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Total anthocyanins, chlorogenic acid concentration, antioxidant and *in ovo* anti-angiogenic activities of rabbiteye blueberries

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Abstract

Anthocyanins are the most abundant phytochemicals found in blueberries and exhibit several biological properties including antioxidant activity. This study investigated total anthocyanin concentration, chlorogenic acid concentration, antioxidant activity using FRAP assay and anti-angiogenic activity of five rabbiteye blueberry varieties ('Centurion', 'Maru', 'Rahi', 'Ono', 'Tifblue'). 'Tifblue' showed the highest total anthocyanin and chlorogenic acid concentrations while 'Rahi' contained the lowest. Interestingly, antioxidant activity was better correlated with chlorogenic acid concentration than anthocyanin. Anti-angiogenic properties of blueberry extracts were determined by chicken chorioallantoic membrane (CAM) assay. Blueberry extracts had macroscopic effects on CAM which were measured both quantitatively and qualitatively. In control CAM, a blood vessel network was formed with fine capillaries while there were fewer, shorter & more branched capillaries in blueberry extract treated CAMs. Even though some blood vessels or capillaries in the blueberry-treated CAMs were elongated, the vessels largely failed to interconnect to form a network. Three varieties, 'Centurion', 'Maru' and 'Tifblue', had the strongest anti-angiogenic impact. The best correlation between composition and anti-angiogenic activity was found with anthocyanin concentration rather than antioxidant activity. Blueberries contain various phytochemicals which contribute to their high antioxidant activity. It is well accepted that berry antioxidant activity plays an important role in the prevention of several diseases. However, this study demonstrated that blueberries exhibited other biological activity that is not necessarily related to their antioxidant activity but may have a role in disease prevention. Moreover, different phytochemicals are responsible for different biological activities.

Introduction

Rabbiteye blueberries *(Vaccinium ashei)* are one of the most cultivated blueberries worldwide. Rabbiteye blueberries were found to exhibit higher antioxidant activity than highbush blueberries (Su and Chien 2007). With their high antioxidant activity, they are believed to contribute to prevention of many chronic diseases including cancers. Water-soluble compounds responsible for color of blueberry fruits are anthocyanins, which are flavonoid polyphenols. Five anthocyanins are found in blueberries including cyanidin, delphinidin, petunidin, peonidin and malvidin (You *et al*., 2011; Li *et al*., 2012). Anthocyanins found in blueberries are glycosylated by glucose, galactose and arabinose. Several solvents such as methanol, ethanol, acetone or ethyl acetate have been used for blueberry polyphenolic extraction (Samappito and Butkup, 2010). Solvent extraction exhibits high extraction yield however, using harsh extracting solvent in food or nutraceutical industries should be avoided because the solvent residue might contaminate the product. There are several techniques used for quantification of total anthocyanin concentration in blueberries (Connor *et al*., 2002; You *et al*., 2011). By using high performance liquid chromatography (HPLC), one of the most common methods, detailed information of anthocyanin composition is provided. Phenolic acids are another subclass of polyphenolic compounds found in blueberries. Chlorogenic acid has been found in several food products such as potato, coffee and berry fruits (Clifford, 1999). Chlorogenic acid exhibits several biological properties such as antioxidant activity; DNA-protection from H_2O_2 induced oxidative stress (Xu *et al*., 2012) and anti-angiogenic effect (Kim *et al*., 2010).

Angiogenesis is the process of generating new blood vessels. At the early stage of life, neovascularisation is very common because blood supply and blood system is required in every part of the body. Angiogenic process is less required when

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mature, but can still be found during menstruation and wound healing. Interestingly, angiogenesis was found to play a key role in several disorders. Tumor metastases and endometriosis (Mathur *et al*., 2011) resulted from high angiogenic activity while reduced angiogenesis led to age-related macular degeneration (Kim *et al*., 2010). In cancers, angiogenesis provides oxygen and nutrient supply for tumor masses (cancer growth) and also a channel for cancer cells to travel to other parts of the body (metastasis). Therefore, inhibition of new blood vessel formation would have a significant role in both cancer prevention and therapy. Blueberries affect tumor growth by reducing the growth of tumor masses (Ravoori *et al.*, 2012) which means blueberries might have anti-angiogenic properties. Various methods have been used for screening of natural compounds with anti-angiogenic activity. Chicken chorioallantoic membrane (CAM) assay is an *in ovo* assay which has been used widely for screening of anti-angiogenic properties of food derived compounds (Veeramani and Veni, 2010).

