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Peroxyl Radical Scavenging Capacity, Polyphenolics, and Lipophilic Antioxidant Profiles of Mulberry Fruits Cultivated in Southern China

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Twenty-seven cultivars of mulberry fruits (*Morus atropurpurea* Roxb) were analyzed for their total phenolic content, total anthocyanin content, and peroxyl radical scavenging capacities. The proanthocyanidin contents of the fruit were also quantified using 4-dimethylamino-cinnamaldehyde assay, and characterization was attempted using electrospray ionization mass spectra. The phenolic compounds of mulberry fruits were characterized using HPLC with ESI-MS and diode array detection. Results showed that the content of mulberry fruits varied with different cultivars with total phenolic content, total anthocyanin content, total proanthocyanidin content, and peroxyl radical scavenging capacities ranging from 0.060−0.244, 0.001−0.056, 0.001−0.015, and 0.301−1.728, respectively. Good correlations were observed among the phenolic, anthocyanin, and proanthocyanidin contents and the radical scavenging capacities of mulberry fruits. Mulberry fruits were found to contain low amount of proanthocyanidins. The high total phenolic content of mulberry fruits were mainly contributed by anthocyanins, rutin, and chlorogenic acids. The lipid soluble antioxidants are profiled by an HPLC method developed in-house, and the results of selected mulberry fruits revealed significant amounts of lutein and δ- and γ-tocopherols but low α-tocopherol. Our results provide useful antioxidant nutritional information of a mulberry cultivar that has potential for large scale plantations.

KEYWORDS: Mulberry fruit; *Morus* spp.; peroxyl radical scavenging activity; polyphenols

INTRODUCTION

Berries as a group of food rich in nutrients have received great attention over the past few years particularly for their health benefits for lowering the risk factors of chronic diseases (*1, 2*). Relatively less studied is mulberry (*Morus* spp.), which is grown wild or cultivated in many countries for its foliage as food for silkworms (*Bombyx mori* L.). In Chinese medicine, its leaf infusion is traditionally used as heat clearing herbal tea to reduce stress and prevent diabetes mellitus, while the root bark of mulberry trees is used for anti-inflammatory, diuretic, antitussive, and antipyretic purposes (*3*). Mulberry leaves and root bark contain many bioactive compounds such as flavonoids (*4, 5*) and polyhydroxylated alkaloids (*6*). The antihyperglycemic property of mulberry leaves and root bark is attributed to its alkaloid content particularly 1-deoxynojirimycin (DNJ), which is known to be a potent α-glycosidase inhibitor (*4*). Mulberry fruits, however, are less commonly used as a tonic, diuretic, and antihypertension agent in traditional Chinese medicine (*3, 7*). The fruits are relatively unfamiliar to consumers as they are not commonly sold in supermarkets because of limited production and the lack of awareness of their health benefits. Some reported work revealed that mulberry fruits are rich in polyphenols and possess antioxidative, anti-inflammatory (*8*), antidiabetic (*3*), and antihyperlipidemic activities (*9*). The high phenolic content in the fruit has been attributed to its anthocyanin pigments, with cyanidin-3-glucoside and cyanidin-3-rutinoside being the major anthocyanins (*7, 10*). Compared to other berries, the bioactive compounds of mulberry fruits has not been extensively studied.

To comprehensively assess the nutritional value of the antioxidants of mulberry, in this study, 27 cultivars of mulberry fruits harvested in southern China were analyzed for their total phenolic, anthocyanin, and proanthocyanidin contents as well as peroxyl radical scavenging capacities to obtain the relationships among these properties. Characterization of the proan-

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thocyanidins and the phenolic compounds, and lipid soluble antioxidants were also accomplished to obtain a holistic profile of the antioxidants and phenolics in mulberry fruits. These compounds may help to substantiate the potential health benefits of mulberry fruits as a potential functional food source for lowering the risk factors of common chronic diseases.

MATERIALS AND METHODS

Reagents. Folin−Ciocalteu's phenol reagent, KCl, sodium acetate, KH_2PO_4, K_2HPO_4, and formic acid were obtained from Merck (Darmstadt, Germany). Na_2CO_3 anhydrous was obtained from Carlo Erba (Milan, Italy). Gallic acid and Trolox were purchased from Acros Organics (Morris plains, NJ). Cyanidin-3-glucoside was purchased from Polyphenols Laboratories AS (Sandnes, Norway). Methanol HPLC grade was obtained from Tedia (Fairfield, OH). 5-Caffeoylquinic acid (CQA) was obtained from Spectrum Chemical (Gardena, CA). Rutin trihydrate, 4-(dimethylamino)-cinnamaldehyde (DMACA), (+)-catechin hydrate, quercetin, fluorescein sodium salt, and 2,2′-azobis(2-methyl propionamidine) dihydrochloride (AAPH) were purchased from Sigma-Aldrich (St. Louis, MO). Ethyl acetate HPLC grade and HCl were obtained from Fischer Scientific (Fair Lawn, NJ). Other solvents were of reagent grade.

Instruments. Total phenolic, anthocyanin, and proanthocyanidin assays as well as the oxygen radical absorbance capacity (ORAC) assay were carried out on a Bio Tek Synergy HT microplate reader (Winooski, VT). The electrospray ionization mass spectra (ESI-MS) were obtained from a Finnigan/MAT LCQ ion trap mass spectrometer (San Jose, CA) equipped with an ESI source. The heated capillary and voltage were maintained at 250 °C and 4.5 kV, respectively. The full-scan mass spectra from *m*/*z* 100 to 1800 were recorded. The same ESI source was used for LC-MS analysis. The Finnigan LCQ system consisted of a P4000 LC pump, AS3000 autosampler, UV6000LP UV detector, and LCQ MS detector. The HPLC chromatograms for phenolic compound identification were acquired using a Shimadzu liquid chromatograph system (LC-10AT VP) with a C18 reversed-phase column (250 × 4.6 mm, Shimadzu VP-ODS), a diode array detector (SPD-M10A VP), and an auto injector (SIL-10AD VP).

