

Antioxidant Activities and Determination of Phenolic Acids from Leaves of *Perilla frutescens*

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Two catecholic phenolic acids (1 and 2) obtained from 80% methanolic extract of *Perilla frutescens* var. *frutescens* leaves through chromatography showed strong DPPH (1: IC₅₀ = 15.5 μM, 2: IC₅₀ = 11.7 μM) and ABTS (1: IC₅₀ = 5.5 μM, 2: IC₅₀ = 4.6 μM) radicals scavenging abilities. Antioxidant compounds contents of 1 and 2 as determined by C₁₈ reversed phase HPLC coupled with diode-array detector were 2.98 and 2.26 mg/g, respectively.

Key words: ABTS, Catecholic phenolic acid, DPPH, HPLC, *Perilla frutescens*

Polyphenols, which are widely distributed in plant and foods, are thought to have positive effects on human health.^{1,2)} In particular, the potentially versatile antioxidant activity of polyphenols is highly effective against several diseases involved in oxidative stress.³⁻⁵⁾ Therefore, polyphenols have been used in many antioxidant capacity assays before testing the properties of a biological system. Several methods have been developed to determine the antioxidant activity, in which DPPH and ABTS radical systems⁶⁻¹⁰⁾ were commonly used to measure the total antioxidant activity.

Perilla frutescens var. *frutescens*, which belongs to the Labiatae family, is an important traditional herbal medicine for treating various diseases including depression, anxiety, tumor, cough, bacterial and fungal infections, allergy, intoxication and some intestinal disorders¹¹⁻¹³⁾ in East Asian Countries such as Korea, China, and Japan. It is also an important ingredient in Korean cooking as a popular garnishes and a food colorant. Several studies have been performed biologically on this plant, a rich source of polyphenolic phytochemicals.^{11,14-17)} As a continuing research on this species, we examined the antioxidant effects of the leaves of this plant and determined the contents of the antioxidant compounds.

In this paper, we wish to report that antioxidant activity-guided fractionation of extract of leaves of this species resulted in the isolation of two phenolic acids (1 and 2), which showed antioxidant activities against DPPH and ABTS

radicals. We also describe their contents were determined by C₁₈ reversed phase HPLC.

Materials and Methods

Plant material. The leaves of *P. frutescens* (Namcheon) were collected in August 22-25, 2004 at the experimental field of Yeongnam Agricultural Research Institute, Miryang, Korea, and air-dried in the shade.

Reagents. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), sodium presulfate, butylated hydroxyl anisol (BHA), (S)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich. All chemicals used were of analytical grade.

Instruments. Melting points were measured on a Thomas Scientific Capillary Melting point Apparatus and were uncorrected. IR and UV spectra were recorded on a Bruker IFS66 infrared Fourier transform spectrophotometer (KBr) and a Beckman DU650 spectrophotometer, respectively. ¹H- and ¹³C-NMRs at 500 and 125 MHz, respectively, and 2D-NMR data were obtained on a Bruker AM 500 spectrometer in CD₃OD. TLC was conducted on precoated Kieselgel 60F₂₅₄ plates (Art. 5715; Merck), and the spots were detected by examining the plates under a UV lamp or by treating the plates with a 10% ethanolic solution of phosphomolybdic acid (Wako Pure Chemical Industries) followed by heating at 110°C. HPLC was performed using an Agilent 1100 series quaternary pump (flow speed: 1 ml/min), Agilent 1100 series diode-array detector (330 nm), and LichroCART 125-4 HPLC-Cartridge (Lichrophore 100 RP-18e, φ 5 μm) column. EIMS were obtained on a JEOLJMS-700 mass spectrometer. Optical rotations were measured on a JASCO DIP-1000

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Abbreviations: HMBC, Heteronuclear Multiple-Bond Connectivity; COSY, Correlation Spectroscopy.

polarimeter.

