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Note

Antioxidative and Antihyaluronidase Activities of Some Constituents from the Aerial Part of *Daucus carota*

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The methanol extract of the aerial part of *Daucus carota* var. *sativus* showed a stronger antioxidative activity than the standard synthetic antioxidant, 3-*tert*-butyl-4-hydroxyanisole (BHA). From this extract, five known compounds, luteolin 7-*O*-β-glucopyranoside (1), chrysoeriol 7-*O*-β-glucopyranoside (2), chlorogenic acid (3), maltol 3-*O*-β-glucopyranoside (4) and benzyl β-glucopyranoside (5) were isolated. Among them, when 1–4 were investigated for their antioxidative activity using the ferric thiocyanate method, 1–3 indicated an antioxidative activity. The scavenging effect of 1–4 on the stable free radical, 1,1-diphenyl-2-picrylhydrazyl was also examined. Compounds 1 and 3 showed a scavenging effect. In addition, 1–3 were assayed for their inhibitory effects on the activation of inactive hyaluronidase induced by compound 48/80. All tested compounds showed this effect.

Keywords: *Daucus carota*, antioxidative activity, ferric thiocyanate method, radical scavenger, antihyaluronidase activity

Antioxidants function to protect against lipid peroxidation which causes the rancidity of fats and oil in food. They can also protect the human body from harmful free radicals which damage unsaturated fatty acids in cell membranes, DNA and enzymes. This damage is believed to induce aging and several diseases, such as cancer, inflammation and atherosclerosis (Yagi, 1987; Yoshikawa *et al*., 1994). It was also reported that some antiallergic drugs showed antioxidative activities due to scavenging of superoxide radicals (Yoshikawa *et al*., 1989). Since hyaluronidase is related to histamine release from mast cells, the inhibitory effect of this enzyme is one of the indexes of the anti-type I allergy (Sakamoto *et al*., 1980; Kakegawa *et al*., 1985)

During the course of our studies on natural antioxidants (Ono *et al*., 1995, 1997, 1998, 1999, 2000; Masuoka *et al*., 1997), the methanol (MeOH) extract of the aerial part of *Daucus carota* var. *sativus* (Umbelliferae), which is used as a foodstuff, showed a stronger antioxidative activity than the standard synthetic antioxidant, 3-*tert*-butyl-4-hydroxyanisole (BHA) using the ferric thiocyanate method. The present paper describes the separation and structure elucidation of five compounds, luteolin 7-*O*-β-glucopyranoside (**1**), chrysoeriol 7-*O*-β-glucopyranoside (**2**), chlorogenic acid (**3**), maltol 3-*O*-β-glucopyranoside (**4**) and benzyl β-glucopyranoside (**5**) from the MeOH extract and the antioxidative activities of **1**–**4**. The inhibitory effect of **1**–**3** on the activation of inactive hyaluronidase induced by compound 48/80 (Maeda *et al*., 1990) is also dealt with.

Materials

α-Tocopherol, BHA and 1,1-diphenyl-2-picrylhydrazyl (DPPH) were obtained from Nacalai Tesque, Inc. (Kyoto). Linoleic acid was purchased from Tokyo Kasei Kogyo Co. (Tokyo). Hyaluronidase (from bovine testis, Type IV-S, 880 units/mg, Lot 94H8000), compound 48/80 and disodium chromoglycate (DSCG) were purchased from Sigma Chemical Co.(St. Louis, MO). Tranilast was obtained from Tocris Cookson, Ltd. (Avonmouth, Bristol, UK). The aerial part of *Daucus carota* var. *sativus* (Kouyo) was collected at Kagoshima prefecture in Japan in April 1995. Luteolin (**6**) [^{1}H-NMR (in dimethylsulfoxide (DMSO)-d_6, 500 MHz) δ: 7.41 (1H, dd, *J* =2.5, 8.0 Hz, H-6′), 7.40 (1H, d, *J*=2.5 Hz, H-2′), 6.89 (1H, d, *J*=8.0 Hz, H-5′), 6.66 (1H, s, H-3), 6.45 (1H, d, *J*=2.0 Hz, H-8), 6.19 (1H, d, *J*=2.0 Hz, H-6), (Harborne, 1986)] was isolated from the fruit of *Vitex tifolia* L.

