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Antioxidative, Antihyaluronidase and Antityrosinase Activities of Some Constituents from the Aerial Part of *Piper elongatum* VAHL.

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Seven known compounds, pyrroside B (1), swertisin (2), isovitexin (3), isoswertiajaponin (4), vomifoliol (blumenol A) (5), (*6S,9R*)-roseoside (6) and angelicoidenol (7) were isolated from the methanol (MeOH) extract of the aerial part of *Piper elongatum* VAHL. and their structures were identified on the basis of physical and spectral data. In addition, the antioxidative activity of 1–4 was evaluated by the ferric thiocyanate method. All these compounds showed stronger antioxidative activity than that of α-tocopherol. Furthermore, the scavenging effects on 1,1-diphenyl-2-picrylhydrazyl (DPPH), the antihyaluronidase and the antityrosinase activities of 1–4, asebogenin (8), 2′,6′-dihydroxy-4′-methoxydihydrochalcone (9), 3-geranyl-4-methoxybenzoic acid (10), 3-geranyl-4-hydroxybenzoic acid (11), nervogenic acid (12) and 2,2-dimethyl-6-carboxyl-8-prenyl-chromene (13), which were previously isolated from the MeOH extract were evaluated. Compounds 4, 8 and 9 showed higher radical scavenging effect than that of L-cysteine, and 4, 8 and 11 exhibited stronger inhibition effect on the activation of hyaluronidase than that of tranilast. Compound 8 indicated almost the same antityrosinase activity as that of kojic acid.

Keywords: *Piper elongatum* VAHL., flavonoid glycoside, antioxidative effect, radical scavenger, antihyaluronidase activity, antityrosinase activity

Antioxidants are used as food additives for the purpose of preventing deterioration of foods, and it is thought that lipid peroxidation is strongly associated with various human diseases, including cancer, inflammation and arteriosclerosis (Yagi, 1987; Yoshikawa *et al*., 1994). It was also reported that some antiallergic drugs show antioxidative actions due to scavenging superoxide radicals or to the inhibition of superoxide production (Yoshikawa *et al*., 1989). Hyaluronidase (EC 3.2.1.35) is a kind of mucopolysaccarase and is related to histamine release from mast cells in the inflammatory reactions. Therefore, 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). Tyrosinase (EC 1.14.18.1) catalyzes two oxidation steps, that is, the hydroxylation of monophenols and the oxidation of *O*-diphenols to quinones (Mayer, 1987), and some antioxidants indicate an antityrosinase activity. Tyrosinase inhibitors such as kojic acid, arbutin and ascorbic acid have therefore been used for prevention and treatment of hyperpigmentation (Emori, 1990; Ikeda & Tsutsumi, 1990).

Piper elongatum VAHL. (Piperaceae) is a small tree commonly found in the lowlands of the Amazon, and its leaves are used as an herb to treat dermatitis and allergy *etc*. in South America (Fournet *et al*., 1994). During the course of our screening for the antioxidative compounds from various herbs (Ono *et al*., 1995, 1997, 1998, 1999, 2000, 2001), the MeOH extract of the aerial part of *P. elongatum* VAHL. showed a stronger antioxidative activity than that of 3-*tert*-butyl-4-hydroxyanisole (BHA). In preceding papers, we have reported the isolation and structure elucidation of nine compounds: asebogenin (**8**), 2′,6′-dihydroxy-4′-methoxydihydrochalcone (**9**), 3-geranyl-4-methoxybenzoic acid (**10**), 3-geranyl-4-hydroxybenzoic acid (**11**), nervogenic acid (**12**), 2,2-dimethyl-6-carboxyl-8-prenyl-chromene (**13**), byzantionoside B (**14**), pipeloside A (**15**) and pipelol A (**16**) from the extract, and the antioxidative activities of **8–13** were evaluated by the ferric thiocyanate method (Masuoka *et al*., 1997, 2002). In a further investigation of this extract, we now report the isolation and structure elucidation of four flavonoid glycosides (**1–4**), two ionone derivatives (**5**, **6**), and a monoterpenoid (**7**). Additionally, we describe the antioxidative activity of **1–4** by the ferric thiocyanate method, the scavenging effects on DPPH, antihyaluronidase activities and antityrosinase activities of **1–4** and **8–13**.

