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Antioxidant properties of anthocyanins extracted from litchi (*Litchi chinenesis* Sonn.) fruit pericarp tissues in relation to their role in the pericarp browning

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Abstract

Anthocyanins were extracted and purified from litchi fruit pericarp and their antioxidant properties were investigated. Effects of exogenous anthocyanin treatments on pericarp browning and membrane permeability of harvested litchi fruit were also evaluated. Anthocyanins from litchi fruit pericarp strongly inhibited linoleic acid oxidation and exhibited a dose-dependent free-radical-scavenging activity against DPPH radical, superoxide anions and hydroxyl radical. The degradation of deoxyribose by hydroxyl radicals was shown to be inhibited by anthocyanins acting mainly as chelators of iron ions rather than directly scavenging hydroxyl radicals. Anthocyanins were also found to have excellent reducing power. The reducing power of anthocyanins, ascorbic acid and butylated hydroxytoluene all at 100 μg/ml were 3.70, 0.427 and 0.148, respectively, indicating that anthocyanins from litchi pericarp had a strong electron-donating capacity. Furthermore, application of anthocyanins to harvested litchi fruit significantly prevented pericarp browning and delayed the increase in membrane permeability. It was therefore suggested that anthocyanins could be beneficial in scavenging free radicals and reducing lipid peroxidation of litchi fruit pericarp.

Keywords: *Litchi chinensis* Sonn.; Anthocyanins; Antioxidant activity; Reducing power; Free radical; Lipid peroxidation

1. Introduction

Oxidation is essential to many living organisms for the production of energy necessary for biological processes. Oxygen-centred free radicals, also known as reactive oxygen species (ROS), including superoxide, hydrogen peroxide, hydroxyl ($HO^{\bullet}$), peroxyl ($ROO^{\bullet}$) and alkoxyl ($RO^{\bullet}$), are produced in vivo during oxidation (Bloknina, Virolainen, & Fagerstedt, 2003). ROS are not only strongly associated with lipid peroxidation, leading to food deterioration, but are also involved in development of a variety of diseases, including cellular aging, mutagenesis, carcinogenesis, coronary heart disease, diabetes, and neurodegeneration (Halliwell & Gutteridge, 1999; Moskovitz, Yim, & Choke, 2002). Although almost all organisms possess antioxidant defence and repair systems to protect against oxidative damage, these systems are insufficient to prevent the damage entirely (Simic, 1988).

Fruits and vegetables contain different antioxidant compounds, such as ascorbic acid, tocopherol, glutathione and carotenoids, which may contribute to protection against oxidative damage (Bloknina et al., 2003). Recent research results have shown that anthocyanins from edible fruits were effective antioxidants in vitro (Einbond, Reynertson, Luo, Basile, & Kennelly, 2004). Antioxidative properties of anthocyanins arise from their high reactivity as hydrogen or electron donors, and from the ability of the polyphenol-derived radicals to stabilize and delocalize the unpaired electron, and from their ability to chelate transition metal

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ions (termination of the Fenton reaction) (Rice-Evans, Miller, & Paganga, 1997). Thus, anthocyanins may play a role in the antioxidant ability related to fruit browning.

Litchi (*Litchi chinensis* Sonn.) is a tropical fruit originating from China, with a bright red attractive pericarp surrounding a white aril (Nakasone & Paull, 1998). Litchi pericarp contains a large amount of anthocyanins, which are responsible for the red colour. In our preliminary study, the anthocyanin content of pericarp tissues of harvested litchi fruit was 0.20 mg/g fresh weight (Duan & Jiang, unpublished data). Cyanidin-3-glucoside (Lee & Wicker, 1991; Zhang, Quantick, & Grigor, 2000), cyanidin-3-rutinoside (Lee & Wicker, 1991; Sarni-Manchado, Le Roux, Le Guerneve, Lozano, & Cheynier, 2000), malvidin-3-glucoside (Lee & Wicker, 1991) and quercetin-3-rutinoside (Sarni-Manchado et al., 2000) were identified as the major monomeric anthocyanins pigments, while cyanidin-3-rutinoside was the most important pigment of litchi (*var.* Huaizhi) pericarp tissues (Zhang, Pang, Yang, Ji, & Jiang, 2004). However, little information on the antioxidant capability of the anthocyanins from litchi fruit peel is available.

The objective of the current research was to evaluate the antioxidant properties of anthocyanins from litchi fruit pericarp and then investigate the effects of exogenous anthocyanin treatments on the pericarp browning of harvested litchi fruit.

2. Materials and methods

2.1. Plant materials

The litchi fruit (*Litchi chinensis* Sonn.) cv. Huaizhi, at about 80% maturity, were obtained from a commercial orchard in Guangzhou, China. Fruits were selected for uniformity of shape and colour, and blemished and diseased fruits were discarded. For in vivo experiments, the fruits were dipped for 3 min in a 0.1% Sportak fungicide solution and air-dried for 2 h at 28 °C prior to anthocyanin treatments.

2.2. Extraction and purification of anthocyanins

Anthocyanins were extracted and purified according to the method of Zhang et al. (2004) with some modifications. A total of 100 g fresh litchi peel tissues was extracted overnight in an extraction medium consisