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Scavenging Effect of Extract from *Perilla frutescens* and Rosmarinic Acid from Free Radical and Lipid Peroxidation

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Abstract

The radical scavenging activity and inhibition effect from lipid peroxidation induced by peroxyl radical of methanol extract from *Perilla frutescens* and its active compound, rosmarinic acid (RA), were investigated *in vitro*. The treatment of extract and RA scavenged 1,1-diphenyl-2-picrylhydrazyl, hydroxyl radical (·OH) and nitric oxide in a concentration-dependent manner. In particular, the extract and RA showed strong radical scavenging activity against ·OH, the most toxic and reactive radical. In addition, *Perilla frutescens* and RA effectively inhibited lipid oxidation induced by sodium nitroprusside and 2,2'-azobis(2-aminopropane) dihydrochloride, determined by the ferric thiocyanate method. The present results suggest that *Perilla frutescens* and RA play a protective role against oxidative stress induced by free radical and lipid peroxidation.

Key words: *Perilla frutescens*, rosmarinic acid, free radical, lipid peroxidation, oxidative stress

INTRODUCTION

The overproduction of reactive oxygen species (ROS)/ reactive nitrogen species (RNS) cause oxidative stress and nitrosative stress in biological systems. ROS and RNS are highly reactive because of their unpaired electrons. The most prominent ROS/RNS are superoxide anion (O_2·), hydrogen peroxide (H_2O_2), hydroxyl ion (·OH) and nitric oxide (NO·) (1). These radicals are formed by all aerobic organisms and are involved in a variety of physiological and biochemical lesions. The overproduction of free radicals contributes to occurrence of degenerative diseases such as diabetes, arteriosclerosis, cardiovascular diseases, cancer, neurodegenerative disorders and the aging process (2-4). Therefore, the ability of antioxidants to attenuate oxidative stress, induced by excessive production of free radicals, has been suggested for the prevention and treatment of diseases. Recently, natural antioxidants have attracted much attention because synthetic antioxidants cause various side effects and toxicities. Therefore, searching for natural antioxidants from herbs or plants, to attenuate oxidative and nitrosative stress, is of growing interest.

Perilla frutescens var. japonica Hera has a long history of cultivation and abundant consumption in Eastern Asia. The plant's biological effects such as antibacterial, antiseptic, antipyretic, spasmolytic, antiasthmatic and anti-allergic functions have been demonstrated in several studies (5-7). In addition, the active components caffeic acid, methyl caffeate, quercetin, rosmarinic acid (RA), and luteolin 7-*O*-glucuronide-6″-methyl ester were isolated from *Perilla frutescens* (8,9). In particular, RA, a polyphenolic phytochemical, is an important constituent of *Perilla frutescens* extract. RA in perilla leaves and seeds functions as an anti-inflammatory and anti-allergic compound (10-13). However, the protective activity of *Perilla frutescens* and RA from oxidative stress and nitrosative stress has not been clearly elucidatated. Therefore, in the present study, we examined the protective effect of *Perilla frutescens* and its major component, RA, against oxidative stress induced by free radicals.

MATERIALS AND METHODS

Materials

Perilla frutescens was obtained from Milyang (Korea). Rosmarinic acid (RA) (M.W.: 260.31) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and the structure was shown in Fig. 1. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), nitroblue tetrazolium (NBT), malondialdehyde (MDA), 2-deoxyribose and Griess reagent were purchased from Sigma-Aldrich Co. 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) and sodium nitroprusside (SNP) were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals used were of analytical grade and obtained from Merck (Darmstadt, Germany) and Sigma-Aldrich Co.

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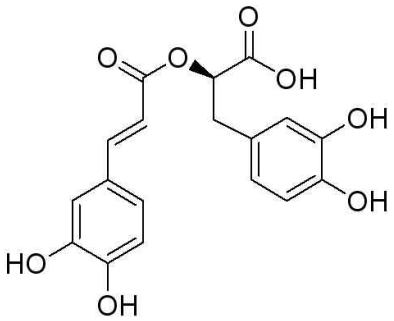

Fig. 1. Structure of rosmarinic acid.

