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Influence of Harvest Season on Antioxidant Activity and Constituents of Rabbiteye Blueberry (*Vaccinium ashei*) Leaves

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ABSTRACT: To select rabbiteye blueberry leaves from an appropriate harvest season to develop functional foods, this paper studied the bioactive secondary metabolites and the antioxidant capacity of rabbiteye blueberry leaves from May, September, and November. The results showed the leaves from May had the highest content of total flavonoids (114.21 mg/g) and the leaves from November had the highest content of total polyphenols and proanthocyanidins (425.24 and 243.29 mg/g, respectively). It was further found that blueberry leaves from different seasons have similar bioactive constituents, but their contents are obviously different by HPLC. The rabbiteye blueberry leaves from November had the highest antioxidant capacity, which was well correlated with their highest proanthocyanidin content. The results clarify that the blueberry leaves from different seasons have different contents of bioactive secondary metabolites and different antioxidant activities, which implied that leaves from November should be selected first for utilization in functional foods.

KEYWORDS: *blueberry leaves, harvest season, polyphenols, flavonoids, proanthocyanidins, antioxidant activity*

INTRODUCTION

In the human body, free radicals and other reactive oxygen species (ROS) are natural byproducts of metabolism, such as hydrogen peroxide (H_2O_2), superoxide anion, and hydroxyl radical. [1] The anthropic body has certain defense capabilities to reduce oxidative damage and keep the balance in normal physiological conditions. However, the reduction of radical scavenging capacity can result in an imbalance between the oxidant and antioxidant systems in vivo. The superfluous ROS could attack biological molecules such as DNA,[2] proteins,[3] and lipids,[4] leading to cell or tissue injury. These oxidative damages are closely related to the occurrence and development of degenerative diseases such as cardiovascular and neurodegenerative diseases,[5,6] There is convincing evidence showing that dietary antioxidants are beneficial for the control of the above-mentioned diseases.[7−10] Consequently, there is considerable interest in the research of natural antioxidants from botanical sources, especially edible plants.[11]

Blueberry (*Vacccinium* L.), a deciduous or evergreen shrub of the Ericaceae plant group, is known for the delicacy and nutritive value of its fruits. Many studies have suggested that blueberry fruits have several biological activities, such as antioxidation,[12,13] neuroprotection,[14] and anti-inflammation.[15] Blueberry fruits are now the main plant parts used commercially, whereas their leaves are considered essentially a waste byproduct of the blueberry industry. Actually, the total polyphenol content in rabbiteye blueberry leaves is 3 times higher than that in rabbiteye blueberry fruits.[16] As early as 1926, blueberry leaf extract given at an oral dosage of 0.3 g/day for 3 months exerted a beneficial effect on 16 volunteers with diabetes mellitus.[17] In recent years, chemical constituents[18] and in vivo/in vitro activities of blueberry leaves, such as antioxidant activity,[19] hypolipidemic activity,[20] antileukemic activity,[21] hypotensive activity,[22] and cancer-preventive activity[23] have been investigated.

As we know, the bioactive plants are concerned with the types and quantities of their secondary metabolites.[24] Harvest season is one of the important factors affecting the secondary metabolites of plants. For example, seasonal changes had an influence on the tannin content in oak leaves,[25] and changes of total polyphenol content in hardwood tree species at different times also have been reported.[26] However, limited information is available on the influence of seasonal changes on the antioxidant activities and contents of bioactive metabolites in blueberry leaves. Therefore, we studied and compared the antioxidant activities and contents of total polyphenols, total flavonoids, and proanthocyanidins in blueberry leaves. Furthermore, the chromatographic profiles of blueberry leaves were analyzed. Our study will provide experimental support for the comprehensive utilization of blueberry leaves.

MATERIALS AND METHODS

Plant Materials and Chemicals. Fresh blueberry leaves were collected respectively in May, September, and November from bushes of *Vaccinium ashei* at the blueberry planting base of Majiang County, Guizhou Province, China. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), Folin−Ciocalteu, linoleic acid, β-carotene, and 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC grade formic acid and acetonitrile were obtained from Merck (Darmstadt, Germany). Polyphenol standards, including chlorogenic acid, caffeic acid, hyperoside, quercitrin, galuteolin, rutin, and gallic acid, were purchased from National Institutes for Food and Drug Control.

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Aluminum trichloride, vanillic, butylated hydroxytoluene (BHT), and other chemicals in the study were from local suppliers (Chengdu, China). The standards for HPLC analysis were dissolved in methanol to a concentration of 1 mg/mL and were stored at 4 °C.

Aqueous Extractions of Blueberry Leaves from Different Seasons. The fresh leaves from different seasons were dried in an oven at 60 °C, respectively, crushed and sieved using a grinder, and passed through a 40 mesh sieve. Three grams of dry coarse powder of blueberry leaves was added to 90 mL of water. Afterward, the mixture was extracted in a water bath at 100 °C for 120 min. The infusion was cooled to room temperature and filtered through filter paper, and the residue was re-extracted under the same condition for complete extraction. The combined filtrate was evaporated by rotary vacuum evaporator at 58 °C, then further dried to a yellow powder using a freeze-dryer at −50 °C, and stored at 4 °C until analysis.

