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Antioxidant Compounds from the Leaves of *Peucedanum japonicum* Thunb

MASASHI HISAMOTO,† HIROE KIKUZAKI,† HAJIME OHIGASHI,‡ AND NOBUJI NAKATANI*,†

Division of Food and Health Sciences, Graduate School of Human Life Science, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi, Osaka 558-8585, Japan, and Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Oiwake, Kitashirakawa, Sakyo, Kyoto 606-8502, Japan

Seventeen compounds were isolated from the *n*-butanol soluble fraction of the leaves of *Peucedanum japonicum* Thunb. On the basis of MS and various NMR spectroscopic techniques, the structures of the isolated compounds were determined as isoquercitrin (**1**), rutin (**2**), 3-*O*-caffeoylquinic acid (**3**), 4-*O*-caffeoylquinic acid (**4**), 5-*O*-caffeoylquinic acid (**5**), cnidioside A (**6**), praeroside II (**7**), praeroside III (**8**), apterin (**9**), esculin (**10**), (*R*)-peucedanol (**11**), (*R*)-peucedanol 7-*O*-*β*-D-glucopyranoside (**12**), L-tryptophan (**13**), uracil (**14**), guanosine (**15**), uridine (**16**), and thymidine (**17**). All compounds except **11** and **12** were isolated for the first time from *P. japonicum.* Several isolated compounds were quantified by high-performance liquid chromatography analysis. In addition, all isolated compounds were examined for radical scavenging on 1,1-diphenyl-2-picrylhydrazyl radical and for inhibition of oxidation of liposome induced by 2,2′-azobis(2-amidinopropane)dihydrochloride. Compounds **2**−**5** were found to be the major potent constituents, which contribute to the antioxidant activity of *P. japonicum* leaves.

KEYWORDS: ***Peucedanum japonicum* Thunb; radical scavenging activity; antioxidant activity; liposome**

INTRODUCTION

In recent years, there is an increasing interest in natural antioxidants from edible plants for food preservation and the prevention of several diseases (*1*). We have been studying efficient antioxidants from natural resources, especially those from spices and herbs (*2*−*4*). Recently, we have focused on the subtropical plants, which grow on the Yaeyama Islands, Okinawa, the southernmost part of Japan. We previously reported the antioxidant activity of the eight species using the linoleic acid autoxidation system (*5*). Their radical scavenging activities against 1,1-diphenyl-2-picrylhydrazyl (DPPH) and superoxide anion (O_2^-) radicals were also evaluated. These results showed that the polar fraction of *Peucedanum japonicum* was found to show the strongest radical scavenging activity, which prompted us to further study this plant.

P. japonicum belonging to the family Umbelliferae is a perennial plant distributed in Japan, the Philippines, China, and Taiwan. In Okinawa, this plant grows on the coast and the cliff and is cultivated as a vegetable in a kitchen garden. The roots of this plant have been used as a folk medicine for the treatment of coughs in the Yaeyama Islands. The leaves are frequently served as a vegetable or a garnish for raw fish. It has been reported that several coumarins isolated from this plant's roots were found to possess some pharmacological activities (*6*−*11*), but little work has been done on the constituents of the aerial part of this plant (*12*, *13*) and their antioxidant activity.

The present paper is concerned with the isolation and structure elucidation of the constituents of *P. japonicum* leaves. Several isolated compounds were quantified by high-performance liquid chromatography (HPLC) analysis. In addition, we examined the antioxidant activities of each extract and soluble fraction from this plant leaf and the isolated compounds against DPPH radical and oxidation of liposome.

MATERIALS AND METHODS

General Procedures. ^{1}H (500 MHz), ^{13}C (125 MHz), and two-dimensional (2D) NMR spectra were recorded with a Varian Unity 500 instrument (Varian Inc., Palo Alto, CA). MS were measured on a HITACHI M-1200AP mass spectrometer (Hitachi Ltd., Tokyo, Japan) with an atmospheric pressure chemical ionization (APCI) interface. Optical rotations were measured using a Jasco P-1030 automatic digital polarimeter (Jasco Co., Tokyo, Japan). Analytical and quantitative HPLC were performed on a JASCO PU-1580 Intelligent Pump equipped with a Jasco MD 910 multiwavelength detector using CAPCELL PAK C_{18} UG80 (4.6 mm × 250 mm) (Shiseido Ltd., Tokyo, Japan). Preparative HPLC was carried out on a HITACHI HPLC system (pump, L-6250; detector, L-4200) using CAPCELL PAK C_{18} UG80 (20 mm × 250 mm) (Shiseido). The DPPH radical scavenging activity was measured using an Arvo 1420sx multilavel counter (PerkinElmer Life

* To whom correspondence should be addressed. Tel: +81-6-6605-2812. Fax: +81-6-6605-3086. E-mail: nakatani@life.osaka-cu.ac.jp.
† Osaka City University.
‡ Kyoto University.

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Sciences Inc., Boston, MA). Column chromatography (CC) was performed using silica gel 60 (70−230 mesh, Merck, Darmstadt, Germany) and Chromatorex ODS DM1020T (100−200 mesh, Fuji Sylisia Chemical Ltd., Tokyo, Japan). Thin-layer chromatography was run using silica gel 60 F_{254} and PR-18 F_{254S} (Merck).

Plant Material. Dried leaves of *P. japonicum* were kindly provided from Yonaguni Commerce and Industry Association (Okinawa, Japan) in April 1998 and identified by Professor Shigetomo Yonemori (Tropical Biosphere Research Center, Iriomote Station, University of the Ryukyus). A voucher specimen has been deposited at the Tropical Biosphere Research Center, University of the Ryukyus, Okinawa, Japan.

