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Identification and Antiradical Properties of Anthocyanins in Fruits of *Viburnum dilatatum* Thunb.

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The fruit of *Viburnum dilatatum* Thunb., called gamazumi, has been shown to prevent oxidative injury in rats given gamazumi crude extract (GCE). Therefore, phenolic compounds in GCE were purified by Sephadex LH-20 column chromatography and preparative high-performance liquid chromatography. Two major anthocyanins were isolated, and their structures were determined by NMR, liquid chromatography/electrospray ionization mass spectrometry/MS and sugar analysis as cyanidin 3-*O*-(2-*O*-β-D-xylopyranosyl)-β-D-glucopyranoside, namely, cyanidin 3-sambubioside (**I**) and that of cyanidin 3-*O*-β-D-glucopyranoside, namely, kuromanin (**II**). This is the first identification of these compounds in *V. dilatatum* fruits, which contain **I** and **II** at 141.3 and 4.5 μg/g of fresh fruit, respectively. By the electron spin resonance method, the superoxide anion radical scavenging activities (IC_{50}) of **I** and **II** were 17.3 and 69.6 μM, and their activities on hydroxyl radicals were 4.3 and 53.2 mM. As a positive control, the activities of ascorbic acid were 74.2 μM on superoxide anion radicals and 3.0 mM on hydroxyl radicals. Our results suggest that these anthocyanins having radical scavenging properties may be key compounds contributing to the antioxidant activity and physiological effects of *V. dilatatum* fruits.

KEYWORDS: ***Viburnum dilatatum*** **Thunb. (gamazumi, snowball tree); cyanidin 3-*O*-(2-*O*-β-D-xylopyranosyl)-β-D-glucopyranoside (cyanidin 3-sambubioside); cyanidin 3-*O*-β-D-glucopyranoside (cyanidin 3-glucoside); radical scavenging activity**

INTRODUCTION

Viburnum dilatatum Thunb., called gamazumi, belonging to the plant family of Caprifoliaceae, is widely distributed in Japan. The fruit has a dark red color and is edible, used by some of the population as a spice in wines and pickles, but is not developed commercially. Recently, the squeezed juice of *V. dilatatum* fruits began to be manufactured in the town of Sannohe. The physiological activities of GCE have been examined in vivo, and a preventive effect on oxidative injury was found in rats subjected to stress (*1*). The results strongly suggested that ingestion of GCE may contribute to reducing the consumption of antioxidant enzymes. In addition, the antioxidant components of GCE exert a direct or supplementary protective action on rats (*2*). More study of the constituents of GCE is required to elucidate the functional components. Although the existence of quercetin, kaempferol, and several anthocyanins in *V. dilatatum* fruit has been reported (*3*−*6*), active compounds for antioxidant have not yet been investigated in this fruit.

Anthocyanins are common plant pigments and are largely responsible for the brilliant orange, pink, scarlet, red, violet, and blue colors of the flower petals and fruits of higher plants (*7*). They belong to the widespread class of phenolic compounds collectively named flavonoids but differ structurally from other flavonoids, except for flavan-3-ols, and do not have a carbonyl group in the C-ring. Recently, anthocyanins have attracted much interest as functional compounds for coloring food (*8*, *9*) and as potent agents against oxidative stress (*10*−*13*). The increasing number of reports on the physiological activities of anthocyanins are a good indication that considerable progress is being made in this field. Numerous products of bilberry have been commercialized on the basis of its antioxidant properties (*14*, *15*). This preventive benefit of anthocyanins and natural sources containing them has resulted in much interest from the botanical supplement industry in manufacturing products.

Therefore, it was necessary to identify the anthocyanins and other phenolic compounds in *V. dilatatum* fruit in order to elucidate the physiological activities of this fruit and to increase the additional value of its products. In this study, we have determined the structures of anthocyanins that appear to be functional components in *V. dilatatum* fruit. The radical scavenging activities of isolated anthocyanins were evaluated

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for one part of the elucidation of the antioxidant properties and physiological functions of this fruit.

