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Chemical Constituents from the Fruit Peels of *Fortunella japonica*

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Abstract Chemical constituents of fruit peels of *Fortunella japonica* Swingle were investigated, and ten compounds were purified and isolated through various chromatographic procedures. Through NMR analysis, isolated compounds were identified as α-tocopherol (**1**), lupenone (**2**), β-amyrin (**3**), α-amyrin (**4**), β-sitosterol (**5**), β-sitosteryl 3-*O*-glucopyranoside (**6**), kaempferide 3-*O*-rhamnopyranoside (**7**), 3',5'-di-*C*-β-glucopyranosylphloretin (**8**), acacetin 7-*O*-neohesperidoside (**9**), and acacetin 8-*C*-neohesperidoside (**10**). Compounds **1-7** were identified for the first time by our group from fruit peels of *F. japonica*.

Keywords: *Fortunella japonica* fruit peels, flavonoid glycoside, tocopherol, triterpenoid, sitosterol

Introduction

Until now, many researchers have focused on the investigation of bioactive components from food materials to clarify their roles in the prevention of diseases (1). Numerous studies indicate that increasing consumption of fruits, vegetables, and tea reduces the risk of diseases (2, 3). Bioactive compounds such as coumarins, flavonoids, and anthocyanins contained in these food materials have been proved to have beneficial health effects such as antioxidative, anti-inflammatory, and anticancer effects (1, 4, 5).

Citrus fruits including oranges, grapefruits, and lemons, which have been consumed by people worldwide have been reported to have various biological activities such as hypotensive and anticancer effects (5-7), with flavonoid glycosides (narirutin, apigenin, naringin, hesperidin, and neohesperidin) identified as biologically active compounds from fruit peels of the genus *Citrus* such as orange, unshiu, and sudachi (7-11). However, only a small portion of citrus fruits are edible, and the larger portions such as peels and seeds are usually wasted without appropriate application. Therefore, to utilize the considerable amount of by-products, much efforts have been placed on their use as resources of functional ingredients such as dietary fiber and bioactive compounds (12, 13). The fruit of *Fortunella* genus, on the other hand, is eaten directly without peeling, and the bioactive compounds contained in the fruit peel of this plant may contribute beneficial health effects. Flavonoid glycosides (acacetin 7-*O*-neohesperidoside, ponicilin, 6,8-di-*C*-glucosylapigenin, 3,6-di-*C*-glucosylacacetin, 2''-*O*-α-L-rhamnosyl-4'-*O*-methylvitexin, etc.) have been isolated and identified from the hot water extracts of *F. japonica* peelings (14, 15). Ogawa *et al.* (16) also isolated 3',5'-di-*C*-β-glucopyranosylphloretin from the ethanol extracts of *F. magrita* fresh fruits and quantitatively analyzed this compound in *Fortunella* species by HPLC. In addition, volatile compounds of *F. japonica* fruit have also been isolated by stream distillation and simultaneous purging/extraction methods and identified by gas chromatography/mass spectrometry (17). However, bioactive compounds in fruit peels of *F. japonica* have not yet been fully investigated.

Therefore, we carried out investigation of the chemical constituents in the methanol extracts of the fruit peel of *F. japonica* to further elucidate various bioactive compounds having beneficial health effects, although several flavonoid glycosides have already been isolated and identified from the hot water and ethanol extracts of the fruit peels (14-16).

Materials and Methods

General experimental procedures Nuclear magnetic resonance (NMR) spectra were obtained with Bruker ARX400 spectrometer (Bruker BioSpin, Tsukuba, Japan) using solvents as the internal standard. Column chromatography was performed using a silica gel (Kieselgel 60 N, 63-210 μm, Kanto Kagaku), Toyo Pearl HW-40 (50-100 mesh, TOSOH, Tokyo, Japan), and Sephadex LH-20 (25-100 mesh, Pharmacia Fine Chemicals, Switzerland). HPLC analysis was carried out using various columns: silica gel column, YMC-Pack SIL-06 (YMC, Tokyo, Japan, *n*-hexane/EtOAc); gel permeation columns (GPC), Shodex H-2001 and H-2002 GP (Shodex, Tokyo, Japan, $CHCl_3$); GPC column, Shodex Asahipak GS 310 2G (Shodex, Tokyo, Japan, MeOH); ODS column, Mightysil RP-18 GP (Kanto Kagaku, Japan, H_2O/MeOH). Compounds analyzed by HPLC were monitored using a UV detector (254 nm, UV 970, JASCO, Japan) and an RI detector (RI 930, JASCO, Japan). Purity of the fractionated compounds during isolation was monitored using TLC-silica gel 60 F_{254} with appropriate developing solvents, and visualized by UV and 1% cerium-sulfate solution spray.

