



*Original Article*

**Antioxidant, free radical scavenging activities and total polyphenolic content of aqueous extracts from seven blueberry cultivars grown in New Zealand**

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**ABSTRACT**

Antioxidant activity in blueberries or other fruits is an appealing characteristic to consumers and food industries. The antioxidant properties of aqueous extracts prepared from the berries of five rabbiteye (*Vaccinium ashei*) and two highbush (*V. corymbosum*) cultivars of blueberry grown in New Zealand, were studied using ferric reducing antioxidant power (FRAP) and scavenging DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical assays. Their ferrous ion chelating activities and total polyphenol contents (TPC) were also determined. Significant differences were found in antioxidant/antiradical activities and TPC among various cultivars of blueberries. The results showed that the highest TPC among the highbush cultivars were detected in the extracts from the berries of Jersey while the lowest TPC were detected in the extracts from the berries of Dixi. Similarly, the antioxidant/antiradical activity was significantly higher in Jersey than in Dixi cultivar which indicates that the antioxidant activity is related to the total phenolic contents. Among the rabbiteye cultivars, Centurion had the highest TPC followed by cultivars Maru, Rahi, Tifblue, and Ono. Regarding the antioxidant activity among the rabbiteye cultivars, it was in accordance with the results of TPC for all cultivars studied in that Centurion had the highest FRAP value and Ono cultivar had the lowest. A similar trend of activity was observed regarding the DPPH radical scavenging capacities. This may confirm that the phenolic compounds found in the blueberry extracts are the main contributors of the antioxidant/antiradical activities. In general, rabbiteye cultivars had significantly higher TPC ( $P < 0.01$ ), higher antioxidant ( $P < 0.05$ ) and free-radical scavenging activity ( $P < 0.05$ ) than highbush cultivars.

**Keywords:** Blueberry cultivars; total phenolic contents; antioxidant activities; ferrous ion chelating activities.

**INTRODUCTION**

It is well known that oxygenated free radicals can damage cellular components [1] and that controlling this oxidative damage may inhibit the onset of carcinogenesis [2]. Some

epidemiological studies have shown an association between the consumption of fruit and vegetables and a reduced risk of human diseases, such as cardiovascular disease and cancer [3-5].

Many studies have suggested that the phytochemical content of vegetables and fruits, such as polyphenols, vitamins and carotenoids that have shown potent antioxidant/free radical scavenging activities contribute to their protective effect against chronic and degenerative diseases [6-10].

In New Zealand, there are more than 10 different blueberry cultivars and their health benefits are not well-studied. As fruits, blueberries contain high concentrations of polyphenolic compounds such as flavonols, catechins and anthocyanins [8, 9, 11, 12].

The objectives of the present study were to compare the phenolic content, ferrous ion chelating activity, antioxidant capacity and free radical scavenging in the berries of seven different blueberry cultivars grown in New Zealand. The association between total phenolic contents, ferrous ion chelating activity, antioxidant/ free radical scavenging activities was also assessed.

## **MATERIALS AND METHODS**

### **Chemicals and standards**

2,4,6-Tripyridyl-s-triazine (TPTZ), sodium acetate, ferric chloride and gallic acid, Folin-Ciocalteu's phenol reagent and ferrous sulfate were purchased from Sigma (Sigma-Aldrich Pty. Ltd., Castle Hill, NSW 1765, Australia).

### **Preparation of extracts**

Blueberry fruits used in this study were grown at commercial farms in Hamilton, New Zealand. All berries were picked at the commercially ripe stage. The berries were maintained in polyethylene bags at -20 °C until extract preparation. Crude aqueous extracts from five rabbiteye blueberry (*Vaccinium ashei*) cultivars (Ono, Rahi, Tifblue, Maru and Centurion) and two highbush (*V. corymbosum*) cultivars (Dixi and Jersey) were prepared by weighing frozen fruits (100 g), mixing with 100 ml of distilled water and then milling with a commercial mini-processor (Braun Miniprimer MR300, Germany).

The crushed berries were put in centrifuge tubes. Tubes were centrifuged (3000g, 15 min) and the clear supernatant fluid was collected and used within 1 h of collection.