Preparation of sample

Freeze-dried *Perilla frutescens* was extracted three times with 20 volumes of 100% methanol at room temperature for 24 hr. The extract was obtained by a rotary evaporator and the yield was 23.43%. The extract from *Perilla frutescens* and RA were dissolved in phosphate buffered saline (PBS) and dimethylsulfoxide, respectively.

DPPH scavenging assay

This assay is based on the capacity of a substance to scavenge stable DPPH radicals. Here, reaction mixtures, containing test samples (100 μL) and DPPH ethanolic solution (100 μL) in 96-well micro plates, were incubated at 37°C for 30 min and absorbance values were then measured at 540 nm (14).

Hydroxyl radical scavenging assay

The reaction mixture contained 0.45 mL of sodium phosphate buffer (0.2 M, pH 7.0), 0.15 mL of 2-deoxyribose (10 mM), 0.15 mL of $FeSO_4$-ethylenediamine tetraacetic acid (EDTA) (10 mM), 0.15 mL of H_2O_2 (10 mM), 0.525 mL of distilled water, and 0.075 mL of sample solution. After incubation at 37°C for 4 hr, the reaction was stopped by adding 0.75 mL of 2.8% trichloroacetic acid and 0.75 mL of 1.0% 2-thiobarbituric acid in 0.05 N NaOH. The solution was boiled for 10 min and then cooled in water. The absorbance was measured at 520 nm. Hydroxyl radical scavenging activity was evaluated as the inhibition rate of 2-deoxyribose oxidation by ·OH (15).

NO scavenging activity

NO was generated from SNP and measured by the Griess reaction, according to Sreejayan and Rao (16). SNP (5 mM), in phosphate buffered saline, was mixed with different concentrations of sample and incubated at 25°C for 150 min. The amount of NO produced by SNP was assayed by measuring the accumulation of nitrite, based on the Griess reaction, using a microplate assay method.

Determination of lipid oxidation

Lipid oxidation was measured by the ferric thiocyanate (FTC) method (17). The solution, containing 1 mL of each sample, 2 mL of 2.51% linoleic acid in ethanol, 4 mL of 0.02 M phosphate buffer (pH 7.0), 0.417 mL of 100 mM AAPH or 12 mM SNP, and 2 mL of distilled water, was mixed and placed in a dark oven at 37°C for 200 min. 0.1 mL of aliquot was taken from a reaction mixture every 24 hr that was first diluted with 9.7 mL of 75% ethanol, then 0.1 mL of 30% ammonium thiocyanate solution, and finally 0.1 mL of 0.02 M ferrous chloride in 3.5% hydrochloric acid. The absorbance of red color was measured at 500 nm every 24 hr until one day after absorbance of the control reached maximum.

RESULTS AND DISCUSSION

The overproduction of free radicals can cause extensive damage to tissues and biomolecules, leading to various pathological conditions and degenerative diseases (1). Many synthetic drugs with antioxidative effects can protect against oxidative damage, but the various adverse side effects are becoming serious issues. Dietary antioxidant intake may be an important strategy for inhibiting or delaying the oxidation of susceptible cellular substrates, by overproduction of free radicals, and relevant to disease prevention in many paradigms. Flavonoids and other phenolic compounds of plant origin have been reported as radical scavengers and inhibitors of lipid peroxidation (18,19). *Perilla frutescens* leaves are known to be rich in flavonoids and RA, the main phenolic acid (20). In addition, evidence suggests that *Perilla frutescens* exhibits are strong anti-inflammatory and anti-allergic biological activities (5,21). However, the protective activity of *Perilla frutescens* and RA against oxidative stress and nitrosative stress has not been clearly elucidated. We investigated the protective activity of *Perilla frutescens* extract and its active compound RA against free radicals.