Extraction and Isolation. The dried leaves of *P. frutescens* (1.2 kg) were pulverized and extracted with 80% MeOH (1 l × 3) at room temperature for 7 days. Filtration and concentration gave the resultant green extract (43 g), which was suspended in H₂O and partitioned with *n*-hexane, CHCl₃, EtOAc, and *n*-BuOH to give *n*-hexane- (8.0 g), CHCl₃- (6.8 g), EtOAc- (7.9 g), and *n*-BuOH-extractable (8.7 g) residues. Subsequently, *n*-BuOH-extractable residues (6.7 g), which showed potent antioxidant activities against DPPH (65% inhibition at 100 μg/ml) and ABTS (70% inhibition at 100 μg/ml) radicals were chromatographed over a silica gel column using CH₂Cl₂: acetone and CH₂Cl₂: MeOH gradients to give 16 fractions (Fr. 1-Fr. 16). The Fr. 10 (1.8 g) was applied to a silica gel column and to be eluted with CH₂Cl₂: MeOH (15:1 → 1:1) to afford 15 subfractions. Subfractions 8-10 were subjected to silica gel chromatography (CH₂Cl₂: MeOH = 10:1 → 1:2) and purified by recrystallization (CHCl₃: MeOH) to yield rosmarinic acid **1** (41 mg). The Fr. 13 (1.2 g) was chromatographed over a silica gel as a stationary phase using CH₂Cl₂: MeOH gradient (10:1) as the mobile phase to afford 12 subfractions. Caffeic acid **2** (49 mg) was purified from subfractions 7-9 by recrystallization in the CHCl₃: MeOH a mixture (3:1) as the solvent system.

HPLC Apparatus and Measurements. The leaves of *P. frutescens* (1.0 g) were extracted with 30 ml of 80% MeOH using a vortex mixer for 1 h at room temperature, and centrifuged. The extracts used for HPLC analysis were passed through a 0.45-μm filter (Millipore, MSI, Westboro, MA, USA) before injection into a reverse phase LichroCART 125-4 HPLC-Cartridge (Lichrophore 100 RP-18e, φ 5 μm) (Merck KGaA), and a 10 μL portion of these solutions was injected into the HPLC system. The mobile phase was acetonitrile (A) and water containing 0.85% (v/v) phosphoric acid (B). The gradient condition was as follows: 5 min, 10% A; 10 min, 20% A; 15 min, 25% A; 20 min, 30% A; 30 min, 40% A, and held for 10 min before returning to the initial conditions. The flow rate was 1.0 ml/min and the detection wavelength was set at 330 nm.

Calibration Curve Preparation and Quantification of Compounds 1 and 2. Standards (10 mg each) of isolated phenolic acids (**1** and **2**) weighed accurately were individually mixed with MeOH in a 10 ml volumetric flask to obtain stock solutions. For calibration curves, the stock solution was diluted with MeOH to obtain the concentration sequence. The linear range and the equations of linear regression were obtained through a sequence of 50, 20, 10, 5, 2, 1, and 0.5 mg/L. All stock solutions were stored at 4°C and brought to room temperature before use.

Compound (1): Yellow powder; $[\alpha]_D^{20}$ +79.7 (*c* 0.50, MeOH), [lit.,¹⁸] $[\alpha]_D^{23}$ +85.0 (*c* 0.12, MeOH); mp 163-165°C (lit.,¹⁸ mp 167-168°C); UV (MeOH) λ_{max} 330, 292, 230, 218 nm; IR (KBr) ν_{max} 3572, 3320, 1650 cm⁻¹; ¹H-NMR (CD₃OD, 500 MHz) δ 3.04 (1H, dd, *J* = 13.8 and 8.8 Hz, H-7 β), 3.10 (1H, dd, *J* = 13.8 and 8.8 Hz, H-7 α), 5.22 (1H, dd, *J* = 8.8 and

