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In Vitro Antioxidant Activity of Some Selected *Prunus* Species in Korea

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In the course of the investigations of natural antioxidants, we examined the antioxidant activities of the methanol (MeOH) extracts of some selected *Prunus* species, including *P. buergeriana, P. davidiana, P. padus, P. pendula* for. *ascendens, P. sargentii, P. serrulata* var. *spontanea* and *P. yedoensis* by three methods as represented by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, total ROS (reactive oxygen species) and the peroxynitrite ($ONOO^-$) scavenging activity tests. We also evaluated the activities of the organic solvent-soluble fractions, including the dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH) fractions and the water (H_2O) layer of *P. serrulata* var. *spontanea* leaves. By means of bioassay-directed fractionation, we isolated eleven known flavonoids (**1**-**11**) from the EtOAc soluble fraction of the MeOH extract of the *Prunus serrulata* var. *spontanea* leaves, exhibiting strong antioxidant activity and characterized as prunetin (**1**), genistein (**2**), quercetin (**3**), prunetin 4′-*O*-β-glucopyranoside (**4**), kaempferol 3-*O*-α-arabinofuranoside (**5**), prunetin 5-*O*-β-glucopyranoside (**6**), kaempferol 3-*O*-β-xylopyranoside (**7**), genistin (**8**), kaempferol 3-*O*-β-glucopyranoside (**9**), quercetin 3-*O*-β-glucopyranoside (**10**) and kaempferol 3-*O*-β-xylopyranosyl-(1→2)-β-glucopyranoside (**11**). Compounds **3** and **10** showed good activities in all tested model systems. Compounds **2** and **8** showed scavenging activities in the DPPH and $ONOO^-$ tests, while compounds **5**, **7**, **9** and **11** were active in the $ONOO^-$ and ROS tests. On the other hand, compounds **1**, **4** and **6** did not show any activities in the tested model systems.

Key words: *Prunus* species, *Prunus serrulata* var. *spontanea*, Antioxidant capacities, 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical, Total reactive oxygen species (ROS), Peroxynitrite ($ONOO^-$)

INTRODUCTION

When humans use oxygen for respiration and combustion, reactive oxygen species (ROS) are formed as by-products including superoxide anion radical ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), hydroxyl radical ($\cdot OH$), singlet oxygen ($^1O_2$) and free radicals of lipids such as alkoxyl radical ($RO\cdot$) and peroxyl radical ($ROO\cdot$) (Singh, 1989; Aruoma, 1996). In addition, peroxynitrite ($ONOO^-$) is the reactive nitrogen species (RNS) formed by the *in vivo* reaction of nitric oxide ($NO\cdot$) and $\cdot O_2^-$ and followed by protonation to be highly reactive peroxynitrous acid (ONOOH) (Sawa *et al.*, 2000; Balavoine and Genleti, 1999). These ROS and RNS may act as potent oxidizing and nitrating agents to damage several cellular components such as proteins, lipids and DNA. Also, these reactive species are likely to be involved in diseases such as Alzheimers disease, cancer (Dreher *et al.*, 1996), aging (Sohal, 2002), arteriosclerosis, rheumatoid arthritis and allergies (Griffiths and Lunec, 1996; Squadrito and Pryor, 1998; Choi *et al.*, 2002).

For several years, many researchers have investigated powerful and nontoxic antioxidants from natural sources, especially edible or medicinal plants to prevent the above reactive species related disorders in human as well as replace synthetic compounds, which may be carcinogenic and harmful to the lungs and liver (Branen, 1975).

In an effort to find these natural antioxidants, we have focused on the *Prunus* species, which has previously reported the biological activities such as sedative, anti-inflammatory, anti-hyperlipidemic, anti-tumor and antioxidant activities (Kritikar and Basu, 1974; Sang *et al.*, 2002;

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Donovan *et al.*, 1998; Nakatani *et al.*, 2000; Kayano *et al.*, 2002; Wang *et al.*, 1999a; 1999b). There are about 25 species of *Prunus* (Rosaceae) grown in Korea, but only 6 species are indigenous. *P. serrulata* var. *spontanea* (Rosaceae) is a large sized tree widely distributed throughout Korea. The red fruits of this plant are edible and are used in traditional folk medicine against heart failure from beriberi, dropsy, mastitis, an emmenagogue and for toothache (Kim, 1996). The bark, so-called Pruni Cortex, have been used for detoxification and relaxation, and as an antitussive in traditional Korea medicine (Kim, 1997).

We investigated the antioxidant activities of the methanol (MeOH) extracts of several parts (leaf, flower, fruit, stem bark and heartwood) of some selected *Prunus* species, including *P. buergeriana, P. davidiana, P. padus, P. pendula* for. *ascendens, P. sargentii, P. serrulata* var. *spontanea* and *P. yedoensis* by the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical, total reactive oxygen species (ROS) generation in kidney homogenates using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) and the peroxynitrite ($ONOO^-$) scavenging/inhibitory activity test.

We also determined the antioxidative activities of the solvent-partitioned fractions, such as the dichloromethane (CH_2Cl_2), ethyl acetate (EtOAc), *n*-butanol (*n*-BuOH) soluble fractions and the water (H_2O) layer of the *P. serrulata* var. *spontanea* leaves. We isolated eleven known flavonoids (**1-11**) from the active EtOAc soluble fraction of the *P. serrulata* var. *spontanea* leaves, and evaluated the antioxidative activities by the same methods as those of the extracts of the selected *Prunus* species and the solvent fractions of the *P. serrulata* var. *spontanea* leaves.

