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1,1-Diphenyl-2-picrylhydrazyl Radical Scavenging Compounds of Fraxini Cortex

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Abstract – The radical scavenging effect of the MeOH extract of Fraxini Cortex on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical was examined. The CH_2Cl_2-and *n*-BuOH-soluble fractions of MeOH extract showed the promising DPPH radical scavenging effects, and further purified by silica gel, Sephadex LH-20 column chromatography, and reversed-phase C-18 MPLC to yield five coumarins, esculetin (**1**), fraxidin (**2**), fraxetin (**3**), fraxidin 8-*O*-β-D-glucopyranoside (fraxin methyl ether) (**5**), esculin (**6**), and a secoiridoid oleuropein (**4**), and a coumarin-secoiridoid escuside (**7**). Compounds **1**, **3**, and **4** showed potent DPPH radical scavenging effects, exhibiting IC_{50} values of 14.68, 9.64, and 22.03 μM, respectively. Compounds **6** and **7** also showed moderate effects with IC_{50} values of 147.79 and 72.73 μM, respectively. L-Ascorbic acid was used as a positive control and exhibited the IC_{50} value of 50.31 μM.
Keywords – Fraxini Cortex, Oleaceae, DPPH, Anti-radical, Coumarin, Oleuropein, Coumarin-secoiridoid

Introduction

Fraxini Cortex is the dry barks of stem or branches of *Fraxinus* species (Oleaceae) include *F. rhynchophylla* Hance, *F. chinensis* Roxb., and *F. stylosa* Lingelsh., and used in traditional Oriental medicine as an astringent for the treatment of diarrhea and as an antiphlogistic in ophthalmology for the treatment of conjunctivitis (Tang and Eisenbrand, 1992). Previous phytochemical studies on the genus *Fraxinus* have led to the isolation of coumarins (Hahn *et al.*, 1976; Hahn and Lee, 1983; Kwon and Kim, 1996; Tsukamoto *et al.*, 1985; Yook *et al.*, 1984; Yook and Moon, 1981), secoiridoids (Damtoft *et al.*, 1992; Shen and Chen, 1995; Takenaka *et al.*, 2000), coumarin-secoiridoid (Iossifova *et al.*, 2002), and lignans (Tsukamoto *et al.*, 1984). The reported biological activities of *Fraxinus* species include inducible nitric oxide synthesis inhibitory activity (Kim *et al.*, 1999), superoxide anion scavenging effect (Chang *et al.*, 1996), anti-inflammatory activity (Stefanova *et al.*, 1995), antimicrobial effect (Kostova *et al.*, 1993), and anti-platelet aggregation activity (Kodaira *et al.*, 1983). In the course of screening for free radical scavengers from medicinal plants, the MeOH extract of Fraxini Cortex was found to have promising free radical scavenging effect on DPPH radical. This paper deals with the isolation and identification of radical scavenging constituents of Fraxini Cortex.

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Experimental

Plant material and isolation – Fraxini Cortex was purchased from the University Oriental herbal drugstore, Iksan, Korea, in November 2004, and the voucher specimen (No. WP 04-399) was deposited at the Herbarium of the College of Pharmacy, Wonkwang University (Korea). Dried and pulverized Fraxini Cortex (1 kg) was extracted twice with MeOH (5 L) under the ultrasonic condition for 3 h. The MeOH extract (109 g) was suspended in H_2O and partitioned successively with *n*-hexane, CH_2Cl_2, and *n*-BuOH. The CH_2Cl_2-soluble extract (10.4 g) was subjected to silica gel column chromatography eluting with CH_2Cl_2-MeOH (16 : 1) to yield four fractions (Fr. A-D). Fr. C (2.1 g) was recrystalized with MeOH to give compound **1** (1.34 g). Fr. A (2.1 g) was subjected to silica gel column chromatography eluting with CH_2Cl_2-MeOH (20 : 1) to afford compound **2** (259 mg). Fr. B (1.76 g) was subjected to Sephadex LH-20 (eluent; CH_2Cl_2-MeOH, 1 : 1) chromatography to yield compound **3** (203 mg). The *n*-BuOH-soluble extract (44.5 g) was subjected to silica gel column chromatography eluting with CH_2Cl_2-MeOH (5 : 1) to give four fractions (Fr. E-H). Fr. F (2.21 g) was chromatographed on silica gel column with CH_2Cl_2-MeOH (8 : 1) to get five subfra-