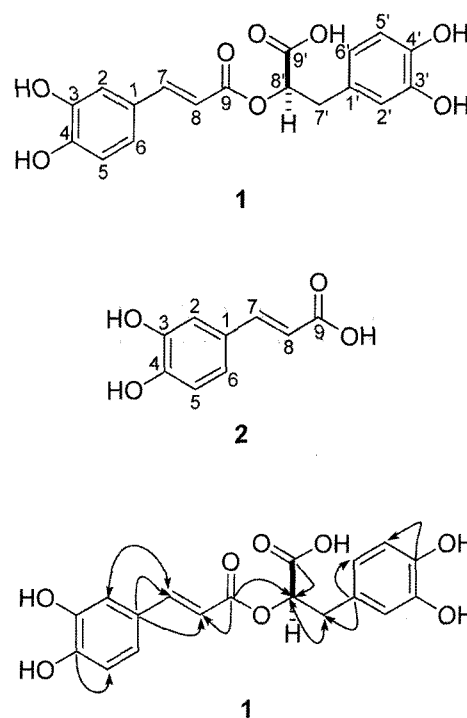


Fig. 1. Structures of isolated compounds **1** and **2** and important HMBC correlations in compound **1**.

4.4 Hz, H-8'), 6.27 (1H, d, *J* = 15.9 Hz, H-8), 6.63 (1H, dd, *J* = 8.1 and 2.0 Hz, H-5), 6.73 (1H, d, *J* = 8.2 Hz, H-6'), 6.77 (1H, d, *J* = 2.0 Hz, H-2'), 6.79 (1H, d, *J* = 8.2 Hz, H-5'), 6.95 (1H, dd, *J* = 8.1 and 2.0 Hz, H-6), 7.06 (1H, d, *J* = 2.0 Hz, H-2), and 7.57 (1H, d, *J* = 15.9 Hz, H-7); ¹³C-NMR data (Table 1).

Compound (2): Yellow powder; mp 215-217°C (lit.,¹⁸ mp 191-195°C, decomp.); UV (MeOH) λ_{max} 320, 282 nm; IR (KBr) ν_{max} 3400, 2910, 1648 cm⁻¹; ¹H-NMR (CD₃OD, 500 MHz) δ 6.24 (1H, d, *J* = 15.9 Hz, H-8), 6.79 (1H, dd, *J* = 6.3 and 2.0 Hz, H-5), 6.94 (1H, dd, *J* = 6.3 and 2.0 Hz, H-6), 7.06 (1H, d, *J* = 1.9 Hz, H-2), and 7.55 (1H, d, *J* = 15.9 Hz, H-7); ¹³C-NMR data (Table 1).

Measurement of DPPH Radical Scavenging Activity: Antioxidant activities of the isolated phenolic acids were measured on the basis of the scavenging activity of the stable DPPH free radical following the method described by Braca *et al.*¹⁹ Various concentrations of the compounds were added to EtOH to form a 0.15 mM mixture which was then shaken vigorously. Absorbance at 517 nm was determined after 30 min, and the radical scavenging effect was calculated as $[A_0 - A_e/A_0] \times 100$, where *A_e* and *A₀* were absorbances of samples with and without isolated phenolic acids, respectively.

Measurement of Trolox Equivalent Antioxidant Capacity (TEAC): TEAC assay is based on the relative ability of antioxidants to scavenge the radical cation 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS^{•+}) in comparison to a standard (Trolox).⁹ The radical cation was prepared by mixing 7 mM ABTS stock solution with 2.45 mM potassium persulfate. The reaction mixture was maintained for 4-8 h until completely mixed and the absorbance stable. ABTS^{•+}

Table 1. ^{13}C -NMR of compounds **1** and **2** at 125 MHz (ppm, m)^a

Position	Compound	
	1	2
1	127.6 (s)	126.4 (s)
2	115.2 (d)	113.8 (d)
3	145.1 (s)	145.7 (s)
4	146.0 (s)	145.3 (s)
5	121.8 (d)	115.1 (d)
6	123.1 (d)	121.6 (d)
7	147.7 (d)	148.0 (d)
8	114.3 (d)	114.1 (d)
9	168.4 (s)	169.8 (s)
1'	129.2 (s)	
2'	117.5 (d)	
3'	146.6 (s)	
4'	149.6 (s)	
5'	116.4 (d)	
6'	116.2 (d)	
7'	37.8 (t)	
8'	74.5 (d)	
9'	173.4 (s)	

