracted in 70% ethanol with sonication (POWERSONIC 420, 700 W, 50/60 Hz, Hwashin Technology Co., Gyeonggi, Korea) for 40 min twice. The LF extracts were filtered, evaporated (EYELA NVC-2000, SB-1000, DPE-1210, CA-1112, Tokyo Rikakikai Co., Ltd., Tokyo, Japan), and freeze-dried (MC FD 5510, IlShinBioBase, Gyeonggi, Korea) to make powder samples. All samples were diluted to a 100 μg/mL concentration prior to antioxidant activities determination.

Measurement of scavenging activity on DPPH radicals

The DPPH free radical scavenging assay was modified using the procedure described by Blois (17). Extracts from each sample were added to a 1.0×10^{-4} M solution of DPPH in ethanol and the reaction mixture was shaken vigorously. The amount of DPPH remaining was measured using a spectrophotometer (SpectraMax M2, Molecular Devices, Sunnyvale, CA, USA) at 525 nm. The following equation was used to determine the radical scavenging activity of each sample:

$$\text{Radical scavenging activity (\%)} = [1-\{(Abs^{ROS}-Abs^{Sample})/Abs^{0}\}]\times100,$$

where Abs^{ROS} is the absorbance of the test solution containing DPPH and the sample, Abs^{Sample} is the absorbance of the sample mixed with ethanol alone, and Abs^{0} is the absorbance of the control. The antioxidant activity of the sample is expressed as DPPH radical scavenging activity (%).

Measurement of antioxidant activity against hypochlorite ions

Myoglobin was dissolved in phosphate buffered saline (PBS, pH 7.2) to give a final concentration of 0.25 mg/mL. The hypochlorite solution was prepared by diluting the sodium hypochlorite solution in distilled water. Then, 0.05 mL of the rest sample was mixed with 0.25 mL of the hypochlorite solution and added to 1.5 mL of the myoglobin solution. The hypochlorite solution generated hypochlorite radicals (CLO).

Measurements of antioxidant activity against hydroxyl radicals

The OH-generating solution was prepared by the Fenton reaction, which is a reaction solution of ferrous sulfate and hydrogen peroxide. Then, 0.49 mL of each test sample and 0.11 mL of the OH generating solution were added to 3.6 mL of a myoglobin solution (0.25 mg of myoglobin/mL).

Measurement of antioxidant activity against peroxyl radicals

Peroxyl radicals were generated by the thermal decomposition of a 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) solution at a predetermined temperature. Briefly, a 50 mM AAPH solution and each test sample were added to a myoglobin solution (0.35 mg myoglobin/mL) and incubated in a water bath at 47°C for 5 min. A test tube containing the AAPH solution, the test sample, and the myoglobin solution were incubated in a water bath at 37°C for 30 min. The value of the peroxyl radicals was expressed as AAPH.

Measurement of antioxidant activity against peroxynitrite ions

A peroxynitrite solution was diluted in a 20 mM NaOH solution. The diluted peroxynitrite solution was then added to a PBS buffer. The concentration of peroxynitrite ions (ONOO) solution was 3.09 mM, as determined by the absorbance at 302 nm and correction by the molar absorbance coefficient for ONOO (1,670 unit). The myoglobin solution (0.25 mg myoglobin/mL) was mixed well with the test sample and peroxynitrite solution.

Determination of antioxidant activity with the myoglobin method

Antioxidant activity assay against CLO, OH, peroxyl radicals, and ONOO were modified using the procedure reported by Terashima et al. (16,18). For each assay, absorbance was measured spectrophotometrically at 409 nm. The myoglobin protective ratio (%) for the antioxidant was determined using the following equation:

$$\text{Myoglobin protective ratio (\%)} = \left[1 - \frac{\text{Abs}^0 - \text{Abs}^{rad}\ \text{(with antioxidant)}}{\text{Abs}^0 - \text{Abs}^{rad}\ \text{(without antioxidant)}}\right],$$

where Abs^0 is the absorbance of the myoglobin solution (control), Abs^{rad} (without antioxidant) is the absorbance of the test solution containing only the reactive oxygen species, and Abs^{rad} (with antioxidant) is the absorbance of the test solution containing both the ROS and the antioxidant.

Statistical analysis

SPSS software (SPSS Inc., Chicago, IL, USA) was used for statistical analysis. The results were expressed as the mean±standard deviation (n=3). A Tukey HSD test of ANOVA was used to determine statistical significance. A P value < 0.05 was considered significant.

