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Polyphenol Composition of Plum Selections in Relation to Total Antioxidant Capacity

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ABSTRACT: Dietary polyphenols are associated with protection against chronic diseases such as cardiovascular disease. Pharmacological studies show a range of bioactivities and efficacy attributable to specific polyphenols. While many fruits are rich in polyphenols, wide cultivar variation of polyphenol composition is common. Our objective was to determine the composition of major bioactive polyphenols in 29 prevarietal selections of Western Australian plums, and Black Amber as an evaluation in developing breeding tools to develop fruit that may have enhanced health-promoting capacities. Total phenolics were quantified colorimetrically; selected polyphenols were quantified by HPLC; and the total antioxidant capacity (TAC) was measured by the antioxidant inhibition of oxygen radicals (AIOR) assay. Total phenolic concentration was significantly correlated with TAC (R = 0.95, P < 0.01). Neo-chlorogenic acid and quercetin glycosides were found to be the predominant polyphenols (mean 29.9 mg·kg^{-1} and 50.7 mg·kg^{-1}, respectively). No significant correlations were found between the composition of predominant polyphenols in plums and the TAC. We argue that the value of in vitro TAC assays to breeding programs may be limited, and future research should focus on the heritability of known bioactive polyphenols.

KEYWORDS: *fruits, breeding, total phenolics, plum, antioxidant, flavonoid*

INTRODUCTION

It is widely accepted that dietary intake of fruits and vegetables is inversely related to the incidence of chronic disease, including cardiovascular disease, cancers, obesity, and type II diabetes mellitus.[1] Government and intergovernment agencies have widely promoted consumption of fruits and vegetables to improve health outcomes and reduce the medical burden. Horticultural breeders and marketers have also seen the commercial opportunity in crop improvement. Dietary polyphenols are principal candidates to explain the health-protective effects of fruit, vegetables and beverages derived from them.[2,3] However, the nexus between in vitro bioactivity and food polyphenol composition is still under debate.[4−6]

Early research focused on the role of polyphenols in attenuating oxidative damage, as they are an abundant form of antioxidant in the human diet.[7,8] Crude antioxidant activity was evaluated from a wide range of foods[9,10] and found to positively correlate to total phenolic concentration.[11−13] Yet, this is recognized as a simplification, as knowledge of the diverse bioactivities of various polyphenols has vastly advanced over the past decade.[4,5,14] Furthermore, antioxidant activity per se may be altered by metabolism and bioavailability.[15] In addition, the activity of quercetin and (−)-epicatechin to enhance nitric oxide production and reduce endothelin-1 in healthy human subjects is not necessarily related to their antioxidant activity.[16] Evidence for roles in modulating gene expression and cell signaling is rapidly accumulating, e.g., the effect of quercetin on the Nrf2/Keap1 pathway[17,18] and of proanthocyanidins on microRNA expression.[19] Increasingly, investigation of cell signaling or other secondary effects of flavonoids is being applied in clinical trials with whole foods such as apple.[20]

Plums are a rich dietary source of polyphenols, are widely consumed,[21] widely available, and a lucrative horticultural crop.[22,23] Predominant polyphenols in plum include the hydroxycinnamic acid derivatives, neo-chlorogenic acid and chlorogenic acid, and the quercetin glycoside rutin.[24−27] Evidence for various bioactivities of chlorogenic acids and quercetin glycosides, in addition to flavan-3-ols, is accumulating.[28−33] However, polyphenol composition is determined by (plant) genetic, environmental, and cultural influences, and hence varies quantitatively and qualitatively among cultivars of plum and other Rosaceae family fruit.[34,35] Thus, in order to manipulate polyphenol content for dietary benefit, it is important to understand the cultivar variation in polyphenols that have potential health promoting activity. Understanding this would aid the agricultural sector in developing and commercializing plum cultivars of high quality, which meets consumer satisfaction and potentially becomes a prospective dietary approach toward prevention and treatment of various diseases.