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Materials and Methods

All the instruments and materials used were the same as cited in the previous report (Masuoka *et al*., 1997) unless otherwise specified. Optical rotations were measured with a JASCO DTP-1000 KUY digital polarimeter (JASCO, Tokyo). Melting points (mp) were determined on a Yanagimoto apparatus and uncorrected. For HPLC column chromatography, Kusano C.I.G. prepacked Si-gel (Kusano Kagakukikai Co., Tokyo, 22 mm i.d.×1000 mm; column 1, 22 mm i.d.×300 mm; column 2), YMC-pack S-5 120A ODS (YMC Co., Kyoto, 20 mm i.d.×250 mm; column 3) were used. Column chromatography was carried out over Diaion HP20 (Mitsubishi Chemical Industries Co., Ltd., Tokyo), Sephadex LH20 (Pharmacia Fine Chemicals. Uppsala, Sweden), silica gel 60 (Merck, Art.7734 and Art. 9385; Merck, Darmstadt, Germany) and Cosmosil $75C_{18}$ ODS (Nacalai Tesque Inc., Kyoto). Tyrosinase (from mushroom, 25,000 units, Lot 24H9542), hyaluronidase (from bovine testes, Type IV-S, 760 units/mg, Lot 100H8270), compound 48/80 and disodium cro-

moglycate (DSCG) were purchased from Sigma Chemical Co. (St. Louis, MO). Tranilast was obtained from Tocris Cookson, Ltd. (Avonmouth, Bristol, UK). Linoleic acid was purchased from Tokyo Kasei Kogyo Co. (Tokyo), and the other reagents were purchased from Nacalai Tesque Inc.

Extraction and isolation The air-dried and powdered aerial part (5.00 kg) of *P. elongatum* VAHL. was extracted with MeOH (13 *l*) under reflux, and the solvent was removed under reduced pressure to afford a brown extract (288 g). This extract was defatted by treatment of hexane (300 ml ×2) to give a hexane soluble fraction (fr.) (7.66 g) and a residue (275 g). The residue was chromatographed over Diaion HP20 (40% MeOH, 60% MeOH, 80% MeOH, MeOH, acetone) to afford fr. 1 (62.6 g), fr. 2 (35.7 g), fr. 3 (37.4 g), fr. 4 (93.1 g) and fr. 5 (39.1 g). A part of fr. 2 (29.0 g) was chromatographed over silica gel [Merck, Art. 7734, $CHCl_3$-MeOH (12 : 1, 10 : 1), $CHCl_3$-MeOH-H_2O (14 : 2 : 0.1, 10 : 2 : 0.1, 8 : 2 : 0.2, 7 : 3 : 0.5, 6 : 4 : 1), MeOH] to give frs. 6–21. The successive chromatography of fr. 7 (952 mg) over Sephadex LH20 (MeOH), silica gel [Merck, Art. 9385, $CHCl_3$-MeOH (15 : 1, 12 : 1, 10 : 1), $CHCl_3$-MeOH-H_2O (14 : 2 : 0.1)] and HPLC (column 3, 50% MeOH) gave **7** (33 mg). The remainder of fr. 2 was subjected to Sephadex LH20 (70% MeOH) to give fr. 22 (4.87 g) and fr. 23 (2.66 g). The chromatography of fr. 23 over silica gel [Merck, Art. 9385, $CHCl_3$-MeOH-H_2O (10 : 2 : 0.1, 8 : 2 : 0.2, 7 : 3 : 0.5, 6 : 4 : 1), MeOH] afforded frs. 24–31. Fraction 27 (584 mg) and fr. 28 (270 mg) were each subjected to HPLC (column 1, $CHCl_3$-MeOH-H_2O, 8 : 2 : 0.2) to give fr. 32 (34 mg), fr. 33 (75 mg), **4** (44 mg) and **1** (89 mg) from fr. 27, and **3** (102 mg) from fr. 28, respectively. Fraction 32 was purified by HPLC (column 3, 50% MeOH) to afford **2** (14 mg). Fraction 22 was successively chromatographed over Cosmosil $75C_{18}$ ODS (50% MeOH, 60% MeOH, 70% MeOH, 75% MeOH) and silica gel [Merck, Art. 7734, hexane-AcOEt (1 : 2, 1 : 4), $CHCl_3$-MeOH (10 : 1), $CHCl_3$-MeOH-H_2O (14 : 2 : 0.1, 10 : 2 : 0.1, 8 : 2 : 0.2, 7 : 3 : 0.5, 6 : 4 : 1), MeOH] to give frs. 34–37. Fraction 34 (218 mg) was successively subjected to Cosmosil $75C_{18}$ ODS (55% MeOH, 65% MeOH, 75% MeOH) and HPLC [column 2, $CHCl_3$-MeOH-H_2O (20 : 1 : 1, lower layer)] to afford fr. 38 (68 mg). Fr. 38 was finally crystallized from a mixture of hexane and AcOEt to give **5** (47 mg), and fr. 36 was purified by HPLC [column 1, $CHCl_3$-MeOH-H_2O (14 : 2 : 0.1)] to afford **6** (24 mg).