Lypophilic antioxidants were analyzed using the HPLC system comprising a Waters (Milford, MA) Alliance 2695 separation module, a Model 996 diode-array UV detector, and a Model 2475 fluorescence detector. An Agilent (Ringoes, NJ) Zorbax SB-C_{18} column (5 μm, 150 mm × 4.6 mm) was used as column-1, protected by a Jour Guard C_{18} cartridge (VICI, Schenkon, Switzerland), and maintained at 30 °C. A Whatman (Maidstone, UK) replaceable Partisphere 5 C_{18} cartridge (5 μm, 110 mm × 4.7 mm) was used as column-2 and chilled to 4 °C. A 2-position six-port Synergi fluid processor (Models AVO-6082, Phenomenex, Torrance, CA) was used for column switching.

Sample Preparation. Mulberry fruits (*Morus atropurpurea* Roxb) were obtained from South China Mulberry Resource Garden in Dafeng Agricultural Experimental Base in Guangdong Academy of Agricultural Sciences (Guangdong, China). The fruits were harvested between February and March of 2002. For each cultivar, 250 to 300 g of full mature mulberry fruits were harvested and stored immediately at 4 °C, and were freeze-dried and kept in freezer for storage. Some basic parameters of these cultivars were reported in an earlier paper by one of us (*11*). The freeze-dried powders were shipped (room temperature) to Singapore for analysis in 2007. The powder (0.25 g) was extracted with acetone/water/acetic acid (AWA 70:29.5:0.5; 3 × 10 mL) for 45, 45, and 30 min, respectively, using an orbital platform shaker at 300 rpm. The mixture was centrifuged, and the supernatant was collected in a 50 mL volumetric flask and topped up to the mark with water for ORAC, total phenolic, total proanthocyanidin, and total anthocyanin assays. For proanthocyanidins and flavonoids identifications, 1 g of the mulberry powder (Guo-2) was extracted three times with 25 mL of AWA each as described above. The supernatant was collected, and the acetone was evaporated under reduced pressure to yield a slurry (8 mL). To obtain the proanthocyanidins, the slurry was liquid−liquid extracted with ethyl acetate, and the ethyl acetate fraction was analyzed using ESI-MS. For flavonoid identification and quantification, the slurry was diluted 4 times with methanol and filtered through a Sartorius Minisart 45 μm porosity and injected into a HPLC and LC-MS system.

Oxygen Radical Absorbance Capacity (ORAC) Assay. The assay was carried out on a fluoroscence microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 525 nm. The temperature of the incubator was set to 37 °C. The procedure was based on the modified $ORAC_{FL}$ method (*12*). AAPH was used as the peroxyl generator, and Trolox as the standard with concentration ranging from 100 to 6.25 μM. Fluorescein solution (160 μL) (9.57 × 10−5 mM), 20 μL of AAPH (81 mM), and 20 μL of sample were mixed in each well, fluorescence readings were taken every 2 min for 2 h, and the area under the curve was calculated. All dilutions were carried out using 75 mM K_3PO_4 buffer at pH 7.04. Data were expressed as mmol Trolox equivalents per gram dry basis (mmol TE/g DW). Experiments were carried out in 5 replicates.

Total Phenolic Assay. Total phenolic content (TPC) was determined using the Folin−Ciocalteau method (*13*). The samples were diluted appropriately with water prior to analysis. The assay was carried out on a microplate reader; 100 μL of FCR (diluted ten times from the original reagent), 80 μL of 75 g/L Na_2CO_3, and 20 μL of sample were mixed in each well, and absorbance was measured at 765 nm after standing for 30 min at 37 °C. Gallic acid was used as the standard with concentration ranging from 1120 to 35 μM. Data were expressed as mmol gallic acid equivalents per gram dry basis (mmol GAE/g DW). Experiments were carried out in 5 replicates.

Total Anthocyanin Assay. Total anthocyanin (TA) content was measured using the pH-differential method (*14*). pH 1.0 buffer was prepared by mixing 25 mL of 0.2 M KCl solution with 67 mL of the 0.2 N HCl solution. pH 4.5 buffer was prepared by dissolving 1.64 g of sodium acetate in 100 mL of deionized water and adjusting the pH to 4.5 with HCl. Samples were diluted 25 times with the buffers. Absorbance was measured at 510 and 700 nm using the plate reader at 25 °C. The standard used was cyanidin-3-glucoside prepared by using the buffers. Data were expressed as mmol cyanidin-3-glucoside equivalents per gram dry basis (mmol CGE/g DW). Experiments were carried out in 5 replicates. The absorbance of each sample was calculated using the following equation:

$$A = (A_{510nm}\text{pH}1.0 - A_{700nm}\text{pH}1.0) - (A_{510nm}\text{pH}4.5 - A_{700nm}\text{pH}4.5)$$

Total Proanthocyanidin Assay. Total proanthocyanidin content (TP) was analyzed using 4-dimethylamino-cinnamaldehyde (DMACA) reagent (*15*). The assay was carried out in a plate reader; 35 μL of sample diluted in methanol was mixed with 175 μL of DMACA reagent (0.1% in 3 M HCl in MeOH). The absorbance was measured at 635 nm after 15 min of standing at 25 °C. (+)-Catechin was used as the standard with concentrations ranging from 80 to 2.5 μM in methanol. Data were expressed as mmol catechin equivalents per gram dry basis (mmol CE/g DW). Experiments were carried out in 5 replicates.

