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Superoxide Radical Scavenging Activity of the Major Polyphenols in Fresh Plums

OCK KYOUNG CHUN, DAE-OK KIM, AND CHANG YONG LEE*

Department of Food Science and Technology, Cornell University, Geneva, New York 14456

The effect of polyphenols among different varieties of plums on superoxide radical scavenging activity (SRSA) was studied by an enzymatic method and their IC_{50} values were determined. We found that the SRSA levels of the polyphenols were closely related to their chemical structures; cyanidin showed the lowest IC_{50} among the polyphenols examined, and aglycones are more effective than their glycosides. BY 69−339 cultivar exhibited the lowest IC_{50} among the eleven plum cultivars, which means the highest antioxidant activity in scavenging superoxide radicals, followed by French Damson, Cacaks Best, Beltsville Elite B70197, Empress, Castleton, Stanley, NY 6, NY 101, Mirabellier, and NY 9. IC_{50} values showed a higher correlation with total flavonoids ($r^2 = 0.8699$) than total phenolics ($r^2 = 0.8355$), which indicated that flavonoids might contribute to the total SRSA more directly than other polyphenols. Anthocyanins in plums appeared to be the major contributors to the total SRSA, except for two yellow cultivars having no anthocyanins. Chlorogenic acid was the predominant phenolic acid, and it also exhibited SRSA significantly in the range of 1.0 to 94.9%. Quercetins were the major flavonols in plums. However, they showed relatively low contribution to the total SRSA.

KEYWORDS: Plums; polyphenols; superoxide radical scavenging activity (SRSA); cyanidin; peonidin; chlorogenic acid; quercetin

INTRODUCTION

Reactive oxygen species (ROS), such as superoxide, hydrogen peroxide, and hydroxyl radical are byproducts of normal metabolism and attack certain biological molecules, leading to destabilization and disintegration of cell membranes and many age-related diseases. Superoxide radical anion ($O_2^{\cdot -}$) originates from the one-electron reduction of free molecular oxygen by nicotinamide adenine dinucleotide phosphate oxidase, which is the membrane-bound enzyme (*1*, *2*). Apart from beneficial effects that are essential for the host defense system, the cytotoxicity of ROS causes severe side effects that must be controlled. Xanthine oxidase catalyzes the oxidation of xanthine and hypoxanthine in the presence of molecular oxygen to yield uric acid and superoxide anion as reaction products (*3*). Xanthine oxidase-derived superoxide radical has been linked to the postischemic tissue injury and generation of neutrophil chemotoxins (*4*). Elimination of superoxide radical anion generated by this enzymatic pathway would be beneficial in the case of ischemia.

Most healthy living species have efficient defense systems against the oxidative stress induced by ROS. The capacity of such protective systems, however, gradually decreases with aging, resulting in disturbances to the normal redox equilibrium established in healthy systems. Therefore, to replenish induced losses, there is a need to provide the body with a constant supply of antioxidants through the regular intake of proper diet. At present, there is overwhelming evidence to indicate that free radicals cause oxidative damage to lipids, proteins, and nucleic acids. Epidemiological studies showed that consumption of fruits and vegetables with high phenolic content correlates with reduced cardio- and cerebrovascular diseases (*5*−*7*) and cancer mortality (*8*−*10*).

Phenolic compounds in fruits and vegetables may produce the beneficial effects by scavenging free radicals. Many of phenolic compounds are known to have antioxidant activity and may help protect cells against the oxidative damage caused by free radicals (*11*). Plant polyphenols have multifunctional properties and can act as reducing agents, hydrogen donating antioxidants, and singlet oxygen quenchers. In the past decades, there has been an increased interest in determining relevant dietary sources of antioxidant phenolics. Thus, fruits such as apples, grapes, and berries have received more attention due to their antioxidant activity.

Although plums are not the most popular fruits, they have been proven to have effective antioxidant activity resulting from their high content of polyphenols (*12*−*19*). It was reported that neochlorogenic acid (3-*O*-caffeoylquinic acid) was a predominant polyphenol in the fresh or dried plum (*20*), and quercetin 3-rutinoside, the major flavonol in plums, existed at relatively low levels (*18*). In addition, all the plum cultivars except yellow plums commonly contained anthocyanins such as cyanidin 3-glucoside and cyanidin 3-rutinoside (*21*).