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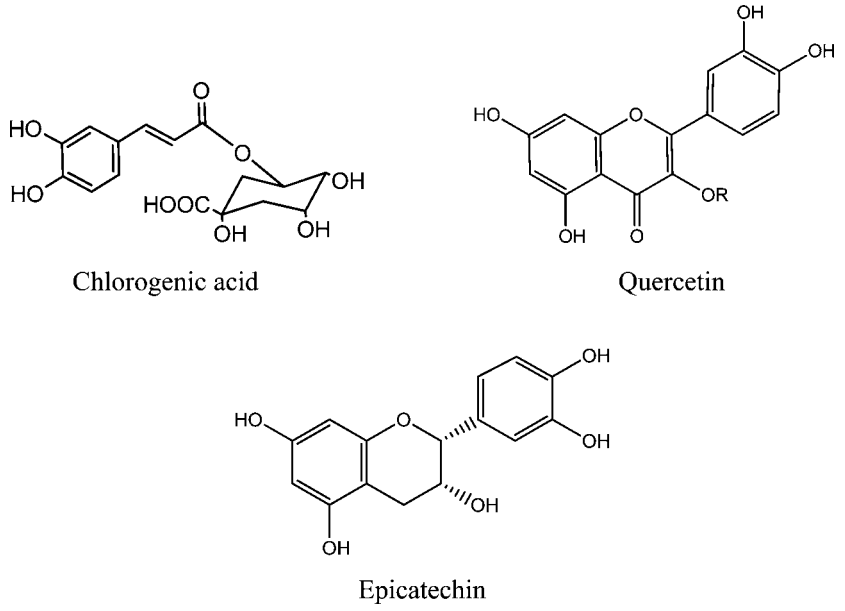

Figure 1. Chemical structures of known bioactive polyphenols detected in the tested plums (R = sugar groups).

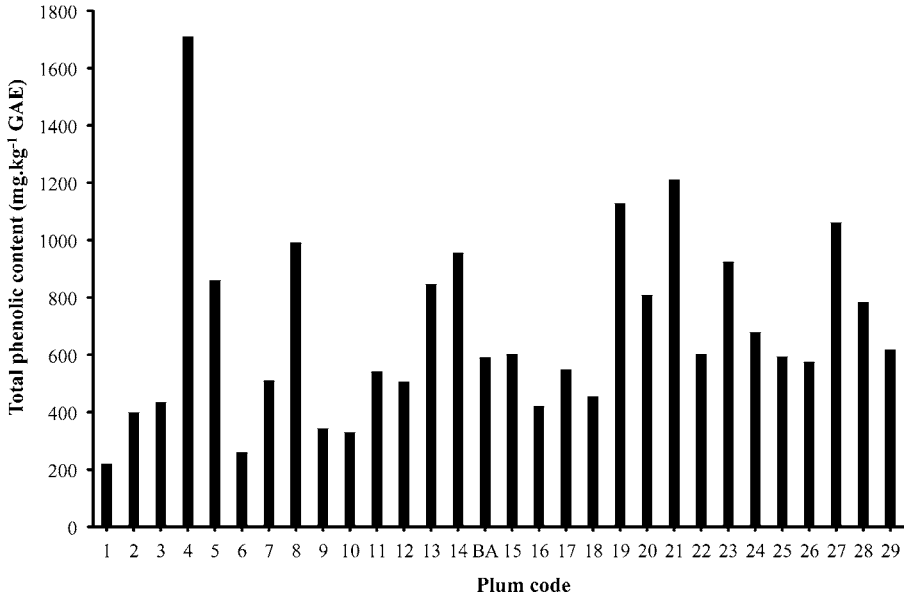

Figure 2. Total phenolic concentration in 29 prevarietal plum selections and Black Amber (BA) expressed as mg·kg^{-1} gallic acid equivalents. Data are mean of duplicate extractions of a pool of 15 fruit from two trees for each plum selection.

The purpose of this study was to evaluate and quantify the diversity of known bioactive polyphenols (Figure 1) in advanced prevarietal plum genotypes, henceforth termed selections, as a foundation for crop improvement and further studies into health-preventative mechanism of whole fruits. The relationships between total phenolics, individual polyphenols, and the total antioxidant capacity (TAC) were assessed, in view of assessing the relevance of TAC for fruit breeding programs.

MATERIALS AND METHODS

Chemicals and Apparatus. Rutin hydrate, chlorogenic acid, neo-chlorogenic acid, quercetin, (±)-catechin, (−)-epicatechin, Folin Ciocalteu's phenol reagent, uroporphyrin I dihydrochloride, Trolox, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) and Brij solution were obtained from Sigma-Aldrich (New South Wales, Australia).

Flat-bottom, 96-well microplates used in total phenolic concentration assay were obtained from Greiner Bio-One (Frickenhausen, Germany). Round-bottom 96-well microplates used in total antioxidant capacity assay were obtained from Alltech Australia (New South Wales, Australia).