MATERIALS AND METHODS

Chemicals

DPPH, L-ascorbic acid and DL-penicillamine (DL-2-amino-3-mercapto-3-methylbutanoic acid) were purchased from Sigma Chemical Company (St. Louis, MO, USA). DCFH-DA and DHR 123 (dihydrorhodamine 123) were of high quality and were purchased from Molecular Probes (Eugene, OR, USA), and $ONOO^-$ from Cayman Chemicals Co. (Ann Arbor, MI, USA).

Plant Materials

The MeOH extracts of *P. buergeriana, P. padus* and *P. pendula* for. *ascendens* were purchased from the Korean Plant Extracts Bank under the influence of the Plant Diversity Research Center, and the stem of *P. davidiana,* the leaves of *P. sargentii, P. yedoensis* and *P. serrulata* var. *spontanea* were collected at Gumjung mountain, Pusan, Korea in April 1999, and authenticated by Dr. Maeng Ki Kim, Korea Environmental and Ecological Services, Pusan, Korea. A voucher specimen has been deposited in the Herbarium of the Medicinal Plant Garden, College of Pharmacy, Pusan National University, Pusan, Korea.

General Experimental Procedures

EI-MS data were carried out on a JEOL JMS-700 spectrometer. Column chromatography was done with silica (Si) gel (Merck, 70-230 mesh) and sephadex LH-20. TLC was carried out on pre-coated Merck Kieselgel 60 F_{254} plate (0.25 mm) and 50% H_2SO_4 was used as spray reagent.

Extraction, Fractionation and Isolation of *Prunus serrulata* var. *spontanea* leaves

The dried leaves of *P. serrulata* var. *spontanea* (1.8 kg) were refluxed with MeOH for three hours. The total filtrate was concentrated to dryness *in vacuo* at 40°C to render the MeOH extract (471 g). This extract was suspended in H_2O and then partitioned with CH_2Cl_2, EtOAc and *n*-BuOH, successively, to afford the CH_2Cl_2 extract (144 g), EtOAc extract (77 g), *n*-BuOH extract (105 g) and the H_2O residue (145 g). The EtOAc extract (77 g) was chromatographed over a Si gel column (12×60, Silica gel 60, Merck, 2 kg) and eluted with EtOAc-MeOH (8 : 1 to 1 : 1) to obtain 14 fractions (Fr. 1–Fr. 14). A portion of fraction 1 (500 mg) was purified by sephadex LH-20 with CH_2Cl_2-MeOH (2 : 1 to MeOH) to obtain compounds **1** (30 mg) and **2** (15 mg). Fraction 3 (7.53 g) was subjected to column chromatography over the Si gel column with *n*-hexane-EtOAc (1 : 1 to MeOH) to give compound **3** (20 mg). Fraction 4 was recrystallized to obtain compound **4** (1.572 g) and the filtrate (5.14 g) was chromatographed on the Si gel column with EtOAc (to pure MeOH) to obtain 20 sub-fractions (Fr. 4-1 to 4-20). Fraction 4-2 (40 mg) was further separated by sephadex LH-20 with MeOH to yield compound **5** (15 mg). Fraction 6 (2.59 g) was subjected to column chromatography over sephadex LH-20 with MeOH to purify compound **6** (40 mg) and compound **7** (80 mg). Fractions 8 and 9 were recrystallized to obtain compound **8** (120 mg and 118 mg, respectively) and both of the filtrates (3.64 g) were further separated to compound **9** (100 mg) by sephadex LH-20 with MeOH. Fraction 14 (4.76 g) was chromatographed over the Si gel column with EtOAc-MeOH (30 : 1 to pure MeOH) to obtain 9 sub-fractions (Fr. 14-1 to 14-9). Fraction 9 (Fr. 14-9, 0.48 g) was further purified to compound **10** (60 mg) by sephadex LH-20 with MeOH. Fraction 4 (Fr. 14-4, 0.48 g) was repeatedly column chromatography over sephadex LH-20 to obtain compound **11** (7 mg).

NMR Analysis

1D (^{1}H- and ^{13}C-NMR) and 2D (HMQC and HMBC) spectra were recorded on a Varian UNITY-400 spectrometer (400 MHz for ^{1}H and 100 MHz for ^{13}C). All isolated

compounds **1**-**10** were measured in DMSO-d_6, except for compound **11** in CD_3OD. Chemical shifts were referenced to the respective residual solvent peaks (δ_H 2.50 and δ_C 39.5 for DMSO-d_6, δ_H 3.30 and δ_C 49.0 for CD_3OD), recordec in values and expressed in ppm. Multiplicities of ^{1}H- and ^{13}C-NMR signals are indicated as s (singlet), d (doublet), brs (broad singlet) and m (multiplet).

