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Antioxidant flavonoid glycosides from the leaves of *Ficus pumila* L.

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Abstract

The Okinawan folks in Japan use *Ficus pumila* L. as a beverage or herbal medicine to treat diabetes and high blood pressure. Four flavonoid glycosides were isolated and identified as rutin (**1** and **3**), apigenin 6-neohesperidose (**2**), kaempferol 3-robinobioside (**4**) and kaempferol 3-rutinoside (**5**). Among these compounds, rutin exhibited the strongest antioxidant activity in DPPH radical scavenging assay and superoxide radical inhibition assay. The preparation of Ooitabi leaves in water provide sufficient amount of flavonoid glycosides to the Okinawan although 50% of aqueous ethanol extracted these flavonoid glycosides more effectively. These results show the potential of Ooitabi leaves as a natural source of antioxidant for health management.

Keywords: *Ficus pumila* L.; Flavonoid glycosides; Antioxidant

1. Introduction

Currently, there is great interest in finding antioxidants from natural sources to minimize oxidative damage to cells. Oxidative damage is caused by free radicals and reactive oxygen species, mostly generated endogeneously (Aniya, 2002). They are recognized to be involved in the pathogenesis of various diseases such as atherosclerosis, cancer, diabetes mellitus and reperfusion disorder (Benzie, 2002). Researchers have demonstrated that appropriate consumption of foods containing antioxidants such as herbs and vegetables can prevent such deleterious effect.

There are many types of antioxidants in the Okinawan vegetables and medicinal plants, which are being consumed for example, Botanbofu (*Peucedanum japonicum* T.), Ryukyuyomogi (*Artemisia campestris* L.), Iriomotekumatakeran (*Alpinia flabellata* R.), and Ryukyubasho (*Musa balbisiana* C.) (Nakatani, 2003). The consumption of such plants made the Okinawans well-known for their exceptional longevity and lowest death rate from lifestyle-related diseases in Japan and perhaps, throughout the world.

Ficus pumila L. or Ooitabi of the Moraceae family is a scandent shrub with evergreen coriaceous leaves that is normally grown between the trees as well as on fragmented surface. The leaves of the plant has been traditionally consumed by some Okinawan elders either as a beverage or used as an invaluable medicinal herb by the folks to treat diabetes, dizziness, high blood pressure, and neuralgia (Mitsuhashi, 1988; Tobinaga, 1989). There is evidence that some of the compounds in the plants regularly consumed by the Okinawans have powerful antioxidant and positive hormonal effects (Nakatani, 1992). Few side effects have been associated with using them as foods, condiments, spices, teas, or home remedies. It is possible that Ooitabi also contains powerful antioxidants that have beneficial effect to the human body.

In this study, we investigate the active antioxidant compounds contained in Ooitabi using DPPH assay as the marker for antioxidant activity.

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2. Materials and methods

2.1. Chemicals and reagents

All chemicals and reagents used were of analytical grade. Bovine serum albumin, DPPH (1,1-diphenyl-2-picrylhydrazyl), MES (2-morpholinoethanesulphonic), NBT (nitroblue tetrazolium), ethanol and methanol used were purchased from Nacalai Tesque (Kyoto, Japan). Copper chloride, EDTA, formic acid, solvents for NMR analysis and xanthine oxidase from buttermilk were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Water (18.3 Ω) was purified using a MilliQ-Labo purification system by Millipore (Osaka, Japan).

2.2. Instruments for chromatography

Medium pressure liquid chromatography (MPLC) by Yamazen Corp. (Osaka, Japan) was consisted of a GR-200 gradient mixer, a Pump 600A pump, and a Prep. UV254 UV detector. The HPLC system was by Shimadzu Corp. (Kyoto, Japan) that consisted of a SCL-6B system controller, a DGU-12A degasser, two units of LC-6A pump, a CTO-6A column oven with 1 ml sample loop injector (Rheodyne, USA), a SPD-6A UV spectrophotometric detector, and a C-R4A chromatopac.

2.3. Plant material and extraction

The leaves of Ooitabi were collected from Motobu-cho of Okinawa prefecture. The leaves were washed and air-dried at 60 °C for an overnight. After that, the dried-leaves were pulverized using a household food processor. Then, 600 g of the ground dried-leaves were weighed and extracted with 50% of aqueous ethanol at room temperature for 24 h. The extract was filtered and the residue was re-extracted for another twice under the same conditions with a total volume of 6 l of 50% of aqueous ethanol.