In this study, we examined total anthocyanin and chlorogenic acid concentrations of 5 rabbiteye blueberry varieties grown in New Zealand. Biological properties of the blueberry extracts including antioxidant and anti-angiogenic properties were studied. The correlations between blueberry phytochemicals and their biological properties were also established.

Materials and Methods

Blueberry extract preparation

One hundred grams of frozen berry fruits were mixed with 100 mL MilliQ water and blended using stand mixer (Sunbeam, SM6200). The fruit mixture was transferred to centrifuge tubes and centrifuged at 10,000 g (Allegra TM 64 R Centrifuge, Beckman Coulter, CA, USA) for 5 minutes at 4°C. The supernatant was collected and kept at -20°C until analysis of antioxidant capacity. The extracts were thawed and kept on ice during antioxidant capacity assay. The supernatant used for HPLC analysis was then filtered through 0.2 µm PVDF filter and transferred into HPLC vials. The supernatant used for anti-angiogenic activity assay was sterilised using 0.22 µm sterilized PVDF filter and kept in sterilised microcentrifuge tube until analysis.

Anthocyanin and chlorogenic acid analysis

Anthocyanin and chlorogenic acid analysis was carried out using a modified high performance liquid chromatography (HPLC) method (Wang *et al*., 2000). The filtered blueberry extracts were placed in a temperature-control (4°C) sampling chamber. The HPLC system consisted of a Shimadzu HPLC CTO-20A (Shimadzu Corp., Kyoto, Japan) coupled with an auto-sampler (SIL-20AC) and a photo-diode array (PDA) detector SPD-M20A. Phenomenex Luna C18 (2) 150 × 4.6 mm (5 µm) reverse phase column (Phenomenex, North Shore City, NZ) was used for anthocyanin separation. Mobile phase consisted of 5% (v/v) aqueous formic acid (solvent A) and 100% HPLC grade methanol (solvent B). A linear gradient profile containing solvent A with the following proportions (v/v) of solvent B: 0-1 min, 14% B; 1-10.24 min, 14-17% B; 10.24-35.28 min, 17-23% B; 35.28-64.59 min, 23-47% B; 64.59-66.59 min, 47-14% B. Total running time was 70 min with 1 mL/min flow rate. Cyanidin 3-O-glucoside chloride was used as the anthocyanin standard. Peak areas of the anthocyanin were quantified at 520 nm. Chlorogenic acid was determined at 280 nm in comparison with the peak area of chlorogenic acid standard. The determination of anthocyanin and chlorogenic acid was done in triplicate. To determine the mean blueberry weight of each cultivar, 30 ripe berries were weighed individually.

Ferric reducing antioxidant power (FRAP)

FRAP assay is a widely used method for determination of antioxidant activity of tested samples (Benzie and Strain, 1996). In this study, the procedure described by Benzie and Strain with some modifications (Molan *et al*., 2009) was followed. Briefly, the FRAP reagent was prepared by mixing 300 mmol/L sodium acetate buffer pH 3.6, 10 mmol/L 2,4,6-tripyridyl-s-triazine (TPTZ) prepared in 40 mmol/L HCl and 20 mmol/L ferric chloride (10:1:1). Aliquots of 8.5 µl of blueberry samples were mixed with FRAP reagent (275 µL) in a 96-well plate. The plate was incubated at 37°C in the dark for 30 minutes and then the absorbance of the mixture was measured at 595 nm. The FRAP values were expressed as mg $FeSO_4$ equivalent /g frozen berries.

Evaluation of anti-angiogenic properties of blueberry extracts

Anti-angiogenesis activity of blueberry extracts was evaluated using Chicken Chorioallantoic Membrane (CAM) assay. The assay was carried out based on the study of Ozgurtas (2008) with some modification. Fertilised eggs were purchased from Golden Coast Commercial, New Zealand. The eggs were incubated in an incubator (R. COM Digital incubator, 20-PRO, United Kingdom) for 5 days at 37°C with fully humidified atmosphere. On embryonic day five, the eggs were arranged

vertically before cutting the egg shell. The egg shell was cut open from the wide end, above the air sac, and the inner membrane was left intact. The blueberry extracts (30 µL) were applied directly on the membrane close to the embryo and then the opening was sealed with parafilm. The eggs were incubated for 2 more days using the same incubator. On day seven, the membrane was removed and angiogenesis was photographically monitored (Olympus, Japan). A 0.25 cm^2 area of CAM, close to the major blood vessel, was selected for blood vessel counting. The blood vessels were counted using gridline intersection method. The blood vessels that intersected the grid vertically and horizontally were counted and calculated for blood vessel density (the number of grid blocks intersected times the linear size of the grid blocks) (Knoll *et al*., 1999).