^aThe chemical shifts of compounds **1** and **2** were determined in CD_3OD

solution was diluted with ethanol, and the absorbance was read at 734 nm. For the photometric assay 0.9 ml ABTS^{•+} solution and 0.1 ml phenolic acids were mixed for 45 s, and the absorbance was measured immediately after 1 min at 734 nm. Antioxidant activity of the phenolic acids were calculated by determining the decrease in absorbance at different concentrations using the following equation: $E = [A_0 - A_e / A_0] \times 100$, where A_e and A_0 are absorbances of samples with and without phenolic acids, respectively. Antioxidant activity was expressed as TEAC value, which expresses the μM of Trolox having the antioxidant capacity corresponding to 1.0 μM of the test substance.

Data Analysis and Curve Fitting: The assay was conducted in triplicate of separate experiments. The data analysis was performed using Sigma Plot 2001. The inhibitory concentration leading to 50% activity loss (IC_{50}) was obtained by fitting experimental data to the logistic curve using the following equation.²⁰⁾

$$\text{Activity (\%)} = 100[1/(1 + ([I]/\text{IC}_{50}))]$$

Results and Discussion

The dried leaves of *P. frutescens* (1.2 kg) were extracted with 80% MeOH. After filtration and concentration the resultant extracts were suspended with H_2O and partitioned with *n*-hexane, CHCl_3 , EtOAc, and *n*-BuOH to give *n*-hexane-, CHCl_3 -, EtOAc-, and *n*-BuOH-extractable residues. Among the extracts, the *n*-BuOH extract showed the highest antioxidant activities against DPPH (65% inhibition at 100

$\mu\text{g}/\text{ml}$) and ABTS (70% inhibition at 100 $\mu\text{g}/\text{ml}$) radicals. Subsequently, bioassay-guided fractionation through antioxidant assay of the *n*-BuOH-extractable residue, by applying successive a silica gel column chromatography and recrystallization, led to the isolation of the phenolic acids **1** and **2** (Fig. 1). These compounds were readily identified as rosmarinic acid (**1**) and caffeic acid (**2**). Compound **1** was obtained as a yellow powder, with a molecular ion peak at m/z 360, as revealed by EIMS. UV spectrum showed absorption maximum at 330 nm and IR spectrum showed strong hydroxyl and carbonyl group absorption bands at 3570 and 1650 cm^{-1} respectively. The ^1H -NMR spectrum of compound **1** showed two doublets at δ 7.57 ppm (7H, d) and 6.27 ppm (8H, d) which on the basis of the observed large proton-proton coupling ($J = 15.9$ Hz) were assigned to a pair of *trans*-olefinic protons. In addition, two ABX-spin systems were observed in the aromatic region were assigned to the two discrete sets of protons of the 3,4-dihydroxyphenyl unit. In addition, three sets of multiplets at δ 3.04 ppm ($J = 13.8$ and 8.8 Hz, H-7 β), 3.10 ppm ($J = 13.8$ and 8.8 Hz, H-7 α) and 5.22 ppm ($J = 8.8$ and 4.4 Hz, H-8') were assigned to three protons coupled in an ABX pattern, consistent with the presence of a -CH(OH)-CH₂- unit. The ^{13}C -NMR spectrum of **1** (Table 1) showed the presence of two carbonyl carbons, of which one was identified with carboxylic acid (δ 173.4) and the other with carboxyl ester (δ 168.4). The presence of two sets of 3,4-dihydroxyphenyl groups were confirmed by the 12 aromatic carbons consisting of six quaternary carbons, of which four were phenoxy carbons [(δ 145.1, C-3), (δ 146.0, C-4), (δ 146.6, C-3'), and (δ 149.6, C-4')]. Two olefinic carbons [(δ 147.7, C-7), (δ 114.3, C-8)], one oxygenated methine carbon (δ 74.5, C-8'), and one methylene carbon (δ 37.8, C-7') were also observed in the spectrum. These chemical shifts, corroborated by ^1H - ^1H , ^1H - ^{13}C COSY and HMBC studies, indicate the structure of compound **1** is rosmarinic acid (Fig. 1).²¹⁾ Compound **2** is a yellow powder with its mass spectrum showing a major ion peak at m/z 180. UV spectrum showed absorption at 320 and 282 nm and IR spectrum exhibited bands for free hydroxyl and carbonyl at 3400 and 1648 cm^{-1} respectively. ^1H -NMR spectrum of compound **2** exhibited three aromatic protons [(δ 6.79, dd, $J = 6.3$ and 2.0 Hz, H-5), (δ 6.94, dd, $J = 6.3$ and 2.0 Hz, H-6), and (δ 7.06, d, $J = 1.9$ Hz, H-2)] and two doublets at δ 7.55 (d, 7H) and 6.24 (d, 8H) which on the basis of the observed large proton-proton coupling ($J = 15.9$ Hz) were assigned to a pair of *trans*-olefinic protons. The ^{13}C -NMR spectrum of **2** (Table 1) showed one carboxylic acid (δ 169.8, C-9), three aromatic carbons [(δ 113.8, C-2), (δ 115.1, C-5), and (δ 121.6, C-6)], two phenoxy carbons [(δ 145.7, C-3) and (δ 145.3, C-4)], and two olefinic carbons [(δ 148.0, C-7) and (δ 114.1, C-8)]. These data indicate that the structure of compound **2** is caffeic acid (Fig. 1).