Chlorogenic acid is widely recognized to be an antioxidant for human low-density lipoprotein (*22*, *23*). It is also known as

* To whom correspondence should be addressed. Chang Yong Lee, Professor and Chairman, Department of Food Science and Technology, Cornell University, Geneva, NY 14456. Tel.: +1-315-787-2271 Fax: +1-315-787-2284. E-mail: cyl1@cornell.edu.

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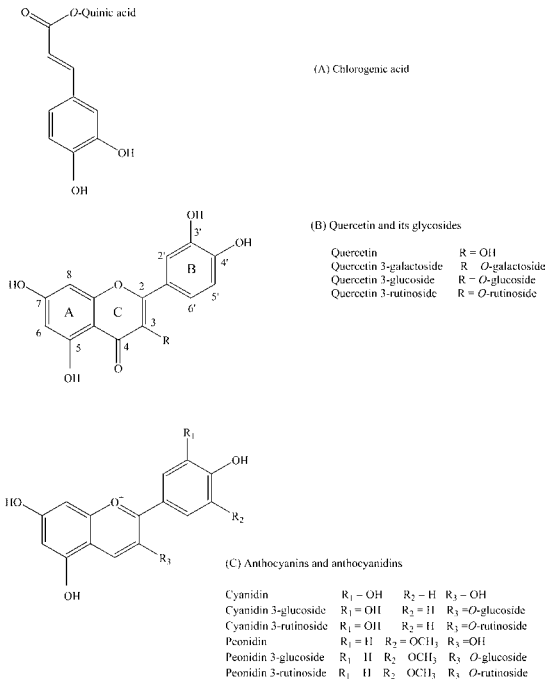

Figure 1. Structure of polyphenolics (A) chlorogenic acid, (B) quercetin and its glycosides, and (C) anthocyanins and anthocyanidins.

a scavenger for reactive species of oxygen and nitrogen (*24*), and an inhibitor against the formation of conjugated diene from linoleic acid oxidation (*25*). Plums contain various kinds of phenolic compounds, including hydroxycinnamic acids, flavonols, and anthocyanins. However, relatively little information is available on the superoxide radical scavenging activity (SRSA), especially on the contribution of individual polyphenols in plums.

Our previous study (*19*) reported that the level of IC_{50} value of SRSA expressed on the basis of vitamin C equivalent antioxidant capacity was variable among different plum extracts. It also showed a weak relationship of SRSA with total phenolic content. Various polyphenolic phytochemicals may react with free superoxide radicals in different ways and thus lead to different scavenging activities. Polyphenols may possess more than one mode of action in the xanthine/xanthine oxidase assay system. They may play a role in either scavenging the generated superoxide radicals or inhibiting the xanthine oxidase activity or both (*3*).

The objectives of this study were to determine the SRSA of major polyphenols in various plum cultivars and to identify the contribution of each polyphenol to the total SRSA.

MATERIALS AND METHODS

Chemicals. (+)-Catechin, chlorogenic acid, Folin−Ciocalteu's phenol reagent, gallic acid, nitro blue tetrazolium (NBT), quercetin, quercetin 3-galactoside (hyperoside), quercetin 3-rutinoside (rutin), xanthine, and xanthine oxidase were purchased from Sigma Chemical Co. (St. Louis, MO). Cyanidin, cyanidin 3-glucoside (kuromanin), cyanidin 3-rutinoside (keracyanin), peonidin, peonidin 3-glucoside, and quercetin 3-glucoside were obtained from Extrasynthese (Genay, France). Peonidin 3-rutinoside was purchased from Polyphenols (Sandnes, Norway). The chemical structures of the phenolic compounds analyzed in this assay are presented in **Figure 1**. All other chemicals used were of analytical grade (Fisher, Springfield, NJ).

Fruits. The eleven plum cultivars (Beltsville Elite B70197, BY 69−339, Cacaks Best, Castleton, Empress, French Damson, Mirabellier, NY 101, NY 6, NY 9, Stanley) used in this study were obtained at commercial maturity between late August and early September in 2002 from the New York State Agricultural Experiment Station orchard in Geneva, New York. All the plums can be categorized as purple plums by the skin color, except for the yellow plums (Mirabellier and NY 101). Immediately after harvest, the plums were stored in a 2−5 °C refrigerator. Plums, cut into several pieces after being pitted, were frozen and freeze-dried. Samples were ground to powder and then stored at −20 °C until analyzed.