Plant Material. Twenty-nine prevarietal plum selections from the Department of Agriculture and Food Western Australia's Manjimup Horticultural Research Institute, and one commercial variety were included in this study. We have referred to individual genotypes as selections. All selections were harvested at storage maturity between December 2008 and February 2009. All fruit were grade 1-equiv. quality. At least 15 fruit per selection, sampled from a range of positions on the tree from at least two trees per selection, were pooled for analysis (one biological replicate). Thus any variance due to environmental or cultural influences should be negligible. Fruits were transported immediately to the University of Western Australia. Upon arrival, the flesh and skin but not the seed were homogenized and stored at −80 °C before analysis.

Extraction of Phenolic Compounds for HPLC Analysis, Total Antioxidant Capacity and Total Phenolic Concentration. Homogenized whole plums (4 g) were extracted with 10 mL of acidic methanol (0.5% v/v acetic acid). The mixture was then vortexed and sonicated for 1 min each. Homogenates were kept on ice between these steps. The homogenates were then left on the bench at room temperature for 45 min. Homogenates were centrifuged (16 000 × *g*) for 15 min at 4 °C. Supernatants were removed and then filtered through a 0.45 μm filter (PALL Life Sciences, Cornwall, UK) and kept at −80 °C until analysis. This extraction was done in duplicate for each plum selection. The extract was directly analyzed by high performance liquid chromatography (HPLC) within 24 h. The extract was diluted 1:5 with cold phosphate buffered saline buffer (PBS, pH 7) for measurement of total antioxidant capacity (TAC). The extract was also used in total phenolic concentration assay after dilution with ultrapurified water.

HPLC-DAD Analyses. Selected polyphenols were quantified by HPLC coupled with a photodiode array detector (DAD; Waters 996, Sydney, Australia). The HPLC system consisted of an autosampler (Waters 717plus) and Waters 600 Solvent Delivery system and equipped with the Empower Software. A reversed phase column, Alltech C_{18} (5 μm; 250 × 4.6 mm, Alltech, Sydney, Australia) was eluted with a mobile-phase gradient program at a flow rate of 1 mL·min^{-1}. Wavelengths at 280, 350, and 520 nm were used for detection. Extracts (20 μL) were injected into the HPLC-DAD. Formic acid (2% in water) (solvent A) and acetonitrile (solvent B) were used in the mobile phase of this system. The solvents were HPLC grade. Elution started with 90% solvent A and 10% solvent B which remained isocratic until 15 min. A gradient was then used to reach 80% A and 20% B between 15 to 35 min. The elution then reached 50% A and 50% B at 35 min, 20% A and 80% B at 38 min, 100% B at 42 min, 90% A and 10% B at 43 min which was held isocratic up to 61 min. Chromatograms were recorded at 280 nm, 350 nm, 420 and 520 nm. The UV spectra of the different compounds were recorded with a diode array detector. Compounds were identified by comparison with authentic standards and UV/vis spectra.

Total Antioxidant Capacity. Total antioxidant capacity (TAC) was measured by the antioxidant inhibition of oxygen radicals (AIOR) method[36] using a Cary Eclipse fluorescence spectrophotometer (Varian Australia, Victoria, Australia). This assay addresses many incongruous assumptions of other TAC methods. In this method, peroxyl radicals trigger decrease in fluorescence of the indicator molecule uroporphyrin I, which is delayed by the presence of antioxidants; Trolox, the vitamin E analogue, was used as an external standard. Plum extracts were diluted 1:5 with cold PBS buffer. Triplicate samples (1 μL) were mixed with 200 μL of uroporphyrin I solution (300 nM) and 40 μL AAPH (291 mM) and then placed in the fluorescence spectrophotometer and detected for 180 min. TAC data are expressed as mol·kg^{-1} fresh weight Trolox equivalents.

