Natural Product Sciences
7(3) : 72~75 (2001)

Antioxidant Activity of Roasted Defatted Perilla Seed

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Abstract – The antioxidant activity of roasted defatted perilla (*Perilla frutescens*) seed was determined by measuring its radical scavenging effect on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals, inhibitory activity on total reactive oxygen species generation in kidney homogenates using 2',7'-dichlorodihydrofluorescein diacetate, and scavenging effect on authentic peroxynitrites. The methanolic extract of roasted defatted perilla seed showed strong scavenging activity in both DPPH and peroxynitrite radicals, and thus fractionated with several solvents. The antioxidant activity potential of the individual fraction was in the order of ethyl acetate>*n*-butanol>dichloromethane>water>*n*-hexane fraction. The ethyl acetate soluble fraction exhibiting strong antioxidant activity was further purified by repeated silica gel and Sephadex LH-20 column chromatography. Luteolin was isolated as one of the active principles from the ethyl acetate fraction, together with the inactive chrysoeriol and apigenin.
Key words – roasted, *Perilla frutescens*, luteolin, antioxidant activity.

Introduction

Perilla (*Perilla frutescens* var. *japonica* Hara) seed is used in herbal medicine. In India the seeds are often used in the curry materials when cooking meat, fish and vegetable (Misra and Husain, 1987). In Korea the seed and its oil are often used in the diet. The roasted seed is used widely as flavouring and as a rich source of nutrients (Yu *et al.*, 1997). Perilla seed oil includes *ca.* 60% of α-linolenate (Okuyama *et al.*, 1992), which has received much attention for its various biological activity. Although perilla seed oil is easily oxidized because of its high degree of unsaturation, the oil from the roasted seed is commonly known to be oxidized less rapidly. This fact indicates that the antioxidants are produced by roasting the perilla seed. Indeed Nagatsu *et al.* (1995) reported the isolation of novel antioxidants from roasted perilla seed. In this paper, we now report the antioxidant evaluation of all extracts and isolated compounds from the roasted perilla seed for its potential to scavenge stable DPPH free radicals, to inhibit total reactive oxygen species, and to scavenge authentic peroxynitrites.

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Materials and Methods

The ^{1}H- and ^{13}C-NMR spectra were recorded at 300 MHz and 75.5 MHz, respectively on a Bruker AM 300 spectrometer with tetramethylsilane as internal standard. Multiplicities of ^{1}H- and ^{13}C-NMR signals are indicated as s (singlet) and d (doublet). Column chromatography was done with silica gel (Merck, 70-230 mesh). TLC was carried out on precoated Merck Kieselgel 60 F_{254} plate (0.25 mm) and spots were detected under UV light using 50% H_2SO_4 spray reagent.

Plant materials – *P. frutescens* seed was purchased from a commercial supplier (Chungbuk Suhan Nonghyup) in 1998 and authenticated by Prof. J. S. Choi of the Faculty of Food Science and Biotechnology, Pukyong National University. A voucher specimen has been deposited in the authors laboratory.

Chemicals – 1,1-Diphenyl-2-picrylhydrazyl (DPPH), L-ascorbic acid, and penicillamine were purchased from Sigma Chemical Company (St. Louis, MO, USA). 2',7'-dichlorofluorescin diacetate (DCHF-DA), dihydrorhodamine 123 (DHR 123), and peroxynitrite were of high quality and were purchased from Molecular Probes (Eugene, Oregon, USA), and Cayman (Ann Arbor, MI, USA), respectively.

Extraction, fractionation and isolation – The perilla seed roasted at 210°C for 20 min was ground

and roasted defatted seed (10 kg) of *P. frutescens* was refluxed with MeOH for three hrs. (9 L×3). The total filtrate was concentrated to dryness *in vacuo* at 40°C to render the MeOH extract (560 g), and this extract was suspended in distilled H_2O and partitioned with *n*-hexane (257.3 g), CH_2Cl_2 (36.5 g), EtOAc (10.4 g), *n*-BuOH (82.1 g) and H_2O (141.2 g) in sequence. The EtOAc fraction showed strong scavenging activity against DPPH radical, peroxynitrite and ROS; Thus, the EtOAc (10 g) fraction was chromatographed on a silica gel column using CH_2Cl_2-MeOH (gradient) to give compound **1** (chrysoeriol, 20 mg), **2** (apigenin, 50 mg) and **3** (luteolin, 500 mg), respectively. The chemical structures of these compounds are shown in Fig. 1.