Chemicals. DPPH, 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH), α-tocopherol, and l-ascorbic acid were bought from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Phosphatidylcholine (PC; 99% grade) from egg yolk and cholesterol were purchased from Sigma Chemical Co. (St. Louis, MO). PC hydroperoxide (PC−OOH) was a generous gift from Professor Junji Terao (Tokushima University, Japan). Just before the preparation of liposomes, PC was purified by a C_8 gel CC to remove contaminant peroxides according to the method of J. Terao et al. (*14*). All solvents and other chemicals used were of the highest analytical grade.

Extraction, Isolation, and Identification of the Constitutes of *P. japonicum* Leaves. The dried powdered leaves of *P. japonicum* (2650 g) were extracted successively with *n*-hexane (6 × 1.3 L), dichloromethane (6 × 1.3 L), and 70% aqueous acetone (6 × 1.3 L) at room temperature. The organic solvent in the 70% aqueous acetone extract was evaporated in vacuo to give the aqueous part, which was partitioned into the ethyl acetate soluble, *n*-butanol soluble, and water soluble parts. Each soluble part was concentrated in vacuo to afford the *n*-hexane (74.8 g), dichloromethane extracts (70.6 g), ethyl acetate soluble (41.8 g), *n*-butanol soluble (79.0 g), and water soluble (428.2 g) fractions, respectively. The *n*-butanol soluble fraction (37.3 g) was fractionated into seven fractions (fractions 1−7) by silica gel CC using stepwise gradients of EtOAc/MeOH/H_2O (from 10:2:1 to 3:2:1, v/v). Fraction 3 (8.8 g) was further chromatographed over ODS with H_2O/MeOH (2:1) to give seven fractions (fractions 3-1 to 3-7). Fraction 3-1 was subjected to ODS CC with H_2O/MeOH (2:1) to afford compound **7** (2 mg). Fraction 3-2 was repeatedly chromatographed on ODS and a preparative HPLC using H_2O/MeOH (3:1), giving **11** (8 mg), **12** (88 mg), **13** (10 mg), and **14** (6 mg). Fraction 3-3 was further purified in the same way as fraction 3-2 to give **6** (2 mg), **8** (80 mg), **9** (68 mg), **10** (8 mg), **15** (5 mg), **16** (8 mg), and **17** (4 mg). Fraction 3-4 was separated with silica gel using EtOAc/MeOH/H_2O (7:2:1) to give **1** (57 mg) and **2** (450 mg). Fraction 5 was rechromatographed on ODS CC [H_2O/MeOH (2:1)] to separate into four fractions (fractions 5-1 to 5-4). Fraction 5-2 was purified on the preparative HPLC [H_2O/MeOH (9:1)] to afford **3** (11 mg), **4** (4 mg), and **5** (57 mg), respectively.

Quercetin 3-*O*-β-D-Glucopyranoside (Isoquercitrin) (1) (*15, 16*). Yellow needles. APCI-MS (negative): *m*/*z* 463 $[M - H]^-$.

Quercetin 3-*O*-α-L-Rhamnopyranosyl-(1→6)-β-D-glucopyranoside (Rutin) (2) (*15, 17*). Yellow needles. APCI-MS (negative): *m*/*z* 609 $[M - H]^-$.

3-*O*-Caffeoylquinic Acid (Neochlorogenic Acid) (3) (*18*−*20*). White powder. APCI-MS (negative): *m*/*z* 353 $[M - H]^-$.

4-*O*-Caffeoylquinic Acid (Cryptochlorogenic Acid) (4) (*18*−*20*). White powder. APCI-MS (negative): *m*/*z* 353 $[M - H]^-$.

5-*O*-Caffeoylquinic Acid (Chlorogenic Acid) (5) (*18*−*20*). White powder. APCI-MS (negative): *m*/*z* 353 $[M - H]^-$.

Cnidioside A (6) (*21*). Amorphous powder; $[\alpha]^{22}_D$ −34.2° (*c* 0.1, H_2O). APCI-MS (negative): *m*/*z* 367 $[M - H]^-$.

Praeroside II (7) (*22, 23*). White powder; $[\alpha]^{24}_D$ −18.3° (*c* 0.1, MeOH). APCI-MS (positive): *m*/*z* 425 $[M + H]^+$. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 1.40 and 1.41 (each 3H, s, *gem*-Me_2), 2.92 (1H, dd, *J* = 9.4, 9.8 Hz, GLC H-4), 3.32 (1H, dd, *J* = 9.4, 9.7 Hz, GLC H-3), 3.48 (1H, dd, *J* = 7.8, 9.7 Hz, GLC H-2), 3.51 (1H, m, GLC H-5), 3.62 (1H, dd, *J* = 5.7, 12.4 Hz, GLC H-6), 3.71 (1H, dd, *J* = 2.1, 12.4 Hz, GLC H-6), 3.90 (1H, d, *J* = 4.3 Hz, H-3′), 4.54 (1H, d, *J* = 7.8 Hz, GLC H-1), 5.17 (1H, d, *J* = 4.3 Hz, H-4′), 6.27 (1H, d, *J* = 9.5 Hz, H-3), 6.77 (1H, d, *J* = 8.8 Hz, H-6), 7.54 (1H, d, *J* = 8.8 Hz, H-5), 7.96 (1H, d, *J* = 9.5 Hz, H-4). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 21.9 and 26.7 (*gem*-Me_2), 58.1 (C-4′), 61.3 (GLC C-6), 70.2 (GLC C-4), 73.8 (GLC C-2), 76.7 (GLC C-3), 77.1 (GLC C-5), 77.6 (C-2′), 78.2 (C-3′), 101.6 (GLC C-1), 110.6 (C-8), 112.0 (C-10), 113.4 (C-6), 112.0 (C-3), 129.1 (C-5), 144.0 (C-4), 152.4 (C-9), 155.6 (C-7), 160.0 (C-2).