Methods

Proton (^{1}H)- and carbon-13 (^{13}C)-nuclear magnetic resonance (NMR) spectra were recorded using a JEOL JNM-GX-400 and a JEOL alpha 500 spectrometer (JEOL, Tokyo), and chemical shifts were given on a δ (ppm) scale with tetramethylsilane (TMS) as an internal standard. The abbreviations used are as follows: s, singlet; br s, broad singlet; d, doublet; dd, double-doublet; br d, broad doublet; ddd, double-double-doublet; t, triplet; m, multiplet. Optical rotations were measured with a JASCO DTP-1000 KUY digital polarimeter (JASCO, Tokyo). Visible absorptions were measured with a Shimadzu UV-140-02 spectrometer (Shimadzu, Kyoto). Column chromatography (CC) was carried out over silica gel 60 (Merck, Art. 9385; Merck, Darmstadt, Germany), Diaion HP20 and MCI gel CHP 20P (both from Mitsubishi Chemical Industries Co., Ltd., Tokyo) and Sephadex LH20 (Pharmacia Fine Chemicals, Uppsala, Sweden).

Extraction and isolation The cut fresh aerial part (2.00 kg) of *Daucus carota* var. *sativus* was soaked in MeOH (4.0 *l*) for 23 days at room temperature. The MeOH extract (71.6 g) was partitioned between hexane (0.6 *l*) and MeOH (0.4 *l*). The MeOH soluble fraction (fr.) was subjected to Diaion HP 20 CC

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(solv., H_2O, 60% MeOH, MeOH, acetone) to afford fr. 1 (58.2 g), fr. 2 (5.3 g), fr. 3 (2.1 g) and fr. 4 (0.2 g). Fraction 2 (1.8 g) was chromtographed over Diaion HP 20 with a gradient of H_2O-MeOH (20%→65%) to afford **1** (139 mg) and frs. 5–15. Fraction 6 (330 mg) was subjected to silica gel CC eluted with $CHCl_3$:MeOH:H_2O (6:4:1) to give frs. 16–19. Fraction 16 (28 mg) was subjected to Sephadex LH-20 CC eluted with 60% MeOH to afford **4** (20 mg). Fraction 19 was chromatographed over MCI gel CHP 20P eluted with MeOH to furnish **3** (47 mg). Fraction 9 (234 mg) was chromatographed over silica gel with a gradient of $CHCl_3$:MeOH:H_2O (14:2:0.1→6:4:1) to give **5** (6 mg). Fraction 13 (82 mg) was subjected to Sephadex LH-20 CC eluted with MeOH to give **2** (20 mg).

Luteolin 7-*O*-β-glucopyranoside (**1**): yellow powder, $[\alpha]_D^{24}$ −49.0° (*c*=1.1, DMSO). ^{1}H-NMR (in DMSO-d_6, 500 MHz) δ: 12.99 (1H, s, OH-5 of aglycone (Ag)), 10.00 (1H, br s, OH of Ag), 9.42 (1H, br s, OH of Ag), 7.46 (1H, dd, *J*=2.0, 8.5 Hz, H-6′ of Ag), 7.43 (1H, d, *J*=2.0 Hz, H-2′ of Ag), 6.92 (1H, d, *J*=8.5 Hz, H-5′ of Ag), 6.80 (1H, d, *J*=2.0 Hz, H-8 of Ag), 6.75 (1H, s, H-3 of Ag), 6.46 (1H, d, *J*=2.0 Hz, H-6 of Ag), 5.09 (1H, d, *J*=7.3 Hz, H-1 of glucose (Glc)). ^{13}C-NMR (in DMSO-d_6, 100 MHz) δ: 181.9 (C-4 of Ag), 164.5 (C-2 of Ag), 163.0 (C-7 of Ag), 161.1 (C-5 of Ag), 157.0 (C-9 of Ag), 149.9 (C-4′ of Ag), 145.8 (C-3′ of Ag), 121.4 (C-1′ of Ag), 119.2 (C-6′ of Ag), 116.0 (C-5′ of Ag), 113.6 (C-2′ of Ag), 105.3 (C-10 of Ag), 103.2 (C-3 of Ag), 99.9 (C-1 of Glc), 99.5 (C-6 of Ag), 94.8 (C-8 of Ag), 77.2 (C-3 of Glc or C-5 of Glc), 76.4 (C-5 of Glc or C-3 of Glc), 73.1 (C-2 of Glc), 69.6 (C-4 of Glc), 60.6 (C-6 of Glc).