ctions (Fr. F1-F5). Fr. F4 (820 mg) was purified by reversed-phase MPLC (column: ODS-S-50 B, 120 Å, 50 μm) eluting with 40% MeOH in H_2O to give compound **4** (167 mg). Fr. F3 (344 g) was chromatographed on reversed-phase MPLC (column: ODS-S-50 B, 120 Å, 50 μm) eluting with 10% MeCN in H_2O to give compound **5** (62 mg). Fr. H (940 mg) was subjected to silica gel column chromatography eluting with CH_2Cl_2-MeOH (5 : 1) to yield compound **6** (73 mg) together with five subfractions (Fr. H1-H5). Fr. H-5 (190 mg) was purified by reversed-phase MPLC (column: ODS-S-50 B, 120 Å, 50 μm) eluting with 30% MeOH in H_2O to give compound **7** (86 mg).

Compound 1 – Light yellow needles; (–)-ESI-MS *m/z* 177 $[M-H]^-$, ^{1}H-NMR (DMSO-d_6, 500 MHz) δ : 7.86 (1H, d, *J* = 9.6 Hz, H-4), 6.97 (1H, s, H-5), 6.74 (1H, s, H-8), 6.16 (1H, d, *J* = 9.6 Hz, H-3).

Compound 2 – Colorless powder; (–)-ESI-MS *m/z* 221 $[M-H]^-$, ^{1}H-NMR (DMSO-d_6, 500 MHz) δ : 7.93 (1H, d, *J* = 9.6 Hz, H-4), 6.83 (1H, s, H-5), 6.36 (1H, d, *J* = 9.6 Hz, H-3), 3.81 (3H, s, 6-OC$\underline{H}_3$), 3.77 (3H, s, 7-OC$\underline{H}_3$); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ : 160.6 (C-2), 115.1 (C-3), 145.2 (C-4), 100.8 (C-5), 149.6 (C-6), 140.6 (C-7), 138.8 (C-8), 139.0 (C-9), 114.9 (C-10), 56.5 (6-O$\underline{C}H_3$), 60.4 (7-O$\underline{C}H_3$).

Compound 3 – Pale yellow prisms; (–)-ESI-MS *m/z* 207 $[M-H]^-$, ^{1}H-NMR (DMSO-d_6, 500 MHz) δ : 7.88 (1H, d, *J* = 9.6 Hz, H-4), 6.78 (1H, s, H-5), 6.21 (1H, d, *J* = 9.6 Hz, H-3), 3.81 (3H, s, 6-OC$\underline{H}_3$); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ : 161.1 (C-2), 112.4 (C-3), 145.6 (C-4), 100.9 (C-5), 145.9 (C-6), 139.8 (C-7), 133.4 (C-8), 139.9 (C-9), 110.8 (C-10), 56.6 (6-O$\underline{C}H_3$).

Compound 4 – Colorless powder; (–)-ESI-MS *m/z* 539 $[M-H]^-$, ^{1}H-NMR (CD_3OD, 500 MHz) δ : 7.50 (1H, s, H-3), 6.68 (1H, d, *J* = 7.8 Hz, H-7"), 6.65 (1H, d, *J* = 1.8 Hz, H-4"), 6.54 (1H, dd, *J* = 7.8, 1.8 Hz, H-8"), 6.07 (1H, br. q, *J* = 7.2 Hz, H-8), 5.91 (1H, br. s, H-1), 4.80 (1H, d, *J* = 6.9 Hz, glc-1), 4.20 (1H, dt, *J* = 11.0, 6.9 Hz, Ha-1"), 4.09 (1H, dt, *J* = 11.0, 6.9 Hz, Hb-1"), 3.96 (1H, dd, *J* = 9.2, 4.4 Hz, H-5), 3.70 (3H, s, COOC$\underline{H}_3$), 2.76 (2H, t, *J* = 6.9 Hz, H-2"), 2.70 (1H, dd, *J* = 14.2, 4.4 Hz, Ha-6), 2.43 (1H, dd, *J* = 14.2, 9.2 Hz, Hb-6), 1.65 (3H, dd, *J* = 7.2, 1.3 Hz, H-10); ^{13}C-NMR (CD_3OD, 125 MHz) δ : 93.9 (C-1), 153.8 (C-3), 108.1 (C-4), 30.5 (C-5), 39.9 (C-6), 171.9 (C-7), 123.5 (C-8), 129.2 (C-9), 12.2 (C-10), 167.4 (C-11), 99.6 (glc-1), 73.4 (glc-2), 77.1 (glc-3), 70.1 (glc-4), 76.6 (glc-5), 61.4 (glc-6), 34.1 (C-1"), 65.6 (C-2"), 129.4 (C-3"), 115.7 (C-4"), 145.5 (C-5"), 143.6 (C-6"), 115.1 (C-7"), 120.0 (C-8"), 50.6 (COO$\underline{C}H_3$).