HPLC and LC-MS Analysis. Identification and quantification of the phenolic compounds was performed on a Shimadzu HPLC system with a C18 reversed-phase column at a column temperature of 25 °C. The mobile phase was a mixture of water−formic acid (99.9:0.1, v/v, pH 2.8) (solvent A) and methanol (solvent B) with the following gradient elution program: 10% B (0−10 min), 10−30% B (10−28 min), 30−45% B (28−35 min), 45−60% B (35−45 min), 60% B (45−50 min), 60−10% B (50−55 min), and reconditioning with 10% B (55−70 min). The flow rate was set at 1 mL/minute, and the injection volume for each sample was 20 μL. The effluent was monitored with DAD at 240−400 nm. The peaks were monitored at 320 nm. Peaks were spiked with available standards for identification. Concentration of individual phenolic compound on one mulberry cultivar was determined by calibration curves of available standards and expressed as mg (μmol) per gram of mulberry powder. Further characterization of the phenolic compounds was done using LC-ESI-MS. The LC conditions for LC-MS^n analysis were the same as those of the HPLC analysis. Full scan mode was used for determination of parent ion and fragment ion *m*/*z*.

Table 1. Total Phenolic Content (TPC), Oxygen Radical Absorbance Capacity (ORAC), Total Proanthocyanidin (TP), and Total Anthocyanin (TA) Content of Mulberry Samples[a]

cultivar	TPC (mmol GAE/g DW)	ORAC (mmol TE/g DW)	TP (mmol CE/g DW)	TA (mmol CGE/g DW)
7403	0.241 ± 0.013 ab	1.399 ± 0.145 b	0.0098 ± 0.0012 efg	0.056 ± 0.005 a
7848	0.189 ± 0.001 ef	1.250 ± 0.079 bcd	0.0090 ± 0.0005 gh	0.042 ± 0.004 bc
Shangshan-6	0.229 ± 0.003 bc	1.334 ± 0.100 bc	0.0115 ± 0.0007 cd	0.039 ± 0.001 bcd
Bei-1−13	0.106 ± 0.002 l	0.491 ± 0.072 lm	0.0044 ± 0.0002 mn	0.012 ± 0.001 jk
Bei-2−5	0.060 ± 0.002 n	0.301 ± 0.026 m	0.0026 ± 0.0003 op	0.013 ± 0.002 jk
Bei-2−8	0.150 ± 0.006 i	0.929 ± 0.080 fgh	0.0069 ± 0.0006 jkl	0.039 ± 0.006 bcd
Bei-3−5	0.145 ± 0.006 ij	0.626 ± 0.040 jkl	0.0081 ± 0.0004 hij	0.013 ± 0.001 jk
Nan-2	0.201 ± 0.005 de	1.266 ± 0.119 bcd	0.0105 ± 0.0011 def	0.031 ± 0.003 defg
Tang-10	0.200 ± 0.003 e	1.389 ± 0.162 bc	0.0129 ± 0.0005 b	0.033 ± 0.001 def
Da-10	0.174 ± 0.004 g	1.132 ± 0.057 cdef	0.0090 ± 0.0003 ghi	0.025 ± 0.003 fgh
Xiaying-1	0.201 ± 0.002 de	1.420 ± 0.154 b	0.0120 ± 0.0005 bc	0.035 ± 0.003 cde
Xiaying-2	0.194 ± 0.005 e	1.037 ± 0.154 defg	0.0110 ± 0.0004 cde	0.028 ± 0.004 efg
Guo-1	0.125 ± 0.005 k	0.825 ± 0.103 ghij	0.0076 ± 0.0004 ij	0.016 ± 0.003 ijk
Guo-2	0.244 ± 0.004 a	1.728 ± 0.194 a	0.0151 ± 0.0008 a	0.045 ± 0.004 b
Guiyou-10−19	0.179 ± 0.010 fg	0.966 ± 0.11 efgh	0.0096 ± 0.0003 fg	0.036 ± 0.004 cde
Guiyou-70	0.081 ± 0.003 m	0.506 ± 0.064 lm	0.0035 ± 0.0003 no	0.003 ± 0.001 lm
Guiyou-154	0.165 ± 0.004 gh	0.790 ± 0.076 hijk	0.0073 ± 0.0004 jk	0.031 ± 0.003 defg
Heipisang	0.105 ± 0.004 l	0.857 ± 0.022 ghi	0.0046 ± 0.0002 mn	0.023 ± 0.002 ghi
Miao-66	0.081 ± 0.002 m	0.546 ± 0.012 klm	0.0036 ± 0.0001 no	0.008 ± 0.001 klm
Xuan-27	0.092 ± 0.004 lm	0.527 ± 0.017 lm	0.0033 ± 0.0002 no	0.018 ± 0.002 hij
Yueyou-18	0.177 ± 0.004 fg	1.038 ± 0.078 defg	0.0111 ± 0.0004 cde	0.026 ± 0.001 fgh
Yueyou-32	0.239 ± 0.006 ab	1.339 ± 0.039 bc	0.0154 ± 0.0011 a	0.043 ± 0.004 bc
Yueyou-34	0.214 ± 0.008 cd	1.176 ± 0.076 bcde	0.0132 ± 0.0007 b	0.029 ± 0.003 efg
Yueyou-36	0.134 ± 0.007 jk	0.667 ± 0.094 ijkl	0.0062 ± 0.0008 kl	0.010 ± 0.001 jkl
Yueyou-51	0.132 ± 0.002 jk	0.873 ± 0.037 ghi	0.0057 ± 0.0002 lm	0.009 ± 0.001 klm
Yueyou-87	0.063 ± 0.003 n	0.450 ± 0.029 lm	0.0015 ± 0.0002 p	0.001 ± 0.001 m
Zhen-1432	0.157 ± 0.002 hi	0.970 ± 0.018 efgh	0.0070 ± 0.0003 jkl	0.029 ± 0.002 efg

