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Phenolic Composition and Antioxidant Activity in Seed Coats of 60 Chinese Black Soybean (*Glycine max* L. Merr.) Varieties

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ABSTRACT: Phenolics in black soybean seed coat (BSSC) are considered to be responsible for the health benefits of black soybean. BSSCs of 60 Chinese varieties were examined for phenolic contents, anthocyanin profiles, and antioxidant activity. Total phenolic and condensed tannin contents ranged from 512.2 to 6057.9 mg gallic acid equivalents/100 g and from 137.2 to 1741.1 mg (+)-catechin equivalents/100 g, respectively. Six anthocyanins (delphinidin-3-glucoside, cyanidin-3-galactoside, cyanidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, and malvidin-3-glucoside) were detected by HPLC. Total anthocyanin contents (TAC) were from 98.8 to 2132.5 mg/100 g, and cyanidin-3-glucoside was the most abundant anthocyanin in all varieties, with a distribution of 48.8−94.1% of TAC. Antioxidant properties detected by DPPH, FRAP, and ORAC methods all showed wide variations ranging from 4.8 to 65.3 μg/100 mL (expressed as EC_{50}), from 17.5 to 105.8 units/g, and from 42.5 to 1834.6 μmol Trolox equivalent/g, respectively. Sixty varieties were classified into four groups by hierarchical clustering analysis, and group 4 consisting of nine varieties had the highest phytochemicals content and antioxidant activity.

KEYWORDS: black soybean, seed coat, anthocyanins, phenolics, antioxidant activity

INTRODUCTION

Black soybean (*Glycine max* L. Merr.) is a soybean cultivar with a black seed coat. It has been used as a herbal material in traditional medicine for hundreds of years in Asia. In the past few decades, proteins and isoflavones are the major health beneficial components in soybean including black soybean that have received the most attention.[1,2] However, they are not enough to explain some health benefits exclusive to black soybean because other soybean cultivars contain equivalent levels of these components. In addition to isoflavones, there are many other phytochemicals in black soybean that are potentially effective in human health, including sterols, phytic acid, saponins, and phenolics.

Recent research showed that black soybean had the highest antioxidant activity compared with other colored seed coat soybeans.[3−6] Furthermore, phenolics had a dominant presence in antioxidants found in black soybean and mainly distributed in the seed coat.[3−6] The total concentrations of 20 phenolic compounds in 5 Korean black soybean varieties were 13−50 times higher in the seed coat than in the cotyledon.[6] Epidemiological studies have associated elevated dietary consumption of phenolics with reduced risk of many chronic diseases such as cardiovascular disease and cancers.[7,8] Phenolics were proven to have distinct antioxidant activity and be responsible for many health-promoting effects of black soybean.[4,5] Considering the superior concentration and activity of phenolics in black soybean seed coat (BSSC), we deduced that BSSC potentially accounts for the medical effects of black soybean.

One of the most important classes of phenolics in BSSC is anthocyanins, which is the predominant reason for its black color and antioxidant activity. More than 99% of the anthocyanins in black soybean are distributed in the seed coat. Anthocyanins in BSSC reduced ischemic reperfusion (I/R)-induced myocardial injury, protected keratinocyte cells from UV damage, and inhibited obesity induced by a high-fat diet.[9−11] In addition to anthocyanins, BSSC is also a good source of other phenolics such as condensed tannins and phenolic acids.[3,6] It is commonly known that the contents and composition of phenolic compounds, especially anthocyanins, are greatly influenced by genetic and environmental conditions. Investigation of phenolic distribution and activity in different varieties is necessary for us to discover black soybean varieties with high health benefits. Furthermore, high contents of protein or fat have been main objectives in traditional soybean breeding. Because of the beneficial properties of BSSC phenolics, the beneficial health-related information of different varieties is also of great importance to breeders.

Researchers have previously reported anthocyanin profiles and contents in black soybean, but they focused efforts on purification and characterization.[12−15] Data on anthocyanin compositions and contents in BSSC of different soybean varieties were limited and restricted to a few varieties. Anthocyanin profiles in BSSC of 10 Korean and 5 Japanese varieties were analyzed previously.[13,15] Studies on other phenolic compounds and antioxidant activity of BSSC in different varieties were even fewer. China is the place of origin for soybean and has developed numerous black soybean varieties. Ecological regions of black

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soybean are distributed all over China. The huge difference of geographic and climatic conditions in different ecological regions may have a great influence on phenolic profiles of black soybeans cultivated there. However, very little is known about the phenolic profiles and antioxidant effects of BSSC from different Chinese varieties until now. Therefore, seed coats of 60 Chinese black soybean varieties were analyzed in this study to determine the contents of individual anthocyanins, total polyphenols, and condensed tannins and antioxidant activity, to investigate correlation among phenolic contents and antioxidant activity of samples tested, and to determine the black soybean varieties with high phenolic contents and antioxidant activity.

MATERIALS AND METHODS

Chemicals. Reference standards of delphinidin-3-glucoside, cyanidin-3-galactoside, cyanidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, and malvidin-3-glucoside were obtained from Polyphenols Laboratories (Sandnes, Norway). Gallic acid, (+)-catechin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), and 3′,6′-dihydroxyspiro[isobenzofuran-1(^{3}H),9′-(^{9}H)xanthene]-3-one, disodium salt (FL), were obtained from Sigma-Aldrich Inc. (St. Louis, MO). HPLC grade methanol, acetonitrile, and formic acid were obtained from Fisher (Suwanee, GA).

Soybean Materials. Sixty varieties of black soybeans (*G. max* L. Merr.) originating in different parts of China were donated by the Institute of Crop Sciences, Chinese Academy of Agricultural Sciences (Beijing, China). All varieties were grown in the experimental station of the Institute of Crop Germplasm Resources, Shanxi Academy of Agricultural Sciences, during May−October in 2008. Soybeans were air-dried and then stored at −20 °C in the dark until used. Seed coats were freeze-dried using a Labconco FreeZone system (Kansas City, MO). All data were reported as the mean ± standard deviation (SD) of experiments run in triplicate. Phenolic contents and antioxidant activity were expressed on the basis of dry weight (DW) of BSSC.

Extraction of Phenolic Compounds. Dried seed coats were ground into fine powder through a 60 mesh sieve. Phenolics were extracted using methods previously described[16] and optimized in our laboratory. Briefly, 500 mg of powder was soaked in 10 mL of petroleum ether for 24 h at room temperature to remove lipids. Degreased powder was extracted twice by suspending in 10 mL of 60% acidified methanol extraction solvent (pH 2.5) for 2 h in a water bath shaker at 40 °C. Extracts were centrifuged at 2000*g* for 10 min, two supernatant aliquots were pooled to produce black soybean seed coat extract (BSSCE), and the extract was stored in the dark at −20 °C until analyzed.

Determination of Total Phenolic Content (TPC). TPC was determined using a modified Folin−Ciocalteu assay.[17] Briefly, 1.0 mL of BSSCE and Folin−Ciocalteu reagent were well mixed. Then, 2.0 mL of 10% Na_2CO_3 aqueous solution was added. The mixture was kept for 1 h at room temperature before measurement at 765 nm using a Shimadzu UV-1800 UV−vis spectrophotometer (Tokyo, Japan). The measurement was compared to a standard curve of prepared gallic acid solutions and expressed as milligrams of gallic acid equivalents per 100 g of sample (mg GAE/100 g).

Determination of Condensed Tannins Content (CTC). Analysis of CTC was carried out according to a modified previous method.[18] To 0.5 mL of suitably diluted sample using methanol were added 2.5 mL of 30 g/L aqueous vanillin solution and 2.5 mL of 30% H_2SO_4 methanol solution. The mixture was kept for 20 min in the dark at 30 °C, and absorbance was measured at 500 nm against methanol as a blank. Condensed tannin was calculated and expressed as milligrams of (+)-catechin equivalents per 100 g of sample (mg CAE/100 g) using the calibration curve of (+)-catechin.