DPPH and ABTS radicals were chosen to test the antioxidant activities of the isolated phenolic acids from the leaves of *P. frutescens*. For measurement of antioxidant activity, UV/Vis spectrophotometry method was used to observe DPPH and

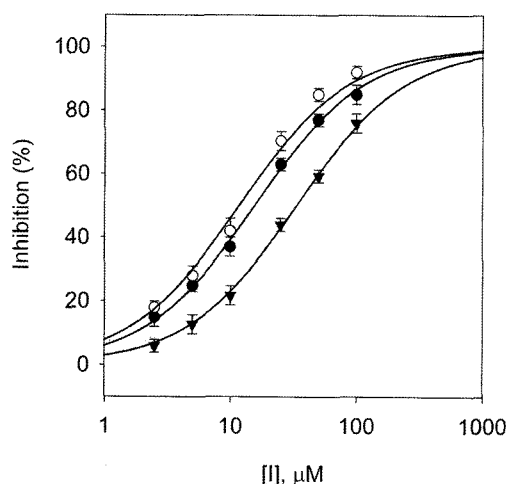


Fig. 2. DPPH radical scavenging activity of compounds **1** and **2**, and BHA. (●): **1**, (○): **2**, (▼): BHA. Each point represents the mean \pm SD of three measurements.

Table 2. Antioxidant activities of isolated compounds **1** and **2** on DPPH and ABTS radicals

Compound	IC ₅₀ (μM) ^a	
	DPPH	ABTS
1	15.5 \pm 2.43	5.5 \pm 2.38
2	11.7 \pm 2.11	4.6 \pm 1.26
BHA/Trolox	33.4 \pm 2.15 (BHA)	13.5 \pm 2.25 (Trolox)

^aInhibitory activity was expressed as the mean of 50% inhibitory concentration of triplicate determinations

ABTS radicals. Anti-radical property of the isolated compounds was examined with DPPH, which is widely used for assessing the ability of polyphenols to transfer labile H-atoms into radicals.²² Using BHA (IC₅₀ = 33.4 μM) as a positive control, catecholic phenolic acids (**1** and **2**), which have a dihydroxyl group, exhibited strong free radical scavenging activities (**1**: IC₅₀ = 15.5 μM, **2**: IC₅₀ = 11.7 μM) on the DPPH radical in a dose-dependent manner (Fig. 2).

The ABTS radical cation was formed immediately after the addition of potassium persulfate to an ABTS solution. Two phenolic acids, **1** and **2** suppressed the absorbance of the ABTS radical, with IC₅₀ values of 4.6 and 5.5 μM, respectively, and when trolox was used as a positive control exhibited IC₅₀ value of 13.5 μM. These compounds showed dose-dependent scavenging activities on the ABTS radical (Fig. 3). The scavenging activities of phenolic acids **1** and **2** against ABTS were three-fold higher compared to the DPPH radical scavenging activities (Table 2).