DPPH radical scavenging activity

DPPH, a stable nitrogen centered free radical, has been widely used to test the ability of compounds or plant extracts to act as free radical scavengers or hydrogen donors (22). The DPPH scavenging activity of *Perilla frutescens* and RA increased in a concentration-dependent manner (Fig. 2). At 100 μg/mL, the DPPH scavenging effect of methanol extract from *Perilla frutescens* was over 80% and RA showed over 90% scavenging activity at 200 μM. *Perilla frutescens* extract and RA had IC_{50} values of 8 μg/mL and 15 μM, respectively, suggesting their promising role as free radical scavengers.

·OH radical scavenging activity

·OH scavenging activity of *Perilla frutescens* and RA

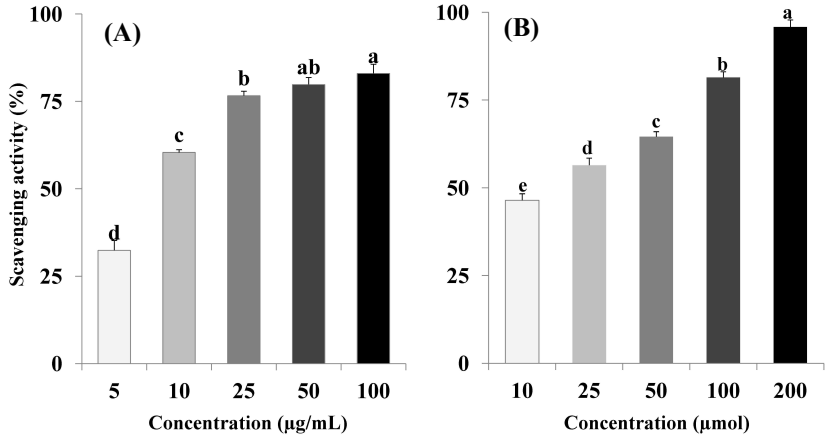

Fig. 2. DPPH scavenging activity of MeOH extract from *Perilla frutescens* (A) and rosmarinic acid (B). Values are mean±SD. [a-e]Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

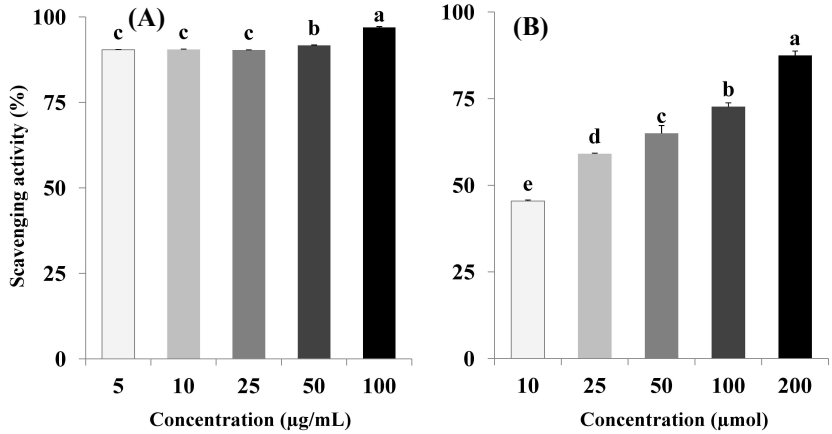

Fig. 3. Hydroxyl radical scavenging activity of MeOH extract from *Perilla frutescens* (A) and rosmarinic acid (B). Values are mean± SD. [a-e]Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

was shown in Fig. 3. ·OH is the major active oxygen species causing lipid peroxidation and enormous biological damage. ·OH is formed from hydrogen peroxide by the Fenton reaction and is considered the most toxic ROS (23,24). The *Perilla frutescens* extract showed high ·OH scavenging activity, even at low concentrations (Fig. 4). At 100 μg/mL, *Perilla frutescens* showed over 90% scavenging effect of ·OH. RA also exerted a strong scavenging effect of ·OH in a concentration-dependent manner. At 200 μM, RA scavenged 87.5% of total ·OH. These results indicated that *Perilla frutescens* and RA may have protective activity from the most toxic and reactive radical, ·OH.

