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Characterization and Activity of Anthocyanins in Zijuan Tea (*Camellia sinensis* var. *kitamura*)

Lihua Jiang,[†] Xiaojia Shen,[†] Toshihiko Shoji,[‡] Tomomasa Kanda,[§] Jiachun Zhou,[†] and Liming Zhao*[,†]

[†]State Key Laboratory of Bioreactor Engineering, R&D Center of Separation and Extraction Technology in Fermentation Industry, East China University of Science and Technology, Shanghai 200237, China

[‡]National Agriculture & Food Research Organization NARO, Plant Physiology & Fruit Chemistry Division, National Institute of Fruit Tree Science, Tsukuba, Ibaraki 305-8605, Japan

[§]Asahi Group Holdings Ltd., Research Laboratories for Fundamental Technology of Food, Moriya, Ibaraki 302-0106, Japan

ABSTRACT: Zijuan tea is a new cultivar produced in Yunnan province of China. Unlike most tea cultivars, Zijuan tea is anthocyanin-rich. The composition and antioxidant activities of anthocyanins of Zijuan tea were studied for the first time in this paper. Anthocyanins were extracted with acidified methanol and quantified as 707 ± 28 μg/g of dry weight (cyanidin-3-*O*-β-D-glucoside equivalent) by high-performance liquid chromatography (HPLC) analysis. Four anthocyanins were successfully identified after Amberlite XAD-7HP adsorption column chromatography and octadecyl silane (ODS) flash chromatography. Among the four, delphinidin-3-*O*-β-D-galactoside (**1**) and cyanidin-3-*O*-β-D-galactoside (**2**) were confirmed by liquid chromatography−electrospray ionization mass spectrometry (LC-ESI-MS) and HPLC. Delphinidin-3-*O*-β-D-(6-(*E*)-*p*-coumaroyl) galactopyranoside (**3**) and cyanidin-3-*O*-β-D-(6-(*E*)-*p*-coumaroyl) galactopyranoside (**4**) were characterized by the high-resolution time-of-flight-mass spectrometry (HRTOF-MS) and nuclear magnetic resonance (NMR) spectrometry. The antioxidant activities of compounds **3** and **4**, which composed approximately 75% of the total anthocyanins content in HPLC analysis, were evaluated by DPPH and FRAP assays. Results showed that both had higher antioxidant activities than commercial antioxidants butylated hydroxytoluene (BHT) used as one of controls in these assays.

KEYWORDS: *Zijuan tea, anthocyanin, antioxidant activity, LC-ESI-MS, NMR*

INTRODUCTION

Tea (*Camellia sinensis*), a popular aromatic beverage, is well-known for its color, flavor, and taste. Catechins, a class of flavonoids largely present in tea, especially green tea, are known to be one of the most important components for tea owing to its health contributing potential.[1] Anthocyanins, responsible for coloring of fruits, vegetables, flowers, and grains, are another major functional flavonoid class. Although anthocyanins are widely distributed in higher plants, anthocyanin-rich tea cultivars are seldom reported. In this decade, three tea plants, Benibana-cha, Sunrouge tea, and Zijuan tea, had been identified as rich in anthocyanins.[2−4]

The chemical structures and properties of anthocyanins had been studied by many researchers' hard work.[5] To data, more than 670 anthocyanins have been identified by nuclear magnetic resonance (NMR) spectral analysis.[6] Anthocyanidins are polyhydroxy and polymethoxy derivatives of 2-phenylbenzopyrylium (flavylium) salts. Most anthocyanins are a composite of six common anthocyanidins: cyanidin, delphinidin, peonidin, pelargonidin, petunidin, and malvidin and sugar moieties. In some cases, sugar moieties may link with aliphatic and/or aromatic acyl groups. It is reported that anthocyanins have various bioactivities such as removing free radicals, antimutagenic activity, and protecting cardiovascular disease.[7] Because oxidation may cause health risks, many investigators have focused on antioxidant activities of anthocyanins. For example, cyanidin-3-*O*-β-D-glucoside, a typical anthocyanin pigment, can increase the oxidation resistance of the serum to lipid peroxidation in rats;[8] delphinidin-3-caffeoylrutinoside-5-glucoside showed high radical-scavenging activities toward both 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical and linoleic acid radical.[9]