Chrysoeriol 7-*O*-β-glucopyranoside (**2**): yellow powder, $[\alpha]_D^{24}$ −33.3° (*c*=0.7, DMSO). ^{1}H-NMR (in DMSO-d_6, 500 MHz) δ: 12.95 (1H, br s, OH-5 of Ag), 7.59 (1H, d, *J*=8.0 Hz, H-6′ of Ag), 7.59 (1H, s, H-2′ of Ag), 6.97 (1H, s, H-3 of Ag), 6.96 (1H, d, *J*=8.0 Hz, H-5′ of Ag), 6.87 (1H, d, *J*=2.5 Hz, H-8 of Ag), 6.46 (1H, d, *J*=2.5 Hz, H-6 of Ag), 5.07 (1H, d, *J*=7.3 Hz, H-1 of Glc), 3.90 (3H, s, OCH_3). ^{13}C-NMR (in DMSO-d_6, 100 MHz) δ: 182.1 (C-4 of Ag), 164.2 (C-2 of Ag), 163.0 (C-7 of Ag), 161.1 (C-5 of Ag), 157.0 (C-9 of Ag), 150.9 (C-3′ of Ag), 148.1 (C-4′ of Ag), 121.4 (C-1′ of Ag), 120.6 (C-6′ of Ag), 115.8 (C-5′ of Ag), 110.3 (C-2′ of Ag), 105.4 (C-10 of Ag), 103.5 (C-3 of Ag), 100.0 (C-1 of Glc), 99.5 (C-6 of Ag), 95.1 (C-8 of Ag), 77.3 (C-3 of Glc or C-5 of Glc), 76.5 (C-5 of Glc or C-3 of Glc), 73.1 (C-2 of Glc), 69.6 (C-4 of Glc), 60.7 (C-6 of Glc), 56.0 (OCH_3).

Chlorogenic acid (**3**): white powder, $[\alpha]_D^{26}$ –33.9° (*c*=1.7, MeOH). ^{1}H-NMR (in MeOH-d_4, 500 MHz) δ: 7.56 (1H, d, *J*=16.0 Hz, H-7 of caffeic acid (Caf)), 7.04 (1H, br s, H-2 of Caf), 6.93 (1H, br d, *J*=8.0 Hz, H-6 of Caf), 6.77 (1H, d, *J*=8.0 Hz, H-5 of Caf), 6.26 (1H, d, *J*=16.0 Hz, H-8 of Caf), 5.35 (1H, m, H-3 of quinic acid (Qui)), 4.20 (1H, br s, H-5 of Qui), 3.73 (1H, br d, *J*=6.7 Hz, H-4 of Qui), ^{1}H-NMR (in acetone-d_6, 500 MHz) δ: 7.59 (1H, d, *J*=16.0 Hz, H-7 of Caf), 7.18 (1H, d, *J*=2.0 Hz, H-2 of Caf), 7.02 (1H, dd, *J*=2.0, 8.0 Hz, H-6 of Caf), 6.88 (1H, d, *J*=8.0 Hz, H-5 of Caf), 6.32 (1H, d, *J*=16.0 Hz, H-8 of Caf), 5.38 (1H, ddd, *J*=5.0, 9.0, 10.5 Hz, H-3 of Qui), 4.27 (1H, ddd, *J*=3.0, 3.0 , 3.0 Hz, H-5 of Qui), 3.80 (1H, dd, *J*=3.0, 9.0 Hz, H-4 of Qui), 2.28 (1H, br d like, *J*=13.0 Hz, H-2a of Qui), 2.14 (2H, m, H_2-6 of Qui), 2.04 (1H, dd, *J*=10.5, 13.0 Hz, H-2b of Qui). ^{13}C-NMR (in MeOH-d_4, 100 MHz) δ: 178.4 (C-7 of Qui), 168.9 (C-9 of Caf), 149.5 (C-4 of Caf), 147.1 (C-7 of Caf), 146.8 (C-3 of Caf), 127.8 (C-1 of Caf), 123.0 (C-6 of Caf), 116.5 (C-5 of Caf), 115.3 (C-2 of Caf), 115.2 (C-8 of Caf), 77.3 (C-1 of Qui), 73.9 (C-4 of Qui or C-5 of Qui or C-3 of Qui), 72.1 (C-4 of Qui or C-5 of Qui or C-3 of Qui), 71.9 (C-4 of Qui or C-5 of Qui or C-3 of Qui), 39.4 (C-2 of Qui), 38.3 (C-6 of Qui).