Compound 5 – Colorless powder; (–)-ESI-MS *m/z* 383 $[M-H]^-$, ^{1}H-NMR (DMSO-d_6, 500 MHz) δ : 7.96 (1H, d, *J* = 9.6 Hz, H-4), 7.12 (1H, s, H-5), 6.40 (1H, d, *J* = 9.6 Hz, H-3), 5.19 (1H, d, *J* = 7.4 Hz, glc-1), 3.84 (3H, s, 6-OC$\underline{H}_3$), 3.84 (3H, s, 7-OC$\underline{H}_3$); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ : 160.4 (C-2), 115.3 (C-3), 145.0 (C-4), 105.4 (C-5), 150.3 (C-6), 145.4 (C-7), 137.1 (C-8), 142.6 (C-9), 114.9 (C-10), 56.6 (6-O$\underline{C}H_3$), 61.4 (7-O$\underline{C}H_3$), 102.8 (glc-1), 74.6 (glc-2), 78.1 (glc-3), 70.4 (glc-4), 77.1 (glc-5), 61.3 (glc-6).

Compound 6 – Colorless powder; (–)-ESI-MS *m/z* 341 $[M-H]^-$, ^{1}H-NMR (CD_3OD, 500 MHz) δ : 7.83 (1H, d, *J* = 9.1 Hz, H-4), 7.42 (1H, s, H-5), 6.80 (1H, s, H-8), 6.21 (1H, d, *J* = 9.3 Hz, H-3), 4.84 (1H, d, *J* = 7.3 Hz, glc-1); ^{13}C-NMR (CD_3OD, 125 MHz) δ : 162.4 (C-2), 111.5 (C-3), 144.7 (C-4), 115.3 (C-5), 143.1 (C-6), 152.0 (C-7), 103.2 (C-8), 151.3 (C-9), 111.8 (C-10), 102.9 (glc-1), 73.5 (glc-2), 77.2 (glc-3), 70.0 (glc-4), 76.3 (glc-5), 61.2 (glc-6).

Compound 7 – Colorless powder; (–)-ESI-MS *m/z* 725 $[M-H]^-$, ^{1}H-NMR (DMSO-d_6, 500 MHz) δ : 7.80 (1H, d, *J* = 9.6 Hz, H-4"), 7.53 (1H, s, H-3), 7.26 (1H, s, H-5"), 6.76 (1H, s, H-8"), 6.13 (1H, d, *J* = 9.6 Hz, H-3"), 5.95 (1H, dd, *J* = 6.4, 0.9 Hz, H-8), 5.84 (1H, s, H-1), 4.82 (1H, d, *J* = 7.5 Hz, H-1"), 4.63 (1H, d, *J* = 7.8 Hz, H-1'), 4.35 (1H, dd, *J* = 11.9, 2.1 Hz, Ha-6'"), 4.07 (1H, dd, *J* = 11.9, 6.4 Hz, Hb-6'"), 4.01 (1H, dd, *J* = 9.2, 4.1 Hz, H-5), 3.64 (1H, dd, *J* = 11.7, 1.2 Hz, Ha-6'), 3.62 (3H, s, COOC$\underline{H}_3$), 3.55 (1H, dd, *J* = 9.1, 7.5 Hz, H-2'"), 3.44 (1H, dd, *J* = 11.7, 6.2 Hz, Hb-6'), 2.69 (1H, dd, *J* = 14.6, 4.1 Hz, Ha-6), 2.39 (1H, dd, *J* = 14.6, 9.2 Hz, Hb-6), 1.64 (3H, dd, *J* = 6.4, 0.9 Hz, H-10); ^{13}C-NMR (DMSO-d_6, 125 MHz) δ : 93.5 (C-1), 154.0 (C-3), 108.3 (C-4), 30.6 (C-5), 40.1 (C-6), 171.1 (C-7), 123.7 (C-8), 129.8 (C-9), 13.6 (C-10), 166.8 (C-11), 99.5 (C-1'), 74.4 (C-2'), 77.9 (C-3'), 70.4 (C-4'), 77.1 (C-5'), 61.6 (C-6'), 161.1 (C-2"), 111.7 (C-3"), 144.9 (C-4"), 115.2 (C-5"), 143.3 (C-6"), 154.8 (C-7"), 103.9 (C-8"), 151.4 (C-9"), 110.5 (C-10"), 102.5 (C-1'"), 73.7 (C-2'"), 76.3 (C-3'"), 70.3 (C-4'"), 73.8 (C-5'"), 64.2 (C-6'"), 51.8 (COO$\underline{C}H_3$).