Total Phenolic Concentration. SPECTRAmax 190 microplate spectrophotometer, Molecular Devices (California, USA) was used for measurement of total phenolic concentration. Total phenolic concentration was measured in plum extracts using 96-well microplate format with Folin Ciocalteu's method.[37] Gallic acid with concentration range between 0 and 200 μg·mL^{-1} was used as standard in this assay. Plum extracts were diluted to fit in the standard curve linear range. 20 μL of standards, blank and extracts prepared were loaded into a flat-bottom 96-well microplate. 100 μL of Folin Ciocalteu's reagent (diluted 1:10 with ultrapurified water) was added to the 96-well microplate and mixed. The microplate was incubated at room temperature for 5 min. Then, 80 μL of 7.5% sodium carbonate was added to the microplate and mixed well. The microplate was covered and then incubated at 45 °C for 30 min in dark. After the incubation, absorbance was read at 765 nm using the microplate spectrophotometer with automix set for 60 s before reading. The area under the curve was calculated and results were expressed in mg·kg^{-1} fresh weight gallic acid equivalents (GAE).

RESULTS

Total Phenolic Concentration. Total phenolics of the 29 prevarietal plum selections and one commercial variety are shown in Figure 2. The greatest total phenolic concentration was found in plum selection #4 with 1711.3 mg·kg^{-1} gallic acid equivalents (GAE) per unit fresh weight, followed by plum selections #21, #18 and #27. Plum selection #4 had a 3-fold greater total phenolic concentration than the commercial variety, Black Amber, and 8-fold higher than selection #1, which had the least total phenolic concentration (221.6 mg·kg^{-1} GAE). In fact, 15 of the prevarietal plum selections tested had a greater total phenolic concentration than Black Amber. The mean phenolic concentration from all plums tested in this study was 684.5 mg·kg^{-1}.

Quantitation of Known Bioactive Polyphenols. The most abundant polyphenols detected by HPLC in the plum selections tested were neo-chlorogenic acid and quercetin glycosides. Polyphenol abundance varied greatly between the 30 plum selections tested, both quantitatively and qualitatively (Table 1). Total quercetins were the most abundant group of polyphenols detected in 23 of the 30 plum selections tested (mean, 48.7 mg·kg^{-1} fresh weight; range, 9.0 to 239.8 mg·kg^{-1}). Rutin was the predominant quercetin glycoside detected, being the most abundant individual polyphenol in selection #23, and presenting a mean of 13.1 mg·kg^{-1} among the plums tested (range, 0 to 63.9 mg·kg^{-1}). The hydroxycinnamic acid,

Table 1. Quantification of Selected Polyphenols in 29 Prevarietal Plum Selections and Black Amber (BA) Using HPLC-DAD at Wavelengths 280 and 350 nm[a]

	polyphenol concentration (mg·kg^{-1} fresh weight)				
plum code	neo-chlorogenic acid	chlorogenic acid	epicatechin	rutin	quercetin (total)
1	0.0	0.0	0.0	9.6	61.8
2	20.8	0.0	0.0	0.0	42.5
3	20.8	0.0	0.0	0.0	46.7
4	0.0	0.0	0.0	0.0	38.5
5	139.4	0.0	154.3	38.7	239.8
6	0.0	0.0	115.8	10.8	39.7
7	0.0	0.0	0.0	0.0	30.5
8	0.0	0.0	0.0	12.3	45.7
9	0.0	0.0	0.0	0.0	18.5
10	0.0	0.0	0.0	0.0	9.0
11	0.0	0.0	0.0	19.6	81.3
12	0.0	0.0	0.0	18.9	76.0
13	24.2	0.0	36.0	0.00	13.0
14	184.5	0.0	0.0	12.9	37.1
BA	0.0	0.0	0.0	14.5	65.4
15	53.6	0.0	0.0	17.0	78.7
16	0.0	0.0	0.0	0.0	45.0
17	0.0	0.0	0.0	9.5	18.4
18	29.0	0.0	0.0	15.7	57.0
19	27.5	0.0	0.0	0.0	30.3
20	25.5	38.4	0.0	11.9	53.4
21	220.5	0.0	74.2	27.4	37.3
22	22.3	0.0	0.0	0.0	9.9
23	0.0	0.0	0.0	63.9	16.2
24	0.0	0.0	0.0	0.0	30.8
25	24.1	0.0	0.0	11.4	34.5
26	85.4	0.0	0.0	21.4	61.8
27	0.0	27.3	0.0	27.0	60.4
28	16.4	0.0	0.0	29.8	57.7
29	34.2	0.0	44.6	30.8	84.0

[a]Data are presented in mg·kg^{-1} of fresh weight plums. Data are mean of duplicate extractions of a pool of 15 fruit from two trees for each plum selections.