Prunetin (1) Colorless needles; m.p. 238-239°C; EI-MS (*m/z*, %): 284 (M^+, 100), 283 (M^+-H, 48), 166 (52), 138 (22), 118 (14); UV_{max} (MeOH): 262 (log 3.29), 334 (sh 2.37) nm; + NaOMe 271 (3.30), 356 (sh 2.63); + NaOAc 261 (3.29), 332 (sh 3.39); + NaOAc + H_3BO_3 262 (3.29), 332 (sh 2.40); + $AlCl_3$ 274 (3.26), 312 (sh 2.54), 377 (2.36); + $AlCl_3$ + HCl 274 (3.25), 313 (sh 2.47), 379 (2.34) nm; ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 3.86 (3H, s, OCH_3), 6.41 (1H, *J* = 2.1 Hz, H-6), 6.66 (1H, d, *J* = 2.1 Hz, H-8), 6.82 (2H, d, *J* = 8.6 Hz, H-3′, 5′), 7.39 (2H, d, *J* = 8.6, H-2′, 6′) 8.41 (1H, s, H-2), 9.60 (1H, brs, 4′-OH), 12.96 (1H, brs, 5-OH); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 180.4 (C-4), 165.2 (C-7), 161.7 (C-5), 157.5 (C-4′), 157.4 (C-9), 154.4 (C-2), 130.1 (C-2′, 6′), 122.5 (C-3), 121.0 (C-1′), 115.0 (C-3′, 5′), 105.4 (C-10), 98.0 (C-6), 92.4 (C-8), 56.1 (OMe) [Farkas *et al.*, 1969; Talukadar *et al.*, 2000].

Genistin (2) ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 6.21 (1H, *J* = 2.1 Hz, H-6), 6.37 (1H, d, *J* = 2.1 Hz, H-8), 6.81 (2H, d, *J* = 8.6 Hz, H-3′, 5′), 7.37 (2H, d, *J* = 8.6, H-2′, 6′), 8.31 (1H, s, H-2), 9.99 (1H, brs, 4′-OH), 12.95 (1H, brs, 5-OH); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 180.1 (C-4), 164.5 (C-7), 162.0 (C-5), 157.6 (C-9, 4′), 157.4 (C-2), 130.1 (C-2′, 6′), 122.2 (C-3), 121.2 (C-1′), 115.0 (C-3′, 5′), 104.3 (C-10), 99.0 (C-6), 93.7 (C-8) [Wang *et al.*, 1999a].

Quercetin (3) EI-MS (*m/z*, %): 302 (M^+, 100) [Young *et al.*, 1991].

Prunetin 4′-*O*-β-glucopyranoside (prunitrin, 4) ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 3.87 (3H, s, OCH_3), 4.91 (1H, d, *J* = 7.3 Hz, H-1″), 6.42 (1H, *J* = 2.1 Hz, H-6), 6.67 (1H, d, *J* = 2.1 Hz, H-8), 7.11 (2H, d, *J* = 8.0 Hz, H-3′, 5′), 7.51 (2H, d, *J* = 8.0, H-2′, 6′), 8.42 (1H, s, H-2), 12.91 (1H, brs, 5-OH); ^{13}C-NMR (75.5 MHz, DMSO-d_6) δ: 180.6 (C-4), 165.6 (C-7), 161.7 (C-5), 157.9 (C-9), 157.6 (C-4′), 155.0 (C-2), 130.5 (C-2′, 6′), 124.4 (C-1′), 122.6 (C-3), 116.5 (C-3′, 5′), 105.7 (C-10), 100.6 (C-1″), 98.4 (C-6), 92.9 (C-8), 77.2 (C-5″), 76.6 (C-3″), 73.4 (C-2″), 70.0 (C-4″), 61.0 (C-6″), 56.5 (OMe) [Talukadar *et al.*, 2000].

Kaempferol 3-*O*-α-arabinofuranoside (juglanin, 5) ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 5.63 (1H, brs, H-1″), 6.21 (1H, d, *J* = 2.0 Hz, H-6), 6.44 (1H, d, *J* = 2.0 Hz, H-8), 6.89 (2H, d, *J* = 8.0 Hz, H-3′, 5′), 8.02 (2H, d, *J* = 8.0, H-2′, 6′), 10.20 (1H, brs, OH), 10.88 (1H, brs, OH), 12.63 (1H, brs, 5-OH); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 177.6 (C-4), 164.2 (C-7), 161.2 (C-5), 159.9 (C-4′), 156.7 (C-9), 156.3 (C-2), 133.4 (C-3), 130.8 (C-2′, 6′), 120.7 (C-1′), 115.4 (C-3′, 5′), 104.0 (C-10), 108.0 (C-1″), 98.6 (C-6), 93.6 (C-8), 86.3 (C-4″), 82.1 (C-2″), 77.1 (C-3″), 60.8 (C-5″) [De Almeida *et al.*, 1998; Kim *et al.*, 1994].

Prunetin 5-*O*-β-glucopyranoside (prunetinoside, 6) ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 3.88 (3H, s, OCH_3), 4.82 (1H, d, *J* = 7.5 Hz, H-1″), 6.89 (1H, *J* = 2.5 Hz, H-6), 6.87 (1H, d, *J* = 2.5 Hz, H-8), 6.80 (2H, d, *J* = 8.6 Hz, H-3′, 5′), 7.32 (2H, d, *J* = 8.6, H-2′, 6′), 8.26 (1H, s, H-2), 9.55 (1H, brs, 4′-OH); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 175.0 (C-4), 163.5 (C-7), 158,7 (C-5), 158.6 (C-9), 157.2 (C-4′), 151.8 (C-2), 130.3 (C-2′, 6′), 124.8 (C-1′), 122.2 (C-3), 114.9 (C-3′, 5′), 109.7 (C-10), 103.5 (C-1″), 102.6 (C-6), 93.8 (C-8), 77.6 (C-5″), 75.8 (C-3″), 73.5 (C-2″), 69.9 (C-4″), 60.9 (C-6″), 56.1 (OMe) [Khalid *et al.*, 1989; Geibel and Feucht, 1991; Geibel *et al.*, 1990].