2.4. Separation by chromatography

The crude extract was chemically filtered using Sep-Pak C18 SPE cartridge (35 cc. reservoir) by Waters. The filtrated extract was evaporated into syrup and then dissolved in water. Then, this extract was fractionated into five fractions using the same cartridge with a stepwise elution from water to ethanol.

The fractions were separated by MPLC using the Ultra Pack ODS-S-50B (300 × 26 mm i.d. column, 50 μm) column by Yamazen Corp. (Osaka, Japan) with UV absorbance at 254 nm. The fractions eluted from MPLC are further separated by HPLC using Cosmosil C18-AR-II Waters (250 × 10 mm i.d. column, 5 μm) column by Nacalai Tesque, Inc. (Kyoto, Japan). A binary mobile phase was used in which mobile phase A was water and mobile phase B was methanol. The elution was started with 2 min of 30% of B and ascended to 50% of B in 28 min and 60% of B in 5 min. The 254 nm UV absorbance was used for monitoring. The fractions obtained were purified by crystallization before they were identified using JNM-α500 NMR spectrometer by JEOL Datum (Tokyo, Japan).

2.5. Antioxidant assays

2.5.1. DPPH radical scavenging assay

The DPPH free radical scavenging assay by Suda (2000) was conducted. The assay consisted of 0.3 ml of 0.4 mM of DPPH solution in ethanol, 0.3 ml of 200 mM of MES buffer (pH 6.0) and 0.3 ml of 50% of aqueous ethanol. Then, 0.3 ml of samples in different concentrations dissolved in 50% of aqueous ethanol were mixed with each of the solution prepared (total volume: 1.2 ml). The assay was allowed to stand for 1 h before measuring the optical absorbance at 520 nm. The SC_{50} (the concentration required to scavenge 50% of DPPH radical) values were determined by linear regression of plots.

2.5.2. Superoxide radical ($\cdot O_2^-$) inhibition activity

The improved assay method for superoxide dismutase described by Imanari, Hirota, Miyazaki, Hayakawa, and Tamura (1977) was used. Briefly, 1 mM of EDTA, 0.05% (w/v) bovine serum albumin, 0.25 mM NBT and test samples in different concentrations were prepared in water. Then, 1 mM of xanthine was prepared in 0.05 M of sodium carbonate buffer (pH 10.2). The reaction mixture was made by adding 0.1 ml of each solution into 2.4 ml of 0.05 M of sodium carbonate buffer (pH 10.2). The reaction mixture was preincubated at room temperature for 10 min and then, 0.1 ml of 0.1 U/ml of xanthine oxidase in water was added to initiate the reaction. After 30 min, 0.1 ml of 6 mM copper chloride was added to stop the reaction. The formation of formazan was observed at 560 nm. Due to the difficulty of dissolving the test samples in water, a maximum concentration of 0.5 mM of each sample was used.

2.6. Quantification

Weighing bottles (40 × 40 mm) were weighed and dried in an oven at 90 °C for an hour. Then, the bottles were transferred into a desiccator contained with silica gel and left for 1–2 h. The weight of the bottles was measured and the procedures were repeated until there was no significant weight change (±0.0003 g) on each bottle.

A hundred milliliter of three different solvents, which were water, 50% of aqueous ethanol and ethanol were used to extract 1 g of dried Ooitabi leaves. The extracts were filtered and filled up to 100 ml. Then, 5 ml of each extract was withdrawn and placed into the weighing bottles separately. The bottles with their contents were dried in the oven and transferred into the desiccator as described. The weight of each bottle was measured and the procedures were repeated until constant weight was attained.

The HPLC system used for the quantification of flavonoid glycosides were described in Section 2.2 using Cosmosil

5C18-AR-II Waters (4.6 × 150 mm, 5 μm) column by Nacalai Tesque, Inc. (Kyoto, Japan). The column temperature was 40 °C. Mobile phase A was of 1% of formic acid (v/v) in water while mobile phase B was 1% of formic acid (v/v) in methanol. The column was developed with a flow rate of 0.8 ml/min using a gradient system as follow: 30% of B (0–1 min) to 60% of B (1–34 min). The column was reconditioned in 30% of B for 15 min before further injection. Detection wavelength was set at 254 nm.