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Materials Fresh fruit of *F. japonica* Swingle (Rutaceae) was provided by Asai Cannery Company (Tokushima, Japan).

Extraction and isolation procedures The fresh peels (1.5 kg) were extracted with MeOH (3 L, three times), a solvent that dissolves various compounds with a broad range of solubility, for 3 hr at 60°C and filtrated. The MeOH solution was combined and concentrated *in vacuo* at 38°C. The MeOH extracts were suspended with the solution of H_2O/MeOH (9:1, v/v, 1 L) and partitioned with *n*-hexane (1 L). The aqueous MeOH layer was concentrated to remove MeOH, and successively fractionated with EtOAc (1 L) and *n*-BuOH (1 L). Each organic layer was evaporated *in vacuo* at 38°C.

The *n*-hexane fraction (12.9 g) was chromatographed on a silica gel column and eluted with an increasing gradient of EtOAc in *n*-hexane to obtain 10 fractions of A~J. Fraction C (*n*-hexane/EtOAc, 8:2, v/v, 269.6 mg) was refractionated into six subfractions (C-a~f) by silica gel column chromatography (*n*-hexane/$CHCl_3$). Fractions C-d (*n*-hexane/$CHCl_3$, 7:3, v/v, 37.1 mg) and C-e (*n*-hexane/$CHCl_3$, 7:3, v/v, 133.6 mg) were subjected to GPC-HPLC with $CHCl_3$ as a mobile phase to give **1** (12.4 mg) and **2** (19.3 mg), respectively. Fraction E (*n*-hexane/EtOAc, 4:1, v/v, 283.9 mg) was successively purified using silica gel-HPLC (*n*-hexane/EtOAc, 8.5:1.5, v/v) and GPC-HPLC ($CHCl_3$) to give **3** (12.5 mg) and **4** (14.5 mg). Fraction F (*n*-hexane/$CHCl_3$, 6:4, v/v, 842.9 mg) was fractionated into six subfractions (F-a~f) by silica gel column chromatography with *n*-hexane/EtOAc (7:3, v/v). Fractions F-d~f [230.0 mg, *n*-hexane/EtOAc (7:3, v/v), the same flow rate on TLC] were purified by GPC-HPLC using $CHCl_3$ as a mobile phase to obtain **5** (157.0 mg). Fraction J (*n*-hexane/$CHCl_3$, 0:10, v/v, 929.2 mg) was subjected to silica gel column using $CHCl_3$/MeOH (9:1, v/v), and six subfractions (J-a~f) were obtained. Fraction J-c (100.8 mg) was purified by GPC-HPLC (MeOH) to obtain **6** (19.6 mg).

A portion of the *n*-BuOH fraction (5.4 g) was subjected to Toyo Pearl HW-40 column with MeOH as a mobile phase. After development on TLC plate, fractions (4.5 g; 50~150 ml) showing the same flow rate on TLC were combined, and a portion of this fraction (82.1 mg) obtained from Toyo Pearl HW-40 column was subjected to GPC-HPLC (MeOH) to obtain compounds **7** (21.9 mg) and **8** (12.5 mg).

EtOAc fraction (2.3 g) was chromatographed on a silica gel column and eluted with an increasing volume of MeOH in $CHCl_3$ to give 11 subfractions (E1-11) and compound **9** (135.2 mg). Fraction E10 ($CHCl_3$/MeOH, 7:3, v/v, 1.1 mg) was subjected to ODS-HPLC (MeOH/H_2O, 1:1, v/v) to yield **9** (44.6 mg), **10** (30.2 mg), and **8** (30.0 mg).