Praeroside III (8) (*23*). White powder; $[\alpha]^{24}_D$ −35.2° (*c* 1.0, MeOH). APCI-MS (positive): *m*/*z* 425 $[M + H]^+$. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 1.40 and 1.47 (each 3H, s, *gem*-Me_2), 2.89 (1H, dd, *J* = 9.5, 9.8 Hz, GLC H-4), 3.18 (1H, dd, *J* = 9.5, 9.8 Hz, GLC H-3), 3.23 (1H, dd, *J* = 7.8, 9.5 Hz, GLC H-2), 3.47 (1H, m, GLC H-5), 3.61 (1H, dd, *J* = 5.6, 12.3 Hz, GLC H-6), 3.68 (1H, dd, *J* = 2.1, 12.3 Hz, GLC H-6), 3.75 (1H, d, *J* = 2.9 Hz, H-3′), 4.36 (1H, d, *J* = 7.8 Hz, GLC H-1), 4.98 (1H, d, *J* = 2.9 Hz, H-4′), 6.26 (1H, d, *J* = 9.5 Hz, H-3), 6.76 (1H, d, *J* = 8.6 Hz, H-6), 7.51 (1H, d, *J* = 8.6 Hz, H-5), 7.95 (1H, d, *J* = 9.5 Hz, H-4). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 23.9 and 24.2 (*gem*-Me_2), 61.2 (GLC C-6), 62.1 (C-4′), 70.0 (GLC C-4), 73.5 (GLC C-2), 76.7 (GLC C-3), 76.9 (GLC C-5), 77.7 (C-2′), 80.8 (C-3′), 104.6 (GLC C-1), 110.4 (C-8), 111.7 (C-3), 111.8 (C-10), 113.9 (C-6), 128.8 (C-5), 144.4 (C-4), 154.3 (C-9), 155.8 (C-7), 160.2 (C-2).

Apterin (9) (*24*). White powder; $[\alpha]^{24}_D$ −186.2° (*c* 0.9, H_2O). APCI-MS (positive): *m*/*z* 425 $[M + H]^+$. ^{1}H NMR (DMSO-d_6, 500 MHz): δ 1.48 and 1.49 (each 3H, s, *gem*-Me_2), 3.02 (1H, dd, *J* = 9.6, 9.8 Hz, GLC H-4), 3.34 (1H, dd, *J* = 9.6, 9.8 Hz, GLC H-3), 3.40 (1H, dd, *J* = 7.8, 9.6 Hz, GLC H-2), 3.50 (1H, m, GLC H-5), 3.63 (1H, dd, *J* = 5.6, 12.2 Hz, GLC H-6), 3.72 (1H, dd, *J* = 2.0, 12.2 Hz, GLC H-6), 4.52 (1H, d, *J* = 6.4 Hz, H-3′), 4.55 (1H, d, *J* = 7.8 Hz, GLC H-1), 5.23 (1H, d, *J* = 8.4 Hz, H-4′ OH), 5.45 (1H, dd, *J* = 6.4, 8.4 Hz, H-4′), 6.25 (1H, d, *J* = 9.5 Hz, H-3), 6.91 (1H, d, *J* = 8.3 Hz, H-6), 7.60 (1H, d, *J* = 8.3 Hz, H-5), 7.99 (1H, d, *J* = 9.5 Hz, H-4). ^{13}C NMR (DMSO-d_6, 125 MHz): δ 22.6 and 24.2 (*gem*-Me_2), 61.2 (GLC C-6), 68.0 (C-4′), 68.7 (GLC C-4), 74.1 (GLC C-2), 76.9 (GLC C-3), 77.1 (GLC C-5), 77.2 (C-2′), 91.9 (C-3′), 97.2 (GLC C-1), 107.3 (C-6), 111.6 (C-3), 112.7 (C-10), 116.6 (C-8), 131.8 (C-5), 144.2 (C-4), 150.6 (C-9), 159.8 (C-2), 162.8 (C-7).

Esculin (10) (*25*). White powder. APCI-MS (positive): *m*/*z* 341 $[M + H]^+$.

(*R*)-Peucedanol (11) (*6, 8, 26*). Amorphous powder; $[\alpha]^{23}_D$ +40.2° (*c* 0.1, EtOH). APCI-MS (positive): *m*/*z* 265 $[M + H]^+$.

(*R*)-Peucedanol 7-*O*-β-D-Glucopyranoside (12) (*8, 26*). Amorphous powder; $[\alpha]^{23}_D$ −14.2° (*c* 1.0, MeOH). APCI-MS (positive): *m*/*z* 427 $[M + H]^+$.

L-Tryptophan (13) (*27*). Colorless needles; $[\alpha]^{20}_D$ −30.2° (*c* 0.1, H_2O). APCI-MS (positive): *m*/*z* 205 $[M + H]^+$.

Uracil (14) (*28*). Colorless needles. APCI-MS (positive): *m*/*z* 113 $[M + H]^+$.

Guanosine (15) (*27*). Colorless needles; $[\alpha]^{20}_D$ −48.2° (*c* 0.1, H_2O). APCI-MS (positive): *m*/*z* 284 $[M + H]^+$.

Uridine (16) (*28*). Colorless needles; $[\alpha]^{21}_D$ +2.2° (*c* 0.1, MeOH). APCI-MS (positive): *m*/*z* 245 $[M + H]^+$.

Thymidine (17) (*28*). Colorless needles; $[\alpha]^{21}_D$ +17.8° (*c* 0.1, MeOH). APCI-MS (positive): *m*/*z* 243 $[M + H]^+$.