[a] Data are expressed on a dry weight basis and presented as mean ± SD ($n = 5$). Means with different letters within a column are significantly different ($P < 0.01$). GAE, gallic acid equivalents; CGE, cyanidin-3-glucoside equivalents; CE, catechin equivalents; TE, Trolox equivalents.

MS^2 operated under negative ion mode with a collision energy of 25−30%, an ionization voltage of 4.5 kV, and a capillary temperature of 280 °C.

Lipophilic Antioxidant Analysis. Sample extraction and HPLC analysis for lipid soluble antioxidants were carried out according to published procedures (*16*). Briefly, 5−15 mg of mulberry powder were mixed with disodium sulfate, acetonitrile, and internal standard (IS) solution in an amber microcentrifuge tube A for 5 min. After centrifugation, the supernatant was transferred to another amber microcentrifuge tube B. The sample in tube A was then mixed with butanol-ethyl acetate (1:1, v/v) and *n*-hexane. After 5 min, the sample was mixed with water and centrifuged for 2 min. The organic layer was then transferred to another amber microcentrifuge tube C and evaporated to dryness under nitrogen. The residue was reconstituted using THF and then mixed thoroughly with the supernatant in tube B for HPLC injection.

IS solution was prepared in ethanol/*tert*-butanol (4:1) added to BHT (0.02%, w/v) and contained α-tocopherol acetate as IS1 for vitamin E vitamers and echinenone as IS2 for carotenoid quantification. For calibration, IS solutions were mixed with standard mixtures of various concentrations. Stock solutions of each standard were prepared individually with appropriate solvents as described earlier (*17*).

The extract (20 μL) was injected into the HPLC system. The mobile phase were acetonitrile (solvent A), methanol (solvent B), and ethanol/*tert*-butanol (4:1, solvent C). Gradient elution was performed at 1 mL/min with the gradient program as follows: 80% A and 20% B (0−8 min); 30% A, 20% B and 50% C (8−32 min); 20% A, 20% B and 60% C (32−39 min); 80% A and 20% B (39−40 min); and reconditioning (40−54 min). Run was performed at both columns for the first 8 min, followed by switching to column-1 only before switching back to both columns at minute 42. Chromatograms were extracted at 440 nm for neoxanthin and violaxanthin and 450 nm for β-cryptoxanthin, lutein, zeaxanthin, and α- and β-carotenes. Tocopherols and tocotrienols were quantified by fluorimetric detection with excitation and emission wavelengths set at 296 and 330 nm, respectively. Data acquisition was performed with the Waters Empower software.

Statistical Analysis. Descriptive statistical analysis was performed using Microsoft Excel. The data were expressed as mean ± standard deviation for samples having sample numbers >2. A one-way analysis of variance (ANOVA) and a Tukey HSD post hoc multiple comparison test were performed using SPSS 12.0 for Windows (SPSS, Chicago, IL).

RESULTS AND DISCUSSION

This is a continuation of a systematic evaluation of the characteristics of mulberry fruits in Guandong province, southern China. Our initial data found that the physical and chemical properties such as sugar content, acidity, and pigment content (*10*) are highly dependent on the cultivars and the weather conditions. To investigate the variation of the growing area's geological conditions and cultivars on the antioxidant nutritional quality of the fruits, the powders were analyzed for oxygen radical scavenging capacity (ORAC), total phenolic content (TPC), total proanthocyanidins, and total anthocyanins (TA), and the results are shown in **Table 1**. For convenience of comparison, all the data were expressed as mmol standard equivalent per gram of dried mulberry fruit. As shown by the data, the content of mulberry fruits varied widely across different cultivars. The Guo-2 cultivar was shown to have the highest ORAC and TPC. The results showed that ORAC values of mulberry fruit correlated very well with its phenolic content. The phenolic content was also found to correlate well with its anthocyanins and proanthocyanidins contents. However, poor correlation was found between the anthocyanins and proanthocyanidins content (Figure S1 in the Supporting Information). The anthocyanin of the mulberry fruit contributed a small fraction (10−25%) of the phenolic content (**Table 1**). Despite the high amount of anthocyanins, mulberry fruits only contained a trace amount of proanthocyanidins and thus were undetected by ESI-MS. ESI-MS of the ethyl acetate fraction in **Figure 1** shows that the MS spectrum lacked the *m*/*z* peaks commonly found in the proanthocyanidins sample at 561, 577, 595, 729, 833, 849, 863, 881, 1121, 1137, 1151, 1409, 1425, and