Compound 1 (chrysoeriol): ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 3.89 (3H, s, OCH_3), 6.20 (1H, d, *J*=2 Hz, H-6), 6.51 (1H, d, *J*=2 Hz, H-8), 6.91 (1H, s, H-3), 6.93 (1H, d, *J*=9 Hz, H-5'), 7.56 (1H, d, *J*=2 Hz, H-2'), 7.57 (1H, dd, *J*=2, 9 Hz, H-2'). ^{13}C-NMR (75.5 MHz, DMSO-d_6) δ: 163.62 (C-2), 103.18 (C-3), 181.76 (C-4), 161.39 (C-5), 98.78 (C-6), 164.09 (C-7), 94.00 (C-8), 157.29 (C-9), 103.67 (C-10), 120.32 (C-1'), 110.18 (C-2'), 150.69 (C-3'), 147.99 (C-4'), 115.73 (C-5'), 121.47 (C-6'), 55.94 (OCH_3).

Compound 2 (apigenin): ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 6.20 (1H, d, *J*=2 Hz, H-6), 6.51 (1H, d, *J*=2 Hz, H-8), 6.78 (1H, s, H-3), 6.93 (2H, d, *J*=9 Hz, H-3', 5'), 7.91 (2H, dd, *J*=9 Hz, H-2', 6'), 12.96 (1H, s, OH). ^{13}C-NMR (75.5 MHz, DMSO-d_6) δ: 164.05 (C-2), 102.75 (C-3), 181.64 (C-4), 161.36 (C-5), 98.73 (C-6), 163.64 (C-7), 93.87 (C-8), 157.22 (C-9), 103.59 (C-10), 121.09 (C-1'), 128.38 (C-2'), 115.85 (C-3'), 161.05 (C-4'), 115.85 (C-5'), 128.38 (C-6').

Compound 3 (luteolin): ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 6.22 (1H, d, *J*=2 Hz, H-6), 6.50 (1H, *J*=2 Hz, H-8), 6.67 (1H, s, H-3), 6.94 (1H, d, *J*=8 Hz, H-5'), 7.41 (1H, d, *J*=2 Hz, H-2'), 7.44 (1H, dd, *J*=2, 8 Hz, H-6'), 12.93 (1H, s, OH). ^{13}C-NMR (75.5 MHz, DMSO-d_6) δ: 164.35 (C-2), 102.79 (C-3), 181.76 (C-4), 161.44 (C-5), 98.97 (C-6), 164.02 (C-7), 94.03 (C-8), 157.40 (C-9), 103.65 (C-10), 119.05 (C-1'), 113.22 (C-2'), 145.75 (C-3'), 149.78 (C-4'), 116.08 (C-5'), 121.54 (C-6').

DPPH radical scavenging effect – The DPPH radical scavenging effect was evaluated according to the method first employed by Blois (Blois, 1958). One hundred and sixty microliters of MeOH solution of varying sample concentrations (0.25-160 μg/ml) was added to 40 μl DPPH methanol solution (1.5×10^{-4} M). After mixing gently and leaving for 30 min at room temperature, the optical density was measured at 520 nm using a spectrophotometer. The antioxidant activity of each fraction and sample was expressed in terms of IC_{50} microgram per ml concentration required to inhibit DPPH radical formation by 50% and calculated from the log-dose inhibition curve.

Assay for the free radical generation – Liver cells (AC_2F) were incubated for 24 h in serum free media in CO_2 incubator at 37°C until confluent, and the cells were transferred to multiwell plates with about 10^5 cells/well and cultured with or without a suspension of test samples (0.5 mg/ml), then incubated with 12.5 μM DCFH-DA at 37 for 30 min. Fluorescence was monitored on a spectrofluorometer with excitation and emission wavelengths of 480 nm and 530 nm, respectively.