Commercially obtained rutin and the other three flavonoid glycoside compounds isolated from Ooitabi that have more than 95% purity were used as standard compounds. Concentrations of 0.0625, 0.125, 0.25, 0.5 and 1 mg/ml of each standard compounds were prepared and subjected to HPLC separately. The injection volume was 5 μl and the column was developed using the same gradient system as described above. The calibration curve was plotted as peak area (arbitrary units) obtained from absorbance at 254 nm against standard (mg). The data points were fitted into a line of best fit by the linear regression method. A concentration of 1 mg/ml of each extract was prepared and 50 μl volume was subjected to HPLC separately. The column was developed using the same gradient system as described above. The amount of the flavonoid glycosides extracted by these three solvents was calculated based on the peak area of the chromatogram of the extract with the calibration curve of the standard compounds.

2.7. Data treatment

The experimental results for the antioxidative assays were expressed as means of ±0.05SD triplicate reactions. Linear regression plots were performed using Microsoft Excel Edition 2003.

3. Results and discussion

The result of the DPPH assay for SPE fractions is shown in Fig. 1. Here, both 25% and 50% aqueous EtOH fraction showed DPPH radical scavenging activity especially the 50% aqueous EtOH fraction showed the strongest DPPH radical scavenging activity. The other fractions did not show significant DPPH radical scavenging radical activity.

Since the two fractions showed antioxidant activity, they were further separated by MPLC. The 25% of aqueous EtOH fraction gave compound **1** after a gradient elution system from 10% to 60% of aqueous methanol in 35 min at a flow rate of 9 ml/min was applied to reversed phase-MPLC. The 50% of EtOH fraction was also separated using MPLC using an isocratic 80% of aqueous methanol eluation to yield three fractions. The second fraction exhibited DPPH radical scavenging activity (data not shown) and therefore, was further separated by HPLC as described in Section 2.5 that gave compounds **2**, **3**, **4** and **5**. These five compounds gave yellow amorphous powder through crystallization. Fig. 2 summarizes the separation scheme of Ooitabi leaves.

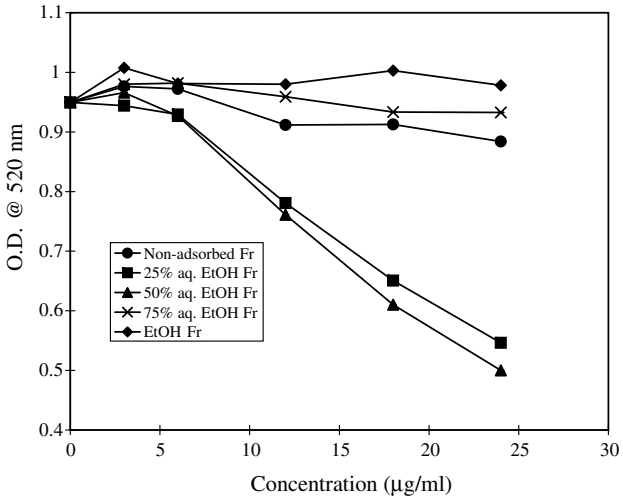

Fig. 1. DPPH radical scavenging activity of fractions eluted from C18 SPE cartridge with a stepwise eluation from water to ethanol.

Analysis of these five compounds by ^{1}H and ^{13}C NMR spectroscopy showed that they were flavonoid glycosides. According to the ^{1}H and ^{13}C NMR spectral, both compounds **1** and **3** were identified as rutin and confirmed with comparison made with authentic compound. For compound **2**, analysis of ^{13}C NMR spectral data identified this compound as apigenin 6-*C*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside or apigenin 6-neohesperidoside. The spectral data corresponded to the results obtained by Saleh, Torgils, & Øyvind, 2005.

The ^{1}H NMR analysis of both compounds **4** and **5** showed similar spectral data as shown in Table 1. However, both compounds showed different ^{13}C NMR spectral. Data comparisons were made with the literatures by Agrawal & Bansal (1989) and Brasseur & Angenot (1986) in which compound **4** was identified as kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside or kaempferol 3-robinobioside while compound **5** identified as kaempferol 3-*O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside or kaempferol 3-rutinoside. The molecular structures of each compound are shown in Fig. 3. Table 2 shows the ^{13}C NMR chemical shifts of these flavonoid glycosides.