Zijuan tea (*Camellia sinensis* var. *kitamura*), an anthocyanin-rich tea cultivar, has been grown in the Yunnan province of China and was classified as a variety for protection (no. 20050031) by the China State Forestry Administration in 2005. This tea plant, having purple-colored stems, leaves, and buds, is a mutant of the broad-leaf variety of the tea plant (*Camellia sinensis* var. *assamica*). Zijuan tea, the leaves of which are sun-dried, has been sold in Chinese tea market and is gradually popular for its characteristic color, taste, and fragrance after being largely cultivated. The compositions of Zijuan tea were simply described to possess abundant anthocyanins, catechins, and theanines.[4] Several Chinese researchers reported that fermented Zijuan tea, owing to its "large molecular pigments" content, had effects on lowering blood lipids in rats consuming a high-lipid diet.[10] However, no research about the chemical composition of anthocyanins in Zijuan tea has been conducted.

In this study, our purpose was to explore the anthocyanins composition and their antioxidant activities in Zijuan tea. Four compounds were isolated and characterized (Figure 1), which is the first report for Zijuan tea. Additionally, an attempt was made to evaluate antioxidant activities of compounds **3** and **4**.

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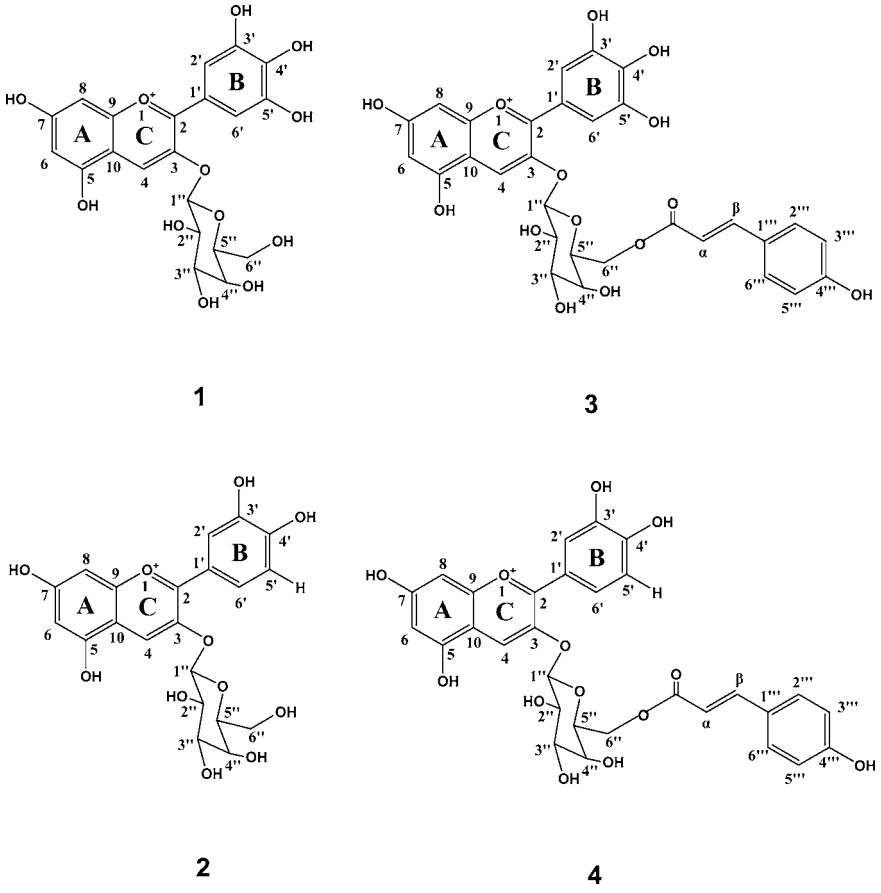

Figure 1. Structures of compounds **1**–**4** from Zijuan tea.

This investigation would provide a fundamental database for further study on anthocyanins from Zijuan tea and help us to realize the value of this unique tea species.

MATERIALS AND METHODS

Safety. No special safety problem needs to be emphasized.