HPLC Analysis of Anthocyanin Profiles. The analysis was performed according to a modified method of Baj et al.[19] All of the samples were analyzed on an Agilent 1200 HPLC system (Waldbronn, Germany) equipped with an Agilent diode array detector and autosampler, using a 4.6 mm × 250 mm i.d., 5 μm, XBridge RP-18 column fitted with a 4.6 mm × 20 mm i.d. guard column of the same material (Waters, Milford, MA).

Column temperature was maintained at 25 °C by an Agilent 1200 column oven. The mobile phase consisted of 10% formic acid in HPLC grade water (solvent A) and methanol/acetonitrile/formic acid/water (25:25:10:40, v/v/v/v) (solvent B). The gradient was programmed as follows: 0−8 min, solvent B 16−20%; 8−11 min, solvent B 20−38%; 11−20 min, solvent B 38−46%. Other chromatographic conditions included a constant flow rate of 1.0 mL/min, an injection volume of 20 μL, and a run time of 20 min. Detection was set at 520 nm. Prior to analysis, all of the samples were filtered through a 0.25 μm membrane filter (Waters).

Identification of anthocyanins was primarily based on comparison of their retention times with known standards and consultation with the literature.[6,12] Six anthocyanin compounds selected as standards were delphinidin-3-glucoside, cyanidin-3-galactoside, cyanidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, and malvidin-3-glucoside with >98% purity. They were used to identify and quantify anthocyanins in BSSC. All standards were dissolved in methanol to produce a stock solution of 1.0 mg/mL. A portion of each stock solution was then diluted using acidified methanol into 1, 10, 50, 100, 200, and 500 μg/mL of working solutions. Standard curves of anthocyanins were generated by injecting 0.02−1 μg of six anthocyanins in 20 μL of acidified methanol. All anthocyanin standard solutions exhibited a linear relationship within that range by plotting area response and injected amounts. The regression coefficients ranged from 0.9987 to 0.9999 for a mixture of pure anthocyanins separated on the C_{18} column. Anthocyanin contents were expressed as milligrams of anthocyanin per 100 g of sample (mg/100 g). Spike recovery ranged from 92.6 to 96.3%. The lower detection limit for all anthocyanin standards was <20 ng/mL.

DPPH Radical Scavenging Activity. DPPH scavenging capacities of BSSCE were evaluated by modification of previously reported methods.[20] Briefly, 2.0 mL of serially diluted BSSCE solutions with 60% acidified methanol were added to 2.0 mL of 200 μmol/L of DPPH methanol solution. The absorbance at 517 nm was recorded after 30 min of incubation in the dark at room temperature. The radical scavenging capacity of each solution was calculated as the percent DPPH radical scavenging effect: DPPH scavenging (%) = $[1 - (Abs_{sample} - Abs_{blank})/Abs_{control}]M \times 100\%$, where methanol (2.0 mL) plus BSSCE (2.0 mL) was used as a blank and DPPH solution (2.0 mL) plus methanol (2.0 mL) was used as a negative control. The median effective concentration (EC_{50}) value of each sample represents the concentration of samples at which 50% DPPH scavenging effect was obtained. EC_{50} was calculated by constructing the percentage of DPPH scavenging versus log(extract concentration expressed as BSSC weight) curves (μg/100 mL).

Ferric Reducing Antioxidant Power (FRAP) assay. A FRAP assay was performed according to the manufacturer's instruction by using a commercially available FRAP kit from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). The FRAP value was expressed as the increased absorbance value of the reaction solution in 1 min by 1 g (DW) of black soybean seed coat in the reaction system (units/g).

Oxygen Radical Absorbance Capacity (ORAC) Assay. The ORAC assay was conducted according to a previous method.[21] Twenty microliters of blank, Trolox standard, or appropriately diluted BSSCE in 75 mmol/L potassium phosphate buffer, pH 7.4 (working buffer), was added to a black clear-bottom 96-well microplate in triplicate. A volume of 200 μL of 0.96 μmol/L FL in working buffer was added to each well

Table 1. Total Phenolic Contents and Condensed Tannins Contents in Seed Coats of 60 Black Soybean Varieties[a]

code	variety	total phenolic content (mg GAE/100 g)[b]	condensed tannins content (mg CAE/100 g)[c]
1	Z09185 Gangbiandou	2848.1 ± 28.4	782.1 ± 34.2
2	Z17513 Heidou	1509.7 ± 56.1	401.9 ± 27.3
3	Z09173 Xiaoheidou	4112.2 ± 69.8	1188.0 ± 37.5
4	Z09202 Heidou	1587.2 ± 9.3	316.9 ± 12.4
5	Z09176 Daheidou	2107.7 ± 9.3	717.9 ± 16.7
6	Z17501 Heidou	4347.7 ± 18.6	1741.1 ± 26.1
7	Z07161 Heijinyuan	3154.9 ± 14.2	852.2 ± 25.2
8	Z17532 Heidou	1897.0 ± 28.4	401.9 ± 15.2
9	Z09190 Biangandou	3517.4 ± 28.4	1022.1 ± 17.3
10	Z17504 Heidou	1739.0 ± 14.2	166.8 ± 18.5
11	Z17494 Heidou	3065.0 ± 24.6	745.6 ± 12.7
12	Z17508 Xiaoheidou	2752.1 ± 32.7	603.3 ± 15.3
13	Z17507 Xiheidou	3873.6 ± 9.3	887.8 ± 28.1
14	Z17496 Heidou	3697.0 ± 16.1	1215.7 ± 37.2
15	Z17492 Xiaoheidou	1472.5 ± 19.4	176.7 ± 13.7
16	Z17530 Heizaodou	1314.5 ± 14.2	356.4 ± 12.1
17	Z09214 Daheirangheidou	4412.7 ± 9.3	1235.4 ± 32.6
18	Z09210 Yaosanxiaodaheidou	2488.7 ± 9.3	895.7 ± 12.7
19	Z17533 Heidou	2079.8 ± 9.3	478.9 ± 19.2
20	Z07177 Fangzhengheidou	3319.1 ± 32.6	1192 ± 49.3
21	Z09195 Daheidou	1004.7 ± 14.2	247.8 ± 11.5
22	Z09172 Xiaoheidou	936.5 ± 27.9	184.6 ± 12.6
23	Z09215 Xiaoheidou	3015.4 ± 69.8	275.5 ± 11.8
24	Z09175 Xiaoheidou	2860.5 ± 9.3	757.4 ± 11.2
25	Z09183 Guangbiandou	3145.6 ± 14.2	909.5 ± 18.3
26	Z17538 Daheidou	1649.1 ± 23.4	328.8 ± 13.6
27	Z17537 Heidou	778.5 ± 9.3	210.3 ± 18.4
28	Z17502 Heihuangdou	2237.8 ± 18.6	459.1 ± 20.8
29	Z09174 Xiaoheidou	2076.7 ± 120.9	419.6 ± 18.9
30	Z17510 Xiaoheidou	1726.6 ± 9.3	431.5 ± 23.7
31	Z09182 Yangyanjing	1890.8 ± 106.9	352.5 ± 12.6
32	Z09199 Xiaoheidou	1550.0 ± 24.6	419.6 ± 8.7
33	Z17515 Heidou	2020.9 ± 142.0	488.8 ± 14.2
34	Z09196 Xiaoheidou	1723.5 ± 5.4	441.4 ± 20.1
35	Z17525 Huangheidou	3058.8 ± 370.7	658.6 ± 25.3
36	Z09191 Erhuangyiheidou	3185.8 ± 37.2	678.4 ± 17.3
37	Z09218 Daheidou	1131.7 ± 9.3	311.0 ± 23.1
38	Z07186 Binxianheidou	1326.9 ± 18.6	457.2 ± 24.6
39	Z17499 Daheidou	2178.9 ± 5.4	474.9 ± 30.5
40	Z17534 Xiaoheidou	2336.9 ± 149.1	488.8 ± 14.2
41	Z09198 Xiaoheidou	732.1 ± 27.9	137.2 ± 21.4
42	Z09216 Xiaoheidou	1866.0 ± 40.5	534.2 ± 32.5
43	Z07171 Humaheidou	905.6 ± 14.2	155 ± 16.3
44	Z17531 Heizaodou	1649.1 ± 5.4	279.4 ± 19.3
45	Z17509 Heizaodou	1453.9 ± 14.2	340.6 ± 7.4
46	Z17522 Wuyunhuangdou	1531.4 ± 18.6	370.3 ± 17.2
47	Z09169 Xiaoheidou	512.1 ± 23.4	176.7 ± 9.7
48	Z17535 Daheidou	1020.2 ± 40.5	220.1 ± 15.6
49	Z09203 Erqiupi	3966.6 ± 85.2	769.3 ± 19.4
50	Z09212 Bawangbian	4428.2 ± 119.5	1004.3 ± 42.7
51	Z09206 Xiaoheidou	3353.1 ± 9.3	826.5 ± 36.7
52	Z09207 Daheidou	1131.7 ± 9.3	297.2 ± 21.4
53	Z09194 Daheiyang	6057.9 ± 37.2	1421.1 ± 28.3
54	Z17540 Xiaoheidou	1612.0 ± 5.4	362.4 ± 16.4