Quantitative Analysis Using HPLC. The standard solutions of compounds **1**−**5**, **8**−**10**, and **12** were prepared at 1 mg/mL in ethanol. They were diluted to make five concentrations (1.0, 12.5, 25.0, 50.0, and 100.0 μg/mL) for calibration curves. The *n*-butanol soluble fraction of *P. japonicum* leaves was dissolved in ethanol (1 mg/mL). All solutions were filtered through a cellulose acetate membrane filter (0.45 μm) (Advantec, Japan), and aliquots of the filtrate (10 μL) were injected to HPLC using CAPCELL PAK C_{18} UG80 (4.6 mm × 250 mm). These tested compounds in the *n*-butanol soluble fraction of this plant leaves were quantified by calibration with the standards. HPLC analysis was carried out according to the method in the previous report (*20*), with a slight modification to the mobile phase: A = 50 mM $NH_4H_2PO_4$ (pH 2.60), B = 80% acetonitrile and 20% A, and C = 50 mM phosphoric acid (pH 2.00). Elution was performed with the linear gradient as follows: 0.0 min, 100/0/0 (A/B/C, %/%/%), 4.0 min, 92/8/0, 10.0 min, 0/14/86, 22.5 min, 2/16/82, 27.5 min, 0/22/78, 45.0 min, 0/50/50, 47.5 min, 100/0/0, 75.0 min, 100/0/0. The flow rate was 1.0 mL/min. The column temperature was 40 °C. The detection was carried out by photodiode array (PDA) (200−600 nm). All of the above experiments were replicated three times each.

Evaluation of Scavenging Activity on DPPH Radical. The method was adopted from the previous report (*29*). DPPH in ethanol (200 μM, 75 μL) was added to the ethanolic sample solution (75 μL) in the 96 well microplate. The absorbance of the reaction mixtures at 520 nm was measured by a multilabel counter. The DPPH radical scavenging activity of each extract, soluble fraction, and positive reference (α-tocopherol and L-ascorbic acid) was determined for 30 min of reaction by comparing the absorbance with that of a blank containing only DPPH and solvent. The activity of the isolated compounds and positive references was evaluated until the reaction reached the steady state at room temperature. All analyses were run in triplicate.

Evaluation of Oxidation of Egg Yolk PC Liposomes Induced by AAPH. The method employed was slightly modified from that of the previous reports (*14*, *30*, *31*). The mixture of purified PC in hexane and cholesterol in chloroform/methanol (95:5, v/v) was evaporated in vacuo for 20 min to make the lipid film in 20 mL round-bottomed flask, and 10 mM Tris-HCl buffer (pH 7.4) containing 0.5 mM diethylenetriamine-*N*, *N*, *N*′,*N*″,*N*″-pentaacetic acid (DTPA) was added. After the suspension was mixed with a Vortex mixer for 1 min, the multilamellar vesicles (MLV) were obtained through ultrasonication for 1 min. The suspension of MLV was extruded 21 times through a polycarbonate filter (100 nm pore diameter) mounted in an extrusion apparatus (LiposoFast) (Avestin Inc., Ottawa, Canada) to prepare large unilamellar vesicles (LUV). The LUV suspension (500 μL), Tris-HCl buffer (350 μL), and ethanol solution of each extract and soluble fraction or isolated compounds (50 μL) were put into a test tube with a screw cap. After they were mixed, the mixture was preincubated at 37 °C for 10 min. The aqueous solution of AAPH (200 mM, 100 μL) was added, and the reaction was performed for 6 h at 37 °C in the dark with continuous shaking. The final concentrations in the incubation mixture were as follows: PC (5 mM), cholesterol (2.5 mM); extracts, fractions (each 20 μg), or isolated compounds (50 μM); and AAPH (20 mM). At regular intervals, 25 μL of each reaction mixture was withdrawn and added to 75 μL of methanol. An aliquot of the mixture (10 μL) was injected into HPLC [column, TSK-gel Octyl 80Ts (4.6 mm × 150 mm, TOSOH, Tokyo, Japan); elution, methanol/water (95:5, v/v); flow rate, 1 mL/min; detection, 235 nm]. The amount of PC−OOH was estimated with calibration curves prepared with standard PC−OOH solutions. All tests were run in triplicate and averaged.

Statistical Analysis. Multiple comparisons were by one way analysis of variance, followed by Scheffe test using StatView (SAS Institute Inc., Cary, NC). Significance was established at $p < 0.01$.

RESULTS AND DISCUSSION

The antioxidant properties of the extracts and fractions obtained from *P. japonicum* leaves together with positive references, α-tocopherol, and L-ascorbic acid were evaluated by the DPPH radical scavenging activity and by the inhibitory effect on oxidation of liposome. The DPPH radical scavenging method is one of the simplest methods to evaluate the hydrogen-donating capacity of antioxidants that are responsible for the chain-breaking activity of oxidation (*29*). As shown in **Figure 1**, the activity at 30 min reaction of the DPPH radical scavenging assay decreased in the order L-ascorbic acid > α-tocopherol > *n*-butanol soluble fraction > ethyl acetate soluble fraction > water soluble fraction > dichloromethane extract > *n*-hexane extract. In the case of the antioxidant assay using AAPH-induced PC liposome peroxidation system, the *n*-butanol soluble fraction showed the highest activity, followed by the ethyl acetate soluble fraction, α-tocopherol, *n*-hexane extract, water soluble fraction, dichloromethane extract, and L-ascorbic acid (**Figure 2**). The *n*-butanol soluble fraction was found to show a strong activity in both assay systems.

The active *n*-butanol soluble fraction was separated into seven fractions (fractions 1−7) by silica gel CC. All fractions except fraction 1 showed a strong activity for the DPPH radical scavenging (**Figure 3**). As the amount of the active fractions 3 and 5 was larger than that of other fractions, both fractions were

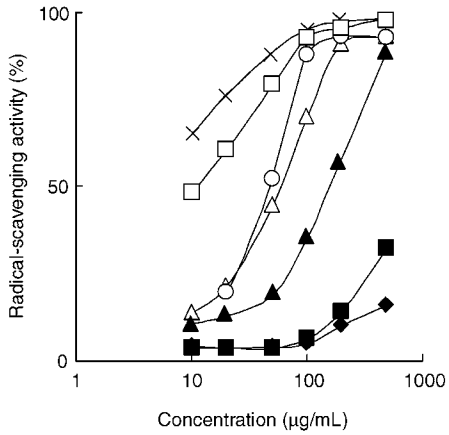

Figure 1. DPPH radical scavenging activity of each extract and soluble fraction from *P. japonicum* leaves, α-tocopherol, and L-ascorbic acid at 30 min of reaction. The reaction mixture consisted of DPPH radical (100 μM) and the following extracts and soluble fractions with different concentrations (10–500 μg/mL): ◆, hexane extract; ■, dichloromethane extract; △, ethyl acetate extract; ○, *n*-butanol soluble fraction; ▲, water soluble fraction; □, α-tocopherol; and ×, L-ascorbic acid. All tests were run in triplicate and averaged.