Percentage inhibition of each extract was calculated as follows:

$$\text{Percentage inhibition} = \frac{(\text{Blood vessel density}_{\text{Control}} - \text{Blood vessel density}_{\text{BB}}) \times 100}{\text{Blood vessel density Control}}$$

Statistical analysis

Anthocyanin and chlorogenic acid analyses were carried out in triplicate. Antioxidant capacity using FRAP assay was done in 3 replicate wells in each of 3 independent experiments. Data are expressed as means ± S.E. Tukey's test was used for mean comparisons and the differences were considered statistically significant at $P < 0.05$. Determination of the correlation between two variables was performed (using Pearson's correlation coefficient, R). All statistical tests were analysed using the SAS program for Windows version 9.2 (SAS Institute Inc., Cary, NC, USA).

Results and Discussion

Anthocyanins and chlorogenic acid

Total anthocyanin concentrations of five rabbiteye blueberry cultivars extracted with MQ water were significantly different from each other ($P < 0.05$) (Table 1). The highest total anthocyanin concentration was 0.61 mg/g frozen berries (FB) in 'Tifblue' while the lowest was found in 'Rahi' at 0.19 mg/g FB. The total anthocyanin concentrations of rabbiteye blueberries reported here were lower than other studies, which is mainly due to the solvent used in this experiment. Generally, non-polar solvents or acidified solvents are used for anthocyanin extraction because they give higher yield and anthocyanins are more stable at low pH condition (Nicoue *et al*., 2007). You and colleagues (2011) demonstrated that total anthocyanin concentration of rabbiteye blueberries extracted by acidified 80% methanol ranged from 1.40 to 2.24 mg/g fresh weight. An aqueous highbush blueberry extract contained approximately 0.85 mg/g berries as measured by spectrophotometric method (Saper *et al.*, 1983).

In this study, HPLC analysis revealed that 13 – 15 glycosylated anthocyanidins were found in rabbiteye blueberries. Five anthocyanidins found were delphinidin, malvidin, cyanidin, petunidin and peonidin. Malvidin was the major anthocyanidin in this study which accounted for approximately 40-50% of total anthocyanins. Blueberries contain wide ranges of anthocyanins in nature, with 15 glycosylated anthocyanins plus additional acylated anthocyanins reported in highbush blueberries (Pranprawit, 2013). Rabbiteye blueberries have previously been reported with 9 or 10-13 anthocyanins (You *et al*., 2011; Li *et al.*, 2012), with malvidin as the major component as in this work.

Chlorogenic acid concentration was the highest in 'Tifblue' cultivar at 0.61 mg/g frozen berries (Table 1). The lowest chlorogenic acid concentration was found in 'Rahi' cultivar. In comparison with other studies carried out at ambient temperature, our study exhibited a similar range of concentration. Chlorogenic acid concentration of highbush blueberries, grown in New Zealand and extracted with 5% formic acid at room temperature (25 °C), ranged from 0.4 to 1.2 mg/g frozen berries (Pranprawit, 2013), while lowbush blueberries extracted with 0.1% HCl in methanol contained approximately 1.1 mg/g fruit weight (Rodriguez-Mateos *et al*., 2011). Chlorogenic acid has high solubility in water therefore solvent or acidified solvent system did not have any effect on chlorogenic acid concentration.

'Tifblue' exhibited the highest FRAP value among 5 cultivars while 'Rahi' showed the lowest antioxidant activity as measured by FRAP assay (Table 1). The FRAP values measured in this study ranged from 3.51 – 6.09 mg $FeSO_4$ equivalent/g frozen berries. FRAP values in other studies varied widely (0.17 – 10.95 mg $FeSO_4$ equivalent/g berries (Remberg *et al*., 2006; Vanzani *et al.*, 2011).