Measurement of peroxynitrite scavenging activity – The fluorescence measurements for the scavenging activity were as described by the method of Kooy *et al.* (Kooy, 1994). DHR 123 (5 mM) in dimethylformamide, which was purged with nitrogen, was stored at 80°C as a stock solution. The buffer used consisted of 90 mM sodium chloride, 50 mM sodium phosphate, 5 mM potassium chloride at pH 7.4 and 100 μM diethylenetriaminepentaacetic acid (DTPA), each of which was prepared with high quality deionized water and purged with nitrogen. The final concentration of DHR 123 was 5 μM. The background and final fluorescent intensities were measured 5 min after treatment with and without the addition of authentic peroxynitrite. DHR 123 was oxidized rapidly by authentic peroxynitrite, and its final fluorescent intensity remained unchanged over time. The fluorescence intensity of oxidized DHR 123 was measured with a microplate fluorescence reader (FL 500, Bio-Tex Instruments) at the excitation and emission wavelengths of 480 nm and 530 nm, respectively. Results were expressed as means±S.E. (n=3) for the final fluorescence intensity minus background fluorescence.

Results and Discussion

It is well known that free radicals and reactive oxygen/or nitrogen species including hydroxyl radical, and peroxynitrites play a role in the etiology of a vast variety of human diseases (Pincemail, 1995).

1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical is a dyed free radical. Due to its odd electron, DPPH

radical gives a strong absorption band at 520 nm (deep violet color). The change in absorbance produced by reduced DPPH was used to evaluate the ability of test samples to act as free radical scavenger. The DPPH radical scavenging effect for the methanol extract and its fractions from the roasted defatted perilla seed are shown in Table 1. The IC_{50} values of the methanol extract and EtOAc, *n*-BuOH, and CH_2Cl_2 fractions obtained from the methanol extract were shown at concentrations of 33.99, 3.30, 11.11, and 16.26 μg/ml, respectively, while the *n*-hexane- and H_2O-soluble fractions showed no activity (>80 μg/ml).

Peroxynitrite ($ONOO^-$), formed from the reaction of superoxide and nitric oxide, is a cytotoxic species that can oxidize several cellular components such as proteins, lipids, and DNA. It has been implicated in diseases such as Alzheimers disease, rheumatoid arthritis, cancer, and atherosclerosis (Squadrito and Pryor, 1998). Reactive oxygen species (ROS) are also implicated in both aging and in various degenerative disorders (Sagar *et al.*, 1992, Ames *et al.*, 1993). ROS, such as hydrogen peroxide, superoxide anion, hydroxyl radical, and nitric oxide, are formed in the body as a consequence of aerobic metabolism, damaging all intracellular components, including nucleic acids, proteins, and lipids (Sagar *et al.*, 1992). In this study, we also investigated the antioxidant effects for potential to inhibit total reactive oxygen species (ROS) generation in kidney homogenates using 2',7'-dichlorodihydrofluorescein diacetate, and scavenge authentic peroxynitrites of the MeOH extract and its solvent partitioned fractions such as *n*-hexane-, CH_2Cl_2-, EtOAc-, *n*-BuOH- and H_2O-soluble fractions derived from roasted defatted perilla meal and further studies are planned that will attempt to identify the active principles form the active fractions. As summarized in Table 1, the inhibition effect of the MeOH extract and its solvent partitioned fractions such as *n*-hexane-, CH_2Cl_2-, EtOAc-, *n*-BuOH-, and H_2O-soluble fractions derived from roasted defatted perilla meal on the peroxynitrite increased in the order of EtOAc>*n*-BuOH>CH_2Cl_2>MeOH>H_2O> *n*-hexane and were 0.7±0.08, 1.6±0.08, 2.8±0.11, 11.9±0.68, 14.2±0.20, and >>100 μg/ml in their IC_{50}, respectively, indicating that the EtOAc soluble fraction of the MeOH extract from roasted defatted perilla meal is sufficient to have a noticeable effect on scavenging peroxynitrites. Especially, the scavenging effect of the EtOAc-fraction on peroxynitrite exceeded that of penicillamine which is a well known peroxynitrite scavenger. The BuOH fraction also showed a strong scavenging activity, which was slightly lower than the EtOAc fraction, on peroxynitrite. However, the hexane-soluble fraction showed inactive even at the higher concentration. In addition, the EtOAc, *n*-BuOH, CH_2Cl_2 fraction were significantly inhibited the total ROS by 6.9±0.44, 25.2±3.03 and 28.4±0.91 μg/ml in their IC_{50}, respectively.

Therefore, the EtOAc-soluble fraction was subjected to further chemical analysis and, after column chromatographic separation, the three compounds (**1**, **2** & **3**) were isolated, and identified as chrysoeriol, apigenin and luteolin, respectively.