MATERIALS AND METHODS

Chemicals. DTPA (Dojindo Laboratories Co., Kumamoto, Japan), DMPO (Labotec Co., Tokyo, Japan), hypoxanthine (Sigma Chemical Co., St. Louis, MO), xanthine oxidase from buttermilk (Sigma Chemical Co.) and L-(+)-ascorbic acid (Tokyo Kasei Kogyo Co., Tokyo, Japan) were used for electron spin resonance (ESR). The highest available grade of each reagent was used. Sephadex LH-20 resin was obtained from Sigma Chemical Co. All other reagents and solvents used in this study were analytical or high-performance liquid chromatography (HPLC) grade.

Preparation of Anthocyanins from *V. dilatatum* Fruit. *V. dilatatum* fruits were harvested in the town of Sannohe and were squeezed to obtain juice with a juice extractor without addition of water or any other material. The GCE was obtained from the squeezed juice of *V. dilatatum* by filtration through cloth, according to our previous method (*1*). The yield of freeze-dried GCE was 50.4 g/kg of fresh fruits. The content of total phenolic compounds in GCE was 53.0 mg/g of gallic acid equivalent by the Folin−Denis method (*12*). The GCE was concentrated with a rotary evaporator at 35 °C for loading onto a 700 mm × 25 mm i.d. Sephadex LH-20 column. The Sephadex LH-20 column was eluted stepwise with H_2O, 20, 60, and 100% methanol. All solvents contained 0.1 N hydrochloric acid to ensure the stability of anthocyanins. The 20% methanol fraction was concentrated at 35 °C and was used for isolation of anthocyanins from GCE by HPLC.

HPLC for Isolation and Quantification of Anthocyanins. Analytical HPLC, using a D-7100S pump (Hitachi Ltd., Tokyo, Japan) was performed on a 250 mm × 4.6 mm i.d. Capcell Pak C_{18} AG120 column (Shiseido Co. Ltd., Tokyo, Japan) with solvent A (5% formic acid) and solvent B (acetonitrile) under gradient elution at a flow rate of 1.0 mL/min. The pigments were analyzed under the following conditions: a linear gradient from 2 to 45% of solvent B in solvent A for 60 min. Anthocyanins were monitored at 500 nm with a L-7450 photodiode array detector (Hitachi Ltd., Tokyo, Japan).

Preparative HPLC with a L-6200 pump (Hitachi Ltd.) was performed on a 250 mm × 20 mm i.d. Capcell Pak C_{18} AG120 column (Shiseido Co. Ltd.) at a flow rate of 4 mL/min under the following conditions with solvent A (5% formic acid) and solvent B (methanol): a linear gradient from 15 to 40% of solvent B in solvent A for 60 min. The collected anthocyanins were monitored at 500 nm with a L-4200 UV−vis spectrophotometer (Hitachi Ltd.).

Quantification of anthocyanins was accomplished under the analytical conditions described above. Each sample was injected in triplicate, and the linear standard calibration curves ($r > 0.9997$) were generated by injection of four concentrations in the range of 1 and 20 μg of purified anthocyanins without an internal standard. The recoveries of anthocyanins at 1 and 20 μg were over 90%.

Identification of Anthocyanins in *V. dilatatum* Fruit. 1. NMR Analysis. The structure of purified anthocyanins from GCE was determined by NMR. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectra were measured on a JNM A-600 instrument (JEOL Ltd., Tokyo, Japan). The purified samples were dissolved in methanol-d_4 containing tetramethylsilane as an internal standard.

2. LC/Electrspray Ionization (ESI)-MS/MS Analysis. LC/ESI-MS/MS analyses were performed on a LCQ Advantage ion trap mass spectrometer equipped with an ESI interface, Xcalibur operating software (ThermoElectron Co., San Jose, CA), and Nanospace SI-II semimicro HPLC system (Shiseido Co. Ltd.). The column used was a 150 mm × 2.0 mm i.d. Capcell Pak C_{18} UG120 (Shiseido Co. Ltd.) at a flow rate of 0.2 mL/min under the following conditions with solvent A (1% formic acid) and solvent B (methanol): a linear gradient from 5 to 70% of solvent B in solvent A for 60 min. Anthocyanins were detected by absorption at 500 nm. The ESI parameters were as follows: sheath gas (N_2) flow rate, 2.5 L/min; auxiliary gas (N_2) flow rate, 0.4 L/min; ion spray voltage, 5 kV; capillary temperature, 290 °C; capillary voltage, 10 V; tube lens offset, 55 V. The ion trap mass spectrometer was operated in positive ion mode scanning from *m*/*z* 50 to *m*/*z* 2000. The mass spectrometer was programmed to obtain the MS and MS/MS spectra using a collision energy of 35.