Pyrroside B (**1**): yellow powder, $[\alpha]_D^{20}$ −92.1° (c=1.0, MeOH). Positive fast atom bombardment mass spectrum (FAB-MS) m/z: 567 $[M+H]^+$, 435 $[M\text{-pentose}+H]^+$, 273 $[M\text{-pentose-hexose}+H]^+$. ^{1}H-NMR (in dimethylsulfoxide (DMSO)-d_6, 500 MHz) δ: 12.15 (1H, s, OH-5-aglycone (Ag)), 7.34 (2H, d, J=8.5 Hz, H-2′-Ag and H-6′-Ag), 6.80 (2H, d, J=8.5 Hz, H-3′-Ag and H-5′-Ag), *ca*. 6.15 (2H, H-6-Ag and H-8-Ag), 5.51 (1H, dd, J=3.0, 13.0 Hz, H-2-Ag), 4.97 (1H, d, J=7.5 Hz, H-1-glucose (Glc)), 4.80 (1H, d, J=3.0 Hz, H-1-apiose (Api)), 3.27 (1H, dd, J=13.0, 17.0 Hz, H-3b-Ag), 2.75 (1H, dd, J=3.0, 17.0 Hz, H-3a-Ag). ^{13}C-NMR (in DMSO-d_6, 125 MHz) δ: 197.3 (C-4-Ag), 165.2 (C-7-Ag), 163.0 (C-5-Ag or C-9-Ag), 162.8 (C-5-Ag or C-9-Ag), 157.8 (C-4′-Ag), 128.7 (C-1′-Ag), 128.4 (C-2′-Ag and C-6′-Ag), 115.2 (C-3′-Ag and C-5′-Ag), 109.3 (C-1-Api), 103.3 (C-10-Ag), 99.5 (C-1-Glc), 96.5 (C-6-Ag), 95.5 (C-8-Ag), 78.8 (C-3-Api), 78.7 (C-2-Ag), 76.2 (C-3-Glc), 76.0 (C-5-Glc), 75.5 (C-2-Api), 73.4 (C-5-Api), 73.0 (C-2-Glc), 70.0 (C-4-Glc), 67.5 (C-6-Glc), 63.4 (C-4-Api), 42.0 (C-3-Ag). ^{13}C-NMR (in pyridine-d_5, 125 MHz) δ: 197.2 (C-4-Ag), 166.5 (C-7-Ag), 164.5 (C-5-Ag or C-9-Ag), 163.6 (C-5-Ag or C-9-Ag), 159.6 (C-4′-Ag), 129.6 (C-1′-Ag), 128.9 (C-2′-Ag and C-6′-Ag), 116.5 (C-3′-Ag and C-5′-Ag), 111.3 (C-1-Api), 104.3 (C-10-Ag), 101.5 (C-1-Glc), 97.7 (C-6-Ag), 96.5 (C-8-Ag), 80.4 (C-3-Api), 79.7 (C-2-Ag), 78.4 (C-3-Glc), 78.0 (C-2-Api), 77.4 (C-5-Glc), 75.2 (C-4-Api), 74.6 (C-2-Glc), 71.3 (C-4-Glc), 68.7 (C-6-Glc), 65.9 (C-5-Api), 43.3 (C-3-Ag).

Swertisin (**2**): pale yellow powder, $[\alpha]_D^{26}$ −6.3° (c=0.4, MeOH). Positive FAB-MS m/z: 447 $[M+H]^+$. ^{1}H-NMR (in DMSO-d_6, 500 MHz) δ: 7.97 (2H, d, J=9.0 Hz, H-2′-Ag and H-6′-Ag), 6.93 (2H, d, J=9.0 Hz, H-3′-Ag and H-5′-Ag), 6.83 (2H, s, H-3-Ag and H-8-Ag), 4.58 (1H, d, J=10.5 Hz, H-1-Glc), 3.88 (3H, s, OCH_3). ^{13}C-NMR (in DMSO-d_6, 125 MHz) δ: 182.2 (C-4-Ag), 163.8 (C-2-Ag or C-7-Ag), 163.7 (C-2-Ag or C-7-Ag), 161.3 (C-4′-Ag), 160.2 (C-5-Ag), 156.7 (C-9-Ag), 128.5 (C-2′-Ag and C-6′-Ag), 120.8 (C-1′-Ag), 115.9 (C-3′-Ag and C-5′-Ag), 109.6 (C-6-Ag), 104.5 (C-10-Ag), 102.9 (C-3-Ag), 90.1 (C-8-Ag), 81.7 (C-5-Glc), 79.0 (C-3-Glc), 72.6 (C-1-Glc), 70.8 (C-2-Glc or C-4-Glc), 69.9 (C-2-Glc or C-4-Glc), 61.7 (C-6-Glc), 56.4 (OCH_3).