NO scavenging activity

NO is an important signaling molecule that acts in many tissues to regulate a diverse range of physiological processes including vasodilatation, neuronal function, inflammation and immune function. In addition, NO can damage DNA, inhibit a variety of enzymes and induce lipid peroxidation. Also, NO plays a role in the regulation of apoptosis (25). Therefore, to investigate the NO

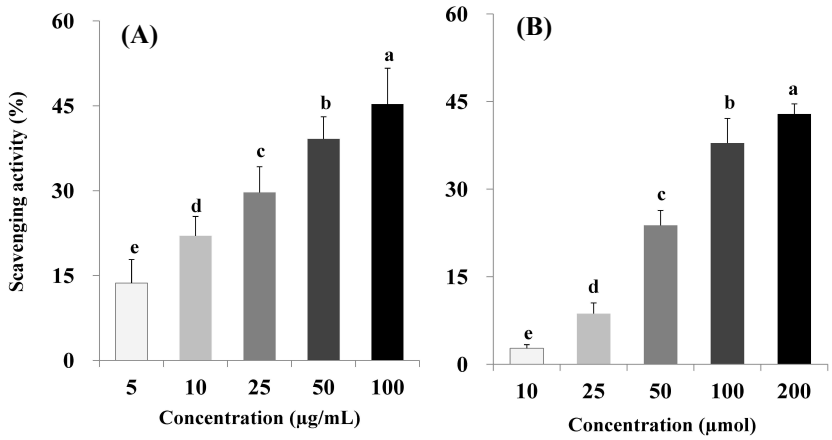

Fig. 4. NO scavenging activity of MeOH extract from *Perilla frutescens* (A) and rosmarinic acid (B). Values are mean±SD. [a-e]Means with the different letters are significantly different ($p<0.05$) by Duncan's multiple range test.

scavenging activity by *Perilla frutescens* and RA, we used SNP as a NO radical generator. Treatment with 100 μg/mL of *Perilla frutescens* exhibited the inhibitory effect against NO over 45% and RA showed a similar result at 200 μM (Fig. 4). Compared to other radicals, the effect of NO scavenging by *Perilla frutescens* and RA was relatively low, but significantly increased in a concentration-dependent manner.

Antioxidant activity from the linoleic acid peroxidation

To determine the antioxidant activity of samples, the FTC method was applied by measuring the peroxide levels during the initial stage of lipid oxidation (26-28). Two different generators, SNP and AAPH, were used to induce NO and peroxyl radicals. SNP is comprised of a ferrous ion center complexed with five cyanide moieties and a nitrosyl group. SNP can interact with oxyhemoglobin, dissociating immediately and forming methemoglobin, and eventually releasing cyanide and nitric acid (29). *Perilla frutescens* and RA inhibited the lipid peroxidation induced by NO (Fig. 5). Control showed the highest absorbance value of 0.38 at 6 days, while after the 7 day incubation period, the formation of peroxides was stopped and the value declined to 0.26 because of non-availability of linoleic acid. The result showed significant decline of absorbance values of *Perilla frutescens* and RA on the last day. Compared with control, the treatment of *Perilla frutescens* showed low absorbance values overall, peaking at 0.019 and gradually declined to 0.004 with 100 μg/mL treatment. Similarly, RA values declined from 0.010 to lower than 0 at the 200 μM concentration. This data indicates that *Perilla frutescens* and RA inhibited lipid peroxidation through elimination of NO. Similarily, AAPH can generate free radicals at a constant rate in a water environment by unimolecular thermal decomposition without the addition of potentially interfering cofactors and transition metals. AAPH was used as the peroxyl radical generator to initiate the reaction (30-32). Peroxyl radicals have been proposed to be mediators of the hydroperoxide-dependent oxidations related to human diseases, and therefore widely accepted that peroxyl radicals are important