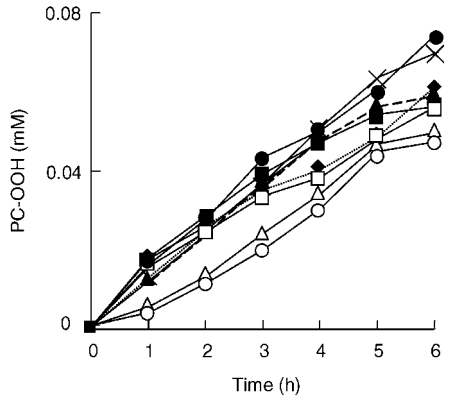

Figure 2. Effect of each extract and soluble fraction from *P. japonicum* leaves, α-tocopherol, and L-ascorbic acid on the AAPH-induced peroxidation of egg yolk PC liposomes. The reaction mixture consisted of egg yolk PC (5 mM), AAPH (20 mM), and extracts and soluble fractions (20 μg) in 10 mM Tris-HCl (pH 7.4) containing 0.5 mM DTPA: ◆, hexane extract; ■, dichloromethane extract; △, ethyl acetate extract; ○, *n*-butanol soluble fraction; ▲, water soluble fraction; □, α-tocopherol; ×, L-ascorbic acid; and ●, control. All tests were run in triplicate and averaged.

purified by repeated chromatographies to afford 17 compounds, quercetin 3-*O*-β-D-glucopyranoside (isoquercitrin) (**1**) (*15*, *16*); quercetin 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside (rutin) (**2**) (*15*, *17*); 3-*O*-caffeoylquinic acid (neochlorogenic acid) (**3**) (*18*−*20*); 4-*O*-caffeoylquinic acid (cryptochlorogenic acid) (**4**) (*18*−*20*); 5-*O*-caffeoylquinic acid (chlorogenic acid) (**5**) (*18*−*20*); cnidioside A (**6**) (*21*); esculin (**10**) (*25*); (*R*)-peucedanol (**11**) (*6*, *8*, *26*); (*R*)-peucedanol 7-*O*-β-D-glucopyranoside (**12**) (*8*, *26*); L-tryptophan (**13**) (*27*); uracil (**14**) (*28*); guanosine (**15**) (*27*); uridine (**16**) (*28*); and thymidine (**17**) (*28*), which were identified on the basis of their NMR and MS spectral data.

Compounds **7**−**9** were found to have the same molecular formula, $C_{20}H_{24}O_{10}$. The UV absorption pattern of **7** given by the PDA detector was very similar to that of 7-oxycoumarin. Signals characteristic of a *Z*-double bond [δ 6.27 and 7.96 (each 1H, d, $J = 9.5$ Hz)], a pair of *ortho* protons [δ 6.77 and 7.54 (each 1H, d, $J = 8.8$ Hz)] in the ^{1}H NMR, and nine carbon

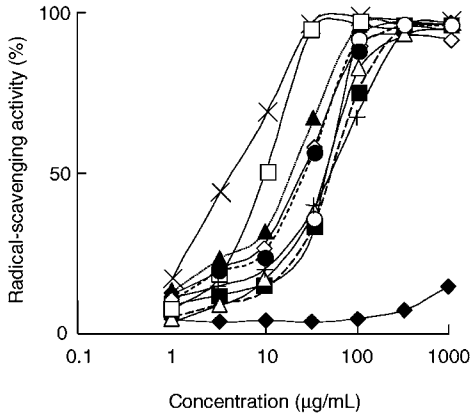

Figure 3. DPPH radical scavenging activity of the *n*-butanol soluble fraction (fractions 1–7) of *P. japonicum* leaves, α-tocopherol, and L-ascorbic acid at 30 min of reaction. The reaction mixture consisted of DPPH radical (100 μM) and fractions (1–1000 μg/mL): ◆, fraction 1; ■, fraction 2; △, fraction 3; +, fraction 4; ▲, fraction 5; ●, fraction 6; ◇, fraction 7; ○, *n*-butanol soluble fraction; □, α-tocopherol; and ×, L-ascorbic acid. All tests were run in triplicate and averaged.

signals in the ^{13}C NMR spectra of **7** suggested the presence of a 7-oxycoumarin skeleton. Furthermore, the signals at δ 3.90 and 5.17 (each 1H, d, J = 4.3 Hz) and δ 1.40 and 1.41 (each 3H, s) were assigned to two oxymethine and *gem*-dimethyl protons, respectively. Complex signals at δ 2.92−3.71 and a large coupling constant (7.8 Hz) of a doublet signal at δ 4.54 suggested the presence of a β-glucopyranosyl residue. These ^{1}H NMR data together with the ^{13}C NMR data indicated that compound **7** was an angular type of dihydropyranocoumarin with a β-glucopyranosyl moiety. In the ^{1}H NMR spectrum, the coupling constant (4.3 Hz) of the oxymethine protons [δ 3.90 (H-3′) and 5.17 (H-4′)] and the relatively close separated singlets (Δ = 0.01) of the 2′-*gem*-dimethyl protons (δ 1.40 and 1.41) indicated a *cis*-configuration at C-3′ and C-4′, according to González et al. (*32*). The heteronuclear multiple bond coherence (HMBC) analysis revealed the glucopyranosyl moiety attached to the hydroxyl group on C-3′ of dihydropyran moiety. All of the signals have been confirmed by the heteronuclear multiple quantum coherence (HMQC) and HMBC data. Compound **7** was thus characterized as *cis*-khellactone 3′-*O*-β-D-glucopyranoside (praeroside II), isolated from the fruits of *Ammi visnaga* (L.) LAM. and the root of *Peucedanum praeruptorum* Dunn. (*22*, *23*).