Table 1. Continued

code	variety	total phenolic content (mg GAE/100 g)[b]	condensed tannins content (mg CAE/100 g)[c]
55	Z09178 Daheidou	1884.6 ± 8.9	465.1 ± 24.2
56	Z09211 Chidingdou	1986.8 ± 18.6	461.1 ± 9.4
57	Z09201 Heidou	3111.5 ± 74.4	390.0 ± 12.6
58	Z09181 Zadou	1333.1 ± 5.4	342.6 ± 21.5
59	Z09204 Xiaoyoudou	4418.9 ± 23.4	1267.0 ± 41.6
60	Z17539 Xiaoheidou	3334.6 ± 112.7	804.8 ± 31.4
	mean	2357.0 ± 1163.6	583.3 ± 359.1
	$LSD_{0.05}$	109.0	32.6

[a] Data are expressed as the mean ± SD (n = 3) on a dry weight basis of black soybean seed coat. [b] mg gallic acid equivalents per 100 g sample. [c] mg (+)-catechin equivalents per 100 g sample.

Table 2. Anthocyanidin Concentrations in Black Soybean Seed Coats of 60 Varieties[a]

		anthocyanin (mg/100 g)						
code	variety	delphinidin-3-glucoside	cyanidin-3-galactoside	cyanidin-3-glucoside	petunidin-3-glucoside	peonidin-3-glucoside	malvidin-3-glucoside	total
1	Z09185 Gangbiandou	21.4 ± 0.7	5.6 ± 0.6	424.4 ± 43.2	38.4 ± 12.9	36.4 ± 12.8	21.6 ± 7.8	547.8 ± 64.3
2	Z17513 Heidou	92.0 ± 5.0	6.1 ± 0.6	591.1 ± 48.8	71.6 ± 15.1	19.1 ± 10.8	24.2 ± 6.5	804.1 ± 36.9
3	Z09173 Xiaoheidou	37.5 ± 1.1	8.6 ± 0.4	747.7 ± 23.6	70.2 ± 16.9	64.8 ± 15.0	30.5 ± 7.1	959.3 ± 6.7
4	Z09202 Heidou	6.7 ± 0.5	ND[b]	157.8 ± 19.4	9.3 ± 1.8	15.3 ± 5.2	7.5 ± 4.3	196.5 ± 19.1
5	Z09176 Daheidou	ND	5.8 ± 0.4	614.9 ± 47.6	9.7 ± 0.8	22.8 ± 7.6	ND	653.2 ± 40.7
6	Z17501 Heidou	ND	7.5 ± 0.2	1049.3 ± 49.3	3.9 ± 0.9	118.5 ± 14.1	ND	1179.2 ± 57.7
7	Z07161 Heijinyuan	ND	6.0 ± 0.5	576.5 ± 65.4	17.1 ± 4.0	33.1 ± 10.2	ND	632.7 ± 79.7
8	Z17532 Heidou	212.9 ± 3.0	7.6 ± 0.3	782.6 ± 50.8	97.1 ± 16.6	19.6 ± 6.8	14.8 ± 3.9	1134.8 ± 58.5
9	Z09190 Biangandou	32.2 ± 0.5	6.2 ± 0.3	600.4 ± 46.1	54.3 ± 7.0	55.7 ± 15.5	26.3 ± 7.8	775.1 ± 62.3
10	Z17504 Heidou	228.1 ± 7.6	5.8 ± 0.2	438.4 ± 37.3	77.3 ± 12.3	16.4 ± 4.6	10.3 ± 2.8	776.3 ± 34.7
11	Z17494 Heidou	166.3 ± 3.3	13.3 ± 0.5	894.2 ± 68.6	85.6 ± 13.0	33.5 ± 8.7	27.7 ± 4.0	1220.6 ± 65.6
12	Z17508 Xiaoheidou	321.9 ± 2.8	11.6 ± 0.4	1058.7 ± 68.6	134.3 ± 19.5	29.6 ± 9.8	19.5 ± 7.0	1575.7 ± 82.9
13	Z17507 Xiheidou	213.9 ± 5.3	16.9 ± 0.4	1336.3 ± 76.1	149.0 ± 16.4	51.7 ± 12.2	47.8 ± 11.9	1815.6 ± 112.5
14	Z17496 Heidou	130.1 ± 2.9	7.1 ± 0.2	726.0 ± 40.3	113.0 ± 13.1	17.3 ± 3.5	31.3 ± 5.6	1024.8 ± 59.2
15	Z17492 Xiaoheidou	168.7 ± 7.9	4.4 ± 0.4	339.3 ± 38.9	66.1 ± 13.0	13.3 ± 3.7	9.5 ± 2.8	601.3 ± 39.5
16	Z17530 Heizaodou	98.9 ± 8.5	3.7 ± 0.2	303.9 ± 36.0	41.1 ± 5.9	11.2 ± 2.7	8.7 ± 2.4	467.6 ± 53.9
17	Z09214 Daheirangheidou	40.1 ± 3.4	7.4 ± 0.4	770.4 ± 58.2	62.1 ± 7.4	55.1 ± 10.7	24.9 ± 6.0	959.9 ± 38.7
18	Z09210 Yaosanxiaodaheidou	49.2 ± 1.7	4.9 ± 0.3	394.0 ± 43.8	58.9 ± 9.3	24.1 ± 4.9	31.6 ± 6.9	562.7 ± 36.1
19	Z17533 Heidou	233.0 ± 4.5	8.8 ± 0.3	663.8 ± 34.8	99.5 ± 13.4	18.1 ± 5.1	15.2 ± 2.9	1038.4 ± 9.4
20	Z07177 Fangzhengheidou	90.5 ± 1.0	4.0 ± 0.3	380.4 ± 34.1	49.3 ± 8.6	14.6 ± 4.2	6.7 ± 2.2	545.6 ± 28.5
21	Z09195 Daheidou	30.9 ± 1.3	ND	203.1 ± 27.4	50.5 ± 6.5	20.7 ± 6.5	31.5 ± 5.4	336.8 ± 21.0
22	Z09172 Xiaoheidou	ND	ND	106.6 ± 16.4	13.6 ± 3.7	18.4 ± 5.3	10.0 ± 2.7	148.7 ± 26.2
23	Z09215 Xiaoheidou	35.6 ± 3.2	5.5 ± 0.3	524.8 ± 17.7	57.5 ± 7.2	44.7 ± 11.2	22.5 ± 5.7	690.7 ± 6.6
24	Z09175 Xiaoheidou	13.6 ± 0.9	4.9 ± 0.3	495.5 ± 39.6	20.2 ± 4.5	23.0 ± 5.5	8.7 ± 3.0	565.9 ± 34.9
25	Z09183 Guangbiandou	17.6 ± 1.1	6.5 ± 0.3	564.4 ± 37.8	41.4 ± 4.7	41.4 ± 5.7	19.6 ± 4.3	690.9 ± 33.9
26	Z17538 Daheidou	170.5 ± 10.2	8.0 ± 0.5	742.3 ± 40.0	64.0 ± 8.5	20.2 ± 5.4	10.7 ± 3.1	1015.8 ± 47.1
27	Z17537 Heidou	93.4 ± 3.1	ND	312.0 ± 22.2	68.4 ± 5.6	6.0 ± 2.2	15.8 ± 4.8	495.6 ± 30.8
28	Z17502 Heihuangdou	233.1 ± 13.7	7.1 ± 0.4	949.7 ± 55.8	161.7 ± 18.3	32.3 ± 8.6	42.0 ± 7.0	1425.9 ± 56.4
29	Z09174 Xiaoheidou	21.6 ± 0.8	3.5 ± 0.3	292.4 ± 34.0	35.0 ± 4.9	24.7 ± 5.6	19.0 ± 5.9	396.2 ± 30.3
30	Z17510 Xiaoheidou	1.8 ± 0.1	7.7 ± 0.3	974.8 ± 83.7	3.8 ± 0.9	44.4 ± 8.0	4.9 ± 1.4	1037.5 ± 75.6
31	Z09182 Yangyanjing	15.0 ± 1.2	3.7 ± 0.2	298.4 ± 34.2	23.2 ± 4.4	24.4 ± 6.9	14.5 ± 3.5	379.2 ± 45.6
32	Z09199 Xiaoheidou	32.3 ± 1.3	3.3 ± 0.2	221.3 ± 25.4	40.1 ± 5.1	16.4 ± 4.4	15.2 ± 3.3	328.6 ± 17.9
33	Z17515 Heidou	139.9 ± 1.0	9.2 ± 0.6	784.1 ± 17.5	119.3 ± 18.3	27.5 ± 6.4	36.5 ± 6.0	1116.6 ± 15.6
34	Z09196 Xiaoheidou	22.5 ± 3.7	ND	279.8 ± 21.1	36.3 ± 5.9	28.7 ± 7.1	17.9 ± 5.6	385.2 ± 22.4
35	Z17525 Huangheidou	269.7 ± 18.3	6.1 ± 0.5	752.5 ± 44.3	125.7 ± 17.4	31.2 ± 7.1	15.3 ± 3.6	1200.3 ± 57.7