Compound 1: yellow oil; ^{13}C-NMR ($CDCl_3$, 100 MHz) δ 74.5 (C-2), 31.5 (C-3), 20.74 (C-4), 118.5 (C-5), 144.5 (C-6), 121.0 (C-7), 122.6 (C-8), 23.8 (C-2a), 117.3 (C-4a), 11.3 (C-5a), 12.2 (C-7a), 145.5 (C-8a), 11.8 (C-8b), 39.8 (C-1'), 21.0 (C-2'), 37.3 (C-3'), 32.7 (C-4'), 37.3 (C-5'), 24.4 (C-6'), 37.4 (C-7'), 32.8 (C-8'), 37.4 (C-9'), 24.8 (C-10'), 39.4 (C-11'), 28.0 (C-12'), 22.7 (C-13'), 19.6 (C-4', -CH_3), 19.7 (C-8', -CH_3), 22.6 (C-12', -CH_3).
Compound 2: white powder; ^{13}C-NMR ($CDCl_3$, 100 MHz), see Table 1.
Compound 3: white powder; ^{13}C-NMR ($CDCl_3$, 100 MHz), see Table 1.
Compound 4: white powder; ^{13}C-NMR ($CDCl_3$, 100 MHz), see Table 1.
Compound 5: white powder; ^{13}C-NMR ($CDCl_3$, 100 MHz), see Table 1.
Compound 6: white powder; ^{13}C-NMR (pyridine-d_5, 100 MHz), see Table 1.
Compound 7: yellow powder; ^{1}H-NMR (CD_3OD, 400 MHz) δ 8.08 (2H, d, J = 8.0 Hz, H-2',6'), 7.10 (2H, d, J = 8.0 Hz, H-3',5'), 6.65 (1H, s, H-8), 6.28 (1H, s, H-6), 3.89 (3H, s, -OCH_3), 5.12 (1H, d, J = 8.0 Hz, H-1"), 4.38-3.40 (4H, H-2"-5"), 0.64 (3H, d, J = 6.0 Hz, H-6"); ^{13}C-NMR (CD_3OD, 100 MHz), δ 161.5 (C-2), 135.0 (C-3), 181.9 (C-4), 164.1 (C-5), 101.4 (C-6), 167.2 (C-7), 94.7 (C-8), 162.8 (C-9), 105.8 (C-10), 122.8 (C-1'), 128.6 (C-2') 114.8 (C-3'), 162.7 (C-4'), 114.8 (C-5'), 128.6 (C-6'), 100.7 (C-1"), 70.9 (C-2"), 70.6 (C-3"), 72.1 (C-4"), 68.5 (C-5"), 18.2 (C-6"), 55.8 (-OCH_3).

Results and Discussion

The peels (1.5 kg) of fruit extracted with MeOH were successively partitioned among *n*-hexane, EtOAc, and *n*-BuOH. After removing the solvents, *n*-hexane fraction, 5.9 g, EtOAc fraction, 2.3 g, and *n*-BuOH fraction, 36.1 g were obtained. Through various chromatographic purifications of these fractions, ten compounds (**1-10**) were isolated and analyzed by NMR to determine their structures.

The ^{13}C-NMR and DEPT spectra of **1** showed 29 carbons including 6 aromatic quaternary carbons (δ 145.5-117.3), 1 oxygenated quaternary carbon (δ 74.5, C-2), 3 methine carbons [δ 32.7 (C-4'), 32.8 (C-8'), 28.0 (C-12')], 11 methylene carbons (δ 39.8-21.0), and 8 methyl carbons (δ 22.6-11.7), suggesting **1** to be tocopherol. More specifically, the presence of six aromatic quaternary carbons including two oxygenated carbons [δ 144.5 (C-6) and 145.5 (C-8a)] and three methylated carbons [δ 118.5 (C-5), 121.0 (C-7), 122.6 (C-8)] indicated that **1** is α-tocopherol. Comparing the spectral data of this compound with those in the literature (18), the structure of **1** was confirmed as α-tocopherol (Fig. 1).

The ^{13}C-NMR (Table 1) and DEPT spectra of **2** showed the presence of 30 carbons including 1 carbonyl carbon (δ 218.3, C-3), 1 quaternary double bond carbon (δ 150.9, C-20), 1 methylene double bond carbon (δ 109.4, C-29), 5 quaternary carbon [δ 47.2 (C-4), 40.8 (C-8), 36.9 (C-10), 42.9 (C-14), 42.9 (C-17)], 5 methine carbons [δ 54.9 (C-5), 49.8 (C-9), 38.2 (C-13), 48.0 (C-18), 48.2 (C-19)], 10 methylene carbons (δ 40.0-21.5), and 7 methyl carbons (δ 26.7, 21.0-14.5). The spectral data suggested that **2** is a pentacyclic triterpenoid. Specifically, the presence of carbon signals of carbonyl (δ 218.3, C-3) and -CH=CH_2 [δ 150.9 (C-20) and δ 109.4 (C-29)] groups indicated that the structure of **2** is lupenone. In addition, the ^{13}C-NMR spectrum of **2** corresponded to that of lupenone isolated from *Tephrosia villosa* (19). Therefore, the structure of **2** was unambiguously identified as lupenone (Fig. 1).

