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Note

Isolation and Measurement of Quercetin Glucosides in Flower Buds of Japanese Butterbur (*Petasites japonicus* subsp. *gigantea* Kitam.)

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Three quercetin glucosides were isolated from flower buds of Japanese butterbur (*Petasites japonicus* subsp. *gigantea* Kitam.) together with caffeic acid as the ingredients that had DPPH radical scavenging activity, using the DPPH-HPLC method for measuring the radical scavenging activity. These quercetin glucosides were identified as quercetin 3-*O*-*β*-D-glucoside, quercetin 3-*O*-*β*-D-6″-*O*-acetylglucoside, and rutin, and the amounts of the glucosides in flower buds were also examined by HPLC. The flower buds were harvested from four different sites, the total amount of quercetin glucosides in each site was 100–170 mg/100 g fr. wt., and there were no great differences of the amounts between growing fields

Key words: *Petasites japonicus*; Japanese butterbur; quercetin glucoside; edible wild plant

Secondary metabolites synthesized by plants are thought to have lots of benefits for human nutrition, health, and disease prevention. Especially antioxidants isolated from natural resources attract considerable attention because several spieces of active oxygens are thought to be harmful for human health and trigger many diseases, for example, diabetes, coronary heart disease, and cancer. But recent epidemical studies have shown quite big possibilities that we could prevent the diseases by taking daily foods which contain high quantities of antioxidants. The recent topic of the ''French paradox'' is a very famous matter associated with these possibilities,[1,2] and it has been revealed that phenolic antioxidants in wine played key roles in preventing these diseases. In order to find health-promoting foods from edible wild plants, we collected the plants and checked DPPH radical scavenging activity, because compounds that had DPPH radical scavenging activity were generally admitted as antioxidants. In the course of our research, we found some plants that showed the DPPH radical scavenging activity. In this paper, we report the ingredients that possessed DPPH radical scavenging activities isolated from flower buds of Japanese butterbur (*Petasites japonicus* subsp. *gigantea* Kitam.) and measurments of quercetin glucosides in the flower buds of *P. japonicus*.

In the course of our survey for the scavengers of DPPH radicals from edible wild plants, DPPH radical scavenging activities of flower buds of *Petasites japonicus* subsp. *gigantea* Kitam., young buds of *Acanthopanax sciadophylloides* and *Evodiopanax innovans*, and aerial parts of *Matteuccia struthiopteris, Allium victorialis, Anemone flaccida, Smilacina japonica, Caltha palustris, Artemisia montana, Symphytum officinale, Cacalia delphiniifolia*, and *Cacalia hastata*, that were well known as edible wild plants in spring, were examined using the DPPH-HPLC method reported by Yamaguchi *et al*.[3] The results are given in Table 1, and among of them the extract of the flower buds of *P. japonicus* showed the best activity, and based on this first screening data, we decided to isolate the active ingredients from the flower buds of *P. japonicus*.

DPPH radical scavenging activities for all fractions purified according to the isolation procedures were examined using the DPPH-HPLC method, and according to the isolation procedure (Fig. 1), compounds **1** (123 mg), **2** (154 mg), and **3** (408 mg) were isolated from the EtOAc-extract of the flower buds (6.5 kg, fresh weight), and compound **4** (118 mg) from the H_2O-extract. Compounds **1** and **4** were identified to be caffeic acid and rutin, respectively,

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Abbreviations: DPPH, 1,1-diphenyl-2-picrylhydrazyl; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile)

Table 1. DPPH Radical Scavenging Activities (%) of Edible Wild Plants

	EtOAc extracts[a)]	H_2O extracts[b)]
Petasites japonicus	69	70
Acanthopanax sciadophylloides	5	10
Evodiopanax innovans	6	3
Matteuccia struthiopteris	4	60
Allium victoriails	1	5
Anemone flaccida	41	50
Smilacina japonica	23	13
Caltha palustris	42	16
Artemisia montana	47	39
Symphytum officinale	27	25
Cacalia delphiniifolia	60	35
Cacalia hastata	45	5

a) a portion (50 mg fr. wt. equivalent) of EtOAc extracts were used.
b) a portion (10 mg fr. wt. equivalent) of H_2O extracts were used.