The antioxidant effectiveness of these flavonoid glycosides were assessed using two different antioxidant assays. For the DPPH assay, compounds **1** and **3** showed the greatest efficiency of scavenging DPPH radicals with a SC_{50} value of 0.05 mM compared to the reference compound, α-tocopherol. The rest of the compounds did not scavenge DPPH radicals as efficient as compounds **1** and **3**. Compounds **1** and **3** also showed inhibition on the formation of NBT formazan caused by the superoxide radical with an IC_{50} value of 0.14 mM. The other compounds also did not show inhibition activity on $\cdot O_2^-$ up to 0.4 mM (Table 2).

By the results shown in Table 2, compounds **1** and **3** showed the strongest antioxidant activity on DPPH and superoxide radicals compared with the other compounds

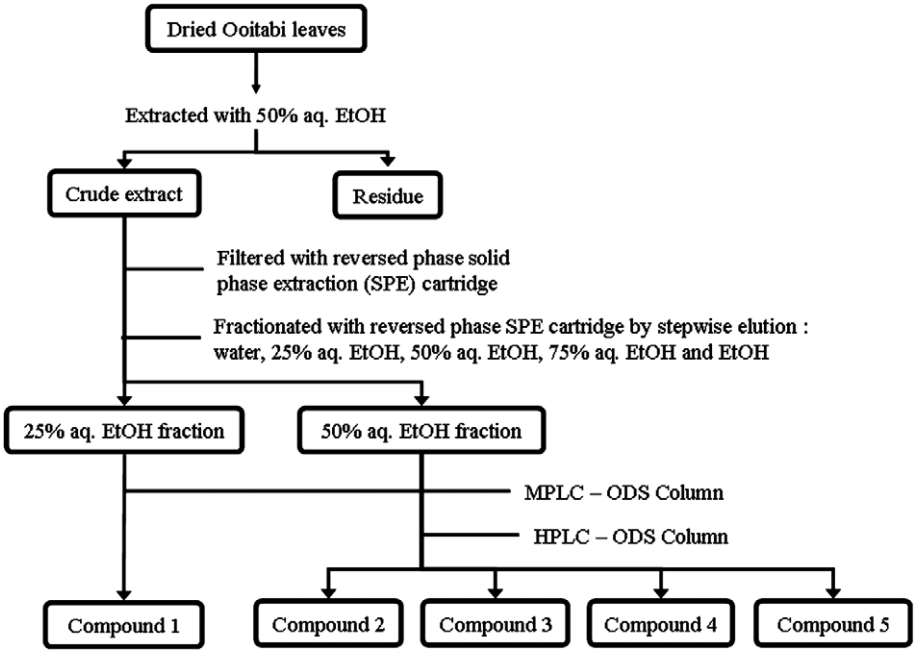

Fig. 2. The separation scheme of dried Ooitabi leaves.

isolated from the leaves of Ooitabi. Based on the molecular structure of each compound, the antioxidant strength is shown to be influenced with the number of the hydroxyl group at the B-ring of the flavonoid structure (Matsuda, Wang, Managi, & Yoshikawa, 2003; van Acker et al., 1996). This is complied with compounds **1** and **3** as it has two hydroxyl groups at the B-ring compared with the other three flavonoid glycosides, which has only one hydroxyl group at the B-ring.

Table 3 shows the quantification results from the dried-leaves of Ooitabi. Water showed the highest yield of dry weight matter followed by 50% of aqueous ethanol and ethanol. Among these three solvents, 50% of aqueous ethanol extracted the most quantity of compound **2** than water and ethanol. The most amount of flavonoid glycoside extracted by water is compounds **1** and **3** followed by compounds **2**, **5** and **4**. Most flavonoid glycoside compounds extracted by ethanol could only be traced. The retention time for compounds **1** and **3**, **2**, **4** and **5** by HPLC analysis is 12.8, 11.2, 14.7 and 16.7 min, respectively.

The strongest antioxidant compound, rutin, can be extracted from the leaves of Ooitabi using the above solvents with 50% of aqueous ethanol extracted more rutin than water and ethanol. Although our experimental results showed that 50% of aqueous ethanol solvent is the best extraction solvent, considerable amount of rutin can still be obtained by using water. Therefore, the preparation of Ooitabi leaves in water consumed by the Okinawan elders is sufficient to provide effective antioxidant for good health.