Extraction of Polyphenols. The polyphenols in plums were extracted from 10 g of ground freeze-dried samples using 80% aqueous methanol in a 250-mL round-bottomed flask by the ultrasound-assisted method (*26*). The mixture of plum powder and aqueous methanol was sonicated for 20 min with continuous nitrogen gas purging. The mixture was filtered and evaporated using a rotary evaporator under reduced pressure at 40 °C. The phenolic concentrate was dissolved in 50 mL of 100% methanol and then made to the final volume of 100 mL with distilled deionized water (ddH_2O) obtained with a NANOpure water system (Barnstead, Dubuque, IA). The solution then was centrifuged in a Sorvall RC-5B refrigerated superspeed centrifuge (Du Pont Company, Biomedical Products Department, Wilmington, DE) at 12000*g* using GSA rotor for 20 min. The final extract was stored at −20 °C until analyzed. Extraction was done in duplicate.

SRSA Assay. The assay is based on the removal rate of xanthine/xanthine oxidase-generated superoxide radical ($O_2^{\cdot -}$) by measuring the reduction of NBT. The enzyme assay of Kweon et al. (*27*) was used with a minor modification. Xanthine, 0.1 mM, and 0.1 mM NBT was dissolved in 50 mM potassium phosphate buffer (pH 7.4) with 0.05 mM EDTA (PBE) to make tetrazolium blue solution. An aliquot (0.9 mL) of tetrazolium blue solution was added to 0.1 mL of sample extract, which was properly diluted with 50% aqueous methanol. The reaction was initiated by the addition of 1 mL of xanthine oxidase solution (0.05 units/mL PBE). The mixture was incubated at 37 °C for 20 min and then terminated by the addition of 2 mL of 2.0 N HCl. The coloration of NBT was measured at 560 nm against a blank that was treated with 2.0 N HCl in advance of the addition of 1 mL xanthine oxidase solution. Chlorogenic acid was prepared up to 500 mg/L of 50% aqueous methanol, quercetin, and its derivatives up to 100 mg/L of 50% aqueous methanol, and anthocyanins and anthocyanidins up to 100 mg/L with 50% aqueous methanol containing 0.15% HCl. All the experiments were replicated six times.

The SRSA was expressed as percent (%) superoxide quenching, which was calculated as $(1 - B/A) \times 100$, where A is the activity of the enzyme without test material, and B is the activity of the enzyme with test material (*27*). This experiment was carried out by three steps to calculate the contribution of individual polyphenols to the total SRSA of plum cultivars; first, we measured the SRSA of individual polyphenol to determine 50% inhibition (IC_{50}) by xanthine/xanthine oxidase system. IC_{50} is defined as the amount of sample required to achieve the NBT reduction by a 50% decrease. To calculate these values, a graphical method was employed from dose−response curves. Second, we extracted polyphenols from fresh plums to determine the total SRSA and IC_{50} of plum cultivars. Third, to evaluate the contribution of individual polyphenols on total SRSA of plums, we calculate the SRSA values of each polyphenolic compound using the concentration of individual polyphenols previously identified by HPLC (*18*) and the IC_{50} of each polyphenols determined by SRSA assay. The relative IC_{50} of individual polyphenols was calculated on the basis of the IC_{50} of cyanidin as 1.00.

Total Phenolics and Total Flavonoids. Total phenolics were determined by the spectrophotometric method (*14*). A 1-mL portion of appropriately diluted extracts was added to a 25-mL volumetric flask filled with 9 mL of ddH_2O. A reagent blank using ddH_2O instead of sample was prepared. A 1-mL sample of Folin−Ciocalteu's phenol reagent was added to the mixture and mixed. After 5 min, a 10 mL of 7% Na_2CO_3 solution was added with mixing. The solution was diluted to the volume (25 mL) with ddH_2O, then, allowed to stand for 90 min, and the absorbance was measured at 750 nm versus the prepared blank. Total phenolics of plums were expressed on a fresh weight basis as mg gallic acid equivalent (GAE)/100 g. Sample of each extraction was analyzed in triplicate.