Materials and Chemicals. Zijuan tea was bought from Yunnan's local market in China; black tea, oolong tea, pu-erh tea, and green tea were bought from a tea store in Shanghai, China. Amberlite XAD-7HP was a product of Rohm and Haas Company (Philadelphia, PA, USA). Silica gel 60 reverse phase C_{18} (ODS, 40–63 μm) was a product of Merck (Darmstadt, Germany). WondaSep C_{18} columns were purchased from GL Sciences, Inc. (Tokyo, Japan). Trifluoroacetic acid (TFA) was purchased from Aladdin Chemistry Co., Ltd. (Shanghai, China). MeOH-d_4 (0.05% tetramethylsilane, TMS) and TFA-d were both purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). Butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), DPPH, and 2,4,6-tripyridyl-2-triazine (TPTZ) were ordered from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). α-Tocopherol and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) were ordered from Sigma-Aldrich Co. (St. Louis, MO, USA). Standards of the cyanidin-3-*O*-β-D-glucoside and delphinidin-3-*O*-β-D-glucoside were products of Polyphenols Laboratories (Sandnes, Norway). All other chemicals were of analytical or high-performance liquid chromatography (HPLC) grade.

Presence of Anthocyanins. The samples of Zijuan tea, black tea, oolong tea, pu-erh tea, and green tea were filtered after being immersed in 0.1% citric acid solution at 90 °C for 5 min and scanned under a Hitachi U-2000 spectrophotometer (Hitachi Ltd., Tokyo, Japan) between 250 to 700 nm.

Quantification. Zijuan tea (20.0 g) was ground into powder and immersed overnight in acidified methanol (0.1% TFA) at 4 °C. This extraction was repeated three times to ensure that anthocyanins were exhaustively extracted. The combined extract was topped up to 500 mL in a volumetric flask with acidified methanol and stored at 4 °C in the dark prior to HPLC quantification.

The quantification of anthocyanin was determined by a HPLC calibration curve of standard cyanidin-3-*O*-β-D-glucoside. The calibration curve was recorded in six different concentrations, 0.50, 0.25, 0.10, 0.05, 0.025, and 0.0125 mg/mL, respectively. Each concentration of the standard solution and the extract of Zijuan tea were analyzed in triplicate. The results are reported as micrograms of cyanidin-3-*O*-β-D-glucoside equivalents per gram dry weight (DW) of Zijuan tea.

Extraction and Isolation. Zijuan tea (250.0 g) was extracted three times using the same process described above. The filtered extract was combined and condensed at below 35 °C to remove most of methanol. Deionized water (500 mL) was added to the methanol concentrate and evaporating continued to remove all methanol. The residual aqueous solution (about 400 mL) was partitioned with diethyl ether (250 mL × 3) to dissolve lipids and volatile compounds and then with chloroform (250 mL × 3) to remove caffeine. The aqueous phase containing the anthocyanins was evaporated and then submitted to Amberlite XAD-7HP adsorption column (30 mm × 550 mm) chromatography. After the free sugars and amino acids had been removed with acidified water, the anthocyanin fraction was successively eluted with acidified methanol (0.1% TFA, 1.5 L). The resulting eluent was concentrated and freeze-dried (20.1 g). The freeze-dried powder was washed with ethyl acetate (200 mL × 3) for separating most of cathechins. The residual solid was freeze-dried again and yielded 4.5 g of red powder.

The red powder (1.0 g) was further fractionated by ODS flash (35 mm × 300 mm, 40–63 μm) chromatography with stepwise elution of 0%, 10%, 20%, 30%, 40%, and 50% MeOH/H_2O (contained 0.1% TFA, each eluent was 1 L). The flow rate was 8 mL/min. Four red-colored fractions (I–IV) were collected and evaporated separately.

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Each fraction was then subjected to a small ODS column (15 mm × 300 mm, 40−63 μm) for further purification. The eluents of fractions I and II were 16% MeOH/H_2O (contained 0.1% TFA), while that of fractions III and IV were 27.5% MeOH/H_2O (contained 0.1% TFA). The flow rate was set at 2 mL/min. Compounds **1**, **2**, **3**, and **4** were obtained from fractions I, II, III, and IV, respectively. Compounds **3** and **4** were passed through WondaSep C_{18} column to remove TFA for NMR analysis.