Table 2. Continued

		anthocyanin (mg/100 g)						
code	variety	delphinidin-3-glucoside	cyanidin-3-galactoside	cyanidin-3-glucoside	petunidin-3-glucoside	peonidin-3-glucoside	malvidin-3-glucoside	total
36	Z09191 Erhuangyiheidou	64.3 ± 4.3	6.7 ± 0.3	738.2 ± 39.1	46.7 ± 9.8	44.6 ± 6.5	15.5 ± 3.7	916.0 ± 47.7
37	Z09218 Daheidou	17.4 ± 0.6	ND	197.6 ± 17.4	27.9 ± 8.2	14.5 ± 3.3	20.8 ± 4.0	278.3 ± 17.1
38	Z07186 Binxianheidou	322.3 ± 6.9	3.6 ± 0.4	335.9 ± 25.9	26.7 ± 6.5	0.3 ± 0.1	ND	688.8 ± 36.2
39	Z17499 Daheidou	192.5 ± 10.4	ND	822.0 ± 41.9	138.2 ± 19.6	23.5 ± 7.1	30.4 ± 5.7	1206.6 ± 25.3
40	Z17534 Xiaoheidou	394.9 ± 5.4	7.3 ± 0.5	690.1 ± 31.5	104.6 ± 15.5	15.7 ± 5.2	10.8 ± 3.4	1223.3 ± 47.7
41	Z09198 Xiaoheidou	12.2 ± 0.3	ND	115.2 ± 15.6	13.1 ± 3.5	7.2 ± 2.8	5.8 ± 2.1	153.5 ± 23.9
42	Z09216 Xiaoheidou	19.7 ± 0.7	3.3 ± 0.3	321.9 ± 20.0	24.2 ± 4.0	17.4 ± 4.1	9.7 ± 2.9	396.2 ± 21.4
43	Z07171 Humaheidou	129.3 ± 1.2	4.4 ± 0.4	375.2 ± 25.4	69.8 ± 16.1	11.3 ± 4.5	17.0 ± 5.4	607.0 ± 44.0
44	Z17531 Heizaodou	107.4 ± 2.2	8.1 ± 0.4	588.4 ± 41.5	198.2 ± 33.8	98.0 ± 12.8	103.8 ± 18.4	1104.0 ± 91.6
45	Z17509 Heizaodou	199.7 ± 9.1	5.5 ± 0.3	606.1 ± 38.2	95.9 ± 21.7	13.3 ± 3.2	14.2 ± 4.7	934.7 ± 47.9
46	Z17522 Wuyunhuangdou	246.7 ± 6.2	5.6 ± 0.5	655.5 ± 45.2	106.7 ± 17.4	12.1 ± 3.6	12.7 ± 3.3	1039.3 ± 60.7
47	Z09169 Xiaoheidou	7.6 ± 1.0	ND	61.8 ± 6.8	13.5 ± 3.8	8.1 ± 2.9	7.7 ± 2.1	98.8 ± 10.0
48	Z17535 Daheidou	103.8 ± 2.3	5.6 ± 0.6	272.1 ± 17.3	51.3 ± 9.4	9.3 ± 2.3	9.6 ± 2.8	451.7 ± 28.3
49	Z09203 Erqiupi	113.7 ± 6.1	7.5 ± 0.5	660.6 ± 50.6	63.9 ± 8.0	30.8 ± 8.1	9.5 ± 2.3	886.0 ± 51.6
50	Z09212 Bawangbian	56.0 ± 1.7	8.6 ± 0.3	844.9 ± 43.0	75.8 ± 10.1	72.6 ± 14.7	29.5 ± 5.9	1087.4 ± 34.1
51	Z09206 Xiaoheidou	27.9 ± 1.0	6.6 ± 0.3	654.5 ± 17.6	40.9 ± 8.2	52.5 ± 9.9	18.7 ± 5.0	801.0 ± 2.9
52	Z09207 Daheidou	23.2 ± 2.0	ND	174.7 ± 15.5	33.8 ± 6.0	12.4 ± 2.8	11.6 ± 3.1	255.6 ± 21.6
53	Z09194 Daheiyang	107.5 ± 1.2	43.9 ± 2.9	1617.4 ± 209.4	112.3 ± 14.6	176.0 ± 24.0	75.3 ± 14.6	2132.5 ± 205.0
54	Z17540 Xiaoheidou	149.9 ± 2.1	6.8 ± 0.5	516.9 ± 38.7	99.0 ± 16.5	20.4 ± 6.3	25.2 ± 5.5	818.2 ± 35.6
55	Z09178 Daheidou	11.4 ± 0.6	3.3 ± 0.3	211.1 ± 21.3	23.2 ± 6.5	19.3 ± 7.1	13.9 ± 3.4	282.2 ± 20.2
56	Z09211 Chidingdou	12.6 ± 0.5	ND	283.3 ± 22.1	18.2 ± 2.0	22.3 ± 6.0	10.8 ± 2.8	347.1 ± 12.8
57	Z09201 Heidou	10.7 ± 0.6	3.5 ± 0.3	310.6 ± 25.8	13.0 ± 3.5	19.9 ± 4.9	6.5 ± 1.8	364.2 ± 20.7
58	Z09181 Zadou	12.6 ± 0.4	ND	184.7 ± 15.8	16.8 ± 3.6	11.8 ± 3.4	6.7 ± 1.9	232.6 ± 13.5
59	Z09204 Xiaoyoudou	36.6 ± 1.9	5.9 ± 0.1	709.4 ± 54.3	44.2 ± 6.5	38.7 ± 9.6	12.7 ± 2.2	847.5 ± 59.0
60	Z17539 Xiaoheidou	47.4 ± 0.8	13.8 ± 0.7	1088.5 ± 92.6	85.0 ± 9.7	83.9 ± 16.5	24.7 ± 5.3	1343.3 ± 73.5
	mean	94.5 ± 96.8	6.0 ± 6.2	556.5 ± 317.7	61.9 ± 41.4	31.8 ± 30.0	19.1 ± 17.1	769.7 ± 425.8
	$LSD_{0.05}$	7.7	0.8	78.8	18.7	13.5	8.7	84.4