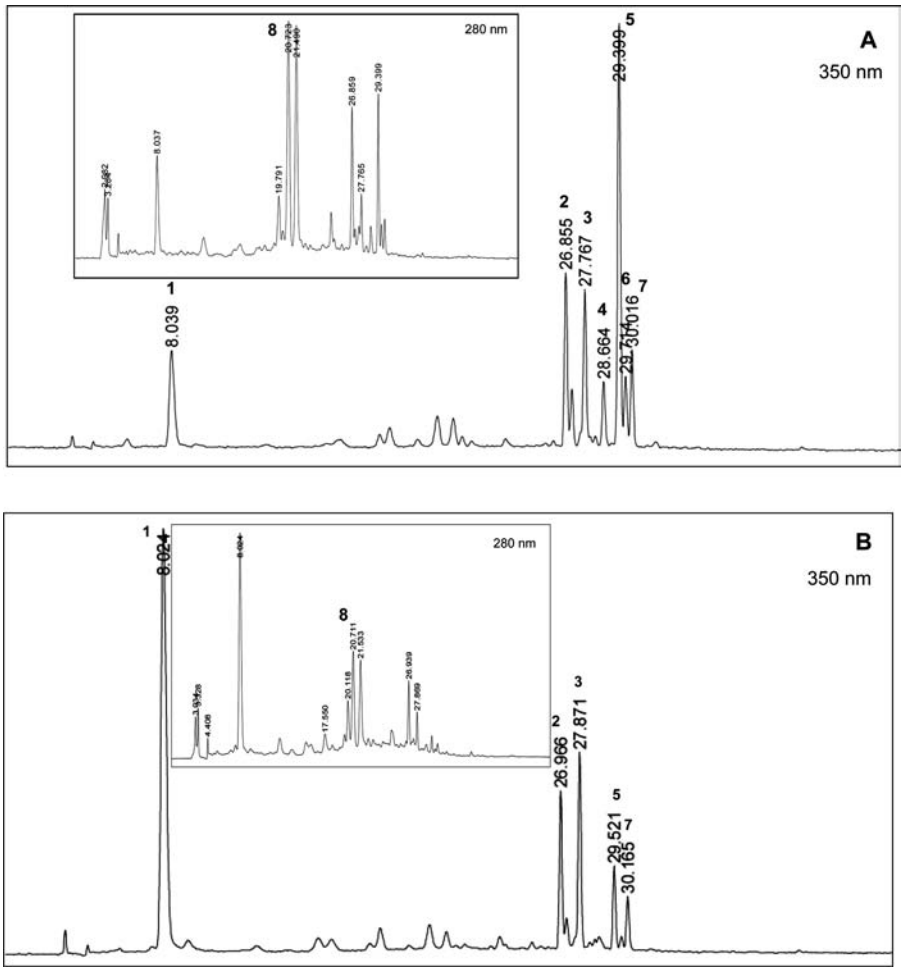

Figure 3. HPLC chromatograms of Plum Cultivar 29 (A) and Plum Cultivar 21 (B) recorded at 350 nm (insert shows chromatogram recorded at 280 nm to detect catechins). Peak 1 represents neo-chlorogenic acid; peak 2 represents rutin; peaks 3−7 represents other quercetin glycosides and peak 8 represents epicatechin (insert).

neo-chlorogenic acid was also detected in abundant levels, being the most abundant polyphenol detected in 4 of the 30 plums tested (mean, 30.9 mg·kg^{-1}; range, 0 to 220.5 mg·kg^{-1}). Chlorogenic acid was also detected in 2 of the 30 plums tested (mean, 2.2 mg·kg^{-1}; range, 0 to 38.4 mg·kg^{-1}). Among flavan-3-ols, epicatechin was only detected in 5 of the 30 plums tested, but was the most abundant polyphenol detected in 1 of the 30 plum selections (selection #13). The mean epicatechin concentration across all 30 plum selections was 14.2 mg·kg^{-1} (range, 0 to 154.2 mg·kg^{-1}). Catechin was not detected in any plum selections in the present study. Figure 3 shows a typical HPLC chromatogram for two of the plum selections, showing neo-chlorogenic acid, rutin, other quercetin glycosides and epicatechin. Interestingly, when only the major detected polyphenols (Table 1) were summed, plum selection #5, followed by plum selection #21, showed the greatest abundance, while plum selection #4, which showed the greatest total phenolic concentration, had less than 1/10th the sum of either selections #5 and #21.