Maltol 3-*O*-β-glucopyranoside (**4**): white powder, $[\alpha]_D^{26}$ −40.8° (*c*=2.1, MeOH). ^{1}H-NMR (in MeOH-d_4, 500 MHz) δ: 8.00 (1H, d, *J*=5.5 Hz, H-6 of Ag), 6.45 (1H, d, *J*=5.5 Hz, H-5 of Ag), 4.80 (1H, d, *J*=7.5 Hz, H-1 of Glc), 3.82 (1H, dd, *J*=2.0, 12.0 Hz, H-6a of Glc), 3.67 (1H, dd, *J*=5.0, 12.0 Hz, H-6b of Glc), 3.40 (2H, H-3 of Glc and H-4 of Glc), 3.34 (1H, dd, *J*=7.5, 9.5 Hz, H-2 of Glc), 3.26 (1H, m, H-5 of Glc), 2.46 (3H, s, H_3-7 of Ag). ^{13}C-NMR (in MeOH-d_4, 100 MHz) δ: 177.2 (C-4 of Ag), 164.6 (C-3 of Ag), 157.2 (C-6 of Ag), 143.7 (C-2 of Ag), 117.3 (C-5 of Ag), 105.5 (C-1 of Glc), 78.5 (C-3 of Glc or C-5 of Glc), 78.0 (C-5 of Glc or C-3 of Glc), 75.4 (C-2 of Glc), 71.1 (C-4 of Glc), 62.5 (C-6 of Glc), 15.8 (C-7 of Ag).

Benzyl β-glucopyranoside (**5**): white powder, $[\alpha]_D^{27}$ −39.2° (*c*=0.7, MeOH). ^{1}H-NMR (in MeOH-d_4, 500 MHz) δ: 7.41 (2H, d, *J*=7.5 Hz, H-2 of Ag and H-6 of Ag), 7.32 (2H, dd, *J*=7.5, 7.5 Hz, H-3 of Ag and H-5 of Ag), 7.27 (1H, t, *J*=7.5 Hz, H-4 of Ag), 4.93 (1H, d, *J*=11.5 Hz, H-7a of Ag), 4.67 (1H, d, *J*=11.5 Hz, H-7b of Ag), 4.35 (1H, d, *J*=7.5 Hz, H-1 of Glc), 3.89 (1H, dd, *J*=2.0, 11.5 Hz, H-6a of Glc), 3.69 (1H, dd, *J*=5.5, 11.5 Hz, H-6b of Glc). ^{13}C-NMR (in MeOH-d_4, 150 MHz) δ: 139.1 (C-1 of Ag), 129.3 ((C-2 of Ag and C-6 of Ag) or (C-3 of Ag and C-5 of Ag)), 129.2 ((C-2 of Ag and C-6 of Ag) or (C-3 of Ag and C-5 of Ag)), 128.7 (C-4 of Ag), 103.3 (C-1 of Glc), 78.1 (C-3 of Glc), 78.1 (C-5 of Glc), 75.2 (C-2 of Glc), 71.8 (C-7 of Ag), 71.8 (C-4 of Glc), 62.9 (C-6 of Glc).

Antioxidative assay by the ferric thiocyanate method Antioxidative assay of the test sample was measured using the previously described ferric thiocyanate method (Ono *et al*., 2000; Kikuzaki & Nakatani, 1993). A mixture of 2.51% linoleic acid ethanol (EtOH) solution (1.00 ml), 0.05 M phosohate buffer (pH 7.0, 2.00 ml) and H_2O (1.00 ml) was added to the EtOH solution (1.00 ml) of each sample in a vial with a cap and placed in the dark at 40°C to accelerate the oxidation. At intervals during incubation, this assay solution (0.05 ml) was diluted with 75% EtOH (4.85 ml), which was followed by adding 30% ammonium thiocyanate (0.05 ml). Precisely 3 min after the addition of 0.02 M ferrous chloride in 3.5% hydrochloric acid (0.05 ml) to the reaction mixture, the absorbance due to the developed red color was measured at 500 nm. The control sample was prepared from the mixture containing all ingredients except a test sample. α-Tocopherol and BHA were used as standard samples.