[a] Data are expressed as the mean ± SD ($n = 3$) on a dry weight basis. [b] Not detectable.

and incubated at 37 °C for 20 min, with intermittent shaking. After prompt addition of 20 μL of freshly prepared 119 mmol/L AAPH in working buffer, the microplate was immediately put into a Thermo Labsystems Fluoroskan Ascent FL plate reader (Pittsburgh, PA) at 37 °C. Decay of fluorescence at 538 nm was measured with excitation at 485 nm every 4.5 min for 2.5 h. The net areas under the curve (net AUC) were calculated by the areas under fluorescence versus time curves for samples minus the AUC for the blank. The ORAC value of BSSC from each variety was obtained by comparison to a standard curve of the AUC for 6.25, 12.5, 25, and 50 μmol/L Trolox standards minus the AUC for blank. ORAC values were expressed as micromoles of Trolox equivalents (TE) per gram of sample (μmol TE/g).

Statistical Analysis. Data were expressed as the mean ± SD for triplicate determinations of each sample. $LSD_{0.05}$ was calculated to analyze the statistical difference among varieties in bioactive substance content and antioxidant activity. Pearson correlation tests were conducted to determine the correlation between variables. Hierarchical clustering analysis was performed using Ward's method to classify the 60 black soybean varieties into 4 groups. The mean data of different groups were analyzed with one-way analysis of variance (ANOVA) followed by SNK-q test. A value of $p < 0.05$ was considered to be statistically significant. All of the statistical analyses were performed using SPSS statistical package version 13.0 (SPSS Inc., Chicago, IL).

RESULTS

Phenolic Content of Black Soybean Varieties. TPC and CTC of seed coats from 60 black soybean varieties are shown in Table 1. The TPC of 60 black soybean samples ranged from 512.2 to 6057.9 mg GAE/100 g with the average of 2357.0 mg GAE/100 g. Z09194 Daheiyang had the highest TPC ($p < 0.05$), followed by Z09212 Bawangbian, Z09204 Xiaoyoudou, Z09214 Daheirangheidou, and Z17501 Heidou with TPC of 4428.2, 4418.9, 4412.7, and 4347.7 mg GAE/100 g, respectively. However, TPC levels of the latter four varieties were not statistically significant ($p > 0.05$). In addition to Z09169 Xiaoheidou with the lowest TPC ($p < 0.05$), there were another four varieties containing <1000 mg GAE/100 g of TPC, which were Z09198 Xiaoheidou (732.1 mg GAE/100 g) = Z17537 Heidou (778.5 mg GAE/100 g) < Z07171 Humaheidou (905.6 mg GAE/100 g) = Z09172 Xiaoheidou (936.5 mg GAE/100 g).

The CTCs of 60 black soybean varieties ranged from 137.2 mg CAE/100 g in Z09198 Xiaoheidou to 1741.1 mg CAE/100 g in Z17501 Heidou. Z09194 Daheiyang had the second highest CTC (1421.1 mg CAE/100 g) ($p < 0.05$) followed by another seven varieties that contained >1000 mg CAE/100 g of condensed tannins, including Z09204 Xiaoyoudou, Z09214 Daheirangheidou,

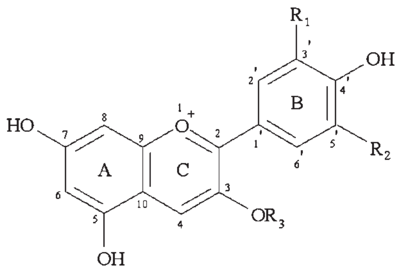

Figure 1. Chemical structures of 6 anthocyanins detected in 60 Chinese black soybean varieties: (1) delphinidin-3-glucoside, $R_1 = R_2 = OH$, R_3 = glucose; (2) cyanidin-3-galactoside, $R_1 = OH$, $R_2 = H$, R_3 = galactose; (3) cyanidin-3-glucoside, $R_1 = OH$, $R_2 = H$, R_3 = glucose; (4) petunidin-3-glucoside, $R_1 = OCH_3$, $R_2 = OH$, R_3 = glucose; (5) peonidin-3-glucoside, $R_1 = OCH_3$, $R_2 = H$, R_3 = glucose; (6) malvidin-3-glucoside, $R_1 = R_2 = OCH_3$, R_3 = glucose.

and Z17496 Heidou, etc. CTCs in Z09198 Xiaoheidou, Z09198 Xiaoheidou, and Z07171 Humaheidou were similar ($p > 0.05$) and ranked equally the last in all varieties ($p < 0.05$).

The coefficients of variation (CVs) of TPC and CTC were 46.5 and 61.6%, respectively, which indicated that there were significant genotype differences in phenolic contents among black soybean varieties.

Anthocyanin Profiles and Contents. Anthocyanin contents of all black soybean varieties tested are presented in Table 2. Six anthocyanins were detected in 60 determined varieties. They were delphinidin-3-glucoside, cyanidin-3-galactoside, cyanidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, and malvidin-3-glucoside. The chemical structures of these standard compounds are shown in Figure 1. Three of them (cyanidin-3-glucoside, petunidin-3-glucoside, and peonidin-3-glucoside) were found in all of the varieties, whereas the other three (delphinidin-3-glucoside, cyanidin-3-galactoside, and malvidin-3-glucoside) were detectable in 56, 56, and 48 varieties, respectively. Neither delphinidin-3-glucoside nor malvidin-3-glucoside was detected in Z09176 Daheidou, Z17501 Heidou, and Z07161 Heijinyuan. Delphinidin-3-glucoside and malvidin-3-glucoside were lacking in Z09172 Xiaoheidou and Z07186 Binxianheidou, respectively. Cyanidin-3-galactoside was not found in 12 varieties, which were Z09202 Heidou, Z09195 Daheidou, Z09172 Xiaoheidou, Z17537 Heidou, Z09196 Heidou, Z09218 Heidou, Z17499 Heidou, Z09198 Xiaoheidou, Z09169 Xiaoheidou, Z09207 Xiaoheidou, Z09211 Chidingdou, and Z09181 Zadou. Forty-four of all 60 varieties analyzed contained all six anthocyanins.