### **Determination of total phenolic content (TPC)**

The total phenolic content (TPC) was quantified according to the method of Molan et al. [13]. Briefly, an aliquot of 12.5 µl of water-soluble extract was mixed with 250 µl of 2% sodium carbonate solution in 96-well microplates and allowed to react for 5 minutes at room temperature. Then 12.5 µl of Folin-Ciocalteu phenol reagent (50 %) was added and allowed to stand for 30 minutes at room temperature before the absorbance of the reaction mixture was read at 650 nm using a plate reader. Calibration was achieved with an aqueous gallic acid solution (100-1000 µg/ml). The TPC of the extract was expressed as mg gallic acid equivalent (GAE) per gram of blueberry fruits and all determinations were performed in duplicate in two separate experiments. All values are reported as mean ± SE of two determinations in all experiments.

### **Evaluation of antioxidant activity as measured by the ferric reducing antioxidant power (FRAP)**

The antioxidant capacity of blueberry extracts was determined using the ferric reducing antioxidant power (FRAP) assay, a colorimetric assay that measures the ability of the tested sample to reduce the intense blue ferric tripyridyl-triazine complex to its ferrous form, thereby changing its absorbance [14]. The working FRAP reagent was prepared by mixing 10 volumes of 300 mmol/L acetate buffer, pH 3.6, with 1 volume of 10 mmol/L TPTZ (2,4,6-tripyridyls-triazine) in 40 mmol/L hydrochloric acid and 1 volume of 20 mmol/L ferric chloride. Briefly, an aliquot of 8.5 µl of extract was added to 275 µl of diluted FRAP reagent using a microplate and the plates were incubated at 37 °C for 30 minutes before measuring the absorbance at 395 nm using a plate reader. A standard curve was prepared using different concentrations (200-2000 µmol/L) of FeSO<sub>4</sub>.7H<sub>2</sub>O. The antioxidant capacity based on the ability to reduce ferric ions of the extract was expressed as micromole FeSO<sub>4</sub> equivalents per litre of aqueous extracts. All solutions were used on the day of preparation and all determinations were performed in duplicate.

#### **Evaluation of free radical scavenging activity using DPPH discoloration assay**

The DPPH assay detects scavenging of free radicals by the tested compound through the scavenging activity of the stable 2, 2-diphenyl-1-picrylhydrazyl (DPPH) free radical. This assay was performed using a previously described method [15] with some minor modifications [13]. Briefly, 25 µL of blueberry extract was allowed to react with 250 µL of 0.2 mM DPPH in 95% ethanol in a 96-well microplate. The plate was then incubated at room temperature (21 °C) for 30 minutes in the dark after which the absorbance was measured at 550 nm using a microplate reader. Scavenging capacity of the sample was compared to that of ascorbic acid as a positive control (0.1- 1.0 mM ascorbic acid).

The antiradical activity was calculated as a percentage of DPPH decolouration relative to a negative control using the following equation:

Antiradical activity (%) = (absorbance of control incubation – absorbance of the blueberry extract/ absorbance of control incubation) x 100. The degree of discoloration indicates the free-radical scavenging efficiency of the substances.

#### **Metal chelating activity assay**

This assay is based on the fact that in the presence of compound possessing chelating activity, the formation of complexes is decreased and the measurement of colour reduction used to estimate chelating activity of the test extract.

The ferrous-ion chelating (FCA) assay used by Singh and Rajini [16] and Chan et al. [17] was followed with slight modifications. Solutions of 2 mM FeSO<sub>4</sub> and 5 mM ferrozine were prepared. Diluted FeSO<sub>4</sub> (100 µl) was mixed with 100 µl of sample, followed by 100 µl of diluted ferrozine. Assay mixtures were allowed to equilibrate for 10 minutes before measuring the absorbance at 560 nm using a plate reader. The ability of the extract to chelate ferrous ions was calculated relative to a negative control using the equation:

Chelating activity % = (absorbance of control incubation – absorbance of blueberry extract/ absorbance of control incubation) x 100.