Anti-angiogenic property of blueberry extracts

Neovascularization from the existing blood vessels of each tested CAM was compared to a control CAM. Chick embryos were found to be well and healthy however, the differences were noticeable between control and blueberry-treated CAMs. Blood vessel network formed on the control CAM while in all blueberry-treated CAM, the blood vessels elongated

Table 1. Total anthocyanins, chlorogenic acid concentration and antioxidant activity (FRAP) of 5 rabbiteye blueberry variety

	'Centurion'	'Maru'	'Rahi'	'Ono'	'Tifblue'
Total Anthocyanin (mg/g frozen berries)	0.32 ± 0.03^{c}	0.51 ± 0.04^{b}	0.19 ± 0.02^{e}	0.29 ± 0.05^{d}	0.61 ± 0.02^{a}
Chlorogenic acid concentration (mg/g frozen berries)	0.24 ± 0.02^{d}	0.28 ± 0.05^{c}	0.18 ± 0.04^{e}	0.33 ± 0.05^{b}	0.61 ± 0.34^{a}
FRAP value ($mgFeSO_4$ equivalent/g frozen berries)	3.80 ± 0.05^{d}	3.91 ± 0.03^{c}	3.51 ± 0.04^{e}	4.53 ± 0.04^{b}	6.09 ± 0.04^{a}

Data expressed as mean ± S.E (n = 3) Different superscript letters indicate significant differences amongst cultivars within a row ($P < 0.05$).

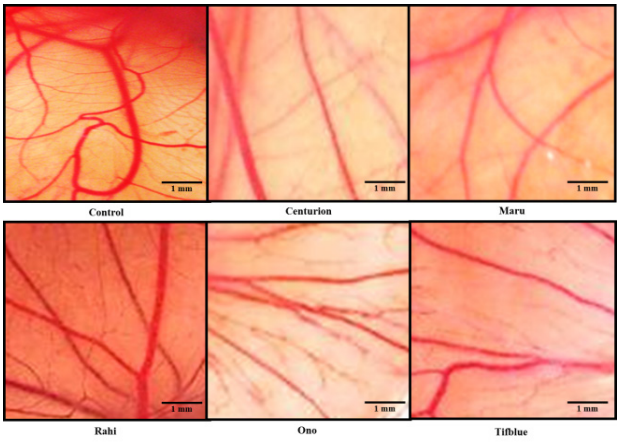

Figure 1. Qualitative observation of anti-angiogenic properties of rabbiteye blueberry extracts (30 µL taken from 180 mL crude extract, crude extract contained 100 g berry fruit and 100 mL MQ water) on CAM

but failed to form a blood vessel network (Figure 1). There are 2 major types of angiogenesis; one is sprouting where the original blood vessel elongate and sprout laterally like plant rooting. Another one is intussusceptive angiogenesis where the original blood vessel diverges and new blood vessels grow into the center. Both types of angiogenesis are found in virtually all tissues and organs and are important for blood network formation (Risau, 1997). The macroscopic observations used here suggested that blueberry extracts have an anti-angiogenic effect. Previously, commercial bilberry extract (100 ng) was used to study anti-angiogenic activity using CAM assay. The researchers found that effect of the extracts on CAM blood vessel was restricted to the area underneath the bilberry solution. The blood vessels under bilberry solution were found to be thinner and less branched (Ozgurtas *et al.*, 2008). In contrast, the effect of the blueberry extracts used in this study was not localised and was found in an area further away from where the blueberry extract was placed.

Not only qualitative changes were observed

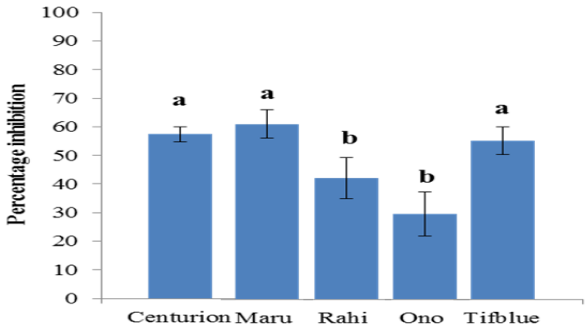

Figure 2. Percentage inhibition of blood vessel density of rabbiteye blueberry extracts. Data are expressed as the mean ± SE (n = 10). Different letters indicate significant difference ($P < 0.05$)

in this study, quantitative determination was also carried out using 10 x 10 grids. The percentage inhibition of blood vessel density is shown in Figure 2. 'Maru', 'Tifblue' and 'Centurion' exhibited high anti-angiogenic activity while 'Rahi' and 'Ono' had significantly lower anti-angiogenic effect on CAM ($P < 0.05$). The anti-angiogenic activity of berry fruit has been reported by several in vitro assays. Vascular endothelial growth factor (VEGF), which has an important role in angiogenic regulation, was inhibited by blueberry extract. The extract also interfered with human microvascular endothelial cell tube formation in Matrigel assay (Roy *et al.*, 2002). Bagchi *et al.* (2004) demonstrated that wild blueberry extract inhibited H_2O_2-induced VEGF by 75% in HaCaT cell assays. However, this is the first report of anti-angiogenic activity of rabbiteye blueberries using CAM assay.