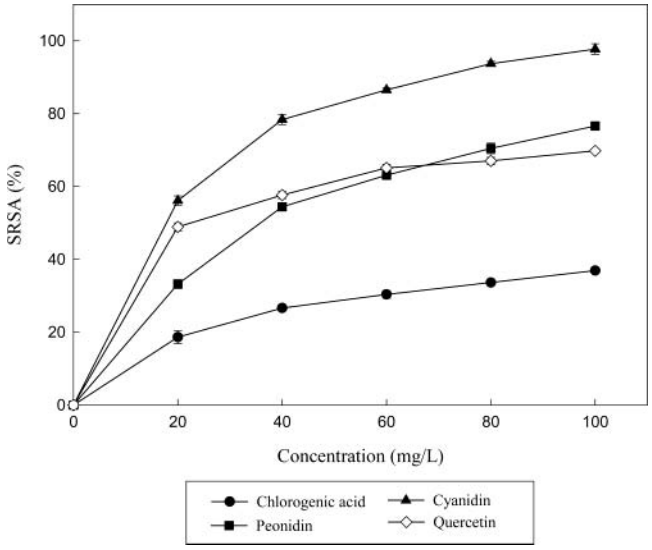

Figure 2. Superoxide radical scavenging activity (SRSA) of individual polyphenols identified in plums. Each point represents the average of six replications. Vertical bars indicate standard deviations.

Total flavonoids were measured using a colorimetric assay developed by Zhishen et al. (*28*). A 1-mL sample of appropriately diluted extracts was added to a 10-mL volumetric flask filled with 4 mL of ddH_2O. A reagent blank using ddH_2O instead of sample was prepared. At 5 min from the starting time, 0.3 mL of 5% $NaNO_2$ was added to the flask, followed by 0.3 mL of 10% $AlCl_3$. At 6 min, 2 mL of 1 M NaOH was added and diluted to volume (10 mL) with ddH_2O then thoroughly mixed. The absorbance of the pink mixture was measured at 510 nm versus the prepared blank. Total flavonoids of plums were expressed on a fresh weight basis as mg catechin equivalent (CE)/100 g. Each extract sample was analyzed in triplicate. Total phenolics and total flavonoids of nine plum cultivars except BY 69−339 and French Damson were previously reported (*18*).

Identification of Polyphenolics Using HPLC. HPLC analysis was performed according to the method of Kim and Lee (*29*). Extracted sample was analyzed using an HPLC system (Hewlett-Packard Model 1100, Palo Alto, CA) equipped with a quaternary pump, a vacuum degasser, and a photodiode array detector. The detector was set at 320 nm for hydroxycinnamic acids, 370 nm for flavonols, and 520 nm for anthocyanins, and the standards were prepared at concentrations of 50, 100, 200, and 400 ng/20 μL to obtain the calibration curves. Each peak of plum extract was identified by comparing retention time, UV−vis spectra given by the photodiode array detector with the standards, and spiking the extract with polyphenolic standards. Due to the unavailability of authentic commercial neochlorogenic acid standard, its identification was accomplished with the comparison of previously reported reversed-phase HPLC separation patterns (*12*, *15*, *21*) and of its UV−vis spectrum with chlorogenic acid. The amount of neochlorogenic acid was expressed as chlorogenic acid equivalent (CAE) (*12*). Acid hydrolysis was used to identify aglycone of quercetin derivatives (*30*). The peak that showed the same UV−vis spectrum with quercetin, but had different retention time in HPLC analysis, was collected repeatedly from analytical column, added with 1.2 N HCl, and refluxed in 90 °C water bath for 2 h. The resulting acid hydrolyzate was analyzed by HPLC to identify the aglycone of the unknown peak. The amount of quercetin derivatives was expressed as quercetin equivalent.

RESULTS AND DISCUSSION

SRSA of Phytochemicals. Figures 2 and **3** show the SRSA of major individual polyphenols found in plums. As seen in **Figure 2**, cyanidin, which is the major anthocyanin of plums, showed particularly high SRSA, followed by peonidin, quercetin, and chlorogenic acid at 100 mg/L. **Figure 3** shows the SRSA of quercetin and its glycosides (**A**) and cyanidin, peonidin, and their glycosides (**B**). Quercetin showed higher SRSA than its glycosides. The higher scavenging activity might be explained by the fact that sugar moieties did not contribute to the SRSA (*31*). Some polyphenols, such as quercetin, may react in two ways, by inhibiting the xanthine oxidase and by scavenging superoxide radicals. The reduction of uric acid production by the inhibition of xanthine oxidase resulted automatically in an equivalent reduction in superoxide (*32*). This means that the inhibition of xanthine oxidase can influence the superoxide radical scavenging reactions and the rate of uric acid reduction equals the oxidase inhibitor without any additional superoxide scavenging activity. The phenolic structure−activity