Chromatographic Fingerprint Analysis of Blueberry Leaves from Different Seasons. Three grams of dry powder of blueberry leaves was extracted with 90 mL of 60% methanol in a water bath at 80 °C for 120 min. The infusion was cooled to room temperature and filtered through filter paper, and the residue was re-extracted under the same condition for complete extraction. The combined filtrate was evaporated by rotary vacuum evaporator at 40 °C. The infusion was made up to volume (100 mL) with methanol and diluted 5 times before detection. The solution was stored at 4 °C until analysis. HPLC analysis was performed using an Agilent 1260 LC system (Santa Clara, CA, USA) equipped with a 150 × 2.1 mm, 3 μm, Welch Ultimate XB-C18 column. The injection volume was 20 μL. Two solvents were used with a constant flow rate of 0.1 mL/min. Solvent A consisted of 100% acetonitrile, and solvent B included 0.2% formic acid in water. Elution was done using a linear gradient from 10 to 20% A in 40 min, from 20 to 26% A in 30 min, and from 26 to 70% A in 10 min and then held constant for 10 min. The wavelength for UV detection was 350 nm.

Determination of the Total Polyphenol Content. The total polyphenol content in the lyophilized extract of blueberry leaves was determined using the Folin−Ciocalteu colorimetric method.[27] Briefly, the mixture of 0.1 mL of aqueous extract (1 mg/mL) and 1 mL of 50% Folin−Ciocalteu reagent was incubated for 2 min; then 2.0 mL of 20% aqueous sodium carbonate and 1.9 mL of distilled water were added to the mixture. The reaction mixture was incubated at 37 °C for 120 min in the dark, and the absorbance was measured at 765 nm against distilled water as blank. Gallic acid was used as the standard, and the results are expressed as milligrams of gallic acid equivalent per gram of extract.

Determination of the Total Flavonoid Content. The total flavonoid content from leaf extract was assayed using a colorimetric method.[28] Briefly, 500 μL of aqueous extract (1 mg/mL) was mixed with 0.1 mL of 0.1 g/L aluminum trichloride and 0.1 mL of 1 M potassium acetate, then diluted with distilled water to 3 mL, and thoroughly mixed. The absorbance of the mixture was measured at 415 nm after 30 min at room temperature. Rutin was used as the standard. The results are expressed as milligrams of rutin equivalent per gram of extract.

Determination of the Proanthocyanidin Content. The proanthocyanidin content of blueberry leaves was determined by using the vanillin−H_2SO_4 method with minor modifications.[29] Briefly, 1.0 mL of aqueous extract (1 mg/mL) was added to 2.5 mL of 1% vanillin solution in methanol and 2.5 mL of 30% H_2SO_4 solution in methanol. The reaction was carried out in the dark at 30 °C for 20 min, and then absorbance was measured at 500 nm. Catechin was used as the standard. The results are expressed as milligrams of catechin equivalent per gram of extract.

DPPH Assay. DPPH radical scavenging activity was measured according to the method with some modifications.[30] The DPPH$^{\bullet}$ solution in ethanol (0.09 mM) was prepared, and 1.9 mL of this solution was mixed thoroughly with 0.1 mL of aqueous solutions of leaf extract. The sample was incubated for 30 min at 37 °C in the dark, and then the decrease in absorbance at 517 nm was measured. BHT, a commercial synthetic antioxidant, was used as the positive control. In this method, the percentage of DPPH radical scavenging activity was calculated using the formula

$$\text{inhibition (\%)} = (1 - A_1/A_0) \times 100 \quad (1)$$

where A_0 is the absorbance of the solution without extract and A_1 is the absorbance of the leaf extract addition. All determinations were done in triplicate.

ABTS Assay. ABTS radical cation scavenging activity was determined according to the method described by Garzon and Wrolstad with slight modifications.[31] ABTS was dissolved in deionized water to 7 mM concentration. ABTS radical cation ABTS$^{\bullet+}$ was produced by reacting ABTS solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand at room temperature in the dark for 12−16 h before use. Then a 0.2 mL solution of aqueous leaf extract was mixed with 0.3 mL of ABTS$^{\bullet+}$ solution and 0.5 mL of distilled water. The mixture was allowed to stand at room temperature for 2 min, and the absorbance at 745 nm was immediately recorded. BHT was used as the positive control. The percentage of ABTS radical scavenging activity was calculated using eq 1, where A_0 is the absorbance of solution without extract and A_1 is the absorbance of the leaf extract addition. All determinations were done in triplicate.

β-Carotene−Linoleic Acid Assay. In this assay, the antioxidant activity was evaluated by the β-carotene−linoleic acid model system.[32] A stock of β-carotene−linoleic acid mixture was prepared as follows: 0.4 mg of β-carotene was dissolved in 2 mL of chloroform, and 25 μL of linoleic acid and 200 mg of Tween-40 were added as emulsifier because β-carotene is not water-soluble. Chloroform was completely evaporated using a vacuum evaporator. Then 50 mL of distilled water was added with vigorous shaking. Twenty-four hundred microliters of the reaction mixture was dispensed into test tubes, and 100 μL of aqueous solution of the leaf extract was added. The tube was incubated for 120 min at 50 °C. The absorbance at 470 nm was taken at zero time (t = 0). Measurement of the absorbance was continued until the color of β-carotene disappeared in the control reaction (t = 120 min). BHT was used as the positive control. The antioxidant activity of the extract was evaluated in terms of the β-carotene bleaching using the formula

$$\text{inhibition activity of } \beta\text{-carotene bleaching (\%)} = [1 - (A_0 - A_t)/(A'_0 - A_t)] \times 100 \quad (2)$$

where A_0 and A'_0 are the absorbance values measured at zero time of the solution for the test sample and the solution without sample, respectively. A_t are the absorbance of the test sample and the solution without sample after incubation for 120 min, respectively. All determinations were done in triplicate.