Compound **8** was identified as a stereoisomer of **7**. The ^{1}H NMR spectrum of **8** showed two largely separated singlets (Δ = 0.07) of the 2′-*gem*-dimethyl group. The signals at δ 3.75 and 4.98 (each 1H, d) with the coupling constant (2.9 Hz) revealed a *trans*-configuration between C-3′ and C-4′. These data suggested compound **8** to be *trans*-khellactone 3′-*O*-β-D-glucopyranoside (praeroside III), previously isolated from the root of *P. praeruptorum* Dunn. (*23*).

The NMR data of **9** were also similar to those of compounds **7** and **8**, except for significant downfield shifted signals at δ 91.9 (C-3′) and 68.0 (C-4′) in the ^{13}C NMR spectrum and the proton resonances at δ 4.52 and 5.45 (each 1H, d, J = 6.4 Hz) attached to C-3′ and C-4′ in the ^{1}H NMR spectrum, respectively. These data suggested **9** to be an angular type of dihydrofuranocoumarin glucopyranoside. The β-glucopyranosyl moiety was linked to the quaternary carbon of the 3′-isopropyl unit. These assignments were confirmed by the 2D NMR spectra data of **9**. This chemical structure was elucidated as apterin by comparing the ^{1}H and ^{13}C NMR data and its optical rotation with those in

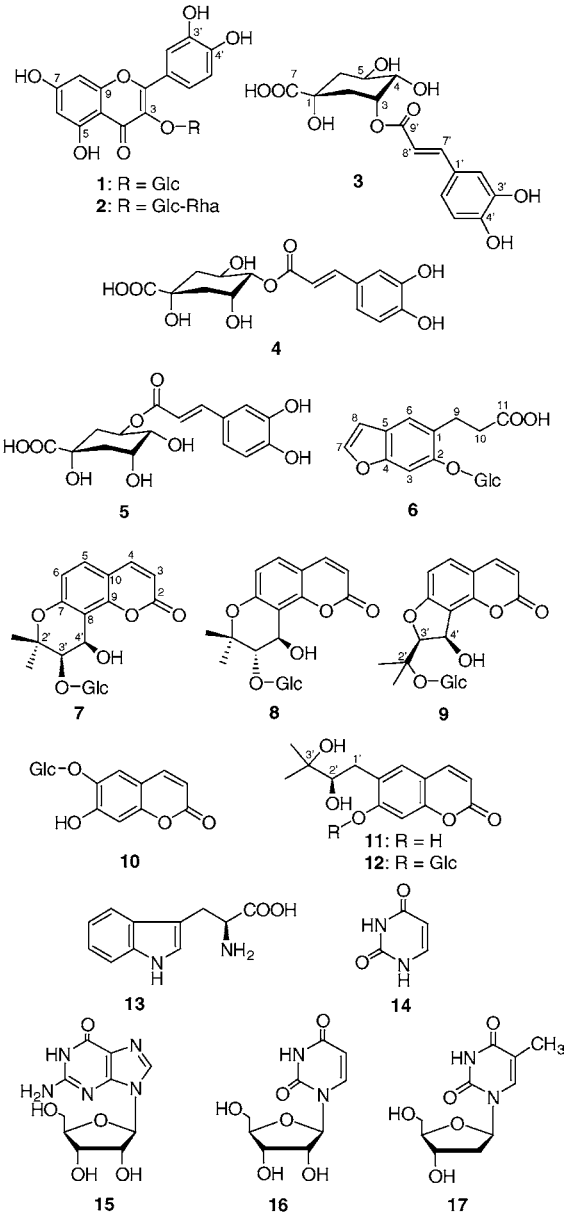

Figure 4. Structure of chemical components isolated from the *n*-butanol soluble fraction of *P. japonicum* leaves.

the literature (*24*). **Figure 4** shows the structures of the isolated compounds from the *n*-butanol soluble fraction. This is the first report of the isolation of compounds **1**−**10** and **13**−**17** from *P. japonicum*.

Seventeen compounds isolated from the *n*-butanol soluble fraction were quantified by HPLC analysis using a C_{18} column. The amount of compounds **1**−**5**, **8**−**10**, and **12** were completely calculated based on the linear relationship between the peak areas and the injected concentrations (**Table 1**). The correlation factors for each compound were high (R^2 = 0.983−0.999). Retention times in the HPLC analysis were 14.1 min for compound **3**, 15.3 min for **10**, 18.4 min for **5**, 19.4 min for **4**, 22.9 min for **12**, 32.0 min for **9**, 33.2 min for **8**, 34.2 min for **2**, and 35.0 min for **1** (**Figure 5**). The *n*-butanol soluble fraction contained the largest amount of **5** (102.70 ± 6.58 mg/g) and was characterized by relatively high amounts of caffeoylquinic acid isomers (**3**−**5**) and **2**.