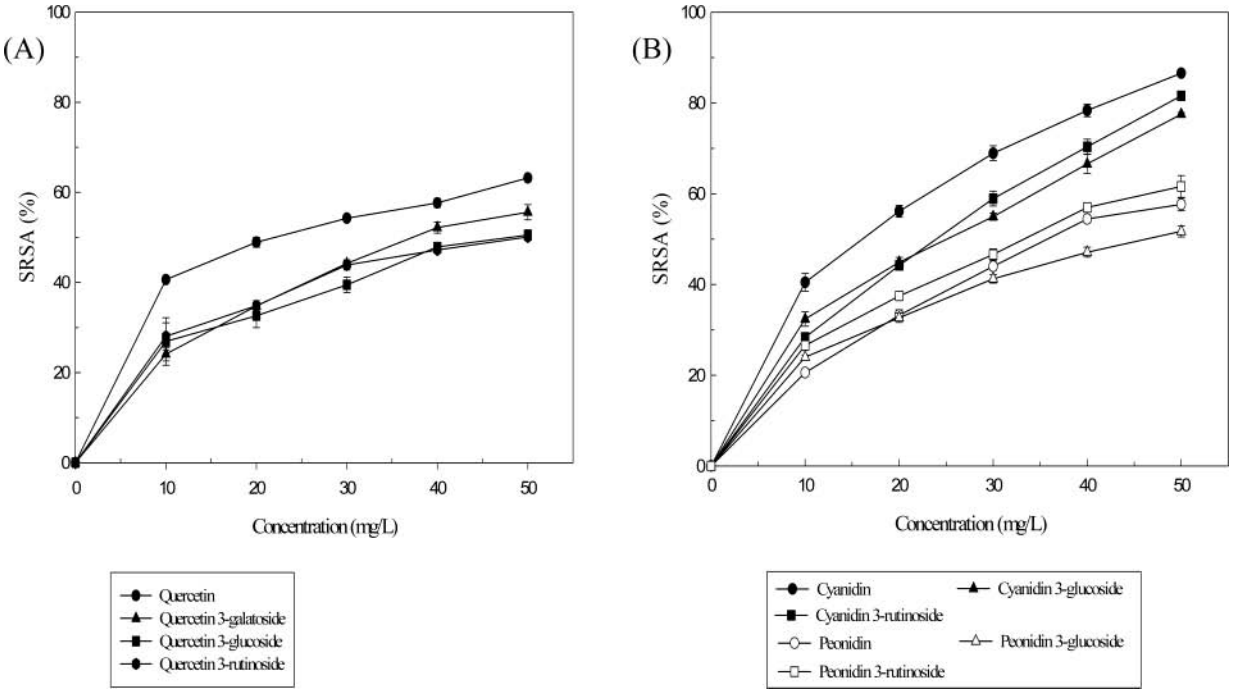

Figure 3. Superoxide radical scavenging activity (SRSA) of (A) quercetin and its glycosides and (B) cyanidin, peonidin, and their glycosides. Each point represents the average of six replications. Vertical bars indicate standard deviations.

Table 1. Superoxide Radical Scavenging Activity Expressed as IC_{50} of Individual Polyphenolics Found in Plum Cultivars

classification	compound	IC_{50}[a] (mg/L)
phenolic acid	chlorogenic acid	225.50 ± 9.77
flavonols	quercetin	22.04 ± 0.56
	quercetin 3-galactoside	37.25 ± 0.65
	quercetin 3-glucoside	47.99 ± 0.48
	quercetin 3-rutinoside	49.64 ± 0.27
anthocyanidins/anthocyanins	cyanidin	16.09 ± 0.30
	cyanidin 3-glucoside	25.08 ± 0.30
	cyanidin 3-rutinoside	23.95 ± 0.36
	peonidin	30.97 ± 0.37
	peonidin 3-glucoside	36.39 ± 1.25
	peonidin 3-rutinoside	33.29 ± 1.25

[a] IC_{50} is defined as the concentration at 50% superoxide radical scavenging activity of each compound.

relationship to inhibition of xanthine oxidase showed a different trend compared with that to superoxide scavenging (*32*). It was reported that flavonols have lower IC_{50} values for the reduction of the superoxide level than for the inhibition of xanthine oxidase, which indicates an additional superoxide scavenging activity (*3*).