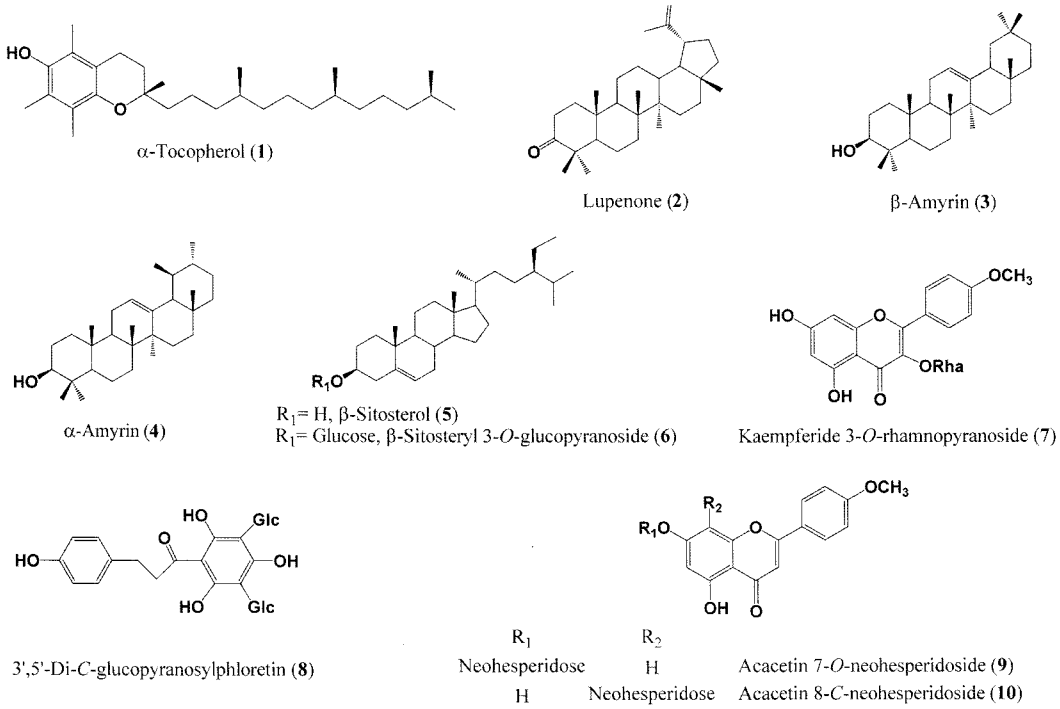

Fig. 1. Structures of the compounds isolated from the fruit peels of *Fortunella japonica*.

The ^{13}C-NMR (Table 1) and DEPT spectra of **3** exhibited the presence of 30 carbons, containing a double bond (δ 145.5, C-13; δ 122.0, C-12) and an oxygenated methine carbon (δ 79.4, C-3), in particular. The ^{13}C-NMR data suggested **3** to be a pentacyclic triterpenoid similar to the structure of **2**. As based on the chemical shifts of the characteristic double bond carbons (δ 145.5, C-13; δ 122.0, C-12) and hydroxyl group (δ 79.4, C-3), the ^{13}C-NMR spectrum of **3**, upon comparison with those of pentacyclic triterpenoids reviewed by Mahato and Kundu (20) confirmed the structure of **3** was β-amyrin (Fig. 1).

The ^{13}C-NMR and DEPT spectra of **4** showed 30 carbon signals of **4** were closely related to those of **3**. The presence of two methine carbons (δ 40.0, C-19 and 20) instead of one methylene carbon (δ 47.2, C-19) and a quaternary carbon (δ 31.4, C-20) indicated that **4** is α-amyrin having vicinal methyl groups in the E ring, which differs from it of **3** having geminal methyl groups. Comparison with the ^{13}C-NMR spectroscopic data in the literature (20) revealed the structure of **4** was α-amyrin (Fig. 1).