Statistical Analysis. Results were expressed as the means ± SD (n = 3). A one-way analysis of variance was used for the data analysis by using SPSS 19.0 software. The IC_{50} values were calculated from linear regression analysis. Differences with a P value of <0.05 were considered significant. All tests were performed in triplicate.

RESULTS AND DISCUSSION

Contents of Total Polyphenols, Total Flavonoids, and Proanthocyanidins. The antioxidant properties of many plants sources are mainly due to polyphenols, which play an important role in neutralizing free radicals, quenching oxygen species, or decomposing peroxides.[33] There were many phenolic constituents including catechin, caffeic acid, quercetin, and kaempferol in blueberry leaves.[18] Flavonoids consist of the largest group of polyphenols in plants. Flavonoids are a class of plant polyphenols with powerful antioxidant properties.[34] Proanthocyanidins are widely distributed in natural plants and act as the active constituent of medicinal plants.[35] Developing blueberry leaves into functional foods is the main application of the leaves studied in this paper, so distilled water was used as the extraction solvent instead of organic solvent. The contents

of total polyphenols, total flavonoids, and proanthocyanidins were determined in blueberry leaves from different months (Table 1). The leaves from November had the highest content

Table 1. Contents of Total Polyphenols, Total Flavonoids, and Proanthocyanidin in Rabbiteye Blueberry Leaf Extracts from Different Months

harvest month	polyphenols[a]	flavonoids[b]	proanthocyanidin[c]
May	300.12 ± 0.07*	114.21 ± 0.03*	102.13 ± 0.06*
September	388.64 ± 0.09*	63.24 ± 0.05*	184.07 ± 0.08*
November	425.24 ± 0.16*	76.62 ± 0.03*	243.29 ± 0.11*

[a]Expressed as mg gallic acid equivalent/g extract. [b]Expressed as mg rutin equivalent/g extract. [c]Expressed as mg catechin equivalent/g extract. An asterisk (*) indicates a significant difference at the $p < 0.05$ level among different harvest months.

of total polyphenols (425.24 ± 0.16 mg/g), followed by the leaves from September (388.64 ± 0.09 mg/g) and May (300.12 ± 0.07 mg/g). The level is comparable to green tea aqueous extract (302.32 mg/g) and black tea aqueous extract (245.88 mg/g).[36] The proanthocyanidin content of blueberry leaves from different months showed similar variation compared with the content of total polyphenols, which ranged from 102.13 ± 0.06 to 243.29 ± 0.11 mg/g, whereas the total flavonoid content of blueberry leaves from different months showed that the leaves from May had the highest (114.21 ± 0.03 mg/g), followed by the leaves from November and September.

Chromatographic Profiles of Blueberry Leaves from Different Seasons. *Detection Wavelength.* The absorbance wavelength (λ) of the leaf extract was analyzed by scanning between 200 and 400 nm on the Perkin Elmer UV-900 to obtain the optimal detection wavelength applied for the chromatography separation. Spectra chromatography displayed that the absorbance peaks of the samples were different; however, they all had a better absorbance peak at 350 nm, and therefore the UV−vis detector was set at 350 nm in this method.

Analysis of Chromatograms. Typical HPLC chromatograms of solutions of blueberry leaves from different seasons are shown in Figure 1. A total of 14 major peaks was obtained (where a major peak was defined as any peak with a peak area that is at least 3% of the chlorogenic acid peak area). On the basis of analysis of the chromatograms, retention times, and percentages of the 14 peaks of the three samples (Figure 2), the

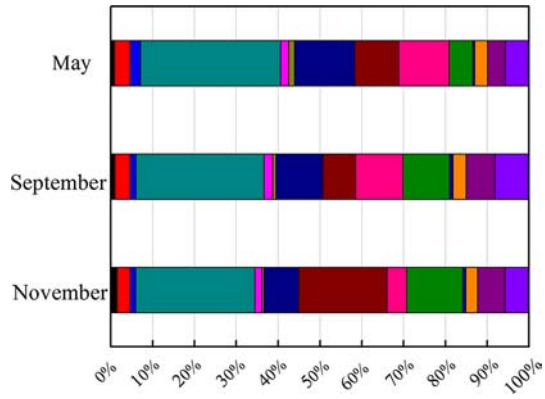

Figure 2. Percentages of the 14 peaks in the HPLC-UV chromatograms of rabbiteye blueberry leaves from different months (from left to right, peaks 1−14).

blueberry leaves from different seasons had similar constituents. However, there were obvious differences in contents, which was in accordance with the contents of active ingredients abovementioned. Figure 3 displays the total areas of these 14 peaks. Obviously, the leaves from May had the largest peak area, following by the leaves from November and September.