Correlation between anthocyanins, chlorogenic acid, antioxidant and anti-angiogenic activities of blueberry extracts

Although assays were all performed in triplicate, the same samples were not used for each assay, so

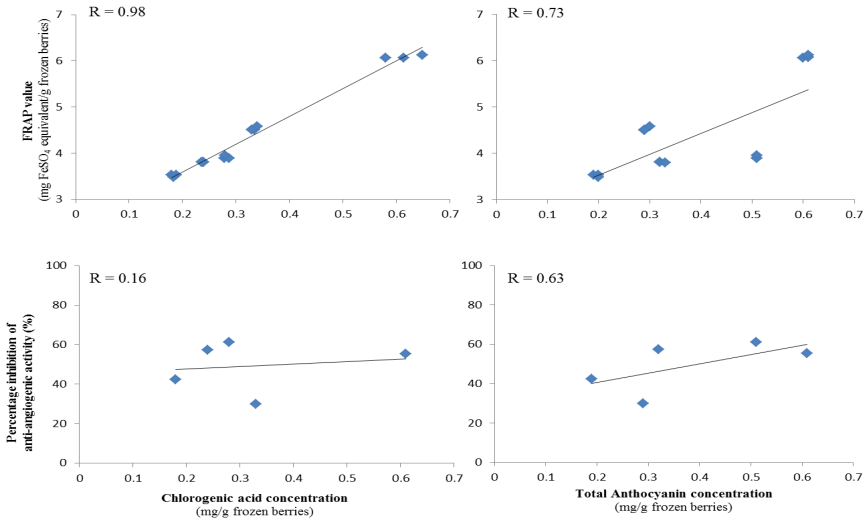

Figure 3. Correlation coefficient (R values) between phytochemicals [total anthocyanin and chlorogenic acid concentration] and antioxidant activity as measured by the ferric reducing antioxidant power (FRAP) or percentage inhibition of anti-angiogenic activity

some correlations are based on only five observations. The chlorogenic acid concentration has a strong and positive correlation (R = 0.98, P < 0.0001) with antioxidant activity of blueberry extracts (Figure 3a). Chlorogenic acid was a major contributor to antioxidant activity of *Lonicera japonica* (Flos Lonicerae) extracts measured by FRAP assay (Wu, 2007). Total anthocyanin content was not as highly correlated (R = 0.73, P = 0.0016) with FRAP value (Figure 3b).

Anti-angiogenic properties of the extracts were better correlated with total anthocyanins (R = 0.63, P = 0.25) than with total antioxidant activity (R = 0.06, P = 0.75) or chlorogenic acid (R = 0.16, P = 0.79). However, with only 5 points, these P values indicate that the correlations were not significant. Previously, antioxidant and anti-angiogenic activities of berry extracts were studied. It was found that grape seed extract which exhibited high antioxidant activity failed to decrease VEGF expression while other berry extracts could lower VEGF expression induced by TNFα (Tumor necrosis factor alpha). The researchers then focused on anti-angiogenic activity of pure flavonoid compounds eg. catechin, ferrulic acid and rutin in comparison with α-tocopherol (as an example of high antioxidant compound). They have found that all pure flavonoid compounds decreased VEGF expression in HaCaT cells in comparison with control and cells treated with α-tocopherol (Roy *et al*., 2002). This study concluded that anti-angiogenic activity does not relate to total antioxidant activity but depends on particular phytochemicals found in berries which is in agreement with our finding.

Conclusion

Total anthocyanin and chlorogenic acid were abundant in rabbiteye blueberries. Rabbiteye blueberries grown in New Zealand were found with 13-15 anthocyanins. The extracts showed antioxidant activities which were similar to other studies. Moreover, we have demonstrated that the extracts possessed anti-angiogenic properties in CAM assay. Chlorogenic acid was found to correlate well with antioxidant activity but not with anti-angiogenesis; while anthocyanin concentration was better related to anti-angiogenic properties than to antioxidant properties. Although blueberries are a good source of antioxidants, other biological activity such as anti-angiogenesis appears to be related to specific bioactives in the fruits.

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