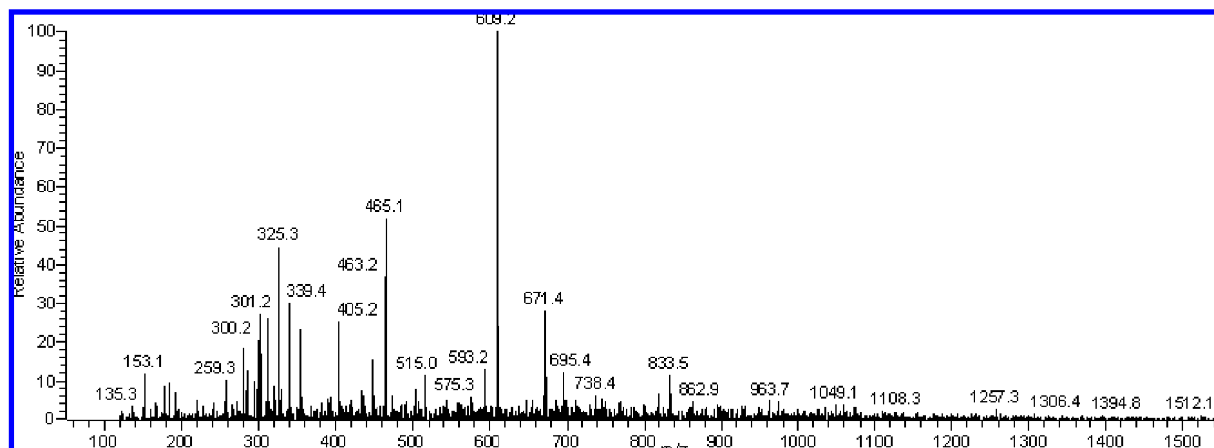

Figure 1. ESI-MS fingerprint of ethyl acetate fraction of Guo-2 extract (negative ion mode). The spectrum lacked the *m*/*z* peaks commonly found in the proanthocyanidins sample at 561, 577, 595, 729, 833, 849, 863, 881, 1121, 1137, 1151, 1409, 1425, and 1439. Other peaks are further characterized in LC-MS shown in **Table 2** and **Figure 2**.

Table 2. Characterization of Phenolic Compounds of Mulberry Guo-2 Extract Using HPLC with PDA and ESI-MS^n Detection

					MS^2 ions (*m*/*z*)		
peak	t_R (min)	λ_{max} (nm)	MW	MS (*m*/*z*); ID	base	secondary peaks	tentative identification
1	18.94	327, 220, 240	354	353; $[M - H]^-$ 355; $[M + H]^+$ 377; $[M + Na]^+$	191	179 (72)[a], 135 (10)	3-caffeoyl quinic acid
2	27.02	327, 218, 243	354	353; $[M - H]^-$ 355; $[M + H]^+$ 377; $[M + Na]^+$	191	179 (9), 135 (1)	5-caffeoyl quinic acid
3	28.43	282	449	449; $[M + H]^+$	287		cyanidin-3-glucoside
3[b]			354	353; $[M - H]^-$ 355; $[M + H]^+$ 377; $[M + Na]^+$	173	179 (85), 191 (15), 135 (10)	4-caffeoyl quinic acid
4	31.01	283, 339	595	595; $[M + H]^+$	287	449 (50)	cyanidin-3-rutinoside
4[b]			433	433; $[M + H]^+$	271		pelargonidin-3-glucoside
5	34.29	318, 215	652	651; $[M - H]^-$ 675; $[M + Na]^+$	593*	325 (3) *MS^3 285	unknown
6	40.53	257, 357	610	609; $[M - H]^-$ 633; $[M + Na]^+$	301	300 (70)	rutin
7	42.98	267, 350, 295	594 617	593; $[M - H]^-$ $[M + Na]^+$	285		kaempferol-3-rutinoside
8	46.21 256, 371	302	301	$[M - H]^-$	179	151 (40)	quercetin

[a] Relative intensity as compared to the base peak. [b] Compound eluting at the same retention time.

1439 (*18, 19*). We suspect that the oligomeric proanthocyanidins are insoluble in ethyl acetate; however, HPLC analysis of the AWA extract did not give any detectable peaks corresponding to proanthocyanidins either. Unlike other berries that contain high amounts of proanthocyanidins (*18*), the proanthocyanidins in mulberry might have depolymerized to anthocyanin during maturation, or they simply were not formed in the first place. We suspect that the mulberry fruit may content hydrolyzable tannins, which may contribute to their radical scavenging capacity. However, the concentrations of hydrolyzable tannins were below the detection limit by the colorization method using potassium iodate reported by Willis and Allen (*20*).

In general, mulberry had comparable total antioxidant capacity and total phenolic contents with other berries including blueberry and cranberry (*21, 22*). However, the variation of these data is deemed to be large as it depends on many factors including cultivar, location, and weather conditions. The individual antioxidant compound profiles in different fruits also need to be taken into consideration for their purported nutritional values. Selecting the cultivar with the highest antioxidant activity (Guo-2) as a representative sample, we analyzed the phenolic compound profiles by HPLC and LC/MS. There were 8 major peaks (**Figure 2**) detected by a PDA detector at 320 nm. The phenolic compounds were characterized by peak spiking, by their UV spectra (210−400 nm), mass spectra, and MS^2 fragmentation patterns. The characteristics of the phenolic compounds in the peaks were summarized (**Table 2**). From their fragmentation patterns, peaks 1, 2, and 3b were identified as 3-caffeoylquinic acid (CQA), 5-CQA, and 4-CQA, respectively, following the scheme by Clifford and co-workers (*23*). 3-CQA was distinguishable from 5-CQA by its intense caffeic acid derived ion (*m*/*z* 179), while 4-CQA produced a distinct dehydrated MS^2 base peak at *m*/*z* 173 (*23*). Spiking with the 5-CQA standard further confirmed the identity of peak 2. Anthocyanins found in peak 3, 4, and 4b produced a strong signal under MS positive mode. With fragments at *m*/*z* 287, peaks 3 and 4 were identified as cyanidin derivatives. From their molecular ions, peaks 3 and 4 were identified as cyanidin-3-glucoside and cyanidin-3-rutinoside, the presence of which had been reported previously (*7, 10*). Peak 4b had molecular ions at *m*/*z* 433, which was much less intense that that of peak 4 at *m*/*z* 595; with fragments at 271, it was identified as pelargonidin-3-glucoside, the main anthocyanin in strawberry (*24*).