The total anthocyanin content (TAC) ranged from 98.8 mg/100 g in Z09169 Xiaoheidou to 2132.5 mg/100 g in Z09194 Daheiyang. The content of the latter was 20.6 times higher than that of the former ($p < 0.05$). The contents of six anthocyanins (delphinidin-3-glucoside, cyanidin-3-galactoside, cyanidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, and malvidin-3-glucoside) in all analyzed varieties were in the ranges 0−394.9, 0−43.9, 61.8−1617.4, 3.8−198.2, 0.3−176.0, and 0−103.8 mg/100 g, respectively. All tested black soybean varieties contained significantly more cyanidin-3-glucoside than the other anthocyanins. Percentage contributions of cyanidin-3-glucoside content to TAC were from 48.8% in Z07186 Binxianheidou to 94.1% in Z09176 Daheidou. Z09194 Daheiyang had the most abundant TAC and also displayed the highest cyanidin-3-glucoside content in all varieties, whereas Z09169 Xiaoheidou had the lowest content of cyanidin-3-glucoside and TAC ($p < 0.05$). Four black soybean varieties possessed >1000 mg/100 g cyanidin-3-glucoside, which were Z17507 Xiheidou (1336.3 mg/100 g) > Z17539 Xiaoheidou (1088.5 mg/100 g) = Z17508 Xiaoheidou (1058.7 mg/100 g) = Z17501 Heidou (1049.3 mg/100 g). Despite the highest percentage contribution, cyanidin-3-glucoside content in Z09176 Daheidou was considerably low, at only 614.9 mg/100 g, due to its low TAC (653.2 mg/100 g). Delphinidin-3-glucoside and petunidin-3-glucoside also contributed 0−46.8 and 0.3−18.0% of TAC, respectively. Ten varieties showed >200 mg/100 g of delphinidin-3-glucoside, where Z17534 Xiaoheidou displayed the highest content of 394.9 mg/100 g ($p < 0.05$). Z17531 Heizaodou (198.2 mg/100 g) had the highest abundance in petunidin-3-glucoside ($p < 0.05$), followed by another 10 varieties containing >100 mg/100 g petunidin-3-glucoside. The concentrations of peonidin-3-glucoside and malvidin-3-glucoside were low in most of the black soybean varieties. Only Z09194 Daheiyang (176.0 mg/100 g) and Z17501 Heidou (118.5 mg/100 g) contained >100 mg/100 g of peonidin-3-glucoside. Z17531 Heizaodou was the only soybean containing >100 mg/100 g of malvidin-3-glucoside in all tested varieties. The content of cyanidin-3-galactose (0−2.1% TAC) was lower than that of the other anthocyanins in all varieties except Z07186 Binxianheidou, Z17501 Heidou, Z17510 Xiaoheidou, Z09176 Daheidou, and Z07161 Heijinyuan.

CVs of TAC, delphinidin-3-glucoside, cyanidin-3-galactoside, cyanidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, and malvidin-3-glucoside were 55.3, 103.0, 103.9, 56.9, 70.5, 92.1, and 88.7%, respectively, suggesting that there were significant genotype differences in anthocyanin contents among black soybean varieties.

Antioxidant Activity. Table 3 shows the antioxidant activity of each BSSC sample determined by FRAP, DPPH, and ORAC methods. The antioxidant activity determined by FRAP ranged from 17.5 units/g in Z09169 Xiaoheidou to 105.8 units/g in Z17501 Heidou, averaging 53.3 units/g. Z09194 Daheiyang (91.7 units/g) and Z17525 Huangheidou (90.9 units/g) both displayed the second highest FRAP values ($p > 0.05$) following Z17501 Heidou. Because the DPPH scavenging activity of BSSC was expressed as EC_{50}, lower values indicated higher antioxidant activity. The EC_{50} of DPPH scavenging activity of 60 tested varieties varied from 4.8 to 65.3 μg/100 mL. Ten varieties (Z09194 Daheiyang, Z09204 Xiaoyoudou, and Z17501 Heidou, etc.) showed higher DPPH scavenging activity than the other varieties ($p < 0.05$), but no significant difference was found among these 10 varieties ($p > 0.05$). The ORAC values ranged from 212.5 to 1834.6 μmol TE/g. Z09194 Daheiyang had the highest ORAC value ($p < 0.05$) followed by Z09212 Bawangbian, Z17501 Heidou, Z09204 Xiaoyoudou, and Z09214 Daheirangheidou with similar values ($p > 0.05$). Z09169 Xiaoheidou uniformly possessed the weakest antioxidant activity by all three methods, and Z09198 Xiaoheidou showed a similarly low FRAP value to it ($p > 0.05$).

Correlation among Antioxidant Activity and Phenolic Content. There were significant linear correlations between variables of phenolic contents and antioxidant activity ($p < 0.001$), as high as $r = 0.988$ between ORAC values and TPC. Distinct correlations were also shown between TPC, TAC, and CTC and between antioxidant activities ($p < 0.001$). EC_{50} values of DPPH scavenging capacity showed statistically negative correlation with all other variables, which means DPPH scavenging capacity was positively correlated with phenolic contents and other antioxidant acitivity.

Table 3. Antioxidant Activity of Black Soybean Seed Coats of 60 Varieties[a]

		antioxidant activity[b]		
code	variety	FRAP (units/g)	DPPH scavenging EC_{50} (μg/100 mL)	ORAC (μmol TE/g)
1	Z09185 Gangbiandou	60.0 ± 1.6	18.1 ± 2.4	1007.2 ± 15.9
2	Z17513 Heidou	35.2 ± 1.7	28.8 ± 3.3	533.0 ± 36.2
3	Z09173 Xiaoheidou	83.4 ± 2.7	10.5 ± 1.2	1201.2 ± 55.3
4	Z09202 Heidou	41.2 ± 4.3	30.1 ± 3.2	567.7 ± 23.2
5	Z09176 Daheidou	51.8 ± 8.0	20.0 ± 1.4	677.1 ± 30.1
6	Z17501 Heidou	105.8 ± 1.1	7.9 ± 0.9	1334.5 ± 47.7
7	Z07161 Heijinyuan	56.6 ± 1.1	13.0 ± 2.2	835.4 ± 12.7
8	Z17532 Heidou	43.2 ± 2.1	22.9 ± 2.4	613.1 ± 18.2
9	Z09190 Biangandou	67.2 ± 0.1	15.2 ± 1.3	1046.9 ± 34.3
10	Z17504 Heidou	34.3 ± 1.7	29.0 ± 2.4	567.3 ± 31.1
11	Z17494 Heidou	52.2 ± 0.6	13.4 ± 1.2	955.3 ± 41.4
12	Z17508 Xiaoheidou	56.2 ± 0.8	18.8 ± 1.8	872.3 ± 38.4
13	Z17507 Xiheidou	84.7 ± 1.5	10.5 ± 0.9	1148.4 ± 44.3
14	Z17496 Heidou	66.4 ± 10.4	12.6 ± 1.2	1100.5 ± 48.4
15	Z17492 Xiaoheidou	33.9 ± 0.2	32.9 ± 1.9	546.8 ± 15.2
16	Z17530 Heizaodou	35.8 ± 0.4	33.5 ± 3.2	566.3 ± 32.2
17	Z09214 Daheirangheidou	77.9 ± 1.3	9.5 ± 1.1	1300.5 ± 55.7
18	Z09210 Yaosanxiaodaheidou	65.5 ± 1.8	16.1 ± 1.3	753.3 ± 19.5
19	Z17533 Heidou	51.0 ± 0.9	19.5 ± 1.7	667.3 ± 20.3
20	Z07177 Fangzhengheidou	74.9 ± 1.4	9.8 ± 0.8	1000.4 ± 45.4
21	Z09195 Daheidou	33.7 ± 1.0	36.8 ± 2.4	372.9 ± 18.5
22	Z09172 Xiaoheidou	25.9 ± 0.4	41.1 ± 3.4	363.8 ± 16.8
23	Z09215 Xiaoheidou	70.1 ± 1.6	12.5 ± 1.2	932.4 ± 56.3
24	Z09175 Xiaoheidou	74.9 ± 0.7	10.4 ± 1.5	887.4 ± 35.6
25	Z09183 Guangbiandou	72.8 ± 0.7	10.8 ± 0.7	971.4 ± 47.8
26	Z17538 Daheidou	51.6 ± 2.1	20.2 ± 1.4	523.3 ± 23.5
27	Z17537 Heidou	23.1 ± 5.4	48.3 ± 3.7	309.4 ± 21.2
28	Z17502 Heihuangdou	49.4 ± 4.1	13.3 ± 1.2	777.4 ± 14.9
29	Z09174 Xiaoheidou	57.5 ± 2.0	16.7 ± 1.4	700.8 ± 40.4
30	Z17510 Xiaoheidou	47.1 ± 6.5	20.8 ± 2.3	586.5 ± 33.6
31	Z09182 Yangyanjing	56.7 ± 0.8	19.3 ± 2.4	578.3 ± 24.6
32	Z09199 Xiaoheidou	44.9 ± 1.3	21.5 ± 2.1	534.4 ± 19.3
33	Z17515 Heidou	59.2 ± 1.8	16.1 ± 1.0	684.8 ± 47.3
34	Z09196 Xiaoheidou	57.2 ± 2.6	18.8 ± 2.3	566.7 ± 31.3
35	Z17525 Huangheidou	90.9 ± 1.0	11.0 ± 1.3	982.8 ± 56.1
36	Z09191 Erhuangyiheidou	70.5 ± 1.4	9.7 ± 0.6	867.9 ± 27.2
37	Z09218 Daheidou	38.8 ± 0.4	31.4 ± 3.2	447.3 ± 27.8
38	Z07186 Binxianheidou	46.9 ± 2.2	24.2 ± 2.0	457.6 ± 23.3
39	Z17499 Daheidou	63.6 ± 1.2	20.8 ± 3.1	706.8 ± 46.5
40	Z17534 Xiaoheidou	59.7 ± 1.3	16.4 ± 1.4	748.7 ± 37.0
41	Z09198 Xiaoheidou	20.3 ± 0.6	56.5 ± 4.3	298.5 ± 24.9
42	Z09216 Xiaoheidou	45.7 ± 2.1	24.3 ± 2.2	653.4 ± 53.2
43	Z07171 Humaheidou	41.0 ± 0.9	33.8 ± 3.1	341.7 ± 16.4
44	Z17531 Heizaodou	25.3 ± 1.0	28.6 ± 2.6	568.2 ± 44.8
45	Z17509 Heizaodou	25.1 ± 1.6	23.1 ± 2.1	526.9 ± 35.9
46	Z17522 Wuyunhuangdou	22.6 ± 3.6	21.7 ± 1.6	561.0 ± 43.2
47	Z09169 Xiaoheidou	17.5 ± 0.4	65.3 ± 3.9	212.5 ± 15.4
48	Z17535 Daheidou	26.0 ± 0.7	42.2 ± 2.6	391.0 ± 11.3
49	Z09203 Erqiupi	59.3 ± 2.2	10.8 ± 1.0	965.2 ± 22.1
50	Z09212 Bawangbian	76.4 ± 1.8	9.6 ± 0.7	1340.6 ± 35.2
51	Z09206 Xiaoheidou	60.1 ± 1.3	11.4 ± 1.4	1000.5 ± 32.2
52	Z09207 Daheidou	25.1 ± 1.4	39.0 ± 3.3	428.4 ± 19.4