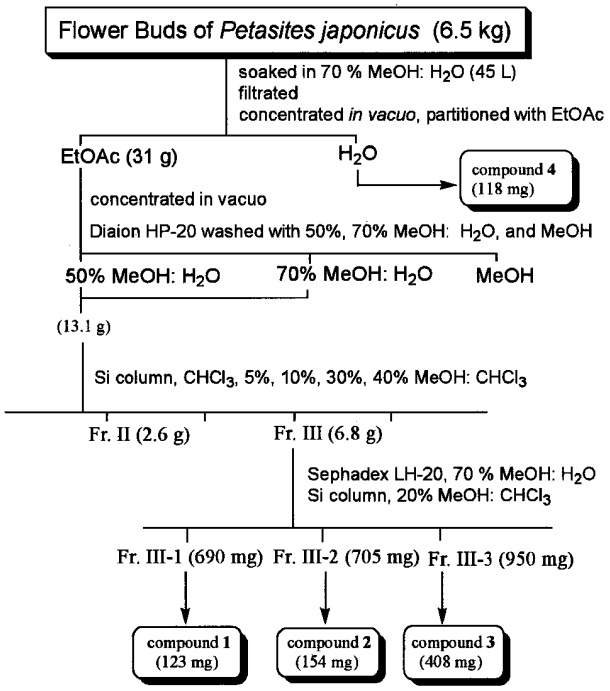
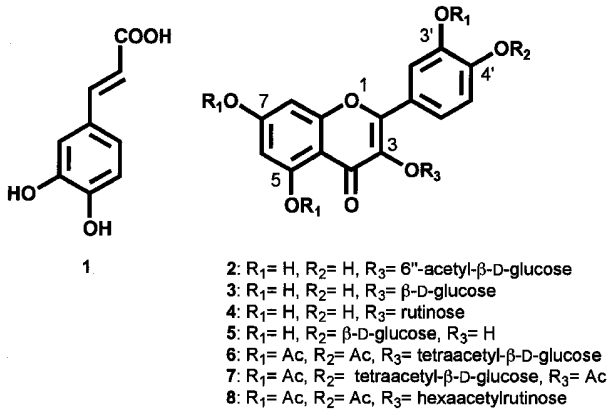


Fig. 1. Isolation Procedure for Compounds **1**–**4**.



1

2: R_1= H, R_2= H, R_3= 6"-acetyl-β-D-glucose
3: R_1= H, R_2= H, R_3= β-D-glucose
4: R_1= H, R_2= H, R_3= rutinose
5: R_1= H, R_2= β-D-glucose, R_3= H
6: R_1= Ac, R_2= Ac, R_3= tetraacetyl-β-D-glucose
7: R_1= Ac, R_2= tetraacetyl-β-D-glucose, R_3= Ac
8: R_1= Ac, R_2= Ac, R_3= hexaacetylrutinose

Fig. 2. Chemical Structures of **1**–**8**.

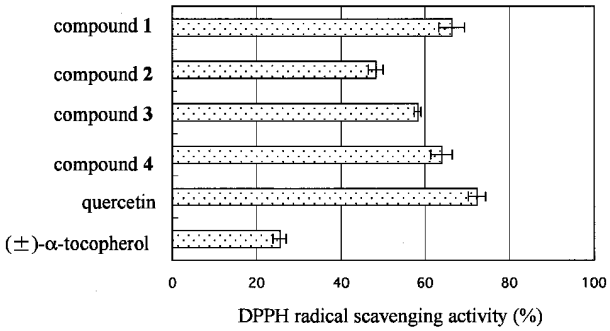


Fig. 3. DPPH Radical-scavenging Activities of 1–4, Quercetin and (±)-α-Tocopherol.