High SRSA of cyanidins and peonidins can be explained by chemical structure−activity relationship. The hydroxyl group at C-5 and C-7 in the ring A and the double bond between C-2 and C-3 in the ring C were essential for a high inhibitory activity on xanthine oxidase (*3*). Anthocyanins and anthocyanidins, including cyanidin, peonidin, and their derivatives, has no double bond between C-2 and C-3 in the ring C. Therefore, the SRSA may have resulted entirely from the superoxide scavenging activity itself. On the other hand, the presence of a hydroxyl group at C-3′ in the ring B is essential for the SRSA and a hydroxyl group at C-3 in the ring C enhances the scavenging ability of flavonoids (*33*). Cyanidin has hydroxyl groups at these positions (**Figure 1**), so it showed relatively higher SRSA than other polyphenolics examined. All glycoside derivatives showed a much lower SRSA than the aglycones. The SRSA decreased when the C-3 hydroxyl group was glycosylated.

Table 1 shows the IC_{50} of individual phenolic compounds using SRSA assay. According to the results, the relative IC_{50} of cyanidin was about 14 times higher than that of chlorogenic acid, and about 3.1 times higher than quercetin 3-rutinoside. In a previous study (*31*), quercetin was reported to possess higher SRSA than chlorogenic acid, quercetin glucoside, and quercetin galactoside. Sichel et al. (*33*) reported that the effectiveness of flavonoids in superoxide radical scavenging, in alkaline medium by ESR spectrometry, was in the order of quercetin > quercitrin > cyanidin > quercetin 3-rutinoside > peonidin. The different aspect of the result with present study may be attributed to different methods of assessment, varying substrate conditions, and differential concentrations of active components. Although there is a wealth of data on the importance of SRSA, the correlation between SRSA and chemical structure is far from clear. The SRSA analyzed in this study is carried out under the similar condition with our body substrate system.

SRSA of Plums. The contents of total phenolics and total flavonoids and the IC_{50} values of eleven plum cultivars are listed in **Table 2**. BY 69−339 variety exhibited the lowest IC_{50} (1.71 g fresh sample in 100 mL of 50% MeOH) among the tested plums, which means powerful antioxidant activity in scavenging superoxide radicals, followed by French Damson, Cacaks Best, Beltsville Elite B70197, Empress, Castleton, Stanley, NY 6, NY 101, Mirabellier, NY 9. BY 69−339 had 4.8 times higher SRSA

Table 2. Total Phenolics, Total Flavonoids, and IC_{50} of Plum Cultivars on the Fresh Plum Basis[a]

cultivars	total phenolics (mg GAE[b]/100 g)	total flavonoids (mg CE[c]/100 g)	IC_{50}[d] (g fresh plum/100 mL)
Beltsville Elite B70197	684.5 ± 2.6	366.0 ± 10.0	2.51
BY 69–339	833.6 ± 14.8	401.3 ± 2.1	1.71
Cacaks Best	571.7 ± 7.6	341.6 ± 9.0	2.45
Castleton	250.5 ± 1.6	152.1 ± 6.5	5.57
Empress	398.7 ± 8.8	227.4 ± 4.9	2.66
French Damson	529.5 ± 2.2	246.2 ± 13.8	2.19
Mirabellier	215.7 ± 2.9	95.4 ± 8.0	7.60
NY 101	196.1 ± 1.5	98.5 ± 6.5	7.44
NY 6	146.6 ± 1.0	71.4 ± 3.1	7.29
NY 9	138.1 ± 2.9	59.3 ± 4.6	8.18
Stanley	236.7 ± 4.5	140.9 ± 9.3	6.21

[a] The content of total phenolics and total flavonoids for the other nine plum cultivars in this study was previously reported (*18*), each value is the mean ± SD ($n = 6$). [b] GAE stands for gallic acid equivalent. [c] CE stands for catechin equivalent. [d] IC_{50} is defined as the concentration at 50% superoxide radical scavenging activity of each compound.