Isovitexin (**3**): yellow powder, $[\alpha]_D^{27}$ +27.5° (c=1.0, MeOH). Positive FAB-MS m/z: 433 $[M+H]^+$. ^{1}H-NMR (in DMSO-d_6, 400 MHz) δ: 13.56 (1H, s, OH-5-Ag), 7.93 (2H, d, J=9.0 Hz, H-2′-Ag and H-6′-Ag), 6.93 (2H, d, J=9.0 Hz, H-3′-Ag and H-5′-Ag), 6.79 (1H, s, H-3-Ag), 6.52 (1H, s, H-8-Ag), 4.60 (1H, d, J=9.5 Hz, H-1-Glc). ^{13}C-NMR (in DMSO-d_6, 100 MHz) δ: 182.0 (C-4-Ag), 163.5 (C-2-Ag), 163.3 (C-7-Ag), 161.2 (C-4′-Ag), 160.7 (C-5-Ag), 156.3 (C-9-Ag), 128.5 (C-2′-Ag and C-6′-Ag), 121.1 (C-1′-Ag), 116.0 (C-3′-Ag and C-5′-Ag), 108.9 (C-6-Ag), 103.4 (C-10-Ag), 102.8 (C-3-Ag), 93.6 (C-8-Ag), 81.6 (C-5-Glc), 78.9 (C-3-Glc), 73.1 (C-1-Glc), 70.6 (C-2-Glc), 70.2 (C-4-Glc), 61.5 (C-6-Glc).

Isoswertiajaponin (**4**): pale yellow powder, $[\alpha]_D^{20}$ −23.2° (c=1.0, MeOH). Positive FAB-MS m/z: 463 $[M+H]^+$. ^{1}H-NMR (in DMSO-d_6, 500 MHz) δ: 13.33 (1H, s, OH-5-Ag), 10.05 (1H, s, OH-3′-Ag or OH-4′-Ag), 9.07 (1H, s, OH-3′-Ag or OH-4′-Ag), 7.55 (1H, dd, J=1.0, 9.0 Hz, H-6′-Ag), 7.50 (1H, d, J=1.0 Hz, H-2′-Ag), 6.88 (1H, d, J=9.0 Hz, H-5′-Ag), 6.68 (1H, s, H-3-Ag), 6.51 (1H, s, H-6-Ag), 4.72 (1H, d, J=10.5 Hz, H-1-Glc), 3.88 (3H, s, OCH_3). ^{13}C-NMR (in DMSO-d_6, 125 MHz) δ: 182.2 (C-4-Ag), 164.5 (C-2-Ag), 163.3 (C-7-Ag), 161.3 (C-5-Ag), 155.1 (C-9-Ag), 149.7 (C-4′-Ag), 145.8 (C-3′-Ag), 121.9 (C-1′-Ag), 119.5 (C-6′-Ag), 115.7 (C-5′-Ag), 114.1 (C-2′-Ag), 105.6 (C-8-Ag), 104.4 (C-10-Ag), 102.3 (C-3-Ag), 94.9 (C-6-Ag), 82.0 (C-5-Glc), 78.7 (C-3-Glc), 73.1 (C-1-Glc), 70.7 (C-2-Glc or C-4-Glc), 70.6 (C-2-Glc or C-4-Glc), 61.6 (C-6-Glc), 56.5 (OCH_3).