The structure of **5** was assigned by a method similar to the one used for **2**, **3**, and **4**. The ^{13}C-NMR (Table 1) and DEPT spectra of **5** showed 29 carbons including 1 quaternary double bond carbon (δ 140.8, C-5), 1 methine double bond carbon (δ 121.7, C-6), 2 quaternary carbons [δ 36.7 (C-10), 42.3 (C-13)], 7 methine carbons [δ 31.9 (C-8), 50.1 (C-9), 56.8 (C-14), 56.1 (C-17), 36.1 (C-20), 45.3 (C-24), 29.2 (C-25)], 1 oxygenated methine carbon (δ 71.8, C-3), 11 methylene carbons (δ 42.3-21.1), and 6 methyl carbons (δ 19.8-11.9), which suggested **5** to be a steroid skeleton. The presence of double bond carbons [δ 140.8 (C-5) and 121.7 (C-6), -C=CH-] and hydroxyl group (δ 71.8, C-3) indicated that the structure of **5** was β-sitosterol. Moreover, the ^{13}C-NMR data of **5** corresponded to those of β-sitosterol isolated from *Typha latifolia* (21). Therefore, the structure of **5** was identified as β-sitosterol (Fig. 1).

The ^{13}C-NMR (Table 1) and DEPT spectral data of **6** displayed 35 carbon signals closely related with those of **5**, except for a sugar moiety [δ 102.3 (78.5-62.7)], which suggested **6** to be β-sitosteryl monosaccharide. Therefore, the structure of **6** was identified as β-sitosteryl 3-*O*-glucopyranoside based on the comparison with the ^{13}C-NMR data of that isolated from *Bauhinia candicans* (Fig. 1) (22).

The ^{13}C-NMR spectrum of **7** exhibited the presence of 22 carbons including 15 carbon signals of aglycone (δ 181.9, carbonyl carbon signal, C-4; δ 167.2-94.7, 14 sp^2 carbon signals), 1 methoxyl signal (δ 55.8, -OCH_3), and 6 carbon signals of monosaccharide (δ 102.3, anomeric carbon signal; δ 79.7-62.9, 4 non-anomeric carbon signals; δ 18.2, methyl carbon signal). Results suggested that **7** is a flavonoid monosaccharide having a methoxyl group. The ^{1}H-NMR spectrum of **7** showed the evidence for aglycone of keampferol including two each proton signals of A ring [δ 6.65 (1H, s, H-8) and 6.28 (1H, s, H-6)] and B ring [δ 8.08 (2H, d, J = 8.0 Hz, H-2', 6') and 7.10 (2H, d, J = 8.0 Hz, H-3', 5')]. The sugar moiety was determined to be rhamnose with an anomeric proton at δ 5.12 (1H, d, J = 8.0 Hz, H-1") and a methyl group at δ 0.64 (1H, d, J = 6.0 Hz, H-6"). In the HMBC spectrum (data not shown), the presence of a cross peak between the methoxyl proton (δ 3.89, -OCH_3) and C-4' (δ 162.7) of aglycone indicated that methoxyl group was substituted at the 4' position of kaempferol. Therefore, the aglycone of **7** was identified as kaempferide (5,7-dihydroxy-4'-methoxyflavonol) (23). In addition, the presence of a cross peak from the anomeric proton (δ 5.12, H-1") and C-3 (δ 135.0) of aglycone established that rhamnose was linked at the 3 position of

Table 1. ^{13}C-NMR data for compounds 2-6 (100 MHz)