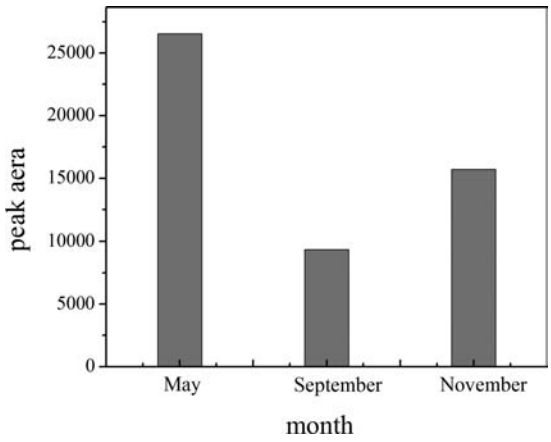

Figure 3. Total peak areas of the tagged 14 peaks of rabbiteye blueberry leaves from different months.

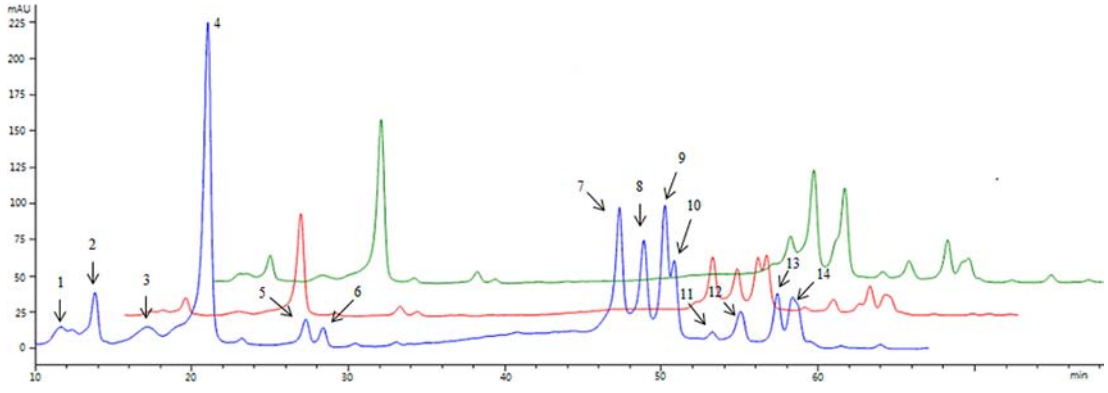

Figure 1. Typical HPLC chromatograms of blueberry leaves, injected volume 20 μL, at λ = 350 nm: blue line, standards for chromatogram of blueberry leaves from May; red line, standards for chromatogram of rabbiteye blueberry leaves from September; green line, standards for chromatogram of blueberry leaves from November. Peaks: 4, chlorogenic acid; 5, caffeic acid; 7, rutin; 8, hyperoside; 9, galuteolin; 14, quercitrin.

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Table 2. Amounts of Chlorogenic Acid, Caffeic Acid, Rutin, Hyperoside, Galuteolin, Quercitrin, and Flavonoids of Rabbiteye Blueberry Leaves from Different Months

	peak area percentage (% total areas)							
harvest month	chlorogenic acid	caffeic acid	rutin	hyperoside	galuteolin	quercitrin	polyphenols[a]	flavonoids[b]
May	33.50	1.96	14.33	10.57	12.11	5.69	78.15	42.69
September	30.62	2.10	11.19	7.88	11.31	8.16	71.26	38.54
November	28.52	1.71	8.38	21.10	4.76	5.76	70.23	33.99

[a]Polyphenols are the sum of peak area percentage of chlorogenic acid, caffeic acid, rutin, hyperoside, galuteolin, and quercitrin. [b]Flavonoids are the sum of peak area percentage of rutin, hyperoside, galuteolin, and quercitrin.

Table 3. Contents of Chlorogenic Acid, Caffeic Acid, Rutin, Hyperoside, Galuteolin, and Quercitrin of Rabbiteye Blueberry Leaves from Different Months

	content of constituents (mg/g)					
harvest month	cholorogenic acid	caffeic acid	rutin	hyperoside	galuteolin	quercitrin
May	33.61 ± 2.12	0.54 ± 0.10	6.50 ± 1.10	3.25 ± 1.22	27.21 ± 2.44	4.22 ± 0.42
September	8.84 ± 0.20	0.09 ± 0.01	2.30 ± 0.03	1.11 ± 0.21	10.45 ± 0.85	1.33 ± 0.03
November	14.07 ± 0.02	0.13 ± 0.01	2.58 ± 0.45	4.79 ± 0.68	20.28 ± 1.07	2.10 ± 0.07