Vomifoliol (blumenol A) (**5**): colorless needles, mp 119–120°C, $[\alpha]_D^{27}$ +173.7° (c=0.9, MeOH). Positive FAB-MS m/z: 225 $[M+H]^+$. ^{1}H-NMR (in chloroform-d, 500 MHz) δ: 5.90 (1H, s, H-4), 5.86 (1H, dd, J=5.5, 15.5 Hz, H-8), 5.79 (1H, d, J=15.5 Hz, H-7), 4.41 (1H, m, H-9), 2.44 (1H, d, J=17.0 Hz, Hb-2), 2.24 (1H, d, J=17.0 Hz, Ha-2), 1.90 (3H, s, H_3-13), 1.30 (3H, d, J=6.0 Hz, H_3-10), 1.08 (3H, s, H_3-12), 1.02 (3H, s, H_3-11). ^{13}C-NMR (in pyridine-d_5, 125 MHz) δ: 197.8 (C-3), 164.6 (C-5), 137.3 (C-8), 129.1 (C-7), 126.7 (C-4), 79.0 (C-6), 67.4 (C-9), 50.3 (C-2), 41.6 (C-1), 24.5 (C-11), 24.5 (C-10), 23.5 (C-12), 19.4 (C-13).

(*6S, 9R*)-Roseoside (**6**): pale yellow syrup, $[\alpha]_D^{30}$ +46.0°

(c=0.9, MeOH). Positive FAB-MS m/z: 387 $[M+H]^+$, 225 $[M-hexose+H]^+$. ^{1}H-NMR (in pyridine-d_5, 500 MHz) δ: 6.33 (1H, dd, J=6.0, 16.0 Hz, H-8-Ag), 6.15 (1H, d, J=16.0 Hz, H-7-Ag), 6.08 (1H, s, H-4-Ag), 4.94 (1H, d, J=8.0 Hz, H-1-Glc), 4.74 (1H, m, H-9-Ag), 4.55 (1H, dd, J=2.0, 11.5 Hz, Hb-6-Glc), 4.29 (1H, dd, J=6.0, 11.5 Hz, Ha-6-Glc), 4.24 (1H, dd, J=9.0, 9.0 Hz, H-3-Glc), 4.15 (1H, dd, J=9.0, 9.0 Hz, H-4-Glc), 4.04 (1H, dd, J=8.0, 9.0 Hz, H-2-Glc), 3.91 (1H, ddd, J=2.0, 6.0, 9.0 Hz, H-5-Glc), 2.68 (1H, d, J=17.0 Hz, Hb-2-Ag), 2.40 (1H, d, J=17.0 Hz, Ha-2-Ag), 2.02 (3H, s, H_3-13-Ag), 1.39 (3H, d, J=6.0 Hz, H_3-10-Ag), 1.26 (3H, s, H_3-12-Ag), 1.16 (3H, s, Ag H_3-11-Ag). ^{13}C-NMR (in pyridine-d_5, 125 MHz) δ: 197.8 (C-3-Ag), 164.2 (C-5-Ag), 134.8 (C-7-Ag), 131.4 (C-8-Ag), 126.9 (C-4-Ag), 102.9 (C-1-Glc), 79.0 (C-6-Ag), 78.7 (C-3-Glc), 78.5 (C-5-Glc), 76.2 (C-9-Ag), 75.4 (C-2-Glc), 71.9 (C-4-Glc), 63.0 (C-6-Glc), 50.3 (C-2-Ag), 41.7 (C-1-Ag), 24.7 (C-11-Ag), 23.5 (C-12-Ag), 21.3 (C-10-Ag), 19.5 (C-13-Ag).

Angelicoidenol (**7**): colorless needles, mp 126-128°C, $[\alpha]_D^{31}$ +10.2° (c=1.1, MeOH). EI-MS m/z: 170 $[M]^+$. ^{1}H-NMR (in MeOH-d_4, 500 MHz) δ: 3.84 (1H, ddd, J=2.0, 3.0, 9.0 Hz, H-2), 3.80 (1H, dd, J=3.5, 8.0 Hz, H-5), 2.32 (1H, dd, J=8.0, 13.5 Hz, H_α-6), 2.24 (1H, ddd, J=5.0, 9.0, 14.0 Hz, H_β-3), 1.66 (1H, d, J=5.0 Hz, H-4), 1.32 (1H, ddd, J=2.0, 3.5, 13.5 Hz, H_β-6), 1.08 (3H, s, H_3-8), 0.87 (3H, s, H_3-9), 0.85 (3H, s, H_3-10), 0.77 (1H, dd, J=3.0, 14.0 Hz, H_α-3). ^{13}C-NMR (in MeOH-d_4, 125 MHz) δ: 77.2 (C-2), 76.7 (C-5), 54.5 (C-4), 52.2 (C-1), 49.6 (C-7), 39.9 (C-6), 37.5 (C-3), 22.5 (C-8), 20.9 (C-9), 14.0 (C-10). ^{13}C-NMR (in pyridine-d_5, 125 MHz) δ: 75.0 (C-2 and C-5), 53.8 (C-4), 50.9 (C-1), 47.9 (C-7), 39.7 (C-6), 37.2 (C-3), 21.9 (C-8), 20.2 (C-9), 13.6 (C-10).