Kaempferol 3-*O*-β-xylopyranoside (7) ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 5.33 (1H, d, *J* = 6.80 Hz, H-1″), 6.21 (1H, d, *J* = 2.0 Hz, H-6), 6.44 (1H, d, *J* = 2.0 Hz, H-8), 6.89 (2H, d, *J* = 8.0 Hz, H-3′, 5′), 8.02 (2H, d, *J* = 8.0, H-2′, 6′), 10.24 (1H, brs, OH), 10.89 (1H, brs, OH), 12.58 (1H, brs, 5-OH); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 177.4 (C-4), 164.3 (C-7), 161.2 (C-5), 160.1 (C-4′), 156.3 (C-9), 156.2 (C-2), 133.1 (C-3), 130.8 (C-2′, 6′), 120.7 (C-1′), 115.3 (C-3′, 5′), 103.9 (C-10), 101.7 (C-1″), 98.8 (C-6), 93.7 (C-8), 75.8 (C-4″), 73.7 (C-2″), 69.4 (C-3″), 65.9 (C-5″) [Agrawal, 1992].

Genistin (8) ^{1}H-NMR (300 MHz, DMSO-d_6) δ: 5.06 (1H, d, *J* = 7.3 Hz, H-1″), 6.47 (1H, *J* = 2.1 Hz, H-6), 6.71 (1H, d, *J* = 2.1 Hz, H-8), 6.82 (2H, d, *J* = 8.6 Hz, H-3′, 5′), 7.40 (2H, d, *J* = 8.6, H-2′, 6′), 8.43 (1H, s, H-2), 9.59 (1H, brs, 4′-OH), 12.94 (1H, brs, 5-OH); ^{13}C-NMR (75.5 MHz, DMSO-d_6) δ: 180.8 (C-4), 163.2 (C-7), 161.6 (C-5), 157.6 (C-9, 4′), 154.8 (C-2), 130.6 (C-2′, 6′), 123.0 (C-3), 121.4 (C-1′), 115.4 (C-3′, 5′), 106.4 (C-10), 100.2 (C-1″), 99.9 (C-6), 95.0 (C-8), 77.4 (C-5″), 76.4 (C-3″), 73.2 (C-2″), 69.8 (C-4″), 60.8 (C-6″) [Wang *et al.*, 1999a].

Kaempferol 3-*O*-β-glucopyranoside (astragalin, 9) ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 5.46 (1H, d, *J* = 7.3 Hz, H-1″), 6.22 (1H, *J* = 2.0 Hz, H-6), 6.44 (1H, d, *J* = 2.0 Hz, H-8), 6.89 (2H, d, *J* = 8.0 Hz, H-3′, 5′), 8.04 (2H, d, *J* = 8.0, H-2′, 6′), 12.61 (1H, brs, 5-OH); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 177.5 (C-4), 164.2 (C-7), 161.3 (C-5), 160.0 (C-4′), 156.4 (C-9), 156.3 (C-2), 133.3 (C-3), 130.9 (C-2′, 6′), 121.0 (C-1′), 115.2 (C-3′, 5′), 104.1 (C-10), 101.0 (C-1″), 98.8 (C-6), 93.7 (C-8), 77.5 (C-5″), 76.5 (C-3″), 74.3 (C-2″), 70.0 (C-4″), 61.0 (C-6″) [Young *et al.*, 1991; Park

et al., 1991].

Quercetin 3-*O*-β-glucopyranoside (isoquercitrin, 10) ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 5.46 (1H, d, J = 7.2 Hz, H-1″), 6.21 (1H, d, J = 2.0 Hz, H-6), 6.41 (1H, d, J = 2.0 Hz, H-8), 6.84 (1H, d, J = 9.0, H-5′), 7.53 (1H, d, J = 2.0 Hz, H-2′), 7.57 (1H, dd, J = 2.0, 9.0 Hz, H-6′), 9.23 (1H, brs, OH), 9.73 (1H, brs, OH), 10.88 (1H, brs, OH), 12.64 (1H, brs, 5-OH); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 177.4 (C-4), 164.1 (C-7), 161.2 (C-5), 156.3 (C-2), 156.2 (C-9), 148.5 (C-4′), 144.8 (C-3′), 133.3 (C-3), 121.6 (C-6′), 121.2 (C-1′), 116.2 (C-5′), 115.2 (C-2′), 104.0 (C-10), 100.9 (C-1″), 98.7 (C-6), 93.5 (C-8), 77.6 (C-5″), 76.5 (C-3″), 72.3 (C-2″), 69.9 (C-4″), 61.0 (C-6″) [Young *et al.*, 1991; Park *et al.*, 1991].