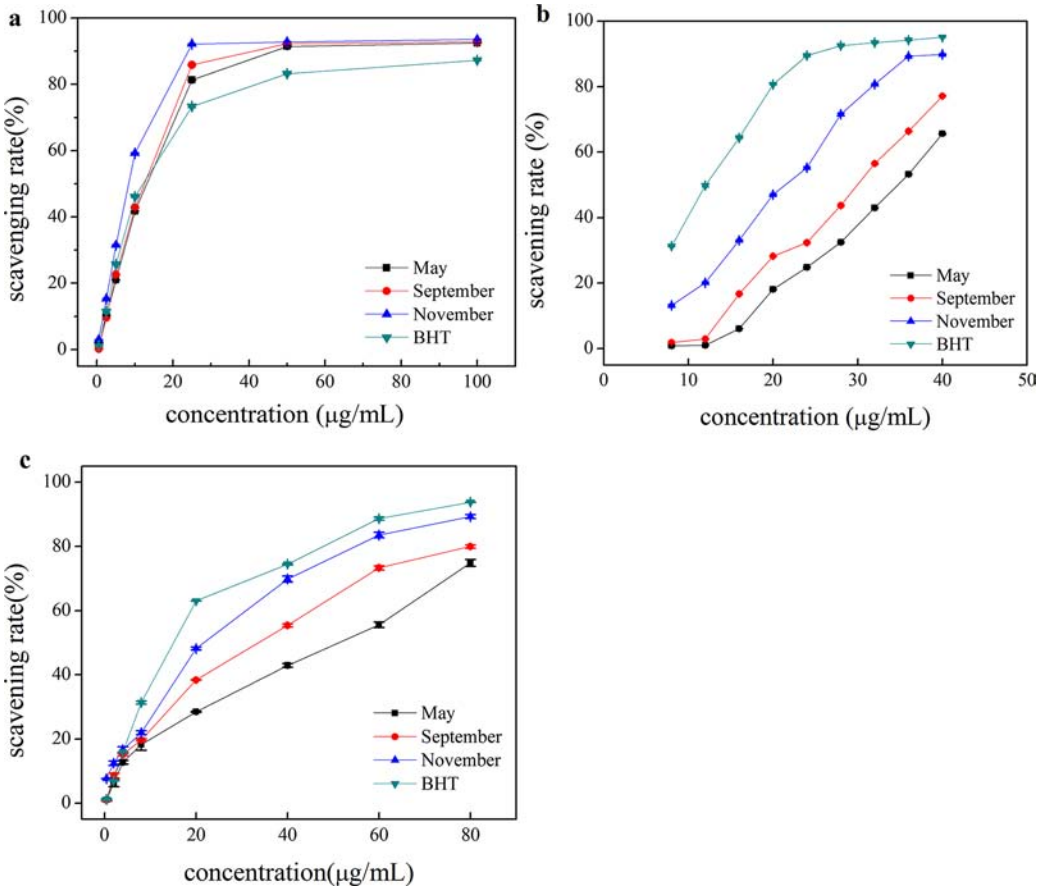

Figure 4. Antioxidant activities of rabbiteye blueberry leaf extracts from different months using the following assays: (a) DPPH$^{\bullet}$ scavenging assay; (b) ABTS$^{\bullet+}$ scavenging assay; (c) β-carotene bleaching assay.

Of the 14 peaks, 6 were identified using commercial standards (chlorogenic acid at 18.98 min, caffeic acid at 25.24 min, rutin at 45.26 min, hyperoside at 46.82 min, galuteolin at 48.16 min, and quercitrin at 56.31 min). For these six constituents, rutin, hyperoside, galuteolin, and quercitrin belong to flavonoids, and all of these are polyphenols. Table 2 shows the peak-area percentage of chlorogenic acid, caffeic acid, rutin, hyperoside, galuteolin, quercitrin, and flavonoids of blueberry leaves from different seasons. Furthermore, we determined the contents of these six constituents in blueberry leaves from May, September, and November (Table 3). For these three samples, the content of hyperoside from November was the highest, and the others from May showed the highest content, whereas the contents of these six constutients from September are lowest. The blueberry leaves from May had the largest peak area and the highest content among these three samples, which was a discrepancy compared with the content of total polyphenols above. The main reason is that some

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polyphenols cannot be detected under this chromatographic condition, such as proanthocyanidins, which are abundant in blueberry leaves.[37]

Seasonal changes have remarkable influence on the process of plant growth, and the kinds and contents of constituents in plants change as time goes on.[38] For blueberry leaves, Percival and MacKenzie had reported that the content of total polyphenols from green leaf tissues at harvest is higher than that of red leaf tissues 2 weeks after harvest.[39] In this study, young blueberry leaves were much more abundant in flavonoids than old leaves, whereas the opposite pattern was observed for the proanthocyanidin content, which was in accordance with the changes in oak leaves.[25]

Antioxidant Activity. *DPPH Radical Scavenging Activity.* Figure 4a shows the DPPH$^{\bullet}$ scavenging capacity of leaves extracts from different months (BHT as the positive control). A dose-dependent relationship was found in DPPH scavenging activity of blueberry leaf extracts from different seasons. The order of DPPH radical scavenging activity for the samples was as follows: November > September and May (no significant difference, $P > 0.05$) > BHT. The EC_{50} values of the leaf extracts from May, September, and November were 11.32 ± 0.11, 12.31 ± 0.18, and 7.63 ± 0.13 μg/mL, respectively (Table 4). Compared with green tea extract (EC_{50} = 23.65 ± 0.20 μg/mL),[40] the extracts of blueberry leaves from different months have strong DPPH$^{\bullet}$ scavenging activity, especially the leaves from November.