The belief that the consumption Ooitabi leaves extract by the Okinawan elders can treat diabetes and high blood pressure might be due to the presence of strong antioxidant compound. There is a report showing that oxidative stress is implicated in the development of diabetic complications (Baynes, 1991). The continuous hyperglycemic condition in diabetes causes non-enzymatic glycation of proteins through Maillard's reaction that results to the formation of advanced glycation end-products (AGEs), which can lead to oxidative stress (Baynes, 1991; Miyata et al., 1997; Vlassara, 1997). Matsuda et al. (2003) stated in their work that flavonoids with strong AGEs inhibitory activity tended to show strong scavenging activity on DPPH radical and/or superoxide radical. Also, naturally occurring flavonoid has been reported to have diminishes the accumulation of diabetes-induced skin fluorescence in rats caused by AGEs (Cervantes-Laurean et al., 2006; Nagasawa et al., 2003), preventing DNA damage from carcinogenesis (Webster, Gawde, & Bhattacharya, 1996) and protection against gastric lesions (La Casa, Villegas, Alarcón de la Lastra, Motilva, & Martín Calero, 2000).

We postulated that rutin contained in the leaves of Ooitabi is a possible compound that is responsible to prevent such diseases. However, further study is still necessary to examine whether Ooitabi can be used to lower blood glucose level, controlling blood pressure and its effectiveness as food in managing diabetes.

In conclusion, flavonoid glycosides in the leaves of Ooitabi are the antioxidant compounds that could be responsible for the good health of Okinawan people. Among them, rutin has been found to possess a highly effective antioxidant compound. Quantitative examination showed that although 50% of aqueous ethanol extracted more rutin than water, the simple preparation of Ooitabi by the Okinawan using water is sufficient to provide them with enough rutin.

Table 1
^{1}H [δ values (ppm) and J values (Hz), 500 MHz] and ^{13}C [δ values (ppm), 125.65 MHz] NMR spectral data for **1** and **3**, **2**, **4** and **5**

Position	**1** and **3**[a]	**2**[b]	**4**[a]	**5**[a]
H-5	12.60 (OH, s)	–	12.56 (OH, s)	12.57 (OH, s)
H-6	6.18 (1H, d, $J = 2.0$ Hz)	–	6.20 (1H, d, $J = 2.0$ Hz)	6.18 (1H, d, $J = 1.3$ Hz)
H-8	6.39 (1H, dd, $J = 7.3$, 1.5 Hz)	6.51 (1H, s)	6.41 (1H, d, $J = 2.0$ Hz)	6.41 (1H, d, $J = 1.3$ Hz)
H-2′	7.65 (1H, dd, $J = 8.5$, 1.9 Hz)	7.83 (2H, d, $J = 8.8$ Hz)	7.98 (2H, d, $J = 8.8$ Hz)	8.05 (2H, d, $J = 8.9$ Hz)
H-3′	–	6.92 (2H, d, $J = 8.8$ Hz)	6.88 (2H, d, $J = 8.8$ Hz)	6.86 (2H, d, $J = 8.9$ Hz)
H-5′	6.83 (2H, d, $J = 8.8$ Hz)	6.92 (2H, d, $J = 8.8$ Hz)	6.88 (2H, d, $J = 8.8$ Hz)	6.86 (2H, d, $J = 8.9$ Hz)
H-6′	7.54 (2H, d, $J = 8.7$ Hz)	7.83 (2H, d, $J = 8.8$ Hz)	7.98 (2H, d, $J = 8.8$ Hz)	8.05 (2H, d, $J = 8.9$ Hz)
H-1″	5.33 (1H, d, $J = 7.4$ Hz)	3.11 (1H, t, $J = 9.3$ Hz)	5.31 (1H, d, $J = 7.8$ Hz)	5.31 (1H. d. $J = 7.6$ Hz)
H-1‴	4.37 (3H, s)	5.22 (3H, s)	4.38 (3H, s)	4.38 (3 H. s)
C-2	156.4	166.2	156.4	156.4
C-3	133.3	104.0	133.2	133.1
C-4	177.4	184.2	177.2	177.3
C-5	156.6	n.d.	161.0	161.1
C-6	98.7	109.5	98.7	98.6
C-7	164.0	164.9	164.0	164.0
C-8	93.6	96.0	93.7	93.6
		94.9		
C-8a	161.2	158.8	156.4	156.7
C-4a	104.0	105.4	103.6	103.9
C-1′	121.2	123.1	120.7	120.8
C-2′	115.2	129.5	130.8	130.8
C-3′	144.7	118.0	114.9	115.0
C-4′	148.4	162.9	159.8	159.8
C-5′	116.2	117.1	114.9	115.0
C-6′	121.6	129.5	130.8	130.8
C-1″	101.2	73.4	101.9	101.2
C-2″	74.1	77.8	71.0	74.1
C-3″	76.4	81.6	72.8	76.2
C-4″	70.0	72.1	67.9	69.8
C-5″	75.9	82.6	73.4	75.6
C-6″	67.0	62.8	65.1	66.8
C-1‴	100.7	102.5	99.9	100.7
C-2‴	70.4	72.4	70.5	70.2
C-3‴	70.5	72.2	70.3	70.5
C-4‴	71.8	73.7	71.8	71.7
C-5‴	68.2	69.9	68.2	68.1
C-6‴	17.7	18.1	17.8	17.6