Peak 5 has an *m*/*z* of 651 (anionic mode). MS^2 fragments gave rise to *m*/*z* 593 by losing a group of 58 from the molecular ion. The MS^3 fragmention of 593 gave rise to a main peak at

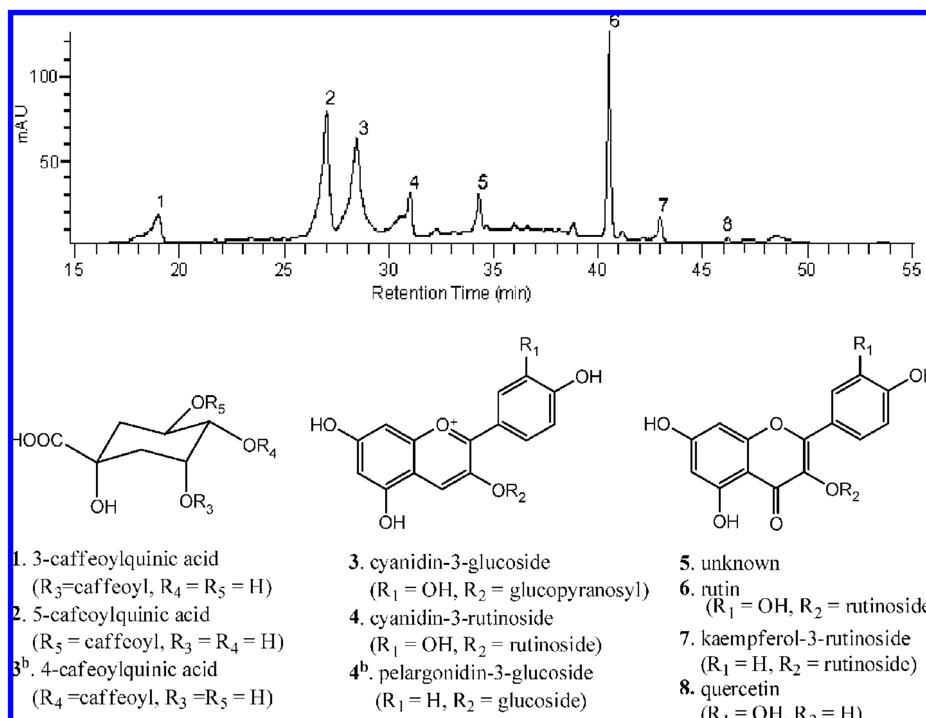

Figure 2. HPLC DAD chromatogram of mulberry Guo-2 extract at 320 nm. Assignments of the chemical structures of phenolic compounds responsible for the peaks are based on secondary or tertiary mass spectra data listed in **Table 2**.

Table 3. Lipophilic Antioxidant Profiles of Selected Mulberry Fruits (μg/g dry weight)[a]

	tocopherols				carotenoids			
cultivar	α-tocopherol	α-tocotrienol	δ-tocopherol	γ-tocopherol	β-carotene	lutein	neoxanthin	violaxanthin
Bei-2−5	2.14	nd	1003	364.1	7.7	13.2	0.9	0.8
Guiyou-154	17.9	6.3	620.6	426.5	5.8	20.8	0.8	0.6
Heipisang	17.1	3.6	1140.7	349.4	7.7	28.9	1.4	0.8
Xuan-27	11.8	9.7	719.2	266.6	5.9	19.8	0.7	0.6
Tang-10	9.2	nd	363.2	224.7	4.7	16.8	0.6	0.5
mean ± RSD	11.6 ± 6.43	6.5 ± 3.0	769 ± 309	326.2 ± 80	6.36 ± 1.3	19.9 ± 5.8	0.9 ± 0.3	0.7 ± 0.1
CV	55%	46%	40%	25%	20%	29%	33%	14%

[a] There is negligible amount of δ- and γ-tocotrienols detected among all cultivars. nd, not detected.

285 corresponding to the molecular ion of cyanidin. The fragment of 593 has the same molecular weight as that of cyanidin rutinoside. Indeed, MS^3 of this anion showed a cyanidin peak at 285 as the main fragment, and this confirmed that 5 is also a cyanidin derivative. The compound has UV−vis maximum absorbance at 318 nm indicating a conjugated structure similar to that of flavones.

Peaks 6, 7, and 8 were identified as flavonols because of their strong UV absorption (λ_{max}) at 350−370. They also had weaker polarity and thus were retained longer under reversed-phase chromatography. Spiking confirmed that peaks 6 and 8 were rutin and quercetin, respectively. Peak 7 was identified as kaempferol-3-rutinoside after comparison with the literature (*25*). The structures of the phenolic compounds found in mulberry fruits are shown in **Figure 2**.

HPLC and LC/MS data showed that cyanidin-3-glucoside and cyanidin-3-rutinoside were the major anthocyanins found in Guo-2 mulberry cultivar. Together with anthocyanins, rutin, and chlorogenic acids were the major contributors to the phenolic contents of mulberry. Using the available standards, the quantity of 3-CQA, 5-CQA, and rutin were determined to be approximately 0.47 mg (1.32 μmol), 1.28 mg (3.62 μmol), and 2.44 mg (3.99 μmol) per gram of dried mulberry fruit powders.