Table 4. EC_{50} and IC_{50} Values of Rabbiteye Blueberry Leaf Extracts from Different Months[a]

harvest month or control	radical scavenging (EC_{50}, μg/mL) DPPH$^{\bullet}$	ABTS$^{\bullet+}$	β-carotene bleaching (IC_{50}, μg/mL)
May	11.32 ± 0.11	33.90 ± 0.11*	39.65 ± 0.01*
September	12.31 ± 0.18	28.84 ± 0.15*	25.54 ± 0.25
November	7.63 ± 0.13*	20.08 ± 0.16*	14.76 ± 0.11
BHT	13.97 ± 0.12*	8.53 ± 0.17*	13.39 ± 0.10

[a]An asterisk (*) indicates a significant difference at the $p < 0.05$ level among different harvest months and control.

ABTS Radical Scavenging Activity. Figure 4b shows the ABTS$^{\bullet+}$ scavenging capacity of leaf extracts from different months (BHT as the positive control). An increase in concentration was synonymous with an increase in ABTS radical scavenging capacity. BHT showed high scavenging activity (EC_{50} = 8.53 ± 0.17 μg/mL, Table 4). The blueberry leaves from different months also had strong scavenging effects. The EC_{50} values of the leaf extracts from May, September, and November were 33.90 ± 0.11, 28.84 ± 0.15, and 20.08 ± 0.16 μg/mL, respectively (Table 4), and all of them showed significant difference.

β-Carotene Bleaching Activity. In the β-carotene bleaching assay, the leaf extracts from different months and BHT showed varied antioxidant activity (Figure 4c). Similarly, they all had a close positive relationship with the extract concentration. Table 4 shows the EC_{50} values of the blueberry leaf extracts in comparison with that of BHT. Their antioxidant capacities decreased in the order BHT > November > September > May, and the β-carotene bleaching activity of the leaf extract from November was not significantly different from that of BHT, which were about 1 and 2 times higher than leaves from September and May, respectively. The EC_{50} value of blueberry leaf extract from November was 14.76 ± 0.11 μg/mL, compared with the *Ginkgo biloba* extract (EC_{50} = 13.1 ± 0.3 μg/mL).[41]

We chose three methods to measure antioxidant capacities to ensure the accuracy of results: the DPPH and ABTS assays based on electron-transfer reaction and the β-carotene bleaching assay based on hydrogen atom transfer reaction. The antioxidant capacities of plant extract vary with assay methods,[42] but all three methods showed that the blueberry leaves from November had the strongest antioxidant capacities.

Correlation between Bioactive Secondary Metabolites and Antioxidant Capacity. The correlation between contents of the total polyphenols, total flavonoids, proanthocyanidins, and antioxidant activities of leaf extracts from different seasons is shown in Table 5. The polyphenols, flavonoids, and proanthocyanidins have certain relevance with antioxidant capacities, especially between proanthocyanidins and DPPH assay (R^2 = 0.938), between proanthocyanidins and ABTS assay (R^2 = 0.926), and between proanthocyanidins and β-carotene−linoleic acid assay (R^2 = 0.890). The lowest correlation was found between flavonoids and antioxidant capacity. Proanthocyanidins, comprising mainly catechins and oligomeric and polymeric proanthocyanidins, seem to be greatly responsible for the antioxidant capacity of the blueberry leaves from different seasons. Catechins and oligomeric proanthocyanidins have free radical scavenging and antilipoperoxidant activities in various antioxidant assay systems.[43,44] The results might explain that although the leaves from May had the highest flavonoid content and the largest total peak area, they had the lowest antioxidant capacity.

Table 5. Correlation between the Contents of Total Polyphenols, Total Flavonoids, Proanthocyanidins, and the Antioxidant Activities of Rabbiteye Blueberry Leaf Extracts

	DPPH$^{\bullet}$ scavenging	ABTS$^{\bullet+}$scavenging	β-carotene bleaching
polyphenols	R^2 = 0.823	R^2 = 0.815	R^2 = 0.794
flavonoids	R^2 = 0.647	R^2 = 0.623	R^2 = 0.549
proanthocyanidins	R^2 = 0.938	R^2 = 0.926	R^2 = 0.890

In conclusion, this study revealed that harvest season has a critical influence on the constituents and antioxidant properties of blueberry leaves, and leaves should be harvested in November for use as important sources of dietary antioxidants. Further work is required to extract and isolate antioxidants from blueberry leaves, to utilize blueberry leaves comprehensively, and to develop functional foods.

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Notes

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REFERENCES

(1) Sorg, O. Oxidative stress: a theoretical model or a biological reality? *C. R. Biol.* **2004**, *327*, 649−662.

(2) Halliwell, B.; Aruoma, O. I. DNA damage by oxygen-derived species. Its mechanism and measurement in mammalian systems. *FEBS Lett.* **1991**, *281*, 9−19.

(3) Dean, R.; Fu, S.; Stocker, R.; Davies, M. Biochemistry and pathology of radical-mediated protein oxidation. *Biochem. J.* **1997**, *324*, 1−18.

(4) Łuczaj, W.; Skrzydlewska, E. DNA damage caused by lipid peroxidation products. *Cell Mol. Biol. Lett.* **2003**, *8*, 391−413.

(5) Aruoma, O. I. Free radicals, oxidative stress, and antioxidants in human health and disease. *J. Am. Oil Chem. Soc.* **1998**, *75*, 199−212.

(6) Mariani, E.; Polidori, M.; Cherubini, A.; Mecocci, P. Oxidative stress in brain aging, neurodegenerative and vascular diseases: an overview. *J. Chromatogr., B* **2005**, *827*, 65−75.