Antioxidative assay by the ferric thiocyanate method Antioxidative activity of the test sample was assayed using the previously described ferric thiocyanate method (Ono *et al.*, 1995). A mixture of 2.51% linoleic acid in ethanol (EtOH) (1.03 ml), 0.05 M phosphate buffer (pH 7.0, 2.00 ml) and H_2O (0.97 ml) was added to the EtOH solution (1.00 ml) of each test 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 (0.10 ml) was diluted with 75% EtOH (9.70 ml), which was followed by the addition of 30% ammonium thiocyanate (0.10 ml). Precisely 3 min after the addition of 0.02 M ferrous chloride in 3.5% hydrochloric acid (0.10 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 for the test sample. A natural antioxidant, α-tocopherol and a synthetic antioxidant, BHA were used as standard samples. Results presented are the means of two experiments.

Assay of scavenging effect on DPPH The method of Uchiyama *et al.* (1968) was applied in a slightly modified manner. Five-tenths mM DPPH in EtOH (0.50 ml) was added to the mixture of EtOH (1.00 ml) and 0.1 M acetate buffer (pH 5.5, 1.00 ml) in a test tube. The solution was 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 for the test sample. α-Tocopherol and L-cysteine were used as standard samples. Test samples and α-tocopherol were dissolved in EtOH, and L-cys-

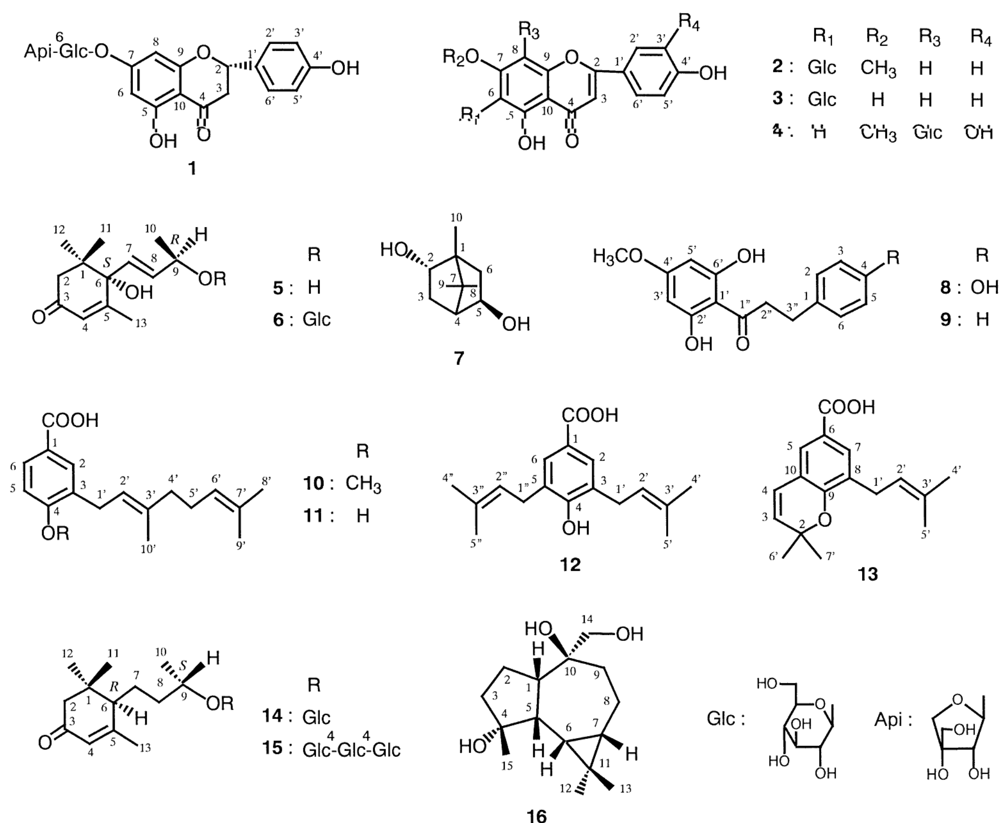

Fig. 1. Structures of **1–16**.