The antiradical efficiency of the isolated compounds was estimated by the DPPH radical scavenging method (*29*). Recent studies indicated that the analysis of kinetics of free radical

Table 1. Amounts of the Isolated Compounds Obtained from the *n*-Butanol Soluble Fraction of *P. japonicum* Leaves

compd	amounts (mg/g[a] ± SD)	compd	amounts (mg/g[a] ± SD)
1	3.30 ± 0.37	**8**	13.75 ± 0.86
2	44.31 ± 2.74	**9**	14.75 ± 1.00
3	42.82 ± 1.84	**10**	3.61 ± 1.60
4	50.40 ± 2.60	**12**	8.75 ± 0.58
5	102.70 ± 6.58		

[a] Dry weight of the *n*-butanol soluble fraction of *P. japonicum* leaves. Values were determined from integration of HPLC signals and response factors calculated from standards. The results are three separate experiments.

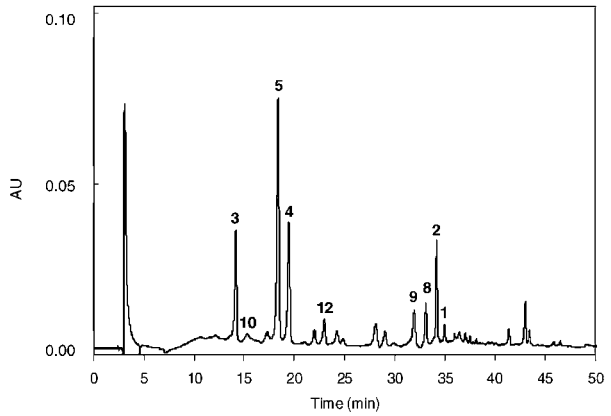

Figure 5. HPLC chromatogram of the *n*-butanol soluble fraction of *P. japonicum* leaves. Isoquercitrin (**1**), rutin (**2**), 3-*O*-caffeoylquinic acid (**3**), 4-*O*-caffeoylquinic acid (**4**), 5-*O*-caffeoylquinic acid (**5**), praeroside III (**8**), apterin (**9**), esculin (**10**), and (*R*)-peucedanol 7-*O*-*β*-D-glucopyranoside (**12**) were detected by PDA (at 316 nm).

scavenging of test compounds is important for a better understanding of their hydrogen-donating capacity as well as the determination of their EC_{50}. Brand-Williams et al. suggested that EC_{50} should be measured at a steady state of the reaction because the kinetic behavior depends on the structure of antioxidant (*33*, *34*). In addition, Sánchez-Moreno et al. proposed that the time to reach a steady state should simultaneously be taken into account to evaluate the antiradical efficiency, considering that not only concentration but also time to reach a steady state are important factors to define antioxidant capacity (*35*). Therefore, we examined the DPPH radical scavenging assay including their kinetic behaviors of the compounds isolated from *P. japonicum* together with positive references, L-ascorbic acid and α-tocopherol. The amount of remaining DPPH radicals was measured at regular intervals until each reaction reached a plateau at every concentration of 12.5, 25.0, and 50.0 μM test compounds. **Figure 6** shows some typical kinetic behaviors of the isolated compounds and the positive references. The DPPH radical scavenging activity at the steady state of reaction and time needed to reach the steady state of test compounds at 25.0 μM was indicated in **Table 2**. The test compounds could be classified into six types (groups A−F) based on their kinetic characteristics. Compounds **1** and **2** belonged to group A, **3**−**5** to B, **6**−**15** to C, **16** and **17** to D, α-tocopherol to E, and L-ascorbic acid to F. It is recognized that the radical scavenging activity largely depends on the number of phenolic hydroxyl groups (*36*) and that preferentially C4−OH on the B-ring and C3−OH on the C-ring contribute to the scavenging activity in the case of flavonoid (*37*). There was no difference in the activity between compounds **1** and **2**, which indicated that the different sugar moiety on C3 might not affect

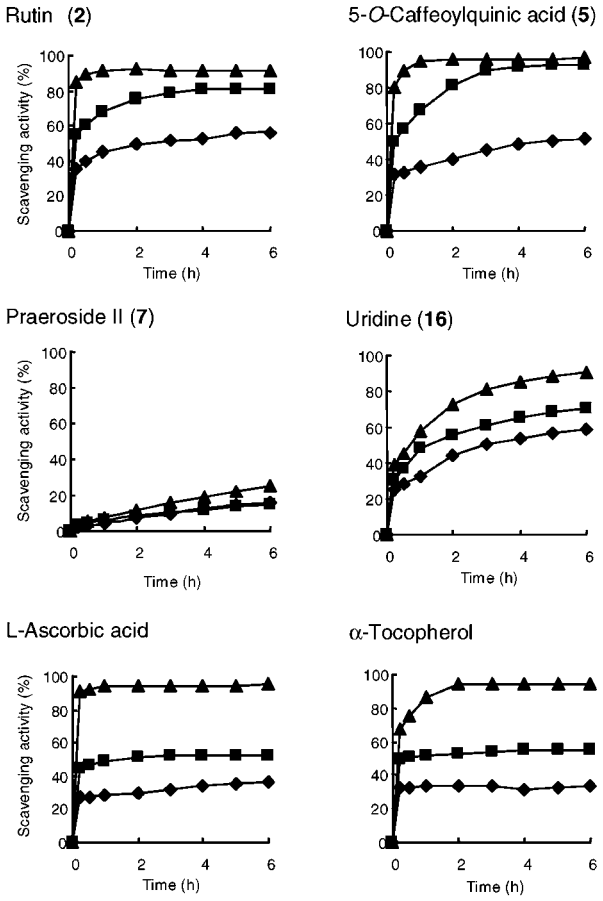

Figure 6. Example of the kinetic behavior of the isolated compounds from *P. japonicum* leaves, α-tocopherol, and L-ascorbic acid on DPPH radical scavenging activity. The final concentration of DPPH ethanol solution was 100 μM. Tested compounds were added at ▲, 12.5 μM; ■, 25.0 μM; and ◆, 50.0 μM. Each value was the mean of triplicate measurement.