Table 3. Continued

code	variety	FRAP (units/g)	DPPH scavenging EC_{50} (μg/100 mL)	ORAC (μmol TE/g)
		antioxidant activity[b]		
53	Z09194 Daheiyang	91.7 ± 1.6	4.8 ± 0.5	1834.6 ± 97.3
54	Z17540 Xiaoheidou	53.9 ± 0.7	27.6 ± 2.3	570.9 ± 26.7
55	Z09178 Daheidou	68.6 ± 2.5	18.6 ± 1.7	620.0 ± 32.1
56	Z09211 Chidingdou	60.5 ± 3.3	20.3 ± 1.2	674.1 ± 38.2
57	Z09201 Heidou	42.0 ± 2.8	18.7 ± 1.4	969.4 ± 52.4
58	Z09181 Zadou	30.6 ± 0.6	29.5 ± 2.7	413.1 ± 29.9
59	Z09204 Xiaoyoudou	60.5 ± 5.3	7.3 ± 0.8	1306.9 ± 66.7
60	Z17539 Xiaoheidou	74.5 ± 1.1	11.3 ± 1.2	1059.9 ± 44.3
	mean	53.3 ± 20.0	21.6 ± 12.2	750.5 ± 317.2
	$LSD_{0.05}$	4.3	5.8	61.8

[a] Data are expressed as the mean ± SD (n = 3) on a dry weight basis of black soybean seed coat. [b] FRAP, ferric reducing antioxidant power; DPPH scavenging EC_{50}, concentration of extract expressed as the dry weight of black soybean seed coat for 50% of DPPH scavenging; ORAC, oxygen radical absorbing capacity.

Table 4. Phenolic Content and Antioxidant Activity of Black Soybean Seed Coats in Four Groups Classified by Hierarchical Clustering Method and the Soybean Varieties Included in Each Group[a]

group	variety codes	TPC (mg GAE/100 g)	CTC (mg CAE/100 g)	TAC (mg/100 g)	FRAP (units/g)	DPPH scavenging EC_{50} (μg/100 mL)	ORAC (μmol TE/g)
1	2, 4, 10, 15, 16, 21, 22, 27, 37, 41, 43, 47, 48, 52, 58 (n = 15)	1140.6 ± 347.9 a	246.7 ± 84.3 a	393.6 ± 228 a	30.8 ± 7.4 a	38.5 ± 10.8 a	424.0 ± 112.4 a
2	5, 8, 19, 26, 29, 30, 31, 32, 34, 38, 42, 44, 45, 46, 54, 55, 56 (n = 17)	1765.4 ± 232.3 b	427.2 ± 98.5 b	704.7 ± 325.0 b	47.6 ± 12.8 b	21.7 ± 3.2 b	592.9 ± 66.0 b
3	1, 7, 11, 12, 13, 18, 23, 24, 25, 28, 33, 35, 36, 39, 40, 49, 51, 57, 60 (n = 19)	2946.8 ± 527.0 c	671.0 ± 190.6 c	988.7 ± 398.8 c	64.3 ± 12.0 c	13.8 ± 3.4 c	901.4 ± 125.4 c
4	3, 6, 9, 14, 17, 20, 50, 53, 59 (n = 9)	4256.8 ± 797.8 d	1254.1 ± 221.2 d	1056.8 ± 444.1 c	78.2 ± 14.0 d	9.7 ± 3.0 d	1274.0 ± 246.5 d

[a] Data are expressed as the mean ± SD on a dry weight basis of seed coats. Means in a row without a common letter are statistically different ($p < 0.05$). TPC, total phenolic content; CTC, condensed tannins content; TAC, total anthocyanin content; FRAP, ferric reducing antioxidant power; DPPH scavenging EC_{50}, concentration of extract expressed as the dry weight of black soybean seed coat for 50% of DPPH scavenging; ORAC, oxygen radical absorbing capacity.

Hierarchical Clustering Analysis of Black Soybean Varieties. Sixty black soybean varieties were classified into four groups by hierarchical clustering analysis with TAC, TPC, CTC, and antioxidant properties of seed coat as variables. Detailed information of each group is presented in Table 4. There were 15, 17, 19, and 9 black soybean varieties included in groups 1−4, respectively. Phenolic contents and antioxidant activity by all methods significantly increased from group 1 through 4 ($p < 0.05$), except that average TACs in groups 3 and 4 were similar ($p > 0.05$). Group 4, consisting of Z09173 Xiaoheidou, Z17501 Heidou, Z09190 Biangandou, Z17496 Heidou, Z09214 Daheirangheidou, Z07177 Fangzhengheidou, Z09212 Bawangbian, Z09194 Daheiyang, and Z09204 Xiaoyoudou, had higher levels of polyphenols and antioxidant capacities compared to the other groups.