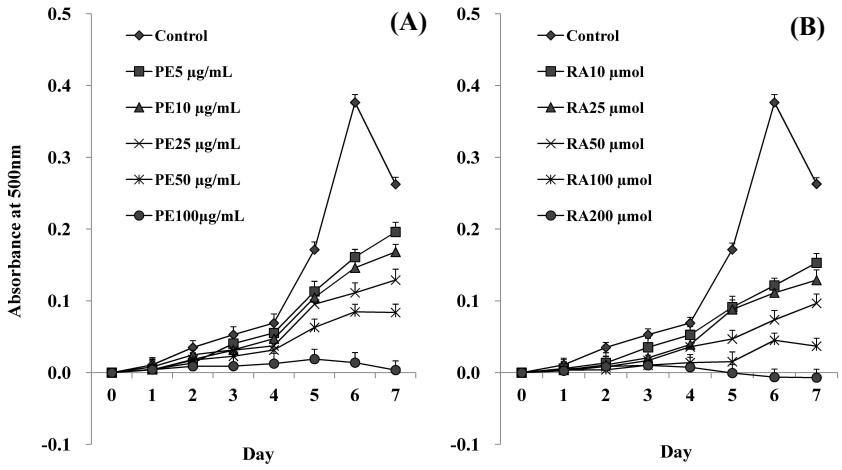

Fig. 5. Protective effect of MeOH extract from *Perilla frutescens* (A) and rosmarinic acid (B) from lipid peroxidation induced by sodium nitroprusside. Values are mean±SD.

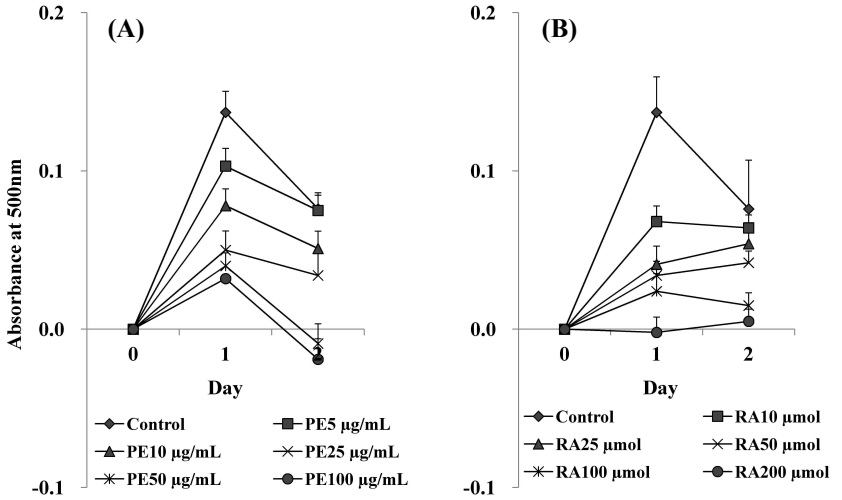

Fig. 6. Protective effect of MeOH extract from *Perilla frutescens* (A) and rosmarinic acid (B) from lipid peroxidation induced by 2,2'-azo-bis(2-amidinopropane) dihydrochloride. Values are mean±SD.

intermediates in the oxidation of fatty acid (33). The treatment with *Perilla frutescens* and RA led to the decrease in absorbance values after the 2 day incubation period (Fig. 6). Control treated with AAPH revealed the value of 0.137 at the 1st day, however the treatment of *Perilla frutescens* and RA at the concentration of 100 μg/mL and 200 μM led to the decline of the values 0.032 and lower than 0, respectively. These results suggested that *Perilla frutescens* and RA exerted antioxidative activity against peroxyl radicals and lipid peroxidation.

In conclusion, the methanol extracts from *Perilla frutescens* and RA exerted strong antioxidative activities against DPPH, ·OH, and NO and inhibited lipid peroxidation induced by NO and the peroxyl radical. These results suggest the promising role of *Perilla frutescens* and RA as effective antioxidants against free radical induced oxidative stress.

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