Assay of scavenging effect on DPPH We applied the method of Uchiyama *et al*. (1968) slightly modified. EtOH solution (1.00 ml) of each tested sample was added to a mixture of 0.1 M acetate buffer (pH 5.5, 1.00 ml) and 0.5 mM DPPH EtOH solution (0.50 ml) in a test tube and left to stand at room temperature for 30 min. The absorbance of the resulting solution was measured at 517 nm. The control sample was prepared from the mixture containing all ingredients except the test sample. α-Tocopherol was used as standard sample.

Assay of antihyaluronidase activity Test samples were dissolved in DMSO, and each solution was diluted with 0.1 M acetate buffer (pH 4.0) to ten volumes. Hyaluronidase, hyaluronic acid potassium salt and compound 48/80 were dissolved with the same buffer. Hyaluronidase activity was determined by the

method of Maeda *et al*. (1990). Antiallergic agents, DSCG and tranilast, which indicated strong antihyaluronidase activity (Maeda *et al*.,1990), were used as standard samples.

Results and Discussion

The aerial part of *Daucus carota* var. *sativus* was extracted with MeOH at room temperature. This extract, which indicated a stronger antioxidative activity than BHA using linoleic acid as the substrate in the ferric thiocyanate method at a concentration of 0.02%, was partitioned between hexane and MeOH. The MeOH soluble fraction showed a stronger antioxidative activity than the hexane soluble fraction, and was fractionated by repeated column chromatographies (Diaion HP20, MCI gel CHP 20P, silica gel, Sephadex LH20) to afford five compounds (**1**–**5**).

*Structure elucidation of **1**–**5*** Compounds **1**–**5** were identified as luteolin 7-*O*-β-glucopyranoside (Harborne & Mabry, 1982; Harborne, 1994), chrysoeriol 7-*O*-β-glucopyranoside (Harborne & Mabry, 1982; Harborne, 1994; Tomás *et al*., 1986), chlorogenic acid (Nishizawa *et al*., 1988), maltol 3-*O*-β-glucopyranoside (Hori *et al*., 1987) and benzyl β-glucopyranoside (Miyase *et al*., 1987), respectively, on the bases of spectral data (Fig. 1). As far as we know, this is the first example of the isolation of **1**–**5** from the aerial part of *Daucus carota* var. *sativus*.

*Antioxidative effect of compounds **1**–**4*** The antioxidative activities of **1**–**4** and luteolin (**6**) which is an aglycone of **1**, were investigated in the same manner as that used for the MeOH extract. All these compounds, except **4**, showed a stronger antioxidative activity than that of BHA at a concentration of 0.5 mM (Fig. 2). Moreover, the scavenging effect of **1**–**4**, and **6** on the stable free radical DPPH was examined. Compounds **1**, **3** and **6** showed the scavenging effect (Fig. 3), which the effects of **1** and **6** being stronger than that of α-tocopherol at a concentration of 0.02 mM. Compounds **1**–**3** were therefore considered to be the antioxidative principles of the MeOH extract of the aerial part of *Daucus carota* var. *sativus*. Recently Yokozawa *et al*. (1998) reported that catechyl (3,4-dihydroxyphenyl) group in the B-ring of flavonoid skeleton is most important for DPPH radical scavenging effect, and our previous study (Ono *et al*., 2000) bore out this structure-activity relationship. The result in this investigation also supported this relationship, because **1** exhibited stronger DPPH radical scavenging effect than that of **2**. We earlier reported (Ono *et al*., 2000) the DPPH radical scavenging effect of five dicaffeoyl quinic acid derivatives whose effects were stronger than that (ΔO. D. 0.212) of **3** at a concentration of 0.01 mM. Therefore, the radical scavenging effect of caffeoyl quinic acid derivatives might depend on the number of caffeoyl residue. Chrologenic acid and its isomer were lately reported as the antioxidants in prune (*Prunus domestica* L.) by Nakatani *et al*. (2000). Moreover, the antioxidative activity of chlorogenic acid using fluorescence spectroscopy was reported by Wang *et al*. (1999).