RESULTS

Antioxidant activities of cooked LF against DPPH radicals compared to those of uncooked LF

As shown in Fig. 1, the uncooked and cooked LF extracts showed DPPH radical scavenging effects. Cooked LF had a greater scavenging activity against DPPH radicals than fresh LF. Importantly, the antioxidant activities

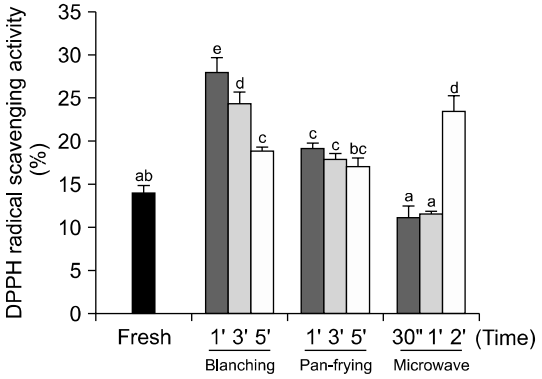

Fig. 1. Free radical scavenging activity of LF against DPPH. Results are presented as mean±SD (n=3). The different letters in each figure indicate significant differences at $P<0.05$.

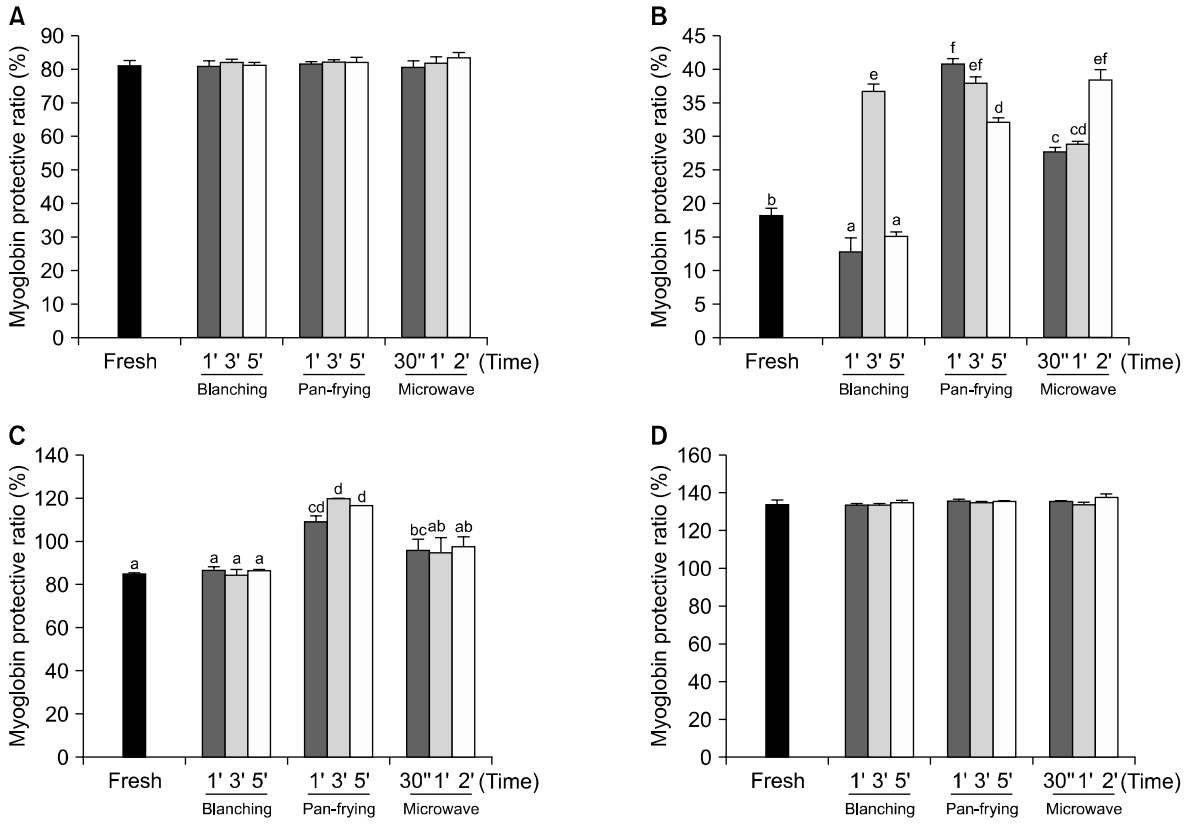

Fig. 2. Myoglobin protective ratio of LF against (A) hypochlorate ions (CLO), (B) hydroxyl radicals (OH), (C) peroxyl radicals (AAPH), and (D) peroxynitrite ions (ONOO). Results are presented as mean±SD (n=3). The different letters in each figure indicate significant differences at $P<0.05$.

of blanched and pan-fried LF were significantly higher ($P<0.05$) than that of fresh LF for all cooking times, while the antioxidant activity of the microwaved LF was not different from that of the fresh LF. Increased blanching and pan-frying time decreased the DPPH scavenging activity of cooked LF. Interestingly, the DPPH scavenging activity of the microwave cooked LF was significantly higher than that of fresh LF at the 2 min time point, while the DPPH scavenging activity of microwaved LF was not different from that of fresh LF at 30 sec and 1 min time point.