the activity. Compounds belonging to groups A and B showed almost the same time to reach a steady state at every concentration, but the kinetic behavior was a little different between groups A and B. In the early stage (0−1 h) of the reaction, group A showed a slightly higher activity than group B, but their activities were reversed during 1−5 h. The kinetic behaviors of caffeoylquinic acid isomers (**3**−**5**) indicated that the position of esterification with caffeic acid on quinic acid moiety did not affect their antiradical activity. Monocaffeoylquinic acids might be explainable by only the capacity of radical scavenging of the caffeoyl moiety. In the case of dicaffeoylquinic acid isomers, it is reported that the position of caffeoyl group affected the activity due to their steric hindrance (*37*). Compounds **6**−**15** belonging to group C did not show the end of reaction even after 24 h, and the scavenging activity was low. L-Ascorbic acid and α-tocopherol reacted rapidly with DPPH radical in a short time. In particular, L-ascorbic acid scavenged the radical very fast in the test compounds. In the case of group D, **16** and **17** showed little activity in the earlier stage of the reaction, but their activity gradually increased to reach a plateau at 48.0 and 24.0 h, respectively. Pyrones and ascorbate derivatives were also reported as slow-reacting scavengers such as **16** and **17** (*38*, *39*). There is no report so far on the antiradical activity of **16** and **17**. This observation requires further experimental verification.

The activity of the isolated compounds was also evaluated by AAPH-induced PC liposome peroxidation system. The

Table 2. DPPH Radical Scavenging Activity at the Steady State of Reaction and Time Needed to Reach the Steady State of the Isolated Compounds from *P. japonicum* Leaves, α-Tocopherol, and L-Ascorbic Acid at 25 μM

compd	DPPH radical scavenging activity[a,b] (% ± SD)	time (h)
1	86.4 ±1.2	5.0
2	80.9 ± 0.8	5.0
3	92.1 ± 2.2	5.0
4	89.8 ± 1.9	5.0
5	91.0 ± 1.6	5.0
6	12.2 ± 3.3	24.0
7	5.9 ± 1.8	>48.0
8	4.5 ± 2.6	>48.0
9	10.2 ± 1.4	>48.0
10	4.4 ± 0.6	24.0
11	4.7 ± 1.0	24.0
12	9.1 ± 2.1	>48.0
13	7.5 ± 1.9	24.0
14	20.3 ± 3.5	>48.0
15	27.8 ± 3.2	>48.0
16	79.5 ± 1.5	48.0
17	42.8 ± 1.3	24.0
α-tocopherol	52.2 ± 0.8	3.0
L-ascorbic acid	51.0 ± 9.5	0.3

[a] The final concentration of DPPH ethanol solution was 100 μM. [b] DPPH radical scavenging activity (%) was the mean of three replications.

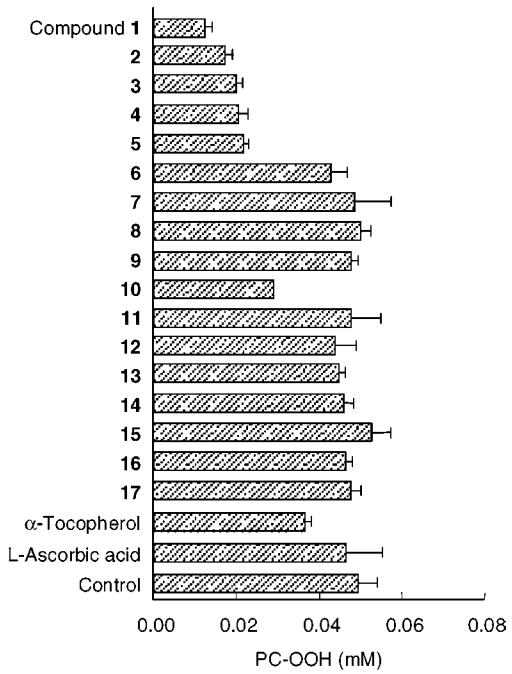

Figure 7. Effect of the isolated compounds from *P. japonicum* leaves, α-tocopherol, and L-ascorbic acid on the AAPH-induced peroxidation of egg yolk PC liposomes. The reaction mixture consisted of egg yolk PC (5 mM), AAPH (20 mM), and tested compound (50 μM) in 10 mM Tris-HCl (pH 7.4) containing 0.5 mM DTPA. Each column expressed the mean of the amounts of PC–OOH after 4 h of incubation based on triplicate measurements.

liposomal system has been recognized as a model of phospholipid bilayers constituting cellular and subcellular membranes (*40*). After 4 h of incubation, compounds **1**−**5** showed a higher activity than α-tocopherol and L-ascorbic acid, as shown in **Figure 7**. There were no significant differences among three caffeoylquinic acid isomers **3**−**5** as shown in the DPPH radical scavenging assay (**Table 2**). Compounds **16** and **17** did not show any activity in the PC liposome system, although **16** and **17** showed the scavenging activity on DPPH radical. This result suggests that slow-reacting scavengers as **16** and **17** might not be available in lipid peroxidation. In the AAPH-induced lipid peroxidation of the PC liposome system, it is recognized that the antioxidant activity depends not only on the presence of *ortho*-dihydroxyphenyl group but also on the hydrophobicity and the affinity of antioxidants to lipid bilayers (*41*). AAPH, an azo compound, has been used as a hydrophilic radical initiator, which produces peroxyl radicals in the aqueous phase, and these radicals attack phospholipids on the membrane surface (*42*). It is suggested that compounds **1**−**5**, hydrophilic antioxidants, are located on the surface of the membrane and showed strong activity toward radicals generated in the aqueous phase of the liposomes (*30*, *43*).

Consequently, 17 compounds were isolated from the *P. japonicum* leaves and 15 compounds (**1**−**10** and **13**−**17**) were newly identified as the constituents of this plant. Rutin (**2**) and caffeoylquinic acid isomers (**3**−**5**) were found to be the major antioxidative constituents and play important roles in the potent antioxidant activity of *P. japonicum* leaves.

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