HPLC Analysis. All samples including extracted, purified, and isolated anthocyanins from Zijuan tea were filtered (0.22 μm) and analyzed by HPLC with an analytical reverse phase C_{18} column (4.6 mm × 250 mm, 5 μm, Inertsil/Wondasil, GL Sciences). The analytical conditions were as follows: two solvents, (A) 10% aqueous acetonitrile (0.1% TFA) and (B) 50% aqueous acetonitrile (0.1% TFA); linear gradient conditions, 0−45 min, (0−75% B); flow rate, 0.8 mL/min; injection volume, 10 μL; column temperature, 28 °C; detection wavelength, 520 and 280 nm.

Spectral Analyses. Liquid chromatography−electrospray ionization mass spectrometry (LC-ESI-MS) analysis was carried out on a LCQ Deca XP ion trap mass spectrometer (ThermoQuest, Finnigan, San Jose, CA) coupled to an Agilent-1100 HPLC equipped with ultraviolet−visible (UV−vis) detector. The HPLC separation condition was the same mobile phase and gradients described above, except with the flow rate at 1.0 mL/min and the injection volume at 20 μL per sample with an Agilent 1100 series microautosampler. The MS parameters were as follows: ESI in the positive ion mode; scan range, m/z 50−1000; spray voltage, 4.8 kV; capillary temperature, 350 °C; capillary voltage, 15.0 V; sheath gas (N_2) flow rate, 60.00 arbitrary units; auxiliary gas (N_2) flow rate, 20.00 arbitrary units. Helium (He) was used as collision gas with automatic collision energy mode. The high-resolution time-of-flight-mass spectrometry (HRTOF-MS) spectral data were obtained with Micromass LCT system (Micromass, Manchester, UK). ^1H (400.13 MHz) and ^{13}C (100.53 MHz) NMR spectra were recorded on a Bruker Avance 400 instrument in MeOH-d_4/TFA-d (9:1, v/v) containing TMS as an internal standard. Chemical shifts were presented in δ (parts per million) and the coupling constants (J) in hertz, respectively.

DPPH Radical Scavenging Activity. The DPPH assay was evaluated according to the method of Brand-Williams et al.[11] Briefly, 1.0 mL of five different concentrations (4, 10, 16, 20, and 30 μg/mL) of ethanolic sample solutions of compounds **3** and **4** were added to 3.0 mL of 0.1 mM ethanolic DPPH solution, respectively. After the mixed solutions were held for 30 min at 25 °C in the dark, the absorbance was measured by a spectrophotometer at 517 nm. The scavenging activity of the DPPH radical was calculated as: DPPH radical scavenging activity (%) = (1 − absorbance of sample/absorbance of control) × 100%, where ethanol (1.0 mL) plus DPPH solution (3.0 mL) was used as a control. The percentage of scavenging activity was plotted against the sample concentration to obtain the IC_{50}, defined as the concentration of sample necessary to cause 50% inhibition.

Ferric Reducing Antioxidant Power (FRAP). The FRAP assay was carried out according to the method of Benzie and Strain.[12] FRAP reagent was freshly prepared by mixing 0.3 M acetate buffer (pH = 3.6), 10 mM TPTZ in 40 mM hydrochloric acid, and 20 mM $FeCl_3 \cdot 6H_2O$ in 10:1:1 ratio. After the sample solutions and FRAP reagent were held separately at 37 °C, 1.0 mL of a 20 μg/mL sample solution and 3.0 mL of FRAP reagent were mixed and held for 4 min at 37 °C. The absorbance of the mixed solution was measured at 595 nm with a control which contained water instead of the sample solution. Trolox calibration solutions (25, 50, 100, 150, and 200 μM) were used to generate the standard curve, and the results are expressed as micromoles of trolox equivalents (TE) per milligram of DW of samples (μmol of TE/mg).

Statistical Analysis. The data are presented as the mean of three replicate determinations and standard deviation (SD). Student's t-test was used for comparison between two means and a one-way analysis of variance (ANOVA) was used for comparison of more than two means. Probability values of $p < 0.05$ and $p < 0.01$ were considered statistically significant and extremely significant respectively. All statistical analyses were completed using IBM SPSS Statistics, version 19.0 (SPSS Inc., Chicago, IL).