#### **Physical and chemical characteristics of blueberries**

The average berry weight was determined by weighing 50 berries from each cultivar. The pH was measured with a digital pH-meter at room temperature (21 °C). Dry matter was determined by drying 5–10 g blueberry sample in a vacuum oven at 70 °C, for 72 hours. The dried blueberries were weighed again and the dried matter that remained was determined using the following equation:

% dry weight = (dry weight/fresh weight) X 100

### Statistical analysis

Data were subjected to one-way analysis of variance for mean comparison, and intercultivar significant differences were calculated according to Tukey's multiple comparison test. Data are reported as mean  $\pm$  standard errors (SE). All tests were carried out in duplicate in two separate experiments. Differences at  $p < 0.05$  were considered significant. Linear regression analysis was carried out using Microsoft Office Excel 2003.

## RESULTS AND DISCUSSION

### Differences in total phenolic content among various cultivars of blueberries.

In Figure 1, the results of the Folin-Ciocalteu assay are shown. Among highbush cultivars, Jersey showed the highest total phenolic content (1103.5  $\mu\text{g/g}$  fresh weight) while Dixi cultivar had the lowest total phenolic contents (TPC) (562.3  $\mu\text{g/g}$ ) and the difference between the two cultivars was statistically significant ( $P < 0.0001$ ). Among the rabbiteye cultivars, Centurion had the highest TPC (1300.2  $\mu\text{g/g}$ ) followed by Maru (1266.4  $\mu\text{g/g}$ ), Rahi (1185.2  $\mu\text{g/g}$ ), Tifblue (1154.3  $\mu\text{g/g}$ ), and Ono cultivar (1061.4  $\mu\text{g/g}$ ). In general, rabbiteye cultivars had significantly higher ( $P = 0.0057$ ) TPC than highbush cultivars.

Cultivar specific variations in TPC have been previously assessed [10, 18, 19]. Ehlenfeldt and Prior [20] studied the chemical composition of 87 highbush blueberry cultivars and varieties and estimated 1.78 mg/g as a mean TPC for these cultivars. Recently, Skupien [21] studied the chemical composition of four highbush blueberry cultivars and reported that the TPC ranged from 1.94-3.07 mg/g fresh fruits.

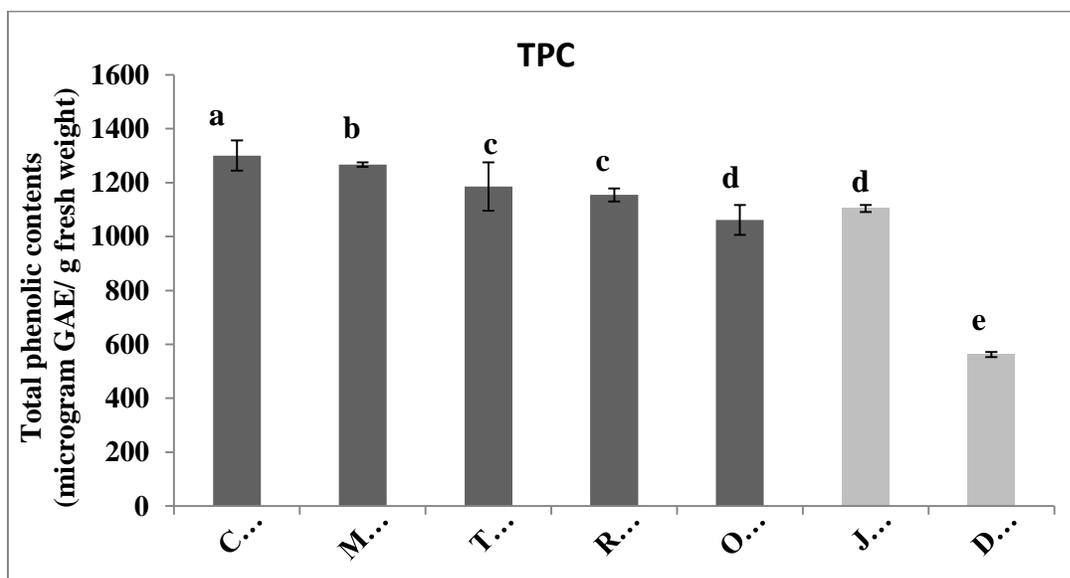


Figure 1. Total phenolic content (TPC;  $\mu\text{g GAE/g}$  fresh berries) of blueberry whole berries. The data were expressed as the means  $\pm$  SEM of duplicate determination in two separate experiments. Different letters indicate significant differences between cultivars.