Lipophilic Antioxidant Profiles. Although mulberry contains significant amounts of lipid (>20%) including ethyl linoleate (*26*), we found that antioxidant capacity of the lipid portion is negligible compared to the water soluble portion. In fact, Prior and co-workers reported the total antioxidant capacity (both lipophilic and hydrophilic fraction) of many fruits including berries, and the results also showed that the ORAC values in the lipophilic portion were insignificant (*21*). However, the individual antioxidant compound profiles can be significant as these compounds may have other bioactivity. Therefore, five mulberry fruits with different ORAC values ranging from high to low were also subjected to solvent extraction to delineate the lipophilic antioxidant profiles. The total phenolic contents of the hexane fractions were rather low and insignificant compared to that of AWA fractions of the same fruits. The lipophilic antioxidant compound profiles are important nutritional information. Using the HPLC method developed in house (*16*), we quantified the selected antioxidant contents in the mulberry fruits as shown in **Table 3**. δ-Tocopherol contents were among the highest in tocopherols followed by γ-tocopherol, whereas, α-tocopherol and α-tocotrienol concentrations were insignificant. Taking into consideration the moisture content of mulberry (~90%), consumption of 100 g of mulberry would provide 11 mg total tocopherols consisting mainly of

δ-tocopherol (70%) and γ-tocopherol (29%). Although the health benefit of δ-tocopherol is not well established, recent research suggested that γ-tocopherol has potent anti-inflammatory and antioxidant properties (*27*). There is no recommended daily intake of γ-tocopherol yet. Chemically, the arene in α-tocopherol is permethylated, whereas its congener γ-tocopherol has one less, and δ-tocopherol has two less methyl groups. The latter has shown to be more reactive toward reactive nitrogen species (*28*), which selectively attack the unmethylated carbons on the arene.

Lutein was the main carotenoid followed by β-carotene (**Table 3**), whereas zeaxanthin levels of five selected mulberry samples were negligible. According to the USDA database (*29*), major sources of lutein and zeaxathanin are leafy greens, corn, and green vegetables such as broccoli, brussel sprouts, green beans, peas, and zucchini. Majority of berries are not known to contain a significant amount of lutein and zeaxanthin. One exception is wolfberry, which has a very high amount of lutein and zeaxanthin (*30*).

In summary, we demonstrated that mulberry fruits contain high amounts of antioxidants and polyphenolic compounds ranging from phenolic acids (chlorogenic acids) anthocyanins, and flavonols. Unlike blueberry, cranberry, or strawberry, the mulberry fruit contains insignificant amount of proanthocyanidins. In addition, we also found that mulberry fruits were also a good source of δ- and γ-tocopherols. The actual concentrations of these antioxidative components are highly dependent on the cultivars. These bioactive compounds may act synergistically *in vivo* to promote health, and the reduction of risk factors of chronic diseases requires further investigation to establish the causative link between mulberry consumption and health promotion. From a water soluble antioxidant capacity perspective, Guo-2 seems to be an ideal cultivar for large scale plantation, but other agro-economical factors including average single fruit size and weight, the number of fruits on a single bud, and flavor attributes will need to be taken into consideration.

ABBREVIATIONS USED

TA, total anthocyanins; TPC, total phenolic content; ORAC, oxygen radical absorbance capacity assay; TP, total proanthocyanidins; CQA, caffeoylquinic acid; DMACA,4-dimethylamino-cinnamaldehyde.

Supporting Information Available: Correlation between ORAC, TPC, TA, and TP of mulberry samples. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Hancock, R. D.; McDougall, G. J.; Stewart, D. Berry fruit as "superfood". Hope or hype. *Biologist* **2007**, *54*, 73–79.

(2) Seeram, N. P. Berry fruits: Compositional elements, biochemical activities, and the impact of their intake on human health, performance, and disease. *J. Agric. Food Chem.* **2008**, *56*, 627–629.

(3) Asano, N.; Yamashita, T.; Yasuda, K.; Ikeda, K.; Kizu, H.; Kameda, Y.; Kato, A.; Nash, R. J.; Lee, H. S.; Ryu, K. S. Polyhydroxylated alkaloids isolated from mulberry trees (*Morus alba* L.) and silkworms (*Bombyx mori* L.). *J. Agric. Food Chem.* **2001**, *49*, 4208–4213.

(4) Du, J.; He, Z. D.; Jiang, R. W.; Ye, W. C.; Xu, H. X.; But, P. P. H. Antiviral flavonoids from the root bark of *Morus alba* L. *Phytochemistry.* **2003**, *62*, 1235–1238.

(5) Zhishen, J.; Mengcheng, T.; Jianming, W. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem.* **1999**, *64*, 555–559.

(6) Asano, N.; Kizu, H.; Oseki, K.; Tomioka, E.; Matsui, K.; Okamoto, M.; Baba, M. N-Alkylated nitrogen-in-the-ring sugars: Conformational basis of inhibition of glycosidases and HIV-1 replication. *J. Med. Chem.* **1995**, *38*, 2349–2356.

(7) Bae, S. H.; Suh, H. J. Antioxidant activities of five different mulberry cultivars in Korea. *LWT Food Sci. Technol.* **2007**, *40*, 955–962.

(8) Kim, S. Y.; Park, K. J.; Lee, W. C. Antiinflamatory and antioxidative effects of *Morus* spp. fruit extract. *Korean J. Med. Crop Sci.* **1998**, *6*, 204–209.

(9) Kim, H. B.; Kim, S. Y.; Ryu, K. S.; Lee, W. C.; Moon, J. Y. Effect of methanol extract from mulberry fruit on the lipid metabolism and liver function in cholesterol-induced hyperlipidemia rats. *Korean J. Seri. Sci* **2001**, *43*, 104–108.