Antioxidant activities of cooked LF against hypochlorite ions and hydroxyl radicals compared to those of uncooked LF

Antioxidant activities against CLO and OH were evaluated based on the protective effect of the specimen against by hypochlorite ions- and hydroxyl radical-induced changes to the structure of myoglobin. Fig. 2A presents the myoglobin protective ratios (%) of LF at various cooking time points. Neither cooking method nor cooking time affected CLO scavenging activity. The myoglobin protective ratios (%) of cooked LF against OH are presented in Fig. 2B. Pan-fried and microwaved LF showed higher antioxidant effects against OH than uncooked LF, while the antioxidant effects of blanched LF were low except at the 3 min cooking time point. Interestingly, the myoglobin protective ratios of pan-fried LF were more than two times that of uncooked LF for all cooking time points.

Antioxidant activities of cooked LF against peroxyl radicals and peroxynitrite ions compared to those of uncooked LF

Fig. 2C shows the myoglobin protective ratio of cooked LF against peroxyl radicals. The myoglobin protective ratios of blanched LF was not different from that of uncooked LF. However, the myoglobin protective ratio of pan-fried LF was dramatically increased compared to that of uncooked LF at 3 and 5 min cooking time points. Microwaved LF had a significantly elevated myoglobin protective ratio compared with that of uncooked LF for all cooking times. As shown in Fig. 2D, the myoglobin protective ratio of cooked LF against ONOO was not affected by microwaving, but not blanching or pan-frying .

Comprehensive evaluation of LF with a 5-axe cobweb chart

In order to characterize antioxidant activities, the myoglobin protective ratios of cooked LF (all methods and cooking times) against five different ROS were plotted on a 5-axe cobweb chart (Fig. 3). As shown Fig. 3A, blanched LF has the highest scavenging activity against DPPH radicals, while the myoglobin protective effect against AAPH was lower than that of other cooking methods. Pan-fried LF shows a higher antioxidant activity against OH than other radicals. As cooking time increased, the antioxidant activities of pan-fried LF decreased except for DPPH radical scavenging activity. Interestingly, the antioxidant activities against CLO, OH, AAPH, and ONOO of pan-fried LF were higher than those of blanched LF (Fig. 3B). Microwaved LF showed high antioxidant activity against all ROS for the 2 min cooking time point (Fig. 3C). Interestingly, antioxidant value of were elevated at the long cooking time point (2 min), but were suppressed at the long cooking time points for blanched LF and pan-fried LF.

DISCUSSION

When vegetables are subjected to cooking processes, variations appear in their antioxidant activities and scavenger capacities. These variations depend upon cutting and chopping of the vegetables, cooking method, time, temperature (19,20). There are four the possible reasons for the increased antioxidant activity after cooking: 1) the release of high amounts of antioxidant components due to the thermal destruction of cell walls; 2) the production of stronger radical-scavenging antioxidants by thermal chemical reactions; 3) the suppression of the

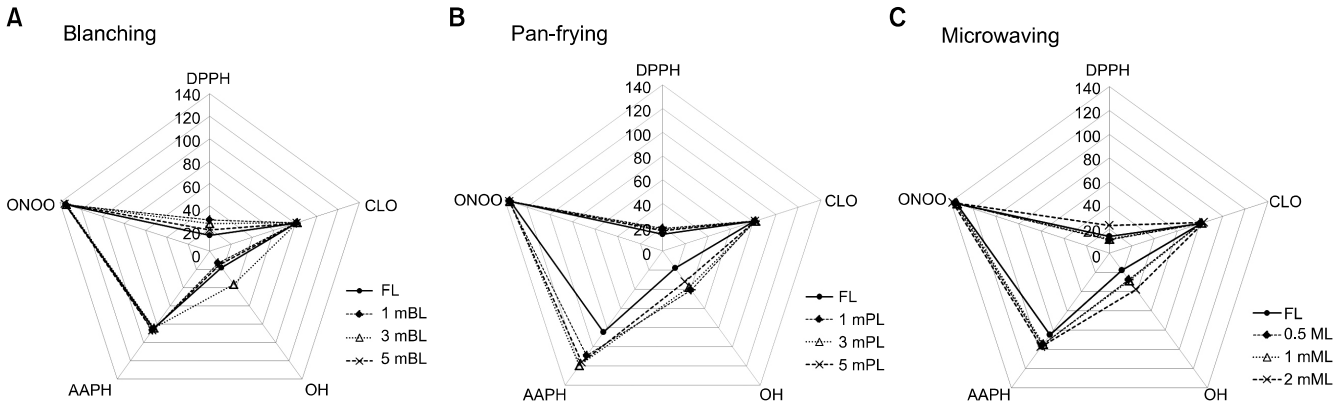

Fig. 3. Antioxidant activity of cooked LF characterized with cobweb chart (A) blanching (B) pan-frying (C) microwaving.