### Differences in antioxidant activities among blueberry cultivars

Significant differences were found in antioxidant activities among various cultivars of blueberries as measured by the ferric reducing antioxidant power (FRAP). Among highbush cultivars, Jersey showed the highest antioxidant activity (8184  $\mu\text{mol/L}$ ) as measured by the FRAP method while Dixi cultivar had the lowest FRAP values (6444  $\mu\text{mol/L}$ ) and the difference was statistically significant ( $P < 0.0001$ ) between the two cultivars (Figure 2). Among the rabbiteye cultivars, Centurion had the highest FRAP value (9448  $\mu\text{mol/L}$ ) followed by Maru (8945  $\mu\text{mol/L}$ ), Tifblue (8004  $\mu\text{mol/L}$ ), Rahi (7840  $\mu\text{mol/L}$ ), and Ono cultivar (7556  $\mu\text{mol/L}$ ). In general, rabbiteye cultivars had significantly higher ( $P < 0.05$ ) FRAP values than highbush cultivars.

In other studies, significant differences in antioxidant activity among strawberry cultivars [19, 22, 23] and blueberry cultivars [10, 13, 24, 25] were observed.

A positive correlation between the TPC and the antioxidant activity (FRAP values) in all blueberry cultivars was observed (Table 1), confirming the findings previously observed for several species of berries [10, 24, 26, 27]. Recently, Castrejon et al. [28] reported that phenolic compounds other than anthocyanins in highbush blueberry fruits have contributed positively to the total antioxidant activity and suggested to implement that in future breeding programs.

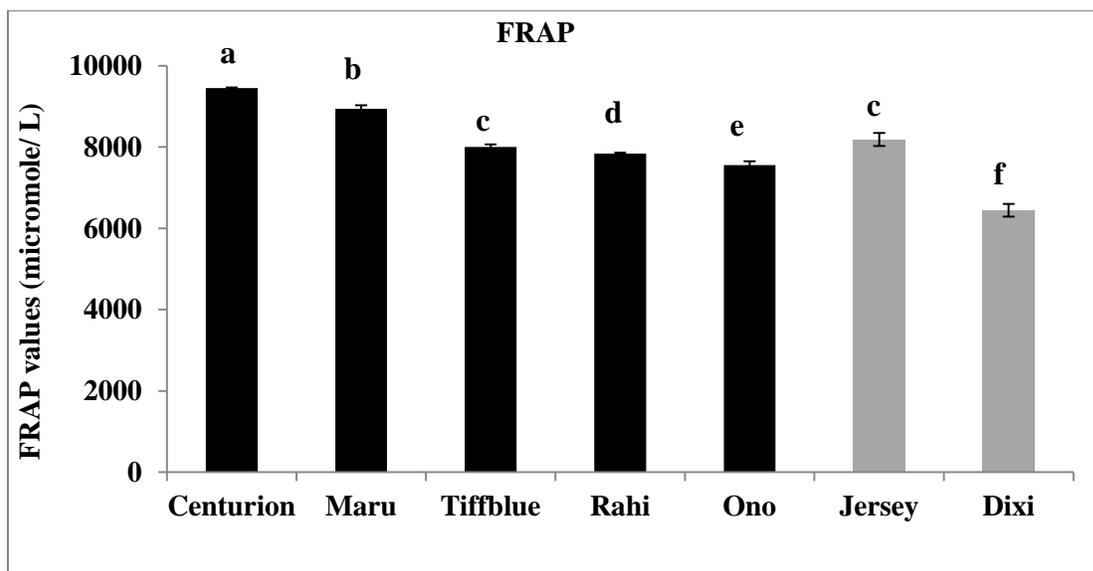


Figure 2. Antioxidant activity of water-soluble extracts from the berries of seven different blueberry cultivars as measured by the ferric reducing antioxidant power (FRAP) method. Data were presented as means  $\pm$  SEM of duplicate determinations in two separate experiments. Different letters indicate significant differences between cultivars.