(10) Liu, X.; Xiao, G.; Chen, W.; Xu, Y.; Wu, J. Quantification and purification of mulberry anthocyanins with macroporous resins. *J. Biomed. Biotech.* **2004**, *5*, 326–331.

(11) Tang, C.; Lou, G.; Liu, X.; Wu, F.; Yang, Q.; Wu, J.; Ren, D. *Canye Kexue* **2003**, *29*, 295–298.

(12) Huang, D.; Ou, B.; Hampsch-Woodill, M.; Flanagan, J. A.; Prior, R. L. High-throughput assay of oxygen radical absorbance capacity (ORAC) using a multichannel liquid handling system coupled with a microplate fluorescence reader in 96-well format. *J. Agric. Food Chem.* **2002**, *50*, 4437–4444.

(13) Singleton, V. L.; Orthofer, R.; Lamuela-Raventos, R. M. Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. *Methods Enzymol.* **1999**, *299*, 152–178.

(14) Giusti, M. M.; Wrolstad, R. E. Characterization of red radish anthocyanins. *J. Food Sci.* **1996**, *61*, 322–326.

(15) McMurrough, I.; McDowell, J. Chromatographic separation and automated analysis of flavanols. *Anal. Biochem.* **1978**, *91*, 92–100.

(16) Lee, B. L.; Su, J.; Ong, C. N. Monomeric C18 chromatographic method for the liquid chromatographic determination of lipophilic antioxidants in plants. *J. Chromatogr., A* **2004**, *1048*, 263–267.

(17) Lee, B. L.; New, A. L.; Ong, C. N. Simultaneous determination of tocotrienols, tocopherols, retinol, and major carotenoids in human plasma. *Clin. Chem.* **2003**, *49*, 2056–2066.

(18) Gu, L.; Kelm, M. A.; Hammerstone, J. F.; Beecher, G.; Holden, J.; Haytowitz, D.; Prior, R. L. Screening of foods containing proanthocyanidins and their structural characterization using LC-MS/MS and thiolytic degradation. *J. Agric. Food Chem.* **2003**, *51*, 7513–7521.

(19) Fu, C.; Loo, A. E. K.; Chia, F. P. P.; Huang, D. Oligomeric proanthocyanidins from mangosteen pericarps. *J. Agric. Food Chem.* **2007**, *55*, 7689–7694.

(20) Willis, R. B.; Allen, P. R. Improved method for measuring hydrolysable tannins using potassium iodate. *Analyst.* **1998**, *123*, 435–439.

(21) Wu, X.; Beecher, G. R.; Holden, J. M.; Haytowitz, D. B.; Gebhardt, S. E.; Prior, R. L. Lipophilic and hydrophilic antioxidant capacities of common foods in the United States. *J. Agric. Food Chem.* **2004**, *52*, 4026–4037.

(22) Wu, X.; Beecher, G. R.; Holden, J. M.; Haytowitz, D. B.; Gebhardt, S. E.; Prior, R. L. Concentrations of anthocyanins in common foods in the United States and estimation of normal consumption. *J. Agric. Food Chem.* **2006**, *54*, 4069–4075.

(23) Clifford, M. N.; Johnston, K. L.; Knight, S.; Kuhnert, N. Hierarchial scheme for LC-MS n identification of chlorogenic acids. *J. Agric. Food Chem.* **2003**, *51*, 2900–2911.

(24) Aaby, K.; Ekeberg, D.; Skrede, G. Characterization of phenolic compounds in strawberry (*Fragaria ananassa*) fruits by different HPLC detectors and contribution of individual compounds to total antioxidant capacity. *J. Agric. Food Chem.* **2007**, *55*, 4395–4406.

(25) Cai, Y.-Z.; Xing, J.; Sun, M.; Zhan, Z.-Q.; Corke, H. Phenolic antioxidants (hydrolyzable tannins, flavonols, and anthocyanins) identified by LC-ESI-MS and MALDI-QIT-TOF MS from *Rosa chinensis* flowers. *J. Agric. Food Chem.* **2005**, *53*, 9940–9948.

(26) Koyuncu, F. Organic acid composition of native black mulberry fruit. *Chem. Nat. Compd.* **2004**, *40*, 367–369.

(27) Dietrich, M.; Traber, M. G.; Jacques, P. F.; Cross, C. E.; Hu, Y.; Block, G. Does gamma-tocopherol play a role in the primary prevention of heart disease and cancer? A review. *J Am Coll. Nutr.* **2006**, *25*, 292–9.

(28) Patel, A.; Liebner, F.; Netscher, T.; Mereiter, K.; Rosenau, T. Vitamin E chemistry. Nitration of non-alpha-tocopherols: products and mechanistic considerations. *J. Org. Chem.* **2007**, *72*, 6504–12.

(29) Holden, J. M.; Eldridge, A. L.; Beecher, G. B.; Buzzard, M.; Bhagwat, S.; Davis, C. S.; Douglass, L. W.; Gebhardt, S.; Haytowitz, D.; Schakel, S. Carotenoid content of U.S. foods: An update of the database. *J. Food Comp. Anal.* **1999**, *12*, 169–196.

(30) Benzie, I. F.; Chung, W. Y.; Wang, J.; Richelle, M.; Bucheli, P. Enhanced bioavailability of zeaxanthin in a milk-based formulation of wolfberry (Gou Qi Zi; *Fructus barbarum* L.). *Br. J. Nutr.* **2006**, *96*, 154–160.

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