(7) Morris, M. C.; Evans, D. A.; Bienias, J. L.; Tangney, C. C.; Bennett, D. A.; Aggarwal, N.; Wilson, R. S.; Scherr, P. A. Dietary intake of antioxidant nutrients and the risk of incident Alzheimer disease in a biracial community study. *JAMA−J. Am. Med. Assoc.* **2002**, *287*, 3230−3237.

(8) Luchsinger, J. A.; Tang, M.-X.; Shea, S.; Mayeux, R. Antioxidant vitamin intake and risk of Alzheimer disease. *Arch. Neurol.−Chicago* **2003**, *60*, 203.

(9) Serafini, M.; Jakszyn, P.; Luján-Barroso, L.; Agudo, A.; Bas Bueno-de-Mesquita, H.; van Duijnhoven, F. J.; Jenab, M.; Navarro, C.; Palli, D.; Boeing, H. Dietary total antioxidant capacity and gastric cancer risk in the European prospective investigation into cancer and nutrition study. *Int. J. Cancer* **2012**, *131*, E544−E554.

(10) Castro, M. M.; Rizzi, E.; Rodrigues, G. J.; Ceron, C. S.; Bendhack, L. M.; Gerlach, R. F.; Tanus-Santos, J. E. Antioxidant treatment reduces matrix metalloproteinase-2-induced vascular changes in renovascular hypertension. *Free Radical Biol. Med.* **2009**, *46*, 1298−1307.

(11) Hu, C.; Zhang, Y.; Kitts, D. D. Evaluation of antioxidant and prooxidant activities of bamboo *Phyllostachys nigra* var. *Henonis* leaf extract in vitro. *J. Agric. Food Chem.* **2000**, *48*, 3170−3176.

(12) Giovanelli, G.; Buratti, S. Comparison of polyphenolic composition and antioxidant activity of wild Italian blueberries and some cultivated varieties. *Food Chem.* **2009**, *112*, 903−908.

(13) Faria, A.; Oliveira, J.; Neves, P.; Gameiro, P.; Santos-Buelga, C.; de Freitas, V.; Mateus, N. Antioxidant properties of prepared blueberry (*Vaccinium myrtillus*) extracts. *J. Agric. Food Chem.* **2005**, *53*, 6896−6902.

(14) Giacalone, M.; Di Sacco, F.; Traupe, I.; Topini, R.; Forfori, F.; Giunta, F. Antioxidant and neuroprotective properties of blueberry polyphenols: a critical review. *Nutr. Neurosci.* **2011**, *14*, 119−125.

(15) Torri, E.; Lemos, M.; Caliari, V.; Kassuya, C. A.; Bastos, J. K.; Andrade, S. F. Anti-inflammatory and antinociceptive properties of blueberry extract (*Vaccinium corymbosum*). *J. Pharm. Pharmacol.* **2007**, *59*, 591−596.

(16) Li, C.; Feng, J.; Huang, W.-Y.; An, X.-T. Composition of polyphenols and antioxidant activity of rabbiteye blueberry (*Vaccinium ashei*) in Nanjing. *J. Agric. Food Chem.* **2013**, *61*, 523−531.

(17) Watson, E. Some observations on the effect of blueberry leaf extract in diabetes mellitus. *Can. Med. Assoc. J.* **1928**, *19*, 166.

(18) Matsuo, Y.; Fujita, Y.; Ohnishi, S.; Tanaka, T.; Hirabaru, H.; Kai, T.; Sakaida, H.; Nishizono, S.; Kouno, I. Chemical constituents of the leaves of rabbiteye blueberry (*Vaccinium ashei*) and characterisation of polymeric proanthocyanidins containing phenylpropanoid units and A-type linkages. *Food Chem.* **2010**, *121*, 1073−1079.

(19) Piljac-Žegarac, J.; Belščak, A.; Piljac, A. Antioxidant capacity and polyphenolic content of blueberry (*Vaccinium corymbosum* L.) leaf infusions. *J. Med. Food* **2009**, *12*, 608−614.

(20) Li, Y. C.; Li, B. X.; Geng, L. J. Hypolipidemic and antioxidant effects of total flavonoids from blueberry leaves. *Eur. Food Res. Technol.* **2011**, *233*, 897−903.

(21) Skupień, K.; Oszmiański, J.; Kostrzewa-Nowak, D.; Tarasiuk, J. *In vitro* antileukaemic activity of extracts from berry plant leaves against sensitive and multidrug resistant HL60 cells. *Cancer Lett.* **2006**, *236*, 282−291.

(22) Sakaida, H.; Nagao, K.; Higa, K.; Shirouchi, B.; Inoue, N.; Hidaka, F.; Kai, T.; Yanagita, T. Effect of *Vaccinium ashei reade* leaves on angiotensin converting enzyme activity *in vitro* and on systolic blood pressure of spontaneously hypertensive rats *in vivo*. *Biosci., Biotechnol., Biochem.* **2007**, *71*, 2335−2337.

(23) Mechikova, G. Y.; Kuzmich, A.; Ponomarenko, L.; Kalinovsky, A.; Stepanova, T.; Fedorov, S.; Stonik, V. Cancer-preventive activities of secondary metabolites from leaves of the bilberry *Vaccinium smallii* A. Gray. *Phytother. Res.* **2010**, *24*, 1730−1732.