### Free radical scavenging activity

The ability of the extracts from different cultivars to donate hydrogen was evaluated using the stable free radical DPPH. In the presence of hydrogen donors, DPPH is reduced and a stable free radical is formed from the scavenger. It is well known that free radicals are the

major cause of various chronic and degenerative diseases such as aging, coronary heart disease, inflammation, stroke, diabetes mellitus and cancer [29, 30] and can cause cellular injuries and initiate peroxidation of polyunsaturated fatty acids in biological membranes [1, 31].

Among highbush cultivars, Jersey showed significantly higher ( $P < 0.0001$ ) free radical scavenging activity in the DPPH assay (55%) than Dixi cultivar (36%). Among the rabbiteye cultivars (Figure 3), Centurion had the highest free radical scavenging activity (71%) followed by Maru (58%), Tifblue (57), Rahi (56%), and Ono cultivar (46%). Centurion showed significantly higher ( $P < 0.05$ ) free radical scavenging activity than the other cultivars while Ono cultivar showed significantly lower ( $P < 0.05$ ) free radical scavenging activity than the other cultivars (Figure 3).

In general, rabbiteye cultivars had significantly higher ( $P < 0.05$ ) free radical scavenging activity than highbush cultivars.

positive correlation between the total phenolic content and DPPH activity was observed (Table 1). Some studies also showed a positive correlation between DPPH activity and total phenolic contents in strawberry cultivars [19, 22]. The presence of variable quantities of diverse phytochemicals, other than polyphenolic compounds with potent antioxidant activities in fruits including blueberries may be behind the differences in the degree of correlation between the total phenolic contents and antioxidant/antiradical activities in different blueberry cultivars.

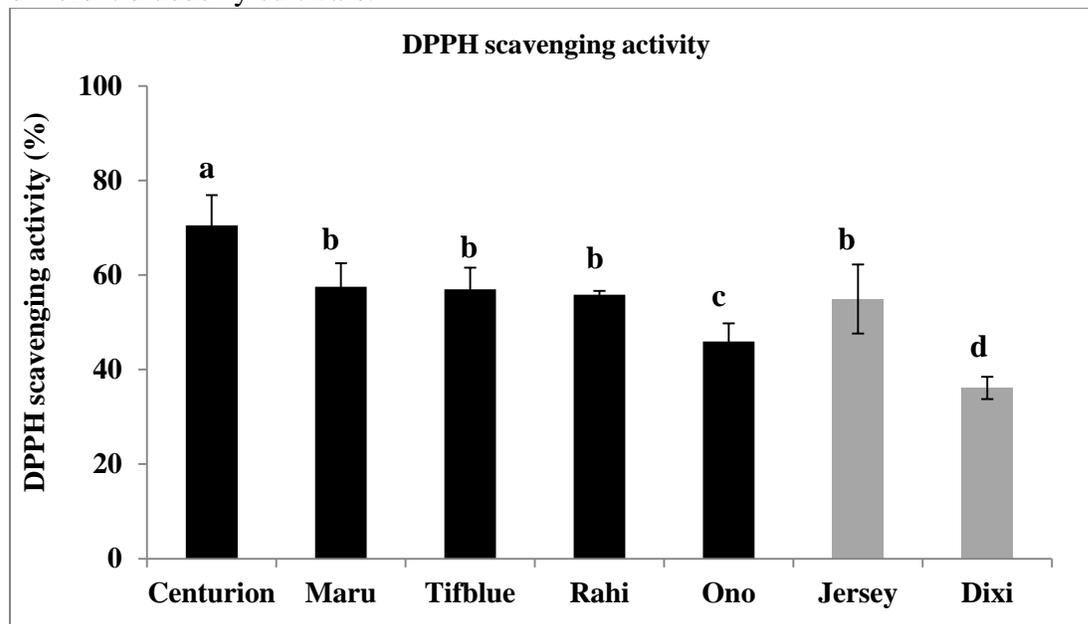


Figure 3. DPPH radical scavenging activity of water-soluble extracts from the berries of seven different blueberry cultivars (means  $\pm$  SEM). Data were presented as the percentage of DPPH radical scavenging and based on duplicate determinations in two separate experiments. Different letters indicate significant differences between cultivars.