DPPH radical scavenging assay – The scavenging action of DPPH radical was measured as follows (Kumar *et al.*, 2000). The reaction mixture contained 1 mL of 0.1 mM DPPH-ethanol solution, 1 mL of ethanol, 0.95 mL of 0.05 M Tris-HCl buffer (pH 7.4) and 50 μL of either test samples or deionized water (control). Reduction of the DPPH free radical was measured by reading the absorbance at 517 nm exactly 30 sec after adding the samples. L-Ascorbic acid was used as a positive control. The absorbance of the sample alone was subtracted as the blank from that of the reaction mixture. DPPH radical scavenging activity of the sample was expressed as the

Table 1. DPPH radical scavenging effects of the MeOH extract of Fraxini Cortex and its fractions

Sample	IC_{50} (μg/mL)[a]
MeOH extract	6.91
n-Hexane Fr.	>100
CH_2Cl_2 Fr.	3.54
n-BuOH Fr.	3.72
Aqueous Fr.	36.95
L-Ascorbic acid	3.28

[a] Concentration giving a 50% decrease of DPPH radical.

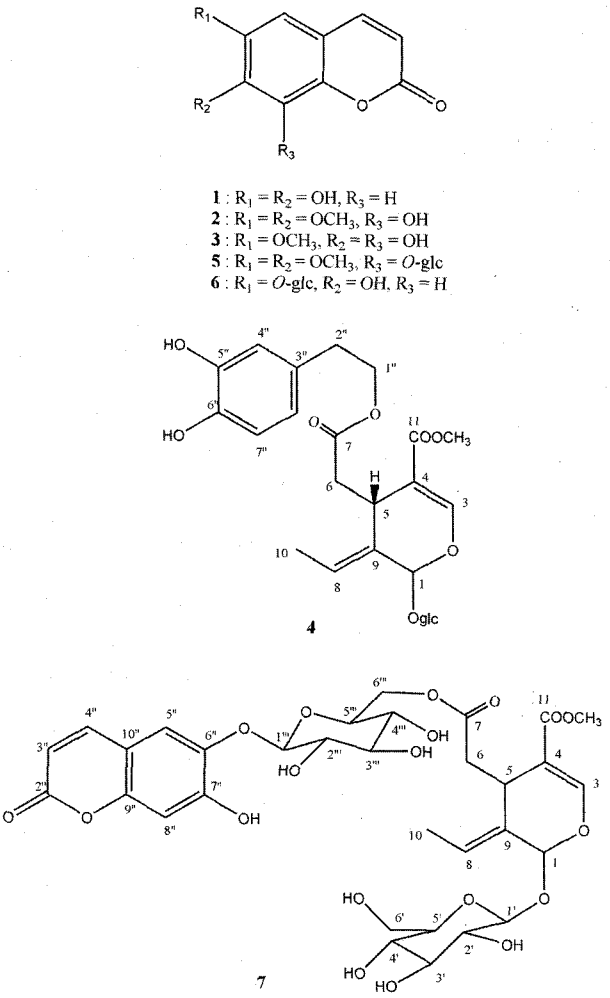

Fig. 1. Chemical structures of compounds **1-7**

IC_{50} value, which required concentration to inhibit DPPH radical formation by 50%, determined from the log dose-inhibition curve.