3. Sugar Analysis. Isolated anthocyanins were hydrolyzed with 4 N hydrochloric acid at 100 °C for 2 h, and then, they were evaporated. After pyridylamination of the sugar fractions, they were analyzed on HPLC by the method of Hase (*16*). The pyridylamination was carried out with the model 4000 Takara Palstation kit (Takara Co., Kyoto, Japan). The HPLC analytical conditions were as follows: pump, L-6200; column, 150 mm × 4.6 mm i.d. Palpak Type A (Takara Co.); column temperature, 65 °C; mobile phase, 0.7 M borate buffer (pH 9.0) containing 10% acetonitrile; flow rate, 0.3 mL/min; detection, fluorescence at 380 nm emission by 310 nm excitation with a F-1050 fluorescent detector (Hitachi Ltd.). In this analysis, commercial D-sugars, except for L-rhamnose and L-fucose, were used as standards.

4. Radical Scavenging Assay. Superoxide anion radical scavenging activity was measured by a modification of the procedure described by Noda et al. (*17*). Superoxide anion radicals were generated by a hypoxanthine−xanthine oxidase reaction. Into a glass test tube, 30 μL of 4.5 M DMPO, 50 μL of 5 mM hypoxanthine, 20 μL of 9.6 mM DTPA, 50 μL of sample solution, and 50 μL of 0.4 U/mL xanthine oxidase were added in that order and were immediately mixed. Reagents were dissolved in 100 mM phosphate buffer (pH 7.4) prepared with deionized water. Samples were diluted with 100 mM phosphate buffer (pH 7.4). Measurement of the ESR spectrum was started at 30 s after addition of xanthine oxidase. The ESR spectra were obtained with a JES-FR30 free radical monitor (JEOL Ltd.) under the following conditions: power, 4 mW; center field, 335.6 mT; sweep width, ±5 mT; modulation frequency, 9.4 GHz; modulation width, 79 μT; sweep time, 1 min; time constant, 0.1 s; and amplification, 200. The spectra were normalized using manganese oxide as an internal standard.

Hydroxyl radical scavenging activity was measured by the spin trapping technique (*17*) according to the following procedure. Into a glass test tube, 20 μL of 0.9 M DMPO, 37.5 μL of 40 mM iron(II) sulfate, 37.5 μL of 1 mM DTPA, 30 μL of sample solution, and 75 μL of 1 mM hydrogen peroxide were added in that order and mixed. Measurement of the ESR spectrum was started 30 s after addition of hydrogen peroxide. The spectrum measurement conditions were as follows: power, 4 mW; center field, 335.5 mT; sweep width, ±5 mT; modulation frequency, 9.4 GHz; modulation width, 0.32 mT; sweep time, 1 min; time constant, 0.1 s; and amplification, 20.

After measurement of all spectra in triplicate, the inhibition values of samples at each concentration were calculated from the ratio of radical signal with or without each sample. The radical scavenging activity was calculated as the median inhibitory concentration (IC_{50}) from the correlation between each mean inhibition value and sample concentration.

Table 1. Radical Scavenging Activities (IC_{50}) of Crude Fractions from GCE

fraction	superoxide anion radical (μg/mL)	hydroxyl radical (mg/mL)
GCE	2.1×10^3	21.8
H_2O	187.6	230.7
20% methanol	81.2	8.3
60% methanol	32.0	4.6
100% methanol	674.3	37.0

RESULTS AND DISCUSSION

Yields and Radical Scavenging Activities of Crude Extracts from *V. dilatatum* Fruit. The yields of the H_2O, 20, 60, and 100% methanol fractions through the Sephadex LH-20 column chromatography were 9.7 g, 173 mg, 109 mg, and 36 mg from 10 g of GCE. The median inhibitory concentrations (IC_{50}) of these fractions as the superoxide anion and hydroxyl radical scavenging activities are shown in **Table 1**. On both radicals, the strongest activity was found in the 60% methanol fraction, and secondary activity was found in the 20% methanol