Kaempferol 3-*O*-β-xylopyranosyl-(1→2)-β-glucopyranoside (kaempferol 3-sambubioside, 11) ^{1}H-NMR (400 MHz, CD_3OD) δ: 3.22 (1H, dd, J = 1.6, 11.6 Hz, Xyl-5‴a), 3.37 (1H, m, Xyl-3‴), 3.38 (1H, m, Xyl-2‴), 3.43 (1H, m, Glu-5″), 3.51 (1H, m, Xyl-4‴), 3.53 (1H, dd, J = 6.3, 12.0 Hz, Glu-6″a), 3.60 (1H, dd, J = 5.6, 12.0 Hz, Glu-6″b), 3.68 (1H, m, Glu-3″), 3.81 (1H, m, Glu-4″), 3.95 (1H, dd, J = 5.0, 12.0 Hz, Xyl-5‴b), 3.98 (1H, m, Glu-2″), 4.72 (1H, d, J = 7.3 Hz, Xyl-1‴), 5.35 (1H , d, J = 7.6 Hz, Glu-1‴), 6.18 (1H, brs, H-6), 6.38 (1H, brs, H-8), 6.87 (2H, d, J = 8.9 Hz, H-3′/5′), 8.09 (2H, d, J = 8.7 Hz, H-2′/6′); ^{13}C-NMR (100 MHz, CD_3OD) δ: 180.6 (C-4), 167.0 (C-7), 163.9 (C-5), 162.3 (C-4′), 159.3 (C-9), 159.0 (C-2), 135.8 (C-3), 133.2 (C-2′, 6′), 123.5 (C-1′), 117.0 (C-3′, 5′), 106.4 (C-10, Xyl-1‴), 102.0 (Glu-1″), 100.7 (C-6), 95.5 (C-8), 81.3 (Glu-2″), 78.0 (Xyl-3‴), 77.8 (Glu-5″), 76.0 (Glu-3″), 75.8 (Xyl-2‴), 71.9 (Xyl-4‴), 71.0 (Glu-4″), 67.6 (Xyl-5‴), 62.8 (Glu-6″) [Jung *et al.*, 1999; Beninger *et al.*, 1998].

Acid hydrolysis of prunetin 5-*O*-β-glucopyranoside (6)

Ten mg of compound **6** was refluxed 5% H_2SO_4 in MeOH (15 mL) for 3 hrs. After concentration to remove the MeOH solvent (5 mL), the reaction mixture was suspended with H_2O and fractionated with EtOAc. The EtOAc soluble fraction was concentrated and recrystallized with MeOH and then identified by ^{1}H- and ^{13}C-NMR (400 MHz and 100 MHz, respectively in DMSO-d_6), which were superimposed on those of prunetin (**1**). ^{1}H-NMR (400 MHz, DMSO-d_6) δ: 3.87 (3H, s, OCH_3), 6.41 (1H, s, H-6), 6.66 (1H, s, H-8), 6.82 (2H, d, J = 8.6 Hz, H-3′, 5′), 7.39 (2H, d, J = 8.6, H-2′, 6′), 8.41 (1H, s, H-2), 9.60 (1H, brs, 4′-OH), 12.96 (1H, brs, 5-OH); ^{13}C-NMR (100 MHz, DMSO-d_6) δ: 180.4 (C-4), 165.2 (C-7), 161.7 (C-5), 157.5 (C-4′), 157.5 (C-9), 154.4 (C-2), 130.1 (C-2′, 6′), 122.5 (C-3), 121.0 (C-1′), 115.1 (C-3′, 5′), 105.4 (C-10), 98.0 (C-6), 92.4 (C-8), 56.1 (OMe).

Measurement of DPPH radical scavenging activity

The DPPH radical scavenging effect was evaluated according to the modified method first employed by Blois (1958). A hundred sixty microliters (μL) of MeOH solution of various sample concentrations (10-320 μL/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 microplate reader spectrophotometer VERSAmax (Molecular Devices, CA, USA). The antioxidant activity of each sample was expressed in terms of IC_{50} (μg/mL or μM required to inhibit DPPH radical formation by 50%) and calculated from the log-dose inhibition curve.

Measurement of the inhibition of the total ROS generation

Rat kidney homogenates prepared from the kidneys of freshly killed male Wistar rats weighing 150-200 g were mixed with or without a suspension of extracts/or compounds, then incubated with 12.5 μM DCFH-DA at 37°C for 30 min. Fifty millimoles (mM) of phosphate buffer at pH 7.4 was used. The fluorescence intensity of oxidized 2′,7′-dichlorodihydrofluorescein (DCF) was monitored on a microplate fluorescence spectrophotometer (Bio-Tek Instruments Inc., Winooski, VT), with excitation wavelength at 460 nm and emission wavelength at 530 nm (Label and Bondy, 1990).

Measurement of the $ONOO^-$ scavenging activity

The $ONOO^-$ scavenging was measured by monitoring the oxidation of DHR 123 by modifying the method of Kooy *et al.* (1994). DHR 123 (5 mM) in dimethylformamide, purged with nitrogen, was stored at -80°C as a stock solution. This solution was then placed in ice and was not exposed to light prior to the study. 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 $ONOO^-$. DHR 123 was oxidized rapidly by authentic $ONOO^-$, 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-Tek Instruments Inc.) at the excitation and emission wavelengths of 480 and 530 nm, respectively. Results were expressed as the mean ± standard error (n = 3) for the final fluorescence intensity minus background fluorescence. The effects were expressed as the percent inhibition of oxidation of DHR 123.