RESULTS AND DISCUSSION

Anthocyanins in Zijuan Tea. Presence of anthocyanins in five tea cultivars: Zijuan tea, black tea, oolong tea, pu-erh tea, and green tea were detected by UV−vis absorption spectra. Only Zijuan tea had the largest adsorption at the 520 nm wavelength (data not shown). This indicated that Zijuan tea was anthocyanin-rich. The amount of anthocyanins in Zijuan tea quantified by HPLC was 707 ± 28 μg/g of DW. Although several anthocyanin compounds in Zijuan tea were detected in HPLC profile, compounds **1**, **2**, **3**, and **4** were major anthocyanins, the relative amounts of which were approximately 6%, 8%, 33%, and 41%, respectively (Figure 2). The four compounds were isolated by Amberlite XAD-7HP adsorption column chromatography and repeated ODS flash chromatography. Compounds **1** and **2** were identified as delphinidin-3-O-β-D-galactoside and cyanidin-3-O-β-D-galactoside by HPLC and LC-ESI/MS analyses. Compounds **3** and **4** were elucidated as delphinidin-3-O-β-D-(6-(E)-p-coumaroyl) galactopyranoside and cyanidin-3-O-β-D-(6-(E)-p-coumaroyl) galactopyranoside through HRTOF-MS and NMR spectroscopy analyses.

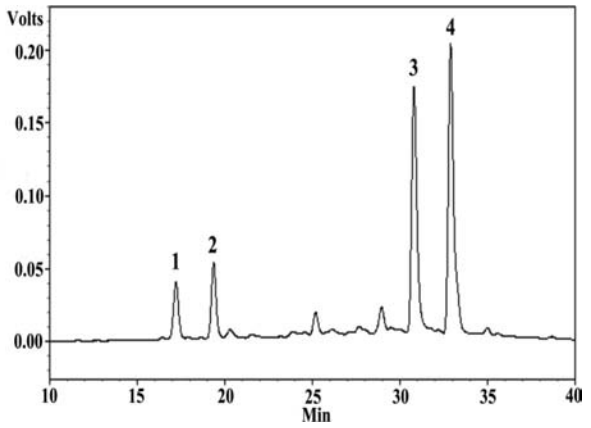

Figure 2. HPLC profile of compounds **1**−**4** from Zijuan tea detected at 520 nm.

Structural Elucidation. The LC-ESI-MS analysis of compound **1** showed a molecular ion $[M]^+$ at m/z 465.0 and a major fragmentation at m/z 303.3 ($[M - 161.7]^+$), which corresponded to a loss of a hexose unit. The major fragmentation at m/z 303.3 indicated the presence of a delphinidin aglycon. The common hexose unit linked to the aglycon was either glucose or galactose. Moreover, the HPLC retention time of compound **1** was anterior to the standard delphinidin-3-O-β-D-glucoside, which is in agreement with the order of elution of the glycosides on the C_{18} column reported previously.[13] Thus **1** was assumed to be delphinidin-3-O-β-D-galactoside compared with the published data in earlier studies.[14]

The LC-ESI-MS analysis of compound **2** showed a molecular ion $[M]^+$ at m/z 449.1 and a major fragmentation at m/z 287.4 ($[M - 161.7]^+$). This data corresponded to cyanidin as aglycon moiety and a hexose as sugar moiety. Similar to compound **1**, the sugar moiety was identified as a galactose by comparison with an authentic cyanidin-3-O-β-D-glucoside by HPLC

analysis.[15] Compound **2** was presumed to be cyanidin-3-*O*-*β*-D-galactoside.