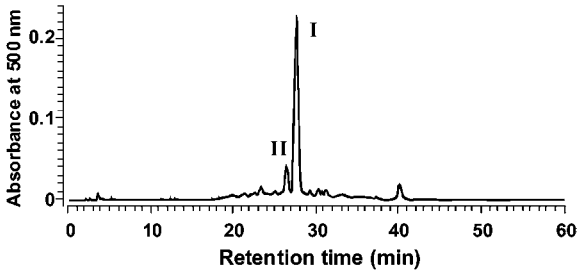

Figure 1. HPLC chromatogram of anthocyanins in the 20% methanol fraction obtained from *V. dilatatum* fruits. Analytical conditions were described in the text.

fraction. The H_2O fraction was the largest amount in GCE, but its radical scavenging activities were weaker than the 20 and 60% methanol fractions. Therefore, the 20 and 60% methanol fractions were selected for HPLC analysis.

Identification and Content of Anthocyanins in *V. dilatatum* Fruit. Each fraction was analyzed by HPLC under gradient conditions with 5% formic acid and acetonitrile as mobile phases. The HPLC profile of the 20% methanol fraction showed three peaks of anthocyanins, which had the absorption maximum in the range of 500 and 510 nm, at retention times of 26.9 (compound **II**), 27.6 (compound **I**), and 39.9 min (**Figure 1**). Among other peaks, two major peaks were also detected in the 60% methanol fraction. Therefore, the major peak pattern, detected by absorption at 500 nm, was the same in the 20 and 60% methanol fractions. However, the anthocyanin peak was small in the H_2O and 100% methanol fractions having lower radical scavenging activities than those of 20 and 60% methanol fractions.

Further isolation of the anthocyanins from GCE was performed by preparative HPLC under the gradient conditions of 5% formic acid/methanol. This procedure allowed **I** and **II** to be separated and obtained in pure form. Then, the anthocyanins were subjected to structural analysis by LC/ESI-MS/MS spectrometry, NMR spectroscopy, and sugar analytical HPLC.

A mass number of *m*/*z* 581 $[M]^+$ was found in the LC/ESI-MS spectrum of **I**, and a mass number of *m*/*z* 287 was obtained in the MS/MS spectrum. It was expected that the *m*/*z* 287 showed $[aglycone]^+$ of cyanidin and these mass spectra indicated **I** as a cyanidin diglycoside. The ^1H NMR spectrum suggested that **I** had cyanidin as an aglycone. Furthermore, these ^1H and ^{13}C NMR spectra also suggested that it was a cyanidin diglycoside (**Table 2**).

The sugar fraction of **I**, obtained by hydrolysis, was subjected to pyridylamination (*16*) and then analyzed by HPLC. The sugars of **I** were determined to be glucose and xylose by comparison with authentic compounds (**Figure 2**). The binding site of position 1 of glucose on position 3 of the aglycone was derived from the HMBC spectrum of **I**. The rather high field position (4.70 ppm, $J = 7.8$ Hz) of the anomeric proton of xylose indicated a sugar−sugar linkage. This linkage, in which position 2 of glucose is bound to position 1 of xylose, was also confirmed by observation of the HMBC spectrum. The assigned NMR data are summarized in **Table 2**. These results indicate that the structure of compound **I** is cyanidin 3-*O*-(2-*O*-*β*-D-xylopyranosyl)-*β*-D-glucopyranoside, namely, cyanidin 3-sambubioside (**Figure 3**). This compound has been identified in *Sambucus nigra* (*18*, *19*), *Sambucus canadensis* (*20*), and *Sambucus ebulus* (*21*). All δ_H values of **I** shifted to high field in 0.1−0.2 ppm as compared to those of cyanidin 3-sambubioside in *S. canadensis* (*20*). Although other papers deal with anthocyanin spectrum in *Viburnum* species using paper chromatographies (*4*, *5*), compound **I** was assigned as cyanidin 3-sambubioside from NMR, MS, MS/MS, and sugar analysis data in detail.