The concentrations for the measurements of DPPH radical scavenging activities were 2×10^{-5} M. Bars represent the means ±SD of four measurements.

because the FDMS and ^{1}H-, ^{13}C-NMR data of **1** and **4** respectively matched those of authentic specimens obtained form caffeic acid and rutin (Fig. 2). Identifications of **2** and **3** were done by comparing physical and spectroscopic data with those of the reported data[4,5] to identify the structures as quercetin 3-*O*-β-D-6″-*O*-acetylglucoside and quercetin 3-*O*-β-D-glucoside, respectively (Fig. 2). The assignments of ^{1}H- and ^{13}C-NMR of **3** were decided by the analysis of HMBC and HMQC experiments, and the assignments of **2** were done by comparing the data with those of **3**. DPPH radical scavenging activity was observed in the fraction of Fr. II, and methyl caffeate was isolated from Fr. II as an active compound. But we decided the isolation of methyl caffeate was due to an artifact, because methyl caffeate could not be obtained when acetone was used for the extraction of the flower buds.

Compounds **1**–**4** were isolated from flower buds of Japanese butterbur (*Petasites japonicus* subsp. *gigantea* Kitam.), and to our best knowledge, this is the first report of the isolation of **2** and **3** from the genus *Petasites*. These compounds are classified into cinnamic acid derivative and flavonoids, which are well known as widely distributed substances in the plant kingdom. DPPH radical scavenging activities of the isolated compounds (**1**–**4**) were examined, using quercetin and (±)-α-tocopherol as positive controls. The activities were measured at the concentration of 2×10^{-5} M, and the results are given in Fig. 3. Using other methods, the antioxidant activities of quercetin glucosides, quercetin, and (±)-α-tocopherol have been examined. Tournaire *et al.*[6] concluded that the presence of a catechol moiety in the B-ring is the main factor for controlling the efficiency of $^1O_2$ scavenging, and also the presence of a 3-hydroxyl moiety is important for the activity of

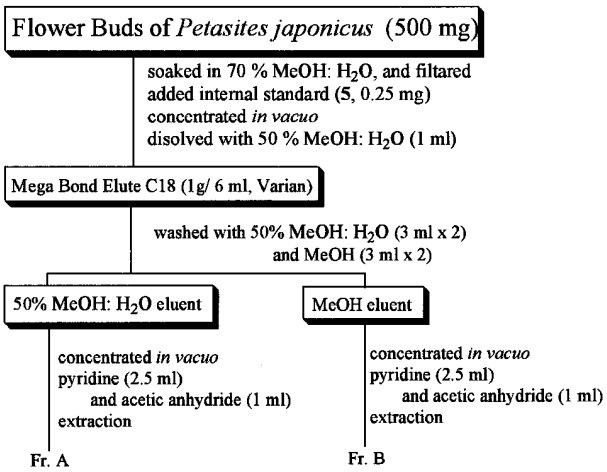


Fig. 4. Procedure for Measurement of Compounds **2**–**4**.

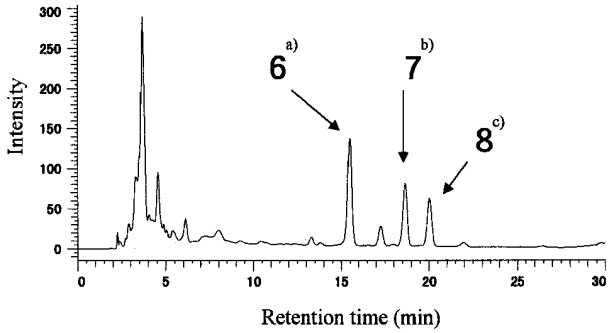


Fig. 5. HPLC Profile Analyzing of Fr. A.
The UV-absorbance was monitored at 310 nm
a) derived from compounds **2** and **3**.
b) derived from internal standard (**5**).
c) derived from rutin (**4**).