P. frutescens contains many secondary metabolites, among which, catecholic phenolic acids (**1** and **2**) were investigated through quantitative analysis using reversed phase HPLC. Figure 4 (A) displays HPLC profiles of 80% methanolic extract from leaves of *P. frutescens*. Although major peaks and other minor peaks were not completely identified, two peaks of the isolated compounds (**1** and **2**) were determined by C₁₈ reversed phase HPLC coupled with diode-array

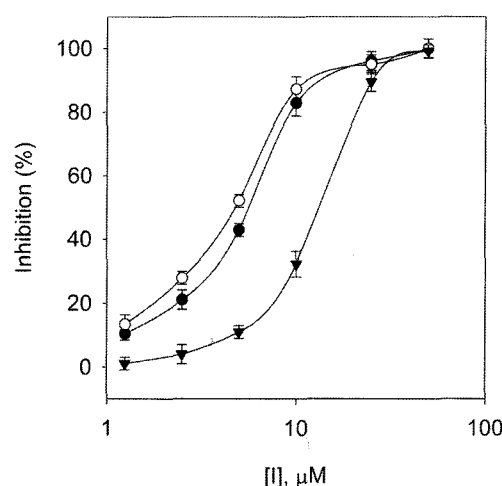


Fig. 3. ABTS radical scavenging activity of compounds **1** and **2**, and Trolox. (●): **1**, (○): **2**, (▼): Trolox. Each point represents the mean \pm SD of three measurements.

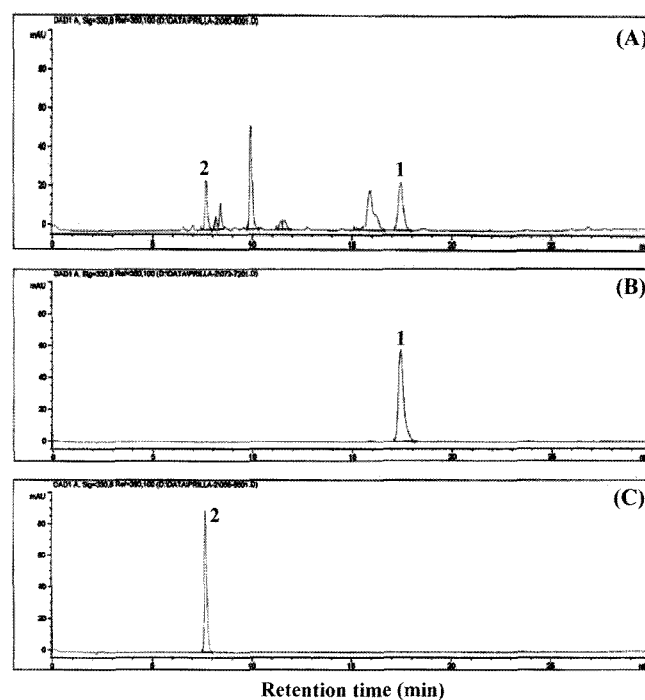


Fig. 4. Characteristic HPLC chromatograms: (A) Profile of 80% MeOH extract; (B) Profile of the isolated compound **1**; (C) Profile of the isolated compound **2**.

detector: Fig. 4 (B) [**1**: retention time (R_t) = 17.4 min] and Fig. 4 (C) [**2**: retention time (R_t) = 7.7 min].

The contents of **1** and **2** were 2.98 and 2.26 mg/g, respectively, in the 80% methanolic extract of *P. frutescens* leaves. These results indicate catecholic phenolic acids were dominant compounds in *P. frutescens*, which belongs to the family Labiatae.

In conclusion, phenolic acids **1** and **2** were isolated from the leaves of *P. frutescens*. Isolated compounds had *ortho*-dihydroxy groups (catechol structure), showing strong antioxidant activities against DPPH and ABTS radical

systems. In addition, catecholic phenolic acids **1** and **2** contents were the most abundant phenolic compounds analyzed by the 80% methanolic leaves extract of *P. frutescens* by HPLC.

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