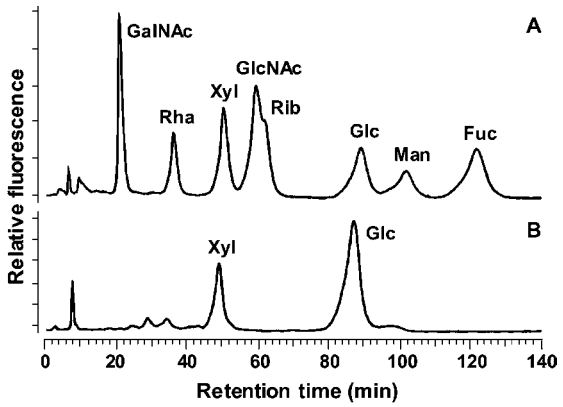

Figure 2. HPLC chromatograms of 2-aminopyridine derivatives: (**A**) standard mixture of sugars; (**B**) sugar fraction of hydrolyzed compound **I**. GalNAc, *N*-acetyl-D-galactosamine; Rha, L-rhamnose; Xyl, D-xylose; GlcNAc, *N*-acetyl-D-glucosamine; Rib, D-ribose; Glc, D-glucose; Man, D-mannose; Fuc, L-fucose. Analytical conditions were described in the text.

Table 2. ^1H (600 MHz) and ^{13}C (150 MHz) NMR Spectroscopic Data for Compounds **I** and **II** (in CD_3OD)[a]

	compound **I**			compound **II**	
atom	δ_C	δ_H (J in Hz)		δ_H (J in Hz)	
		Cyanidin			
2	163.91				
3	145.24				
4	135.96	8.81	s	8.98	s
5	159.09				
6	103.32	6.55	d (1.9)	6.65	d (1.8)
7	170.21				
8	95.08	6.79	d (1.9)	6.89	s
9	157.39				
10	113.09				
1′	121.14				
2′	119.34	7.89	d (2.2)	8.06	d (2.4)
3′	147.04				
4′	155.79				
5′	117.34	6.89	d (8.9)	7.02	d (9.0)
6′	128.79	8.18	dd (2.4, 8.5)	8.28	d (2.4)
		3-*O*-*β*-Glucopyranoside (A)			
1	101.42	5.45	d (7.8)	5.40	d (7.8)
2	81.59	3.95	dd (1.2, 11.1)	3.88	dd (3.0, 9.6)
3	79.69	3.59	m	3.81	dd (5.4, 3.6)
4	70.69	3.51	d (11.4)	3.60	m
5	77.85	3.45	dd (9.6, 9.0)	3.77	dd (5.4, 3.6)
6a	62.35	3.92	t (5.4)	4.21	dd (9.6, 7.8)
6b		3.77	dd (10.8, 7.2)	3.96	d (3.0)
		O-*β*-Xylopyranosyl (B)			
1	105.59	4.79	d (7.6)		
2	75.67	3.19	dd (9.0, 9.6)		
3	79.20	3.81	t (9.0)		
4	70.79	3.59	m		
5a	67.20	3.72	dd (4.2, 6.6)		
5b		3.07	dd (unresorved)		

[a] Abbreviations: s, singlet; d, doublet; dd, double doublet; m, multiplet; t, triplet.

The summarized assignment of ^1H NMR spectroscopic data of compound **II** is shown in **Table 1**. The ^1H NMR spectrum of **II** was similar to that of **I**, except for the portion relating to the sugars. This compound also appeared to have cyanidin as an aglycone but was a monoglycoside. The coupling constants of the anomeric protons ($J = 7.8$ Hz) indicated the sugar to be

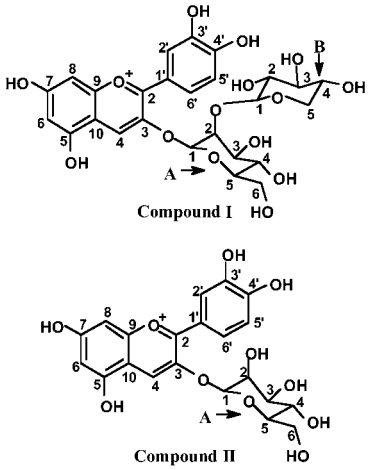

Figure 3. Structures of cyanidin 3-sambubioside (**I**) and cyanidin 3-glucoside (**II**).