Position	2[a]	3[a]	4[a]	5[a]	6[b,c]
1	39.6	38.9	39.1	37.3	36.9
2	34.2	27.6	27.6	31.7	30.3
3	218.3	79.4	79.4	71.8	71.8
4	47.2	39.1	39.1	42.3	39.4
5	54.9	55.5	55.5	140.8	140.9
6	19.7	18.7	18.7	121.7	121.8
7	33.6	33.0	33.3	31.9	32.1
8	40.8	38.9	40.3	31.9	32.1
9	49.8	48.0	48.0	50.1	50.4
10	36.9	37.5	37.2	36.7	36.4
11	21.5	26.9	23.6	21.1	21.3
12	25.2	122.0	124.7	39.8	40.0
13	38.2	145.5	139.9	42.3	42.3
14	42.9	40.1	42.4	56.8	56.3
15	27.4	26.5	29.1	24.3	24.5
16	35.5	27.3	26.9	28.2	28.5
17	42.9	32.8	33.7	56.1	56.9
18	48.0	47.7	59.4	12.0	12.2
19	48.2	47.2	40.0	19.4	19.4
20	150.9	31.4	40.0	36.1	36.4
21	29.8	35.0	31.6	18.8	19.4
22	40.0	37.3	41.9	34.0	34.3
23	26.7	28.4	28.4	26.1	26.5
24	21.0	15.8	16.0	45.3	45.3
25	15.8	15.9	15.9	29.2	30.0
26	16.0	17.1	17.2	19.8	20.0
27	14.5	26.3	23.7	19.1	19.2
28	18.0	28.7	28.4	23.1	23.4
29	109.4	33.7	17.8	11.9	11.9
30	19.3	24.0	21.7	-	-

The solvent was used with $CDCl_3$[a] and pyridine-d_5[b]. [c]The carbon signals of glucose for compound **6** were assignable to δ 102.3 (C-1'), 75.3 (C-2'), 78.5 (C-3'), 71.6 (C-4'), 78.3 (C-5'), 62.7 (C-6').

kaempferide. Therefore, the structure of **7** was determined to be kaempferide 3-*O*-rhamnopyranoside (Fig. 1).

Compounds **8-10** were identified as 3',5'-di-*C*-β-glucopyranosylphloretin (**8**), acacetin 7-*O*-neohesperidoside (**9**), and acacetin 8-*C*-neohesperidoside (**10**) by NMR analysis (data not shown) based on comparison with the NMR spectral data of those of previous reports (14-16).

Ten compounds isolated from MeOH extracts of *F. japonica* fruit peels were identified as α-tocopherol (**1**), lupenone (**2**), β-amyrin (**3**), α-amyrin (**4**), β-sitosterol (**5**), β-sitosteryl 3-*O*-glucopyranoside (**6**), and kaempferide 3-*O*-rhamnopyranoside (**7**) including 3',5'-di-*C*-β-glucopyranosylphoretin (**8**), acacetin 7-*O*-neohesperidoside (**9**), and acacetin 8-*C*-neohesperidoside (**10**) (Fig. 1). Ogawa *et al.* (16) reported the result of structural elucidation of 3',5'-di-*C*-β-glucopyranosylphloretin (**8**) isolated from the ethanol extracts of *Fortunella margarita* fresh fruits. Moreover, they also carried out quantitative analysis of this compound in the peel, juice sac, and leaves of the genus *Fortunella* and *F. japonica*. Matsuno *et al.* (14) and Kumamoto *et al.* (15) also reported that acacetin 7-*O*-neohesperidoside (**9**) and acacetin 8-*C*-neohesperidoside (**10**) were identified from the peels of *F. japonica*. We report here the occurrence of kaempferide 3-*O*-rhamnopyranoside, which is substituted for the methoxyl group and rhamnose in kaempferol, for the first time in this plant. Although α-tocopherol (**1**), lupenone (**2**), β-amyrin (**3**), α-amyrin (**4**), β-sitosterol (**5**), and β-sitosteryl glucopyranoside (**6**) are usually present in many plants (24-26), as far we know, the isolation and identification of these compounds from the fruit peels of *F. japonica* are also reported for the first time in this paper.

With respect to the biological activities of flavonoids isolated from this plant, flavonoid glycosides such as kaempferide 3-*O*-rhamnopyranoside (**7**), 3',5'-di-*C*-β-glucopyranosylphloretin (**8**), acacetin 7-*O*-neohesperidoside (**9**), and acacetin 8-*C*-neohesperidoside (**10**) have not yet been investigated. However, aglycones (kaempferide, acacetin, and phloretin) of these compounds exert various biological activities: kaempferide, for antioxidative and anti-estrogenic activities (27, 28); acacetin, for antioxidative (29) and anti-inflammatory effects (30), and inhibitory effect against human liver cancer cell (31); phloretin, for anticancer effect (32). Therefore, flavonoid glycosides isolated from *F. japonica* fruit peels may also contribute to biological activities. In future studies, the biological activities of these compounds should be clarified to better understand the beneficial effects of *F. japonica*.

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