Results and Discussion

In the present study aiming at the identification of antioxidative components from traditional medicine, the MeOH extract of Fraxini Cortex was investigated. The CH_2Cl_2- and *n*-BuOH-soluble fractions of MeOH extract showed the promising DPPH free radical scavenging effects (Table 1), and subsequent activity-guided fractionation led us to the isolation of seven compounds (**1-7**). The structures of **1-6** were identified as esculetin (Razdan *et al.*, 1987), fraxidin (Tsukamoto *et al.*, 1985), fraxetin (Tsukamoto *et al.*, 1985), oleuropein (Damtoft *et al.*, 1992; Inoue *et al.*, 1982), fraxidin 8-*O*-β-D-glucopyranoside (Tsukamoto *et al.*, 1985), and esculin (Kwon and Kim, 1996), respectively, on the basis of comparisons of MS, ^{1}H-NMR, and ^{13}C-NMR data with those reported in the literature (Fig. 1).

Compound **7** was obtained as a colorless powder. Its ESI-MS showed $[M-H]^-$ at *m/z* 725. The ^{1}H-NMR spectrum of compound **7** exhibited the signals of a set of two *ortho*-coupled methine protons at δ 7.80 (d, J = 9.6 Hz) and δ 6.13 (d, J = 9.6 Hz) together with two methine protons at δ 7.26 (s) and δ 6.76 (s) which implied the presence of di-substituted coumarin skeleton. The presence of secoiridoid skeleton in compound **7** was obvious from the set of signals consisting of one-proton singlet at δ 7.53, three-protons singlet at δ 3.62, and one-proton doublet of doublet at δ 5.95 (J = 6.4 and 0.9 Hz). The ^{1}H-NMR spectrum also showed two anomeric protons of sugar moiety at δ 4.63 (1H, d, J = 7.8 Hz) and δ 4.82 (1H, d, J = 7.5 Hz). These results and detailed analysis of its ^{13}C-NMR, HMQC, and HMBC spectra indicated the presence of two structural units, one secoiridoid glucoside and one coumarin glucoside. The identity of compound **7** was confirmed as escuside, a type of compound consisting of one coumarin glucoside unit linked to a secoiridoid moiety, on the basis of comparison of its data with those of reported in the literature (Iossifova *et al.*, 2002).

Free radical scavenging activity of compounds **1-7** was evaluated by the interaction with the stable free radical DPPH (Table 2). Among the seven compounds, compounds **1**, **3**, and **4** showed potent scavenging effects on DPPH radical, exhibiting IC_{50} values of 14.68, 9.64, and 22.03 μM, respectively. Compounds **6** and **7** also showed moderate effects with IC_{50} values of 147.79 and 72.73 μM, respectively. L-Ascorbic acid, well known for its anti-radical efficiency, was used as a positive control and exhibited the IC_{50} value of 50.31 μM.

There is increasing evidence that free radicals and active oxygen species are involved in a variety of pathological events (Halliwell, 1994). Free radical-mediated cell damage and attack on polyunsaturated fatty acids

Table 2. DPPH radical scavenging effects of compounds **1-7**

Sample	IC_{50} (μM)[a]
Compound **1**	14.68
Compound **2**	>200
Compound **3**	9.64
Compound **4**	22.03
Compound **5**	>200
Compound **6**	147.79
Compound **7**	72.73
L-Ascorbic acid	50.31

[a] Concentration giving a 50% decrease of DPPH radical.

results in the formation of lipid radicals. These react readily with molecular oxygen to produce peroxy radicals, responsible for initiating lipid peroxidation. The peroxidation of cellular membrane lipids can lead to cell necrosis and considered to be implicated in a number of pathological conditions (Kappus, 1985). DPPH is known to abstract labile hydrogen and the ability to scavenge the DPPH radical is related to the inhibition of lipid peroxidation (Matsubara *et al.*, 1991; Rekka and Kourounakis, 1991). Therefore, it would be interesting to widen the investigations for compounds **1**, **3**, and **4** isolated from Fraxini Cortex with pharmacological models examining the anti-radical effiency in a more specific way.

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