Table 3. IC_{50} Value of Radical Scavenging Activities of Compounds **I** and **II** by ESR

	superoxide anion radical		hydroxyl radical	
	μg/mL	μM	mg/mL	mM
I	10.1	17.3	2.5	4.3
II	31.3	69.6	23.9	53.2
ascorbic acid	13.1	74.2	0.5	3.0

the β-form of glucose. In the LC/ESI-MS/MS analysis of **II**, the MS spectrum showed a mass of *m*/*z* 449 $[M]^+$, and the MS/MS spectrum showed a mass of *m*/*z* 287 $[aglycone]^+$; these mass units are indicative of cyanidin hexose. From these results, the chemical structure of **II** was confirmed as cyanidin 3-*O*-β-D-glucopyranoside, namely, kuromanin or cyanidin 3-glucoside, **II** (**Figure 3**). The MS/MS spectrum of **II** corresponded to that of the commercial authentic compound. Kuromanin is widely distributed in edible plants. Identification of the minor anthocyanin is under way.

The contents of these anthocyanins were determined by HPLC with monitoring of absorption at 500 nm. The concentrations of **I** and **II** in GCE were 2804.4 ± 334.9 and 89.6 ± 14.3 μg/g, respectively, which correspond to 141.3 and 4.5 μg/g, respectively, in fresh fruit.

Radical Scavenging Activities of Compound I and II. Our previous study suggested that the ingestion of GCE by rats subjected to oxidative stress attenuated the reduction of antioxidant enzymes, presumably because of the presence of antioxidant compounds in GCE (*2*). To elucidate the antioxidant activity of the compounds in GCE, the superoxide anion and hydroxyl radical scavenging activities of compound **I** and **II** were evaluated by the ESR method and compared with those of ascorbic acid as a positive control. In this study, **I** and **II** were dissolved in distilled water as stock solutions and diluted with physiological buffer (pH 7.4) to the appropriate concentrations just before the ESR measurements. The IC_{50} values of these compounds under these experimental conditions are shown in **Table 3**. Compounds **I** and **II** showed the dose-dependent radical scavenging activities (data not shown). The specific scavenging activity on superoxide anion radicals was in the order **I** > **II** > ascorbic acid, the two anthocyanins showing more potent activity than ascorbic acid. The hydroxyl radical scavenging activity was in the order ascorbic acid > **I** > **II**; however, the activity of **I** was similar to that of ascorbic acid and superior to that of **II**.

Cyanidin 3-sambubioside, compound **I**, has been found in several varieties of *Sambucus* (*18*−*21*). However, little has been published regarding the antioxidant potencies of the purified anthocyanin. Kuromanin has been extensively examined for its physiological functions and is known to have strong antioxidant activity (*11*, *22*). It has also been reported that cyanidin-based anthocyanins are effective quenchers of free radicals (*23*). Noda et al. (*24*) suggested that delphinidin, cyanidin, or pelargonidin directly scavenged superoxide anion radicals, not via inhibition of enzymes, whereas they indirectly scavenged hydroxyl radicals by chelating with ferrous ion in the hydroxyl radical generation system. We also found that the yield of DMPO−OOH spin adducts with each sample concentration shifted with the change of DMPO concentration in a dose-dependent manner (data not shown). Therefore, compounds **I** and **II** are suggested to directly scavenge superoxide anion radicals. Whereas, direct scavenging actions of **I** and **II** on hydroxyl radicals were uncertain, because the dose-dependent yield of DMPO−OH with the change of DMPO concentration was not found.

These results suggest that compound **I**, the main anthocyanin in the fruit of *V. dilatatum*, contributes most to the antiradical effect of GCE by its direct radical scavenging activity and, together with the previous study, indicate that anthocyanins may play an important role as antioxidants under physiological conditions. The phenolic compounds other than anthocyanins in *V. dilatatum* fruit are currently being purified and identified.

ABBREVIATIONS USED

GCE, gamazumi crude extract; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DTPA, diethylenetriamine-*N*,*N*,*N*′,*N*′′,*N*′′-pentaacetic acid.

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