Statistical analysis

Values were expressed as the mean ± standard error of three or five experiments.

RESULTS AND DISCUSSION

It is well known that free radicals and ROS or RNS including H_2O_2, $\cdot O_2^-$, ·OH, NO· and $ONOO^-$ play a role in the etiology of a vast variety of human degenerative diseases (Fincemail, 1995; Beckman *et al.*, 1990). These reactive species are formed in the body as a consequence of aerobic metabolism, and damage all intracellular components, such as nucleic acids, proteins and lipids. ROS are also implicated in both aging and various degenerative disorders (Sagar *et al.*, 1992; Ames *et al*, 1993). DPPH is a free radical compound and has been widely used to test the free radical scavenging ability of various chemicals. In this study, we investigated the general antioxidant effects for the potential to scavenge stable DPPH free radicals, inhibit total ROS generation in kidney homogenates using DCFH-DA and scavenge authentic $ONOO^-$ of the MeOH extracts of the selected *Prunus* species such as *P. buergeriana, P. davidiana, P. padus, P. pendula* for. *ascendens, P. sargentii, P. serrulata* var. *spontanea* and *P. yedoensis*.

As summarized in Table I, most of the MeOH extracts tested showed significant antioxidant activities on the DPPH radical with IC_{50} (50% inhibition concentration) <10 μg/mL, comparable to L-ascorbic acid (IC_{50} 1.7 μg/mL) as a well-known antioxidant, especially like the stem bark of *P. pendula* for. *ascendens* with IC_{50} 1.6 μg/mL. The inhibition activities of the stem bark of *P. pendula* for. *ascendens*, the stem of *P. yedoensis* and the heartwood of *P. serrulata* var. *spontanea* on total ROS were as good as that of the positive control Trolox (73.6 ± 0.3%) at concentration of 40 μg/mL with 75.8 ± 0.9%, 72.1 ± 1.4% and 71.2 ± 0.6%, respectively. In the $ONOO^-$ system, the stem of *P. davidiana*, and the leaves and stem of *P. sargentii* showed stronger inhibition activities than penicillamine (74.1 ± 0.5%) at a concentration of 10 μg/mL with 92.8 ± 0.2%, 91.9 ± 0.9% and 91.6 ± 0.4%, respectively. Table II showed the antioxidative activities of the MeOH extract and several solvent partitioned fractions such as the CH_2Cl_2-, EtOAc-, *n*-BuOH-fractions and the H_2O layer derived from the *P. serrulata* var. *spontanea* leaves. As summarized in Table II, the scavenging activity of the MeOH extract and its fractions on the DPPH increased in the order of EtOAc > H_2O > MeOH > *n*-BuOH > CH_2Cl_2 and were 8.0, 19.0, 21.9, 48.6 and 65.7 μg/mL in their IC_{50}, respectively. In addition, the inhibition ratio of the EtOAc soluble fraction exhibited 95.4 ± 0.1%, which exceeded that of penicillamine (74.1 ± 0.5%) on the $ONOO^-$ at a concentration of 10 μg/mL. These results imply that the EtOAc fraction of the MeOH extract as well as the MeOH extract of the *P. serrulata* var. *spontanea* leaves are scavengers of free radicals, the $ONOO^-$ and ROS and their capacity to scavenge these radicals may contribute to their antioxidant activity. It is interesting to note that the EtOAc fraction had high scavenging activities. The comparative scavenging effects of fractions derived from the MeOH extract of the *P. serrulata* var. *spontanea* leaves on a variety of *in vitro* model systems can be explained on the basis of compositional difference. The results suggest that there are likely to be many antioxidants in the EtOAc soluble fractions, and then we have a lot of attention placed on isolating antioxidative compounds from the

Table I. Antioxidant activities of the MeOH extracts of some selected *Prunus* species

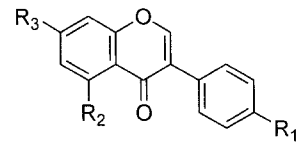
Plants	Parts of plant used	DPPH[a] IC_{50} μg/mL	Total ROS[b] Inhibition ratio (%) mean ± SE[d]	$ONOO^-$[c] Inhibition ratio (%) mean ± SE[d]
*P. buergeriana**	Leaf	145.7	-1.5 ± 8.3	8.2 ± 2.8
	Stem bark	3.4	58.4 ± 0.3	55.8 ± 0.8
	Heartwood	4.0	51.3 ± 0.4	42.4 ± 0.9
*P. davidiana***	Stem	3.6	13.2 ± 12.9	92.8 ± 0.2
*P. padus**	Leaf	2.8	60.6 ± 0.8	46.7 ± 1.9
	Stem	4.5	31.0 ± 1.0	40.3 ± 2.0
	Flower	5.4	39.1 ± 1.0	35.3 ± 3.0
P. pendula for. *ascendens**	Leaf	5.4	57.6 ± 2.2	57.2 ± 0.7
	Stem bark	1.6	75.8 ± 0.9	76.3 ± 0.2
	Heartwood	3.4	64.3 ± 1.2	49.2 ± 1.1
*P. sargentii***	Leaf	19.0	58.8 ± 1.9	91.9 ± 0.9
	Fruit	79.1	39.6 ± 2.4	61.4 ± 1.4
	Stem	8.0	57.9 ± 1.1	91.6 ± 0.4
	Stem bark	15.7	30.8 ± 3.9	47.4 ± 1.8
P. serrulata var. *spontanea*	Leaf**	21.9	6.9 ± 2.7	67.8 ± 3.2
	Flower**	14.3	12.2 ± 1.6	80.6 ± 0.8
	Stem bark**	67.2	1.2 ± 2.2	34.6 ± 8.0
	Heartwood*	4.1	71.2 ± 0.6	61.4 ± 1.9
P. yedoensis	Branch*	3.2	72.1 ± 1.4	67.8 ± 1.5
	F & L*	6.4	53.1 ± 3.8	41.6 ± 1.8
	Leaf**	27.1	48.3 ± 3.3	72.9 ± 3.9
L-ascorbic acid		1.7		
Trolox			73.6 ± 0.3	
Penicillamine				74.1 ± 0.5