$^1O_2$ scavenging. This tendency coincides with that of the result of **2**–**4** and quercetin in Fig 3. This result was also supported by the reports written by Masuda *et al.*[7], in which the activities of the quercetin glucosides were compared with that of quercetin using the method of measuring the inhibitory activities of AMVN-induced oxidation of methyl linoleate, and in that case quercetin showed higher inhibitory activity than quercetin glucosides. DPPH radical scavenging activities have been also demonstrated for caffeic acid and (±)-α-tocopherol by other groups.[8,9] They have reported that caffeic acid showed greater scavenging activity than (±)-α-tocopherol, but less activity on the inhibition of the formation of conjugated diene derived from linoleic acid than (±)-α-tocopherol. As far as DPPH radical scavenging activity, the results of Fig. 3 led the conclusion that compounds **1**–**4** had higher DPPH radical scavenging activities than that of (±)-α-tocopherol, but were less active than quercetin. Although we reported as above, this conclusion should be limited as DPPH radical scavenging activity, because we did not evaluate **1**–**4** by other methods and conditions.

In recent years, a lot of biological activities of flavonoids in foods such as antioxidative activity,[10] antimicrobial and antiviral activities,[11] anti-inflammatory activities,[12] anti-allergic activities,[13] and antitumor activities[14] have been demonstrated, and recent topics of flavonoids were reviewed by Harborne and Williams.[15] These reports urged us to examine the amounts of the quercetin glucosides in flower buds in *P. japonicus*. In order to quantify the amounts and inspect the difference depending on the growing fields, the samples were collected from four different sites (Nopporo area of Sapporo, Megumino area of Eniwa, Naganuma town, and Rawan area of Ashoro town) in Hokkaido. According to Fig. 4, the purification and derivatization of quercetin glucosides were done. Since there were inseparable compounds accompanying **2** and **3**, the derivatizations of **2**–**4** were done using a solution of pyridine and acetic anhydride. When portions of Fr. B were analyzed, there were no peaks that corresponded to the acetylated quercetin glucosides, but the peaks that corresponded to the acetylated quercetin glucosides (**6**–**8**) were observed when the portions of Fr. A were analyzed (Fig. 5). The recovery of each experiment was calculated from the ratio of the areas of the acetylated internal standard (**7**), and the recovery rates were always more than 65%. The amount of the each acetylated compound was evaluated from the ratio of the peak area that corresponded to each acetylated compound, and the values given in Table 2 were recalculated based on the recovery rates and represented the amounts of **2** plus **3**, and **4**. The total amounts of the glucosides were estimated to be 100–170 mg/100 g fr. wt., and there were no great differences of the amount between the growing fields. Because of the relatively high amounts of quercetin glucosides, the flower bud of Japanese butterbur might have the possibility to become a health-promoting food. But we also have to pay attention to the carcinogenic activity of flower buds of *P. japonicus*. A carcinogenic compound was isolated from *P. japonicus* Maxim. and named petasitenine.[16] Hirano *et al.* reported as unpublished results that petasite-

Table 2. The Contents of **2**–**4** in the Flower Buds of Japanese Butterbur

(*P. japonicus* subsp. *gigantea* Kitam.)

collecting areas	amount (mg/100 g)[a]	
	2+**3**	**4**
Nopporo area[b] of Sapporo	99.8±3.4	73.7±3.7
Megumino area[b] of Eniwa	98.5±4.5	79.9±5.0
Rawan area of[b] Ashoro town	69.9±3.6	48.8±2.1
Naganuma town[c]	51.1±4.8	51.1±1.0

a) mean±SD. b) n=9. c) n=3

nine was usually isolated in quantities of less than 5.17 g/53 kg of dry flower stalk of *P. japonicus* Maxim., but the measurement of petasitenine has not been done. In order to determine whether the flower buds of *P. japonicus* can be accepted as a health-promorting food, other experiments are required.