[a] Solvent: DMSO-d_6. δ in ppm from TMS.
[b] Solvent: Methanol-d_4. δ in ppm from TMS.

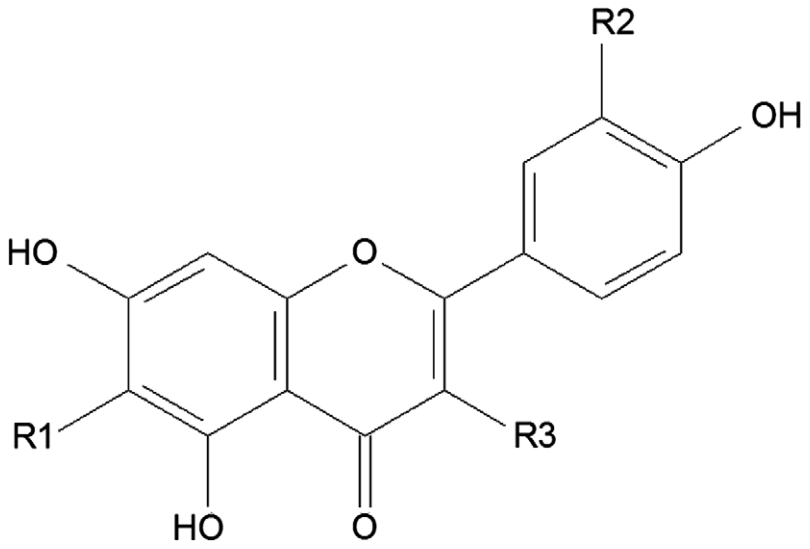

Fig. 3. Molecular structure of the isolated compound from Ooitabi: (**1** and **3**) R1 = H, R2 = OH, R3 = *O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside, (**2**) R1 = *C*-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside, R2 = H, R3 = H, (**4**) R1 = H, R2 = H, R3 = *O*-α-L-rhamnopyranosyl-(1→6)-β-D-galactopyranoside, (**5**) R1 = H, R2 = H, R3 = *O*-α-L-rhamnopyranosyl-(1→6)-β-D-glucopyranoside.

Table 2
The SC_{50} and IC_{50} of each flavonoid glycosides determined by DPPH radical scavenging assay and xanthine oxidase inhibitory assay, respectively

Compound	DPPH	XOD
	SC_{50} (mM)	IC_{50} (mM)
1 and **3**	0.05	0.14
2	>0.4	>0.4
4	>0.4	>0.4
5	>0.4	>0.4
α-Tocopherol	0.17	–

Table 3
The quantity of each flavonoid glycoside compounds extracted by three different solvents

Extract	mg/g of dry weight of Ooitabi extract				
	Dry weight matter	**1** and **3**	**2**	**4**	**5**
Water	12.6	1.69	0.90	Trace	0.21
50% aq. EtOH	10.4	2.38	3.15	0.16	0.01
EtOH	1.5	Trace	0.01	Trace	Trace

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