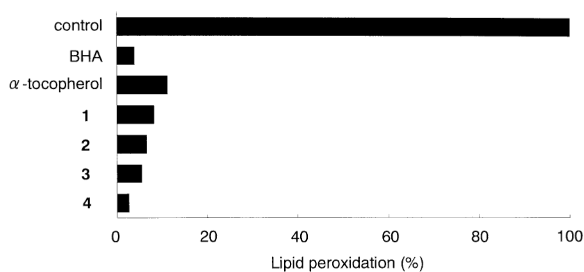

Fig. 2. Antioxidative activity of **1–4**, α-tocopherol and BHA after 5 days of lipid peroxidation. The final concentration of each sample tested was 0.5 mM. The value of control represents 100% lipid peroxidation.

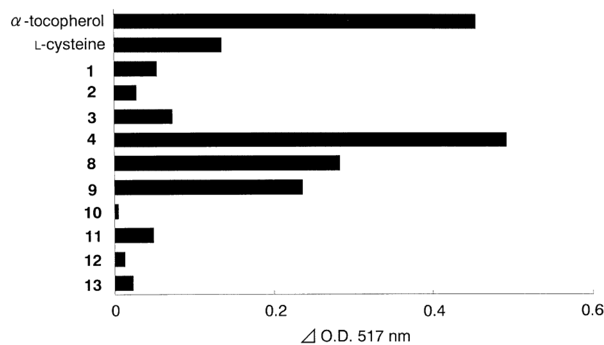

Fig. 3. DPPH radical scavenging effects of **1–4**, **8–13**, L-cysteine and α-tocopherol. The final concentration of each sample tested was 0.02 mM. ΔO.D.=O.D. of control at 517 nm (1.122)−O.D. of sample. DPPH; 0.1 mM.

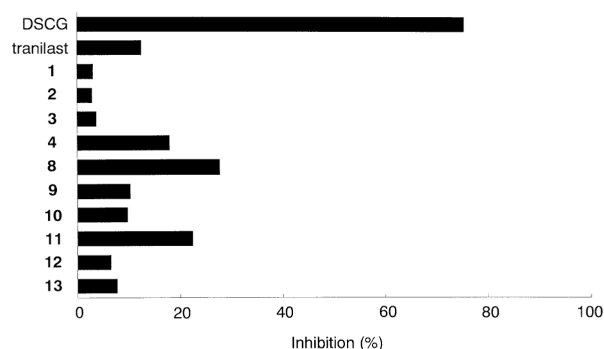

Fig. 4. Inhibitory effects of **1–4**, **8–13**, tranilast and DSCG on the activation of hyaluronidase. The final concentration of each sample tested was 0.2 mM.

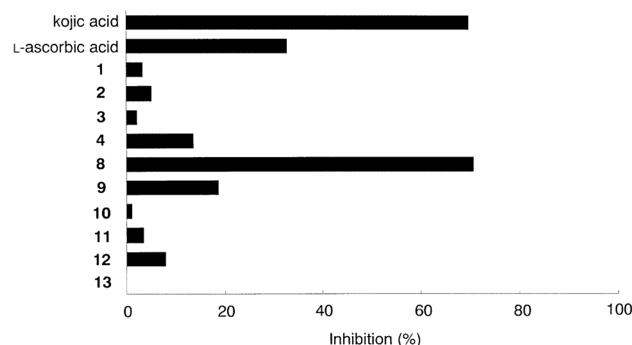

Fig. 5. Inhibitory effects of **1–4**, **8–13**, L-ascorbic acid and kojic acid on tyrosinase. The final concentration of each sample tested was 0.1 mM.

teine was dissolved in 0.1 M acetate buffer (pH 5.5). Results presented are the means of two experiments.

Inhibition of hyaluronidase activity Inhibitory effect of test sample was measured using the previously described method (Ono *et al.*, 1997). 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. A mixture of sample solution (0.20 ml) and 2000 units/ml of hyaluronidase solution (0.10 ml) was preincubated at 37°C for 20 min. Then 0.10 mg/ml of compound 48/80 solution (0.20 ml) was added and each mixture was incubated at 37°C for 20 min. After incubation, the assay was commenced by adding 0.8 mg/ml of hyaluronic acid potassium salt solution (0.50 ml) to each tube and incubated at 37°C for 40 min. Reactions were terminated by the addition of 0.4 N NaOH solution. Hyaluronidase activity was determined by the Morgan-Elson method (Aronson & Davidson, 1967) as modified by Maeda *et al.* (1990). Antiallergic agents, DSCG and tranilast, which indicated strong antihyaluronidase activity (Maeda *et al.*, 1990) were used as standard samples.