(24) Fiáková, S.; Šturdíková, M.; Múčková, M. Bioactive secondary metabolites produced by microorganisms associated with plants. *Biologia* **2007**, *62*, 251−257.

(25) Feeny, P. Seasonal changes in oak leaf tannins and nutrients as a cause of spring feeding by winter moth caterpillars. *Ecology* **1970**, *51*, 565−581.

(26) Schultz, J. C.; Nothnagle, P. J.; Baldwin, I. T. Seasonal and individual variation in leaf quality of two northern hardwoods tree species. *Am. J. Bot.* **1982**, 753−759.

(27) Li, H.; Zhang, M.; Ma, G. Radical scavenging activity of flavonoids from *Trollius chinensis* Bunge. *Nutrition* **2011**, *27*, 1061−1065.

(28) Awah, F. M.; Uzoegwu, P. N.; Ifeonu, P.; Oyugi, J. O.; Rutherford, J.; Yao, X.; Fehrmann, F.; Fowke, K. R.; Eze, M. O. Free radical scavenging activity, phenolic contents and cytotoxicity of selected Nigerian medicinal plants. *Food Chem.* **2012**, *131*, 1279−1286.

(29) Lee, H.-R.; Bak, M.-J.; Jeong, W.-S.; Kim, Y.-C.; Chung, S.-K. Antioxidant properties of proanthocyanidin fraction isolated from wild grape (*Vitis amurensis*) seed. *J. Korean Soc. Appl. Biol. Chem.* **2009**, *52*, 539−544.

(30) Dudonne, S.; Vitrac, X.; Coutiere, P.; Woillez, M.; Mérillon, J.-M. Comparative study of antioxidant properties and total phenolic content of 30 plant extracts of industrial interest using DPPH, ABTS, FRAP, SOD, and ORAC assays. *J. Agric. Food Chem.* **2009**, *57*, 1768−1774.

(31) Garzón, G.; Wrolstad, R. Major anthocyanins and antioxidant activity of nasturtium flowers (*Tropaeolum majus*). *Food Chem.* **2009**, *114*, 44−49.

(32) Obame, L.; Edou, P.; Bassole, I.; Koudou, J.; Agnaniet, H.; Eba, F.; Traore, A. Chemical composition, antioxidant and antimicrobial properties of the essential oil of *Dacryodes edulis* (G. Don) HJ Lam from Gabon. *Afr. J. Microbiol. Res.* **2008**, *2*, 148−152.

(33) Rice-Evans, C. A.; Miller, N. J.; Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933−956.

(34) Pietta, P.-G. Flavonoids as antioxidants. *J. Nat. Prod.* **2000**, *63*, 1035−1042.

(35) Ferreira, D.; Slade, D. Oligomeric proanthocyanidins: naturally occurring O-heterocycles. *Nat. Prod. Rep.* **2002**, *19*, 517−541.

(36) Gramza, A.; Khokhar, S.; Yoko, S.; Gliszczynska-Swiglo, A.; Hes, M.; Korczak, J. Antioxidant activity of tea extracts in lipids and correlation with polyphenol content. *Eur. J. Lipid. Sci. Technol.* **2006**, *108*, 351−362.

(37) Takeshita, M.; Ishida, Y.-i.; Akamatsu, E.; Ohmori, Y.; Sudoh, M.; Uto, H.; Tsubouchi, H.; Kataoka, H. Proanthocyanidin from blueberry leaves suppresses expression of subgenomic hepatitis C virus RNA. *J. Biol. Chem.* **2009**, *284*, 21165−21176.

(38) Grace, S.; Logan, B.; Adams, W. Seasonal differences in foliar content of chlorogenic acid, a phenylpropanoid antioxidant, in *Mahonia repens*. *Plant Cell Environ.* **1998**, *21*, 513−521.

(39) Percival, D.; MacKenzie, J. L. Use of plant growth regulators to increase polyphenolic compounds in the wild blueberry. *Can. J. Plant Sci.* **2007**, *87*, 333−336.
(40) Yu, F.; Sheng, J.; Xu, J.; An, X.; Hu, Q. Antioxidant activities of crude tea polyphenols, polysaccharides and proteins of selenium-enriched tea and regular green tea. *Eur. Food Res. Technol.* **2007**, *225*, 843−848.
(41) Zhu, L.; Tan, J.; Wang, B.; He, R.; Liu, Y.; Zheng, C. Antioxidant activities of aqueous extract from *Agrimonia pilosa* Ledeb and its fractions. *Chem. Biodivers.* **2009**, *6*, 1716−1726.
(42) Sun, T.; Ho, C.-T. Antioxidant activities of buckwheat extracts. *Food Chem.* **2005**, *90*, 743−749.
(43) Scott, B. C.; Butler, J.; Halliwell, B.; Aruoma, O. I. Evaluation of the antioxidant actions of ferulic acid and catechins. *Free Radical Res.* **1993**, *19*, 241−253.
(44) Salah, N.; Miller, N. J.; Paganga, G.; Tijburg, L.; Bolwell, G. P.; Rice-Evans, C. Polyphenolic flavanols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. *Arch. Biochem. Biophys.* **1995**, *322*, 339−346.