*Some of samples tested were purchased and **the others of samples were collected and then extracted with hot MeOH (see Materials and Methods). The flower and leaf of *P. yedoensis* are abbreviated to F & L. [a]DPPH is the free radical scavenging activity (IC_{50}: μg/mL). [b]Total ROS is the inhibition percent of total ROS generation in kidney postmicrosomal fraction at the concentration of 40 μg/mL. [c]$ONOO^-$ is the inhibition percent of peroxynitrite at the test concentration of 10 μg/mL. [d]Values of total ROS and $ONOO^-$ were expressed as the mean ± standard error of three or five experiments.

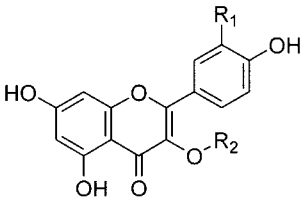
Table II. Antioxidant activities of the MeOH extracts and its fractions of the *Prunus serrulata* var. *spontanea* leaves

MeOH and its fractions used	DPPH[a] IC_{50} μg/mL	Total ROS[b] Inhibition ratio (%) mean ± SE[d]	$ONOO^-$[c] Inhibition ratio (%) mean ± SE[d]
MeOH	21.9	6.9 ± 2.7	67.8 ± 3.2
CH_2Cl_2	65.7	-176.2 ± 4.9	65.4 ± 0.5
EtOAc	8.0	27.7 ± 4.2	95.4 ± 0.1
n-BuOH	48.6	15.3 ± 3.5	83.9 ± 1.2
H_2O	19.0	8.9 ± 2.8	41.3 ± 6.0
L-ascorbic acid	1.7		
Trolox		73.6 ± 0.3	
Penicillamine			74.1 ± 0.5

[a]DPPH is the free radical scavenging activity (IC_{50}: μg/mL). [b]Total ROS is the inhibition percent of total ROS generation in kidney postmicrosomal fraction at the concentration of 40 μg/mL. [c]$ONOO^-$ is the inhibition percent of peroxynitrite at the test concentration of 10 μg/mL. [d]Values of total ROS and $ONOO^-$ were expressed as the mean ± standard error of three or five experiments.



Prunetin (1) : $R_1 = R_2 = OH$, $R_3 = OMe$
Genistein (2) : $R_1 = R_2 = R_3 = OH$
Prunetin 4′-*O*-β-glucopyranoside (4) : R_1 = O-Glu, $R_2 = OH$, $R_3 = OMe$
Prunetin 5-*O*-β-glucopyranoside (6) : R_1=OH, R_2=O-Glu, R_3=OMe
Genistin (8) : $R_1 = R_2 = OH$, R_3 = O-Glu



Quercetin (3) : $R_1 = OH$, $R_2 = H$
Kaempferol 3-*O*-α-arabinofuranoside (5) : $R_1 = H$, R_2 = Ara
Kaempferol 3-*O*-β-xylopyranoside (7) : $R_1 = H$, R_2 = Xyl
Kaempferol 3-*O*-β-glucopyranoside (9) : $R_1 = H$, R_2 = Glu
Quercetin 3-*O*-β-glucopyranoside (10) : $R_1 = OH$, R_2 = Glu
Kaempferol 3-*O*-β-xylopyranosyl-(1→2)-β-glucopyranoside (11) : $R_1 = H$, R_2 = Xyl-(1→2)-Glu

Fig. 1. Structures of isolated compounds **1-11**.

EtOAc soluble fraction of the *P. serrulata* var. *spontanea* leaves. Flavonoids and phenolic compounds, which are of great interest for the radical-scavengers are expected to be present in the EtOAc fraction.