Inhibition of tyrosinase activity We applied the method of Masamoto *et al.* (1980) slightly modified. Test samples were dissolved in DMSO. A mixture of sample solution (0.03 ml) and 1/15 M phosphate buffer (pH 6.8, 1.77 ml) was preincubated at 30°C for 10 min. Then 250 units/ml of mushroom tyrosinase solution (0.20 ml) was added to the mixture which was incubated at 30°C for 20 min. Three-hundredths percent of DOPA solution (1.00 ml) was added to the mixture. After incubation at 30°C for 10 min, the amount of dopachrome in the reaction was measured as O.D. at 475 nm. Kojic acid and L-ascorbic acid were used as the standards.

Results and Discussion

The MeOH extract of the aerial part of *P. elongatum* VAHL. was defatted by treatment of hexane. The residue was successively subjected to Diaion HP20, Sephadex LH20, silica gel, Cosmosil $75C_{18}$ ODS column chromatography as well as HPLC on ODS and silica gel to give seven compounds (**1–7**).

*Structure elucidation of **1–7*** Compounds **1–7** were identified as pyrroside B (Hori *et al.*, 1988), swertisin (Fujita & Inoue, 1982), isovitexin (Harborne & Mabry, 1986), isoswertiajaponin (Fujita & Inoue, 1982), vomifoliol (blumenol A) (Fukui *et al.*, 1977, Okamura *et al.*, 1981), (*6S,9R*)-roseoside (Otsuka *et al.*, 1995, Okamura *et al.*, 1981) and angelicoidenol (Mahmood *et al.*, 1983), respectively, on the bases of physical and spectral data (Fig. 1). As far as we know, this is the first example of the isolation of **1–7** from the aerial part of *P. elongatum* VAHL.

*Antioxidative activity of **1–4** by the ferric thiocyanate method* The antioxidative activity of **1–4** was evaluated using linoleic acid as the substrate by the ferric thiocyanate method. All these compounds showed stronger antioxidative activity than α-tocopherol at a concentration of 0.5 mM, especially **4** exhibited higher antioxidative activity than that of BHA (Fig. 2).

*Scavenging effects of **1–4** and **8–13*** The scavenging effects of **1–4** and **8–13** on the stable free radical DPPH were

examined. Compounds **4**, **8** and **9** exhibited potent scavenging effects (Fig. 3). In particular, the effect of **4** was similar to that of α-tocopherol at a concentration of 0.02 mM. Recently, Yokozawa *et al*. (1998) reported that the catecyl (3,4-dihydroxyphenyl) group in the B-ring of flavonoid skeleton is most important for the DPPH radical scavenging effect, and our previous studies (Ono *et al*. 2000, 2001) bore out this structure-activity relationship. The result in this study supported their reports, because **4** exhibited a higher effect than those of **1**–**3**. In addition, 2′,6′-dihydroxy-4′-methoxyl benzyl group in **8** and **9** seemed to be one of the important structures in relation to radical scavenging effect.

*Antihyaluronidase activity of **1**–**4** and **8**–**13*** Compounds **1**–**4** and **8**–**13** were assayed for their inhibitory effects on the activation of inactive hyaluronidase induced by compound 48/80. **4**, **8** and **11** showed stronger antihyaluronidase activity than that of tranilast at a concentration of 0.2 mM (Fig. 4). Since the activity of **9** is weaker than that of **8**, it is speculated that 4-hydroxyl group in dihydrochalcone skeleton is important for the activity. In addition, the activity of **11** was considered to be related to hydroxyl group at C-4.

*Antityrosinase activity of **1**–**4** and **8**–**13*** Inhibitory effects of **1**–**4** and **8**–**13** on tyrosinase were examined. Compound **8** showed the strongest activity among the test compounds, and had almost the same antityrosinase activity as that of kojic acid at a concentration of 0.1 mM (Fig. 5). It was presumed that the hydroxyl group at C-4 for the dihydrochalcone contributed to a potent inhibitory effect, because the activity of **8** was three times that of **9**. The structure-activity relationships of benzoic acid derivatives and cinnamic acid derivatives for antityrosinase activity were reported by Tazawa *et al*. (2001) and Shirota *et al*. (1994). In their report, the 4-hydroxyphenyl group is important for the activity. Our results in this study supported their findings, because **8**, **11** and **12** exhibited stronger antityrosinase activity than **9**, **10** or **13**, respectively.

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