The result of ^{1}H and ^{13}C NMR analyses for compounds **3** and **4** are summarized in Table 1. The ^{1}H and ^{13}C NMR spectra indicated that **3** consisted of three moieties. In the ^{1}H NMR spectrum, the observation of a singlet signal of a symmetric B ring at *δ* 7.76 (H-2′/ H-6′) and three aromatic protons at *δ* 8.87 (H-4), *δ* 6.53 (H-6), and *δ* 6.78 (H-8) showed that the aglycon moiety of **3** was a delphinidin nucleus, and the structure was suggested by the published data.[16] The sugar moiety of **3** was presumed to be a hexose from analyzing the ^{1}H and ^{13}C NMR spectra. From the appearance of a broad doublet signal (*J* = 3.4 Hz) of the H-4″ at *δ* 4.02, it was ascertained that the hexose unit was a galactosyl.[17] The anomeric proton signal of galactosyl at *δ* 5.31 with large coupling constant of 7.7 Hz indicated a *β*-linkage duo to axial−axial coupling between H-1″ and H-2″.[18] Moreover, the ^{1}H and ^{13}C NMR spectra analyses showed the existence of *p*-coumaroyl unit, and the large coupling constants (*J* = 16.0 Hz) for H-*α* and H-*β* revealed the *E*-configuration of *p*-coumaroyl.[19] On the basis of the ^{1}H and ^{13}C spectra of the delphinidin unit and a comparison with reference data it demonstrated that the linkage between the aglycon and galactosyl was at the delphinidin 3-hydroxyl. The downfield NMR shifts of H-6″a (*δ* 4.32), H-6″b (*δ* 4.59), and C-6″ (*δ* 64.6) of the galactosyl suggested that *p*-coumaroyl unit was linked at galactosyl 6″-hydroxyl.[17] The molecular ion was confirmed as $C_{30}H_{27}O_{14}^+$ by the HRTOF-MS analysis, giving a molecular ion peak at *m*/*z* 611.1411 (the exact mass of $C_{30}H_{27}O_{14}^+$ was *m*/*z* 611.1401). These spectral data elucidated compound **3** to be delphinidin-3-*O*-*β*-D-(6-(*E*)-*p*-coumaroyl) galactopyranoside, a structure that corresponded with the published data.[3]

Table 1. ^{1}H (400.13 MHz) and ^{13}C (100.53 MHz) NMR Data for Compounds 3 and 4 in CD_3OD/TFA-*d* (9:1, v/v)

	^{1}H *δ* (ppm), *J* (Hz)		^{13}C *δ* (ppm)	
	3	4	3	4
aglycon	delphinidin	cyanidin		
2			164.4	164.7
3			145.5	145.3
4	8.87 s	8.94 s	135.9	137.2
5			159.2	159.1
6	6.53 d (1.6)	6.54 d (2.0)	103.7	103.8
7			170.4	170.6
8	6.78 d (1.6)	6.83 d (2.0)	95.1	95.3
9			157.6	157.8
10			113.2	113.4
1′			120.1	121.3
2′	7.76 s	8.06 d (2.3)	112.9	118.7
3′			147.6	147.5
4′			144.8	156.0
5′		7.03 d (8.7)	147.6	117.6
6′	7.76 s	8.25 dd (2.3, 8.7)	112.9	128.4
galactosyl				
1″	5.31 d (7.7)	5.29 d (7.7)	103.8	103.8
2″	4.07 dd (7.7, 9.6)	4.05 dd (7.7, 9.6)	72.0	72.0
3″	3.76 dd (3.3, 9.8)	3.73 dd (3.4, 9.6)	74.8	74.8
4″	4.02 brd (3.4)	4.00 brd (3.2)	70.2	70.2
5″	4.13brdd (3.7, 9.0)	4.13brdd (3.5, 9.2)	75.2	75.3
6″a	4.32 dd (3.6, 11.9)	4.31 dd (3.5, 11.6)	64.6	64.6
6″b	4.59 dd (8.8, 12.0)	4.58 dd (8.9, 11.7)		
p-coumaroyl				
1‴			127.1	127.1
2‴, 6‴	7.28 d (8.6)	7.30 d (8.6)	131.3	131.3
3‴, 5‴	6.79 d (8.4)	6.80 d (8.6)	117.0	117.0
4‴			161.4	161.4
α	6.24 d (16.0)	6.24 d (15.9)	114.8	114.8
β	7.44 d (16.0)	7.45 d (15.9)	147.0	147.0
COO			169.1	169.1

The high-resolution MS analysis of compound **4** gave a molecular ion peak at *m*/*z* 595.1456, which is in good accordance with the calculated mass for $C_{30}H_{27}O_{13}^+$ (*m*/*z* 595.1452). The ^{1}H NMR spectrum showed almost identical resonances to those for **3**, except for a set of ABX-type aromatic signals at *δ* 8.06 (d, *J* = 2.3 Hz, H-2′), *δ* 7.03 (d, *J* = 8.7 Hz, H-5′), and *δ* 8.25 (dd, *J* = 2.3, 8.7 Hz, H-6′), suggesting that the structure is a cyanidin nucleus.[20] On the basis of these observations, **4** was characterized as cyanidin-3-*O*-*β*-D-(6-(*E*)-*p*-coumaroyl) galactopyranoside. This compound was also in good accordance with the reference data.[21]