DISCUSSION

Anthocyanins are the second most important phytochemicals in black soybean besides isoflavones. It has been shown that anthocyanins predominantly account for the difference in polyphenols between black soybeans and other soybean cultivars[4,22] and that anthocyanins in BSSC displayed many health benefits. Therefore, our study chiefly focused on identifying the profiles of anthocyanins in various varieties. Six anthocyanins, including delphinidin-3-glucoside, cyanidin-3-galactoside, cyanidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside, and malvidin-3-glucoside, were identified in this study. Anthocyanin profiles of black soybeans reported in previous studies were highly distinct. Among 10 Korean black soybean varieties, only cyanidin-3-glucoside was detected in 2 varieties, whereas delphinidin-3-glucoside or petunidin-3-glucoside or both of them were also found in the other 8 varieties.[13] Cyanidin-3-glucoside and peonidin-3-glucoside were found by Xu et al. in 2 black soybean varieties grown in the North Dakota−Minnesota region and petunidin-3-glucoside, as well, was found in a commercially available American variety.[3,14] However, as many as 10 anthocyanins, consisting of 6 aglycones and 4 glycosides, were identified in 1 of 5 varieties in another study.[6] Lee et al.[12] claimed the presence of 9 anthocyanins in black soybean, among which catechin-cyanidin-3-glucoside, pelargonidin-3-glucoside, delphinidin-3-galactoside, and cyanidin were not detected in this study. Five of 6 anthocyanins detected in our experiment, except malvidin-3-glucoside, were also found by them. Cyanidin-3-

galactoside was detected in most samples (48 varieties) despite trace concentrations in this research. Only Lee et al.[12] previously reported its presence in black soybean. Varietal discrepancies of anthocyanin profiles in these studies possibly contribute to the difference in black soybean varieties subject to analysis and to different sensitivities of methods by which anthocyanins were separated and characterized.

As revealed in previous studies, TAC in BSSC exhibited broad ranges of variation from <1.0 to 20.4 mg/g.[3,14,15] Wide variation was also found in our current study from 98.8 to 2135.2 mg/100 g. The highest TAC here is close to the highest content reported in Japanese (20.4 mg/g)[15] and Korean (20.18 mg/g)[13] varieties. Three Japanese black soybeans analyzed in Yoshida's research[15] all exhibited high TAC (20.4, 16.9, and 15.3 mg/g). In contrast, Choung et al.[13] found a much higher range of 1.58−20.18 mg/100 g in 10 varieties, which is close to our data. Soybeans tested in other studies usually contained <5.0 mg/100 g of total anthocyanin.[3,6,14] In our current and the majority of previous studies, cyanidin-3-glucoside was the predominant anthocyanin in black soybean.[3,6,15] Therefore, the high TAC in some black soybean varieties, to a large extent, was attributed to their high cyanidin-3-glucoside content. Z09194 Daheiyang (1617.4 mg/100 g) analyzed in this study contained more cyanidin-3-glucoside than all previously reported varieties with the exception of a variety cultivated in Japan in Yoshida's research.[15] No other anthocyanins but 20.4 mg/g cyanidin-3-glucoside were detected in this Japanese variety.

In addition to anthocyanins, highly polymerized proanthocyanidins, which are also known as condensed tannins, were reported to be an important class of phenolics in soybean seed coat.[23] Therefore, CTC and TPC were measured in this study. To our best knowledge, CTC and TPC were determined in whole black soybean but not seed coat in most previous studies.[24] However, in a study conducted by Xu et al.,[3] they compared the phenolic distribution in various black soybean seed parts and measured about 70 mg GAE/g of TPC and 50 mg CAE/g of CTC. These values are even higher than the highest TPC (6057.9 mg GAE/100 g) and CTC (1741.1 mg CAE/100 g) in the varieties analyzed in this study. In addition to varietal differences, another possible reason why lower CTC and TPC but much higher TAC was determined in our varieties is that our extraction conditions were different from theirs. The structure of condensed tannins was not characterized in this study, whereas Todd et al.[25] pointed out that procyanidin was the major subclass of proanthocyanidin in BSSC. Takahata et al.[23] demonstrated that the degree of polymerization (DP) of proanthocyanidins in the brown or black soybean seed coat was as high as 30. It is presently not clear whether there are differences in DP and subclass of proanthocyanidins in diverse black soybean varieties.

Significant positive correlation existed among TPC, TAC, and CTC, which indicates that both anthocyanins and condensed tannins are the main polyphenols in BSSC. Additional phenolic compounds detected in BSSC in previous research such as phenolic acids and isoflavones[3,6,14] also contributed to TPC.

Antioxidant activity is the basic bioactivity of phenolic compounds and the main mechanism by which they exert health promotion effects. It is commonly known that antioxidants can scavenge radicals by two major mechanisms: hydrogen atom transfer (HAT) and single electron transfer (SET). Because no single present antioxidant activity assay is able to accurately reflect all radical sources or all antioxidants in a mixed or complex system, the ORAC method using a HAT reaction mechanism together with FRAP and DPPH methods using the SET reaction mechanism were utilized simultaneously in this study to estimate the antioxidant properties of BSSC. Correlation analysis indicated that all antioxidant assay methods were well-correlated, which means these methods have good consistency in the estimation of antioxidant activity of BSSC. It is difficult to compare our values of FRAP and DPPH with those reported previously because different substrate concentrations and end points were used.[6,14] Using Trolox as a standard in the ORAC method makes it possible to compare the values obtained in different laboratories with each other. To the best of our knowledge, only Xu et al.[3] determined the antioxidant activity of BSSC by the ORAC method and reported an ORAC value of approximately 450 μmol TE/g in a commercially available American black soybean variety. Fifty-one of 60 analyzed varieties in this study showed >450 μmol TE/g of antioxidant activity by ORAC, which indicated most varieties in our study possessed higher antioxidant potential than this variety.

The correlation results between phenolic contents and antioxidant activities revealed that TPC, TAC, and CTC were well-correlated with overall antioxidant activity of BSSC. This indicated that anthocyanins and condensed tannins were major antioxidants in BSSC. Anthocyanins showed distinct antioxidant capacity in many studies in vivo and in vitro;[26,27] meanwhile, they were the main components contributing to the many effects of black soybeans based on antioxidant activity. Condensed tannins have been proven to be antioxidative in many studies.[28] As mentioned above, there were other minor phenolic compounds, such as phenolic acids, existing but not identified in BSSC in this study.[3]

Grape seed and many other berry seeds have been used as valuable sources of antioxidants in nutraceutical production because of their plentiful phytochemicals and high antioxidant activity. A study showed that Chardonnay grape seed flour displayed the strongest antioxidant capacity (1076.4 μmol TE/g) by ORAC in tested fruit seed flours of raspberry, blueberry, cranberry, and grapes.[29] According to clustering analysis results, nine varieties with the highest phenolic contents and antioxidant potential comprised one group (group 4) in this study. It is noticeable that seven varieties in this group showed higher antioxidant activity than the above-mentioned grape seed. These valuable properties make some black soybean varieties potential sources for nutraceutical development. This study also provides useful information for black soybean breeders. Varieties in group 4 can be used as parents for breeding new black soybean varieties with high bioactivity as well as high nutrition. Additionally, with increasingly more attention paid to food safety and health, consumers have many more concerns about the potential adverse effects of synthetic food colorants than they ever had. Anthocyanins are popular natural colorants due to their vivid colors and health benefits. TAC values in half of the tested black soybean varieties are even higher than those in blueberries (7.2 ± 0.5 mg/g DW),[30] which have been considered a high-anthocyanin-containing fruit. These black soybean varieties can be used to extract anthocyanins for nutraceutical development or food colorants.

In conclusion, 6 anthocyanins including cyanidin-3-glucoside, etc. were detected in 60 black soybean varieties cultivated in China. Anthocyanin profiles, phenolic contents, and antioxidant properties largely varied among tested varieties. Significantly correlative relationships existed between phenolic contents and antioxidant activity determined by FRAP, DPPH, and ORAC. Clustering analysis classified nine varieties into one group with more phytochemicals and stronger antioxidant capacities than the other three groups. These varieties have the potential to be used in functional foods and black soybean breeding as well as food colorant development.

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ABBREVIATIONS USED

BSSC, black soybean seed coat; BSSCE, black soybean seed coat extract; TPC, total phenolic content; CTC, condensed tannins content; TAC, total anthocyanin content; DPPH, 2-diphenyl-1-picrylhydrazyl radical; FRAP, ferric reducing antioxidant power; ORAC, oxygen radical absorbing capacity; AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; AUC, area under the curve; DP, degree of polymerization.

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