oxidation capacity of antioxidants by thermal inactivation of oxidative enzymes; 4) the production of new, non-nutrient antioxidants or the formation of novel compounds such as Maillard reaction products (21). This study evaluated the antioxidant activities of uncooked and cooked LF and compared the antioxidant activities of LF following different cooking methods and durations. The blanching time increased, the radical scavenging activities of blanched LF decreased. This may be due to the release of phenolic compounds, these affect antioxidant activities from disrupted plant cell walls. The blanching of LF for 1 and 3 min resulted in the softening or disruption of plant cell wall, which may result in an increase in antioxidant activities against various ROS. Parr and Bolwell (22) reported that phenolics, which are hydroxyl groups with have high antioxidant activities, might be transformed via oxidation to quinones during the storage or processing of plant foods. Oboh (23) reported that blanching some tropical green leafy vegetables for 5 min was associated with an increase in total phenol content. The antioxidant activity of blanched LF against DPPH radical was greater than that of fresh LF for all cooking times (Fig. 1). Thus, the boiling of LF may increase total phenol contents, resulting in increased antioxidant activity against DPPH radicals. Minimal heat treatment through the blanching process has been recommended to prevent a major loss of antioxidant properties by some *cruciferous* vegetables (24). However, in this study, the antioxidant properties against DPPH of LF that had been blanched for an extended period (i.e., 10 min) were not different from those of fresh LF (Fig. 3A).

DPPH radicals have been widely used to evaluate the efficacy of various antioxidants. However, the physiological meaning of high activity against DPPH radicals is not clear because these radicals do not exist in the living systems (25). Since peroxyl radicals generated from AAPH primarily oxidized lipids in cell membranes and generate lipid peroxides, substances with high antioxidant activities are thought to possess protective effects on cell membranes (16). In this study, pan-fried LF had higher antioxidant effects against peroxyl radicals (AAPH) than against other ROS (Fig. 2C). These data suggest that the process of pan-frying LF may release substances with lipophilic antioxidant capacity. The andioxidant activity of pan-fried LF against hydroxyl radicals was greater than that of fresh and that of LF that had been cooked by other methods (Fig. 2B). Substances with high antioxidant activities against OH are considered to be effective in eliminating these highly reactive radicals that react with other all substances (16). As shown in Fig. 2, pan-frying LF significantly decreased all ROS except hydrochlorite ions. In contrast, microwaving LF was associated with an increase in antioxidant activity. LF that was microwaved for 2 min had the highest radical scavenging activity against DPPH, OH, AAPH, and ONOO. These increases in antioxidant activities may be attributed to: 1) the high heat breaking down plant cell walls, and 2) the enrichment of antioxidative substances due to evaporation by the microwaving process. Past studies have showed that processing enhances the antioxidant potential of fruits and vegetables by improving the antioxidative properties of naturally occurring compounds or by the formation of Maillard reaction products that display antioxidant activities (26,27). Although microwaved LF is classified as a weak antioxidant by the CLO and the ONOO methods, it showed very strong antioxidant activity against OH. These results reveal variable antioxidant activities of microwaved LF against different ROS. The positive effect of cooking on the antioxidant activities of LF may be explained by matrix softening, leading to increased antioxidant availability, and by the formation of new compounds with antioxidant activities, particularly in the case of the microwaving method. Furthermore, the use of oil may prevent leaching out and induce high antioxidant activity when vegetables are pan-pried. Although vegetables are frequently used in salads or chutney, they are more often consumed in a cooked form. Blanching, pan-frying, and steaming are common vegetable cooking methods. It is well known that cooking can cause considerable changes in the appearance, aroma, and taste of a vegetable. In our previous sensory evaluation of *gomchwi*, blanched *gomchwi* (namul) was preferred over fresh *gomchwi* (28). The blanching process may result in a leaching out of antioxidative substances from the vegetable into the cooking water. Therefore, a short blanching time may be more suitable for maintaining antioxidant activity. Further study is needed to correlate total phenolic content and antioxidant activity using myoglobin methods.

ACKNOWLEDGEMENT

This research was supported by the Globalization of Korean Foods R&D program, funded by the Ministry of Agriculture, Food, and Rural Affairs, Republic of Korea (No. 912024-1) in part from the Priority Research Centers Programs through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2011-0031386).

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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