### Metal chelating ability of extracts from different blueberry cultivars

Although both oxygen and iron are essential for life, because they are required for respiration and the activity of many enzymes [32], they are double edged swords. Iron, for example, has the ability to generate free radicals from peroxides by the Fenton reaction.

Consequently, minimizing of the ferrous ion concentration in the Fenton reaction can protect the body against oxidative damage [33, 34]. Oxygen, on the other hands, exists as a stable triplet biradical in the nature but when inhaled it is reduced gradually and finally metabolized to water. During the reduction process, variable amounts of reactive components, such as superoxide anion radicals, hydroxyle radicals, nitric oxide and the singlet oxygen are generated which are collectively called reactive oxygen species (ROS) [35, 36].

Extract from the berries of all cultivars showed 100% chelating effect of ferrous ions after 30 min incubation when diluted 1-2 folds (data not shown). In order to show the differences between the different cultivars, the extracts have been diluted 4 fold so the results in Figure 4 are based on 4-fold-diluted extracts.

Jersey cultivar showed significantly higher ferrous ion chelating activity than Dixi cultivar ( $P < 0.001$ ). Among rabbiteye cultivars, Centurion cultivar showed significantly higher ( $P < 0.005 - 0.001$ ) ferrous ion chelating activity than the other 4 cultivars. No significant differences were detected between Maru, Rahi, Tifblue and Ono cultivars (Figure 4) which showed a chelating activity ranging from 16-19%.

The importance of this finding is that the ferrous ion is the most powerful prooxidant among the various species of metal ions [37, 38]. It has been reported that extracts or compounds with chelating activity are believed to inhibit lipid peroxidation by stabilizing transition metals [39].

A positive correlation was observed between the chelating activity and the TPC in all cultivars (Table 1).

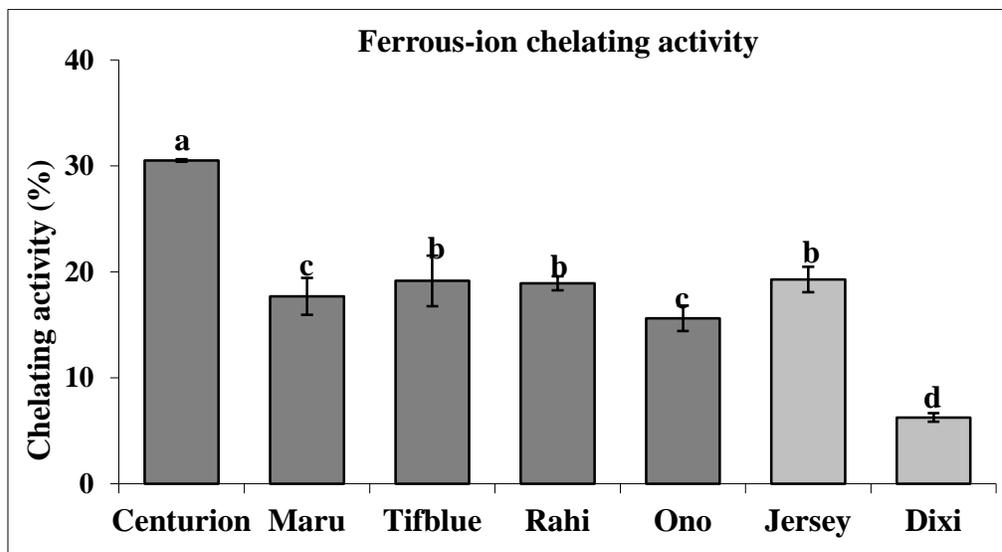


Figure 4. Metal chelating activity of seven different blueberry extracts. The results are expressed as means  $\pm$  SEM of duplicate determination in two separate experiments. Different letters indicate significant differences between cultivars.

Table 1. Correlation Coefficients between antioxidant activities [FRAP, DPPH radical scavenging activity and ferrous ion chelating activity (FICA)] and total phenolics contents (TPC) in fruits of different blueberry cultivars.