*Antihyaluronidase activities of **1**–**3*** Compounds **1**–**3** and **6** were assayed for their inhibitory effects on the activation of inactive hyaluronidase induced by compound 48/80 (Maeda *et al*., 1990). Compounds **1** and **3** showed almost the same inhibitory activity as that of tranilast, and the activity of **2** was one half

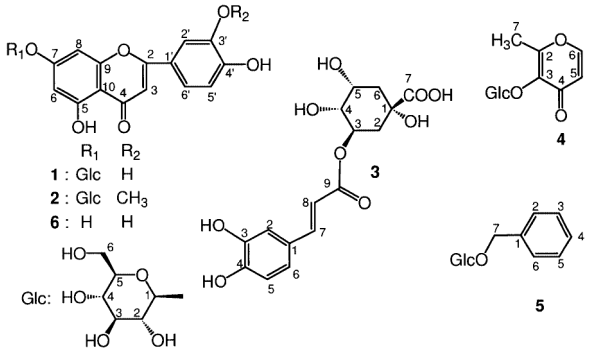

Fig. 1. Structures of 1–6

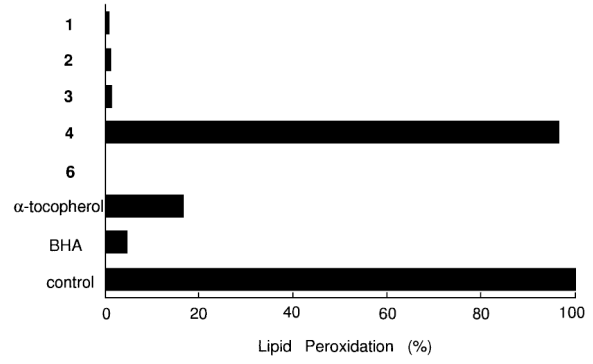

Fig. 2. Antioxidative activity of **1**–**4**, **6**, α-tocopherol and BHA after 5 days of lipid peroxidation. The final concentration of each sample tested was 0.5 mM. A control containing no added samples or standards on its values represents 100% lipid peroxidation.

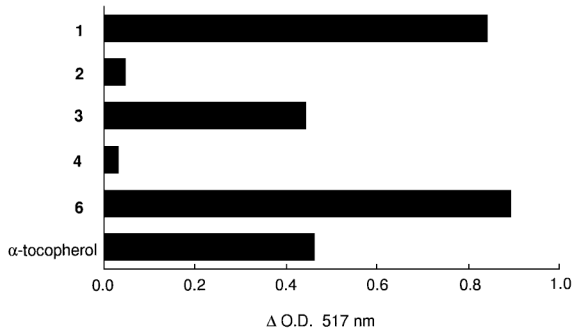

Fig. 3. DPPH radical scavenging effect of **1**–**4**, **6** and α-tocopherol. The final concentration of each sample tested was 0.02 mM. ΔO. D.=O.D. of control at 517 nm (1.120) −O.D. of sample. DPPH; 0.1 mM.

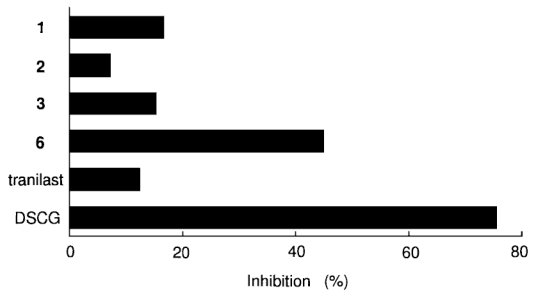

Fig. 4. Inhibitory effect of **1**–**3**, **6**, tranilast and DSCG on the activation of hyaluronidase. The final concentration of each sample tested was 0.2 mM.

that of **1** at a concentration of 0.2 mM (Fig. 4). Therefore, it was presumed that the catechyl group showed a stronger hyaluronidase inhibitory activity than the guiacyl (4-hydroxy-3-methoxyphenyl) group. Structure-activity relationship of flavonoids as inhibitors of hyaluronidase was reported by Kuppusamy *et al.* (1990). In their report, it was stated that the inhibitory activity of flavonoid glycosides is less than those of the corresponding aglycone. Our result in this study supported their report, because **6** exhibited stronger inhibitory activity than did **1**. The inhibitory effects of chlorogenic acid on the histamine release from the mast cells which induced compound 48/80 (Kimura *et al.*, 1985) and 5-lipoxygenase (Nishizawa *et al.*, 1988) were previously reported. Therefore, chlorogenic acid may be effective as an anti-allergic drug.

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