Total Antioxidant Capacity. The total antioxidant capacity (TAC) of the 30 plum selections tested showed a 7.5-fold range; from 4795 to 36 187 mol·kg^{-1} Trolox equivalents (TE) (mean, 13 281 mol·kg^{-1} TE; Figure 4). Plum selection #4 presented the greatest TAC value, apparently greater than 2-fold the value found in many other plum selections. Plum selection #4 was already noted as that with the greatest total phenolic concentration among the 30 selections.

Relationships of Total Phenolics, Individual Polyphenols and Total Antioxidant Capacity. The total phenolic concentration of the 30 plums tested was significantly correlated with TAC ($R = 0.95$, $P < 0.01$; Figure 5). However, there were no significant relationships between the abundance of individual monomeric polyphenols and TAC ($R = 0.29$ and $R = 0.11$ for neo-chlorogenic acid and quercetin glycosides, respectively).

DISCUSSION

It is widely accepted that increased consumption of fruit and vegetables is associated with reduced incidence of chronic disease. The pharmacological and food science industries have interacted well to enable a better understanding of the relationship between food composition and bioactivity, with the goal of improving dietary health through new products and/or promoting consumption of "healthy" foods. However, there is evidence for benefits of eating whole foods, particularly fruit and vegetables, rather than isolated components.[38] Plant breeders have so far been slow to capitalize on the genetic influence for breeding high polyphenol foods. Here we sought to bridge the gap between plant breeders and the food and pharmacological sciences by investigating biomarkers in prevarietal plum breeding germplasm. The knowledge would provide a foundation

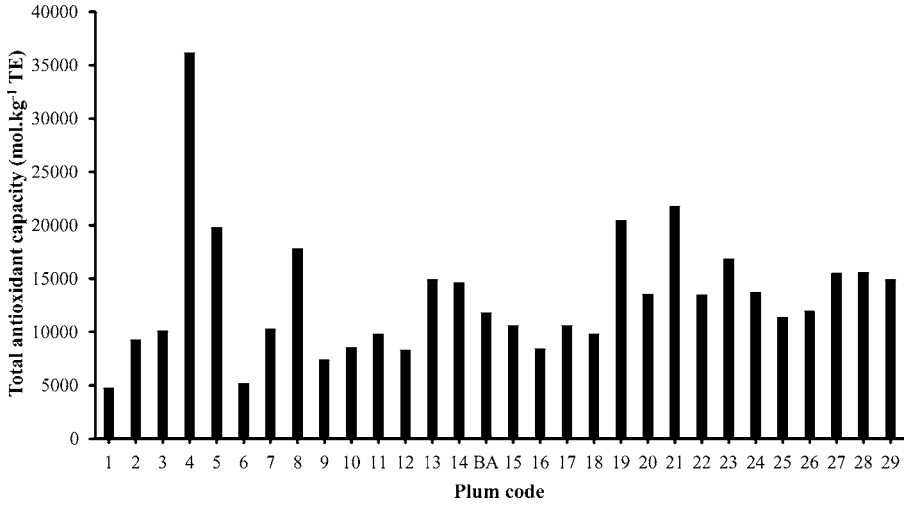

Figure 4. Total antioxidant capacity in 29 prevarietal plum selections and Black Amber (BA). Data represent total antioxidant capacity (AIOR) in whole plum extracts expressed as mol·kg^{-1} trolox equivalent. Data are mean of duplicate extractions of a pool of 15 fruit from two trees for each plum selections.