The EtOAc soluble part of the MeOH extract from the leaves of *P. serrulata* var. *spontanea* was repeatedly chromatographed over the Si gel and sephadex LH-20 to yield compounds **1-11** (Fig. 1). The structural identifications of these compounds were elucidated by 1D (^{1}H- and ^{13}C-NMR) and 2D NMR (HMQC and HMBC) spectral data and by comparison with the published spectral data. These isolated compounds **1-11** were readily elucidated as prunetin (**1**), genistein (**2**), quercetin (**3**), prunitrin (**4**), kaempferol 3-*O*-α-arabinofuranoside (**5**), prunetin 5-*O*-β-glucopyranoside (**6**), kaempferol 3-*O*-β-xylopyranoside (**7**), genistin (**8**), kaempferol 3-*O*-β-glucopyranoside (**9**), quercetin 3-*O*-β-glucopyranoside (**10**) and kaempferol 3-*O*-β-xylopyranosyl-(1→2)-β-glucopyranoside (**11**), respectively (see text). Four compounds (**5**, **7**, **9** and **11**) showed similar chemical shifts, corresponding to the aglycone kaempferol. This indicated the compounds were kaempferol glycosides, such as kaempferol 3-*O*-α-arabinofuranoside (**5**), kaempferol 3-*O*-β-xylopyranoside (**7**), kaempferol 3-*O*-β-glucopyranoside (**9**) and kaempferol 3-*O*-β-xylopyranosyl-(1→2)-β-glucopyranoside (**11**) by comparison with the published NMR spectral data of several monoglycosides (Agrawal, 1992). For the structural elucidation of **6**, we measured the 1D and 2D NMR spectroscopy and it suggested the possible positions of one glucose unit at C-5 or C-7 of prunetin (**1**). To confirm the position of glucose, we accomplished the acid hydrolysis of **6** and compared the NMR (^{1}H- and ^{13}C-NMR) of **6** with those of the product. In the ^{1}H-NMR data of the product, there appeared the signal at δ_H 12.96 (brs), corresponding to 5-OH, which was not in that of **6**. Also, in the ^{13}C-NMR data of the product, the ketone group next to the free hydroxyl-bearing carbon signal shifted downfield (from δ_C175.0 to δ_C 180.4), indicating that the position of glucose was at C-5. On the basis of these results, compound **6** was established as prunetin 5-*O*-β-glucopyranoside. In the HMBC spectrum of **11**, the signals of a glucose anomeric proton at δ_H 5.35 (d, J = 7.6 Hz) and a xylose anomeric proton at δ_H 4.72 (d, J =7.3 Hz) were correlated with that of kaempferol C-3 at δ_C135.8 and that of glucose C-2 at δ_C 81.3, respectively. These results suggested that the order of sugars was β-xylopyranosyl-(1→2)-β-glucopyranoside, further supported by the coupling constant (J = 7.3 Hz for xylose) (Agrawal, 1992).

The antioxidant activities of eleven isolated compounds **1-11** are shown in Table III. In the case of the antioxidant activities of flavonols, compound **3** and its glucoside **10** exhibited strong antioxidant activity with IC_{50} 3.5 μM and 4.6 μM on the DPPH radical, as well as 0.9 ± 0.1 μM and 1.1 ± 0.1 μM on the $ONOO^-$, respectively. Compounds **5**, **7**, **9** and **11** were marginal activities in the tested model systems. Compounds **2** and **8** showed scavenging activity on the $ONOO^-$ by IC_{50} 24.4 ± 1.2 μM and 44.5 ± 6.1 μM, respectively. The activity of **8** was lower than that of **2**. The antioxidant activity of *P. serrulata* var. *spontanea*

Table II. Antioxidant activities of isolated compounds **1-11** derived from the *P. serrulata* var. *spontanea* leaves

Compounds	DPPH[a] IC_{50} μM	Total ROS[b] Inhibition ratio (%) mean ± SE	$ONOO^{-}$[c] IC_{50} μM
1	NS[d]	–*	NS
2	NS	–	24.4 ± 1.2
3	3.5	40.8 ± 0.4	0.9 ± 0.1
4	NS	–	NS
5	100.4	36.8 ± 0.5	10.5 ± 0.8
6	NS	–	NS
7	100.4	38.9 ± 1.0	16.8 ± 4.0
8	NS	–	44.5 ± 6.1
9	107.6	39.6 ± 0.5	7.0 ± 0.4
10	4.6	72.5 ± 0.2	1.1 ± 0.1
11	NS	53.1 ± 0.6	10.3 ± 1.2
L-ascorbic acid	11.5		
Trolox		73.6 ± 0.3	
Penicillamine			3.2 ± 0.4

[a]DPPH is the free radical scavenging activity (IC_{50}: μM). [b]Total ROS is the inhibitory activity of total free radical generation in kidney postmicrosoma fraction at 40 μg/mL (Inhibition ratio: %). *No inhibition effects showed at 40 μg/mL on ROS. [c]$ONOO^{-}$ is the inhibitory activity of peroxynitrite (IC_{50}: μM). [d]NS represent as no effect up to a concentration of 100 mM.

leaves may be correlated with their active components, such as flavonols and isoflavones. On the other hand, prunetin (**1**), and its glucoside **4** and **6** showed no activities even at higher concentrations.

Flavonoids, hydroxycinnamates and related phenolic acids are reported to function as potent antioxidants by virtue of their hydrogen-donating properties (Rice-Evans *et al.*, 1996) and metal-chelating properties (Morel *et al.*, 1993; Salah *et al.*, 1995). Several studies have also shown that these compounds can prevent $ONOO^{-}$-mediated nitration of protein-bound and free tyrosine and can inhibit $ONOO^{-}$-mediated oxidation of DHR 123 and DNA (Oshima *et al.*, 1998). These results shows that structural requirements for the observed activity are needed for the scavenging activities of different classes of compounds on free radicals and ROS or RNS.

The present work would tend to screen the antioxidant capacities of some selected *Prunus* species, as well as indicate that the MeOH extract, its various fractions and components of the *P. serrulata* var. *spontanea* leaves 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*. Investigations of further antioxidant principles are now in progress.

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