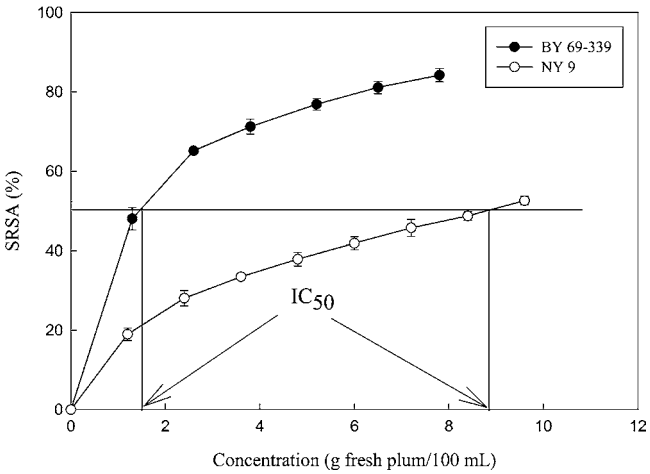

Figure 4. Superoxide radical scavenging activity (SRSA) of two plum varieties: BY 69–339 and NY 9. Each point represents the average of six replications. Vertical bars indicate standard deviations.

than NY 9 (**Figure 4**). This study clearly showed that there is a wide range of difference in the antioxidant activity among different varieties of plums. In terms of relationship between antioxidant activity (SRSA) and phenolic content, IC_{50} values showed higher correlations with total flavonoids ($r^2 = 0.8699$) than total phenolics ($r^2 = 0.8355$) (**Figure 5**), which indicated that flavonoids might contribute to the total SRSA more than other polyphenols in plums.

The major polyphenolics in BY 69−339 and French Damson identified by HPLC analysis are shown in **Table 3**. For the other nine plum cultivars in this study, we used the quantification data of previous study (*18*), which reported that chlorogenic acids were the major polyphenolics, followed by cyanidin glycosides except Early Magic, and that the composition of polyphenolics, as well as the total phenolic amount itself, affected on the total antioxidant capacity of plum cultivars. SRSA also reflects the composition of polyphenolics as well as the amount of total polyphenolics in plums. The amount of cyanidin glycosides in BY 69−339 was 208.9 mg/100 g, which was 7 times higher than that in French Damson (30.5 mg/100 g), whereas the amount of chlorogenic acids in French Damson (20.0 mg/100 g) was 4.7 times higher than that in BY 69−339 (94.7 mg/100 g). Because SRSA of cyanidin glycosides is 9

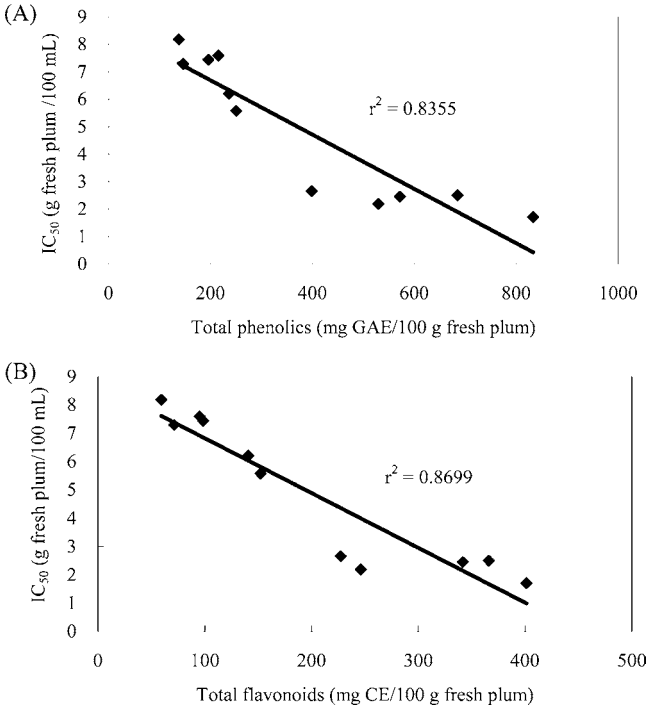

Figure 5. Relationships between IC_{50} values and the content of total phenolics and total flavonoids in the fresh plums: (A) correlation of IC_{50} with the total phenolics as gallic acid equivalents (GAE); (B) correlation of IC_{50} with the total flavonoids as catechin equivalent (CE).