Experimental

Measurement of DPPH radical scavenging activity. Measurement of DPPH radical scavenging activities for all fractions were done by the method reported by Yamaguchi *et al.*[3] In briefly, a portion of the crude material dissolved in a solution of EtOH (200 μl) was mixed with the 100 mM Tris-HCl buffer (pH 7.4, 800 μl) and then added to 1 ml of 500 μM DPPH in EtOH. The mixture was shaken vigorously and left for 20 min at room temperature in the dark, and then a sample (60 μl) of the reaction mixture was put through a reversed-phase HPLC analysis to measure the DPPH radical scavenging activity. Analyses were done in a TSKgel Octyl-80TsQA column (4.6×250 mm, Tosoh, Tokyo) at ambient temperature with a mobile phase of MeOH/H_2O (7:3, v/v) at flow rate of 0.8 ml/min. The peaks were monitored using a UV detector set at 517 nm. The DPPH radical scavenging activity was evaluated from the difference in the peak-area decrease of the DPPH radical detected at 517 nm between a blank and a sample. The data were calculated according to the following numerical formula:

(DPPH radical scavenging activity) = [(A-B)/A] × 100 (%)

A: a peak area of the blank correspondence with DPPH

B: a peak area of the sample correspondence with DPPH

Authentic compounds. Authentic compounds of caffeic acid and rutin were purchased from Wako Pure Chem. (Osaka).

Purification of caffeic acid (1) and quercetin glucosides (2 and 3). Flower buds of Japanese butterbur (6.5 kg, fresh weight) were collected from the campus of Hokkaido University, Sapporo in late April and early May, 1999 and soaked in 70% MeOH (45 liters). The extract was partitioned with EtOAc and H_2O. The EtOAc-soluble phase was purified as shown in Fig. 1 to give three active fractions (Fr. III-1 (690 mg), III-2 (705 mg) and III-3 (950 mg)). Fr. III-1 was purified by HPLC (YMC, YMC-Pack ODS-AM, 300×10 mm, 55:45 = H_2O: 80% aq. MeOH, flow rate: 2.5 ml/min, $A_{254\,nm}$) to give **1** as a pale brown oil (caffeic acid, 123 mg). The eluent of Fr. III-2 was concentrated, and the resulting yellow oil was dissolved in 56% aq. MeOH (10 ml) and left at 5°C to give **2** as yellow powders (quercetin 3-*O*-β-D-6″-*O*-acetylglucoside, 154 mg). Fr. III-3 was purified by a medium pressure liquid chromatography. The preparative column was a Lobar LiChroprep RP-18 (Merck, 440×37 mm), and the mobile phase was in isocratic mode, as 40:60:0.1 = MeOH: H_2O: AcOH (v/v/v). Compound **3** was isolated using this chromatography system as yellow powders (quercetin 3-*O*-β-D-glucoside, 408 mg).

Compound **2**: Mp 185–188°C; $[\alpha]_D^{25}$ 4.7° (*c* 0.52, MeOH); IR ν_{max} (KBr) cm^{-1}: 3280, 1650, 1600, 1500, 1380, 1340, 1180, 1050, 850, 690; FDMS: 507 $[M+H]^+$ (100), 506 (62), 302(45); ^{1}H-NMR δ_H (270 MHz, CD_3OD): 7.55–7.40 (2H, m, H-2′, 6′), 6.73 (1H, d, J=8.6 Hz, H-5′), 6.29 (1H, d, J=1.8 Hz, H-8), 6.10 (1H, d, J=1.8 Hz, H-6), 5.04 (1H, d, J=7.3 Hz, H-1″), 4.08 (1H, br. d, J=11.9 Hz, H-6″a), 3.94 (1H, dd, J=11.9, 5.4 Hz, H-6″b), 3.42–3.20 (4H, m, H-2″, 3″, 4″, 5″), 1.73 (3H, s, -CO-CH_3): ^{13}C-NMR ν_C (67.5 MHz, CD_3OD): 179.2 (C-4), 172.4 (-*C*O-CH_3), 165.9 (C-7), 162.8 (C-5), 159.2 (C-8a), 158.3 (C-2), 149.6 (C-4′), 145.7 (C-3′), 135.3 (C-3), 123.2 (C-6′), 122.9 (C-1′), 117.3 (C-2′), 115.7 (C-5′), 105.5 (C-4a), 104.3 (C-1″), 99.8 (C-6), 94.7 (C-8), 77.9 (C-3″ or 5″), 75.6 (C-3″ or 5″), 75.5 (C-2″), 71.2 (C-4″), 64.2 (C-6″), 20.5 (-CO-CH_3).