The antioxidant activities of isolated components from the EtOAc fraction of the methanol extract of roasted defatted perilla meal was shown in Table 2. Among three isolated compounds, luteolin significantly scavenged the peroxynitrite at the IC_{50} of 1.43±0.27

Table 1. Antioxidant activity of extracts derived from *P. frutescens* on DPPH, $ONOO^-$ and ROS

Extract	DPPH[a]	$ONOO^-$[b]	ROS[c]
MeOH	33.9	11.9±0.68	–
n-Hexane	>120	>>100	–
CH_2Cl_2	16.3	2.8±0.11	28.4±0.91
EtOAc	3.3	0.7±0.08	6.9±0.44
n-BuOH	11.1	1.6±0.08	25.2±3.03
H_2O	86.8	14.2±0.20	79.3±6.60
Penicillamine	<0.63	2.91±0.64	
L-Ascorbic acid			

[a]DPPH: DPPH free radical scavenging activity (IC_{50}: μg/ml).
[b]$ONOO^-$: Inhibition percent of peroxynitrite (IC_{50}: μg/ml).
[c]ROS: Inhibitory activity of total free radical generation in kidney postmicrosomal fraction (IC_{50}: μg/ml).

Table 2. Antioxidant activity of isolated compounds derived from *P. frutescens* on DPPH, $ONOO^-$ and ROS

Samples	DPPH[a]	$ONOO^-$[b]	ROS[c]
Chrysoeriol	>100	NS[d]	5.0±0.3
Apigenin	>100	NS	5.0±0.5
Luteolin	1.7	1.43±0.27	53.0±2.2
Penicillamine		2.91±0.64	
L-Ascorbic acid	11.5		

[a]DPPH: DPPH free radical scavenging activity (IC_{50}: μM).
[b]$ONOO^-$: Inhibition percent of peroxynitrite (IC_{50}: μM).
[c]ROS: Inhibition percent of total free radical generation in kidney postmicrosomal fraction at the concentration of 20 μg/ml).
[d]NS: represent as no effect up to a concentration of 100 μM.

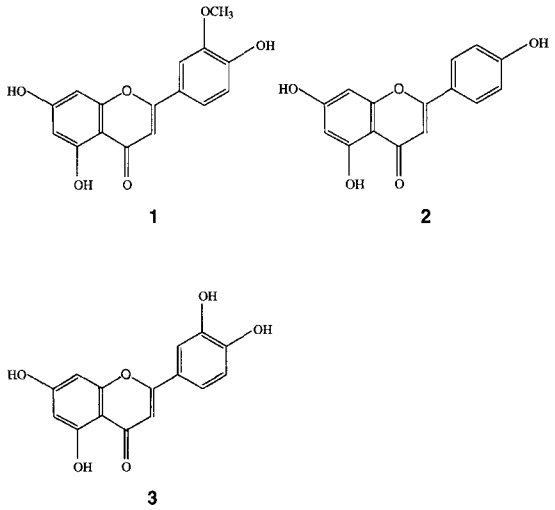

Fig. 1. Structures of compounds **1-3**.

μg/ml, and total ROS by 53 % at concentration of 20 μg/ml, respectively. The peroxynitrite scavenging effect of luteolin was two-fold more potent than that of penicillamine, which is a well known peroxynitrite scavenger. Luteolin also exhibited potent DPPH scavenging activity with the IC_{50} value of 1.7 μM. The radical scavenging effect of a luteolin **3** on DPPH radical exceeded that of L-ascorbic acid which is a well known antioxidant. However, compounds **1** and **2** were found to have no activity. These results suggest that the antioxidative activity in the original methanol extract of roasted defatted perilla meal was partially attributable to luteolin.

Luteolin was reported as an antioxidant (Torel *et al.*, 1986). As luteolin has a catechol moiety in its structure, the antioxidative potency of this compound may be attributable to this moiety (Choi *et al.*, 2001).

The present work would tend to indicate that the methanolic extract of roasted defatted perilla meal and its various fractions, and its components, may be useful for the treatment of oxidative damage. It will be interesting to further investigate the antioxidative activity of these natural compounds in preventing various radical-mediated injuries in pathological situations *in vivo*. Investigation of further antioxidant principles are now in progress.

Acknowledgements

This research was supported by a grant (PF002201-07 and -08) from Plant Diversity Research Center of 21st Century Frontier Research Program funded by Ministry of Science and Technology of Korean Government.

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(Accepted August 16, 2001)