Table 3. Content of Polyphenolics in Plum Cultivars on the 100-g Fresh Sample Basis[a]

	cultivars	
polyphenolics	BY 69–339 (mg)	French Damson (mg)
3-*O*-caffeoylquinic acid	18.2 ± 1.4[b]	84.1 ± 5.5
5-*O*-caffeoylquinic acid	1.8 ± 0.4	10.6 ± 0.5
cyanidin 3-glucoside	64.1 ± 5.3	5.3 ± 0.3
cyanidin 3-rutinoside	144.8 ± 12.5	25.2 ± 1.7
peonidin 3-glucoside	ND[c]	0.6 ± 0.1
peonidin 3-rutinoside	ND	9.7 ± 0.7
quercetin 3-glucoside	3.7 ± 0.3	0.5 ± 0.0
quercetin 3-rutinoside	8.1 ± 0.7	3.8 ± 0.3
quercetin derivative 1	0.7 ± 0.1	ND
quercetin derivative 2	2.3 ± 0.2	ND
quercetin derivative 3	0.6 ± 0.0	ND
total	244.4	139.6

[a] The content of polyphenolics for the other nine plum cultivars in this study was previously reported (*18*). [b] Each value is the mean ± SD ($n = 4$). [c] ND stands for not detected.

times higher than that of chlorogenic acids (**Table 1**), BY 69−339 demonstrated much higher SRSA than French Damson.

Figure 6 shows the contribution of each polyphenolic group to the total SRSA of fresh plums. The SRSA value of each plum cultivar was calculated from the concentration of polyphenols identified by HPLC and the relative IC_{50} of each polyphenol determined by this SRSA assay. As seen in **Figure 6**, anthocyanins were the major contributors to the total SRSA in plums except for two yellow plums. The anthocyanins contributed an average of 76.6% of the total SRSA of purple plums. Sato et al. (*34*) reported that there was a positive relationship of the SRSA with the color of grape wine, which means the anthocyanins are the important components in the total SRSA in red fruits and red fruit products. In the yellow plums, chlorogenic acid was the major contributor to SRSA (Mirabellier as 60.5% and NY 101 as 94.9%). Quercetins were the major flavonols

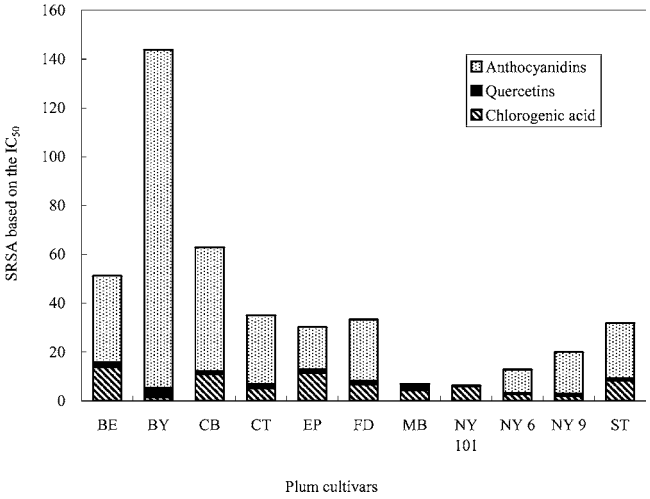

Figure 6. Contribution of each polyphenolic group to the total superoxide radical scavenging activity (SRSA) of fresh plums. BE, Beltsville Elite B70197; BY, BY 69–339; CB, Cacaks Best; CT, Castleton; EP, Empress; FD, French Damson; MB, Mirabellier; ST, Stanley.

existing in plums. However, they showed relatively low contribution to the total SRSA at the range of 2.0−39.5%.

In our previous study (*18*), chlorogenic acids were the major source of antioxidant capacity in plums, and anthocyanins showed the second highest contribution to the total antioxidant capacity. This study demonstrated that in purple plums, however, the effect of anthocyanins on the total SRSA of plums was superior to that of chlorogenic acids.

In conclusion, polyphenols in fresh plums showed relatively higher SRSA than other fruits, and anthocyanins and chlorogenic acids were the major contributors to the total SRSA.

ABBREVIATIONS USED

CAE, chlorogenic acid equivalent; CE, catechin equivalent; ddH_2O, distilled deionized water; GAE, gallic acid equivalent; NBT, nitro blue tetrazolium; PBE, potassium phosphate buffer containing EDTA; SRSA, superoxide radical scavenging activity

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