Compound **3**: Mp 210–213°C; $[\alpha]_D^{25}$ −9.6° (*c* 0.40, MeOH); IR ν_{max} (KBr) cm^{-1}: 3280, 1650, 1600, 1500, 1380, 1340, 1180, 1050, 850, 690; FDMS: 464 $[M]^+$ (30), 302 (100); ^{1}H-NMR δ_H (270 MHz, CD_3OD): 7.60 (1H, br.s, H-2′), 7.48 (1H, br.d, J=8.6 Hz, H-6′), 6.71 (1H, d, J=8.6 Hz, H-5′), 6.28 (1H, br.s, H-8), 6.09 (1H, br.s, H-6), 5.15 (1H, d, J=7.3 Hz, H-1″), 3.60 (1H, br.d, **J**=11.9 Hz, H-6″a), 3.47 (1H, dd, J=11.9, 5.1 Hz, H-6″b), 3.40–3.00 (4H, m, H-2″, 3″, 4″, 5″); ^{13}C-NMR δ_C (67.5 MHz, CD_3OD): δ 179.2 (C-4), 165.8 (C-7), 162.8 (C-5), 158.8 (C-8a), 158.2 (C-2), 149.6 (C-4′), 145.7 (C-3′), 135.4 (C-3), 123.1 (C-6′), 122.9 (C-1′), 117.4 (C-2′), 115.9 (C-5′), 105.5 (C-4a), 104.2 (C-1″), 99.8 (C-6), 94.6 (C-8), 78.3 (C-3″), 78.0 (C-5″), 75.7 (C-2″), 71.1 (C-4″), 62.5 (C-6″).

Purification of rutin (4). The H_2O-soluble phase was obtained as shown in Fig. 1 and concentrated under reduced pressure, and the resultant black oil was put through a column chromatography using Diaion HP-20 resins (Mitsubishi Chem., 700 g). The resins were washed by H_2O (3 liters) and 70% aq. MeOH (3 liters), successively. The 70% aq. MeOH eluents were purified using a Sephadex LH-20 (Amersham Pharmacia Biotech, 250 g, 7:3, MeOH: H_2O, v/v) column chromatography. The eluents of active fractions were concentrated, and to the resultant oil was added a solution of 45% aq. MeOH (4 ml). The solution was left at 5°C to afford **4** as yellow powders (rutin, 118 mg).

Measurement of the quercetin glucosides in the flower buds. Before purification, quercetin 4′-*O*-*β*-D-glucoside (**5**), which had been isolated from fruits of onion and identified by comparing the spectral data with those reported,[17] was added as an internal standard. Each portion of the roughly purified extract of Frs. A and B (10 μg fr. wt. equivalent, Fig. 4) was analyzed by HPLC. The HPLC system (Hitachi) for the measurement consisted of L-7100 pump (flow rate: 1.2 ml/min), L-7300 column oven (temperature: 40°C), L-7200 auto-sampler and L-7455 DAD detector. The column (Kanto Chem., Mightysil RP-18 GP Aqua, 4.6×250 mm) was eluted with a mixed solvent of 80% aq. MeOH (solvent A) and H_2O (solvent B), using a linear gradient with the flow rate of 1.2 ml/min. The combination of A and B was linearly converted from 0 min to 15 min, 7:3 to 8:2: from 15 min to 23 min, 8:2 to 8:2: from 23 min to 25 min, 8:2 to 1:0. The typical retention times for 3′,4′,5,7-*O*-tetraacetylquercetin 3-*O*-*β*-D-2″, 3″, 4″, 6″-*O*-tetraacetylglucopyranoside (**6**), 3′,3,5,7-*O*-tetraacetylquercetin 4′-*O*-*β*-D-2″,3″,4″,6″-*O*-tetraacetylglucoside (**7**) and decaacetylrutin (**8**) were 15.63, 18.80 and 20.19 min, respectively. These acetylated compounds were synthesized from each corresponding compound by treating it with a mixture of pyridine and acetic anhydride, and the structures were confirmed by ^{1}H-NMR and FDMS spectra.

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