The isolation and identification of four anthocyanins were the first time for Zijuan tea. Although compounds **1** and **2** were previously identified in many plants, compounds **3** and **4** were only reported in Benibana-cha, Sunrouge tea, and red flowers of *Camellia hongkongensis* Seem.[2,3,21] It seems that there is a specific biosynthesis route of **3** and **4** in genus *Camellia*. Compounds **1**−**4** have galactose as a sugar moiety, which implied that the biosynthesis pathway of galactose bound to aglycon may be characteristic to Zijuan tea. In addition, the difference of structures of **1**and **2** between **3** and **4**, just lacking one *p*-coumaroyl unit, infers that **1** and **2** may be the biodegradation products of **3** and **4** in Zijuan tea.

Antioxidant Activity. It was apparent that compounds **3** and **4** were the major anthocyanins by HPLC analysis, being nearly 75% of the total anthocyanins in Zijuan tea. However, any bioactivities of **3** and **4** are not clear up to now. Their antioxidant activities were evaluated with DPPH and FRAP assays in this present study.

The DPPH radical scavenging activity of **3** and **4** was both extremely significant higher than BHT ($p < 0.01$) but lower than BHA (Figure 3). All samples showed a dose-dependent manner in scavenging DPPH radical. **3** had slightly higher DPPH radical scavenging activity than **4** by comparing the IC_{50} value of **3** (26.4 *μ*g/mL) with **4** (29.8 *μ*g/mL). This result may

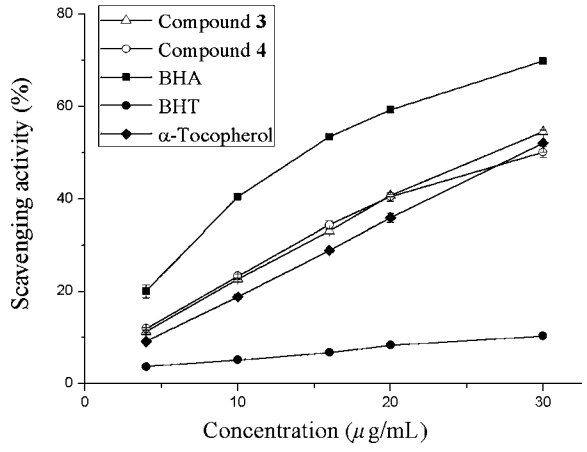

Figure 3. Comparison of DPPH radical-scavenging activities of the isolated compounds **3** and **4** with the commercial antioxidants.

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be caused by that anthocyanins with three hydroxyl groups on the B ring had slightly higher antioxidant activity than that with two hydroxyl groups.[22]

The FRAP value of **3** and **4** compared with BHA, BHT, and α-tocopherol as controls were concluded in Table 2. The FRAP value of **3** and **4** were both higher than that of BHT and α-tocopherol ($p < 0.01$) but inferior to that of BHA. Furthermore, the FRAP value of **3** was also slightly higher than that of **4**, which was similar to the result of DPPH assay.

Table 2. Comparison of the Antioxidant Activities of the Compounds 3 and 4 Isolated from Zijuan Tea with the Commercial Antioxidants using the FRAP Assay

sample	FRAP (μmol TE/mg)[a]
compound **3**	5.94 ± 0.21
compound **4**	5.81 ± 0.17
BHA	8.13 ± 0.24
BHT	4.39 ± 0.16
α-tocopherol	2.66 ± 0.18

[a]FRAP, ferric reducing antioxidant power.

In conclusion, the isolation of four anthocyanins was the first instance for Zijuan tea. Antioxidant activities of compounds **3** and **4** were evaluated. We think that further study on anthocyanins of Zijuan tea and their activities is worth doing in the future. Especially, the synergistic effect of anthocyanins and catechins in this purple tea also needs to be researched. We expect that drinking Zijuan tea could bring some new potential health benefits.

AUTHOR INFORMATION

Corresponding Author

*Phone: +86-021-64250829. Fax: +86-021-64250829. E-mail: zhaoliming@ecust.edu.cn.

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Notes

The authors declare no competing financial interest.

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