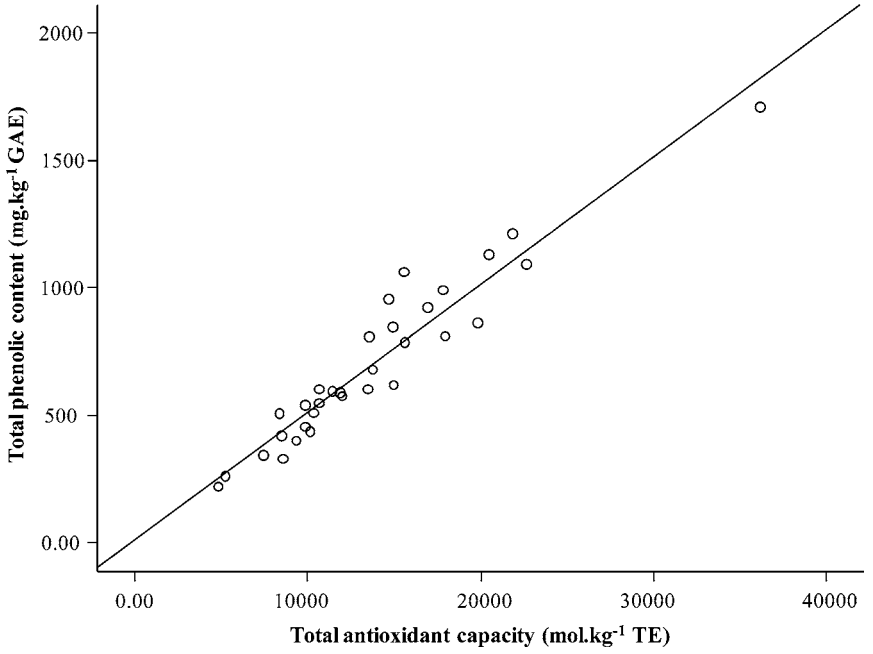

Figure 5. Correlation of total phenolic concentration with total antioxidant capacity in 29 prevarietal plum selections and Black Amber (BA).

for further research toward breeding of elite lines of plum and other Rosaceae fruit.

We focused our research on polyphenols that were abundant in plums and for which there was pharmacological evidence for their protective dietary function. There is good evidence that quercetin glycosides have cardiovascular protective effects.[16−18,30,39] Quercetins were the most abundant polyphenol detected in over two-thirds of the plum selections tested, with rutin being the most abundant glycoside, although not detectable in all selections. The qualitative and quantitative variation between plum selections is consistent with other reports.[26,27,40]

Hydroxycinnamic acids are an abundant class of polyphenol in fruits, vegetables, and coffee, in particular.[2] Evidence for antihypertensive activities is accumulating,[33,41] together with population studies suggesting reduced risk of type 2 diabetes.[42] While both chlorogenic acid and its isomer neo-chlorogenic were detected among the plum selections, more than half of the plum selections tested lacked detectable levels of either. Neo-chlorogenic acid was more abundant across the plum selections than chlorogenic acid, similar to previous reports in plum and other Rosaceae fruit including peaches, nectarines and cherries.[27,40,43] However, at least one study reported greater abundance of chlorogenic acid than its isomer in plums.[25]

Flavan-3-ols were the third target class of polyphenol in this study, due to the well establish protective effects on vascular function and blood pressure.[29] Epicatechin was only detected in 5 of the 30 plums tested, and catechin was undetectable across the plum selections. This is consistent with previous reports.[40] Furthermore, levels detected were low by comparison to those found in tea and cocoa.

In addition to investigating select polyphenols, we assessed total phenolics and an in vitro measure of total antioxidant capacity (TAC). The "antioxidant capacity" has been a major focus in the food sciences; "antioxidant-rich" has become a

commercial proxy for "healthy". Numerous TAC measures are available and their relevance is a subject of continued debate.[4,5] Currently, ORAC[44] is the predominant method; this assay does address some of the technical reservations some researcher have about TAC assays, such as the percentage inhibition and length of inhibition, but is highly pH- and temperature-sensitive, and requires separate assays for hydrophilic and lipophilic components.[45] Further, it is laborious to optimize and impractical for application in a breeding program. We chose the Antioxidant Inhibition of Oxygen Radicals assay (AIOR),[36] as it addresses each of these problems. Nonetheless, our data show strong correlation between total phenolics (hydrophilic) and the AIOR values. However, we found no correlation between TAC and any or all of the select polyphenols we targeted. While the debate on the validity and choice of TAC assay continues, the weight of available evidence suggests that investing further research in developing biomarkers from polyphenols with abundant pharmacological evidence will progress the quest to breed elite lines of fruit and vegetable more rapidly. On the data presented, we suggest that quercetin, epicatechin, and chlorogenic acids content be used in the breeding program as primary targets for biomarker development and application. A breeding approach focusing on single or a few target metabolites of a common biosynthetic pathway is consistent with recent studies identifying genetic markers for flavonoids, such as in grape[46] and apple.[47,48]

Our study deliberately sought to minimize variance in polyphenols due to environment, cultural practice, or postharvest storage. This suits our present purpose of seeking biomarkers for breeding, but other influences will ultimately have to be addressed. We judge that individual breeding programs may seek a similar process in identifying the genetic influence first and following up with further study. However, the next line of study for plum and other Rosaceae fruit should be the heritability of select polyphenols, e.g., quercetin glycosides.

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Notes

The authors declare no competing financial interest.

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