Cultivar	Correlation coefficient		
	FRAP vs TPC	DPPH vs TPC	FICA vs TPC
<u>Rabbiteye cultivars (<i>Vaccinium ashei</i>)</u>			
Centurion	0.8312	0.9988	0.9019
Maru	0.7823	0.9611	0.9110
Tifblue	0.7841	0.8356	0.8701
Rahi	0.9882	0.9994	0.7493
Ono	0.9452	0.9463	0.9327
<u>Highbush cultivars (<i>Vaccinium corymbosum</i>)</u>			
Jersey	0.9499	0.9696	0.9989
Dixi	0.7863	0.8355	0.8414

### Physical and chemical characteristics

Fruit weight, pH, and dry matter are presented in Table 2. Among the highbush cultivars, the weight of 50 berry sample showed that the berries of Dixi were significantly larger (1.63 vs 0.89 g/berry;  $P < 0.001$ ) than those of Jersey cultivar. Among rabbiteye cultivars, Maru has the largest fruits (1.85 g) whereas Tiffblue has the smallest ones (1.01 g) and the differences between these cultivars were significant ( $P < 0.05 - 0.001$ ). It seems that the TPC and antioxidant activity are negatively correlated with the weight of the berries. Similarly, Krüger et al. [40] quantified the total phenols, total anthocyanins, ascorbic acid, and antioxidant capacity in fruit juice of 23 black currant cultivars grown at Geisenheim, Germany and reported that the total phenols, total anthocyanins and antioxidant activity were negatively correlated with berry weight. The authors concluded that as long as the anthocyanins and other bioactive compounds are mainly localized in the skin of black currant fruit, the amounts of the bioactive compounds will be reduced when the ratio between surface area and fruit volume become too wide. Accordingly and from a health point of view, the berry fruit size should not be regarded as an important breeding aim of the breeders in the future.

The pH of Jersey blueberries (pH 3.41) is significantly higher ( $P < 0.0001$ ) than that of the Dixi blueberries (pH 3.17). Among rabbiteye cultivars, Rahi blueberries showed the highest pH (3.62), followed by Centurion (pH 3.59), Tifblue (pH 3.33), Ono (pH 3.3), and Maru (pH 3.27).

Moisture content in blueberries is estimated at 80.1-87.7% [21, 41]. It can be seen from Table 1 that within highbush cultivars, Jersey had significantly more ( $P < 0.0001$ ) dry weight content (19.02) than Dixi cultivar (16.3). Among rabbiteye cultivars, Centurion berries had the highest dry weight content (20.6%), followed by Ono (18.97%), Tifblue (18.8%), Rahi (17.99%), and Maru (14.57%) berries (Table 2). Recently, Skupien [21] estimated the dry weight content of Jersey cultivar grown in Poland to be 15.83% which is much lower than the value obtained in the present study. The difference may be attributed to the differences in environmental factors and growing conditions.

Table 2. Berry size and pH values of highbush (*Vaccinium corymbosum*) and rabbiteye blueberry cultivars (*Vaccinium ashei*).

Cultivar	Fruit weight (g/berry)	pH value	Dry weight (%)
<b>Rabbiteye cultivars (<i>Vaccinium ashei</i>)</b>			
Centurion	1.35 ± 0.001	3.59	20.60
Maru	1.85 ± 0.0008	3.27	14.57
Tifblue	1.01 ± 0.002	3.33	18.80
Rahi	1.48 ± 0.0002	3.62	17.99
Ono	1.55 ± 0.0007	3.3	18.98
<b>Highbush cultivars (<i>Vaccinium corymbosum</i>)</b>			
Jersey	0.89 ± 0.0007	3.41	19.02
Dixi	1.63 ± 0.0001	3.17	16.31

### Conclusions

In conclusion, our results clearly demonstrate that the fruits of highbush blueberry commonly consumed in New Zealand are excellent source of antioxidants. The results of the present study show that extracts from the berries of different blueberry cultivars have highly potent antioxidant/free radical scavenging and metal chelating activities under *in vitro* conditions, and might provide protection against oxidative damage. These activities are influenced significantly by the amount of phenolic compounds present in the berries of each cultivar.

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### CONFLICT OF INTEREST STATEMENT

The author declares that there is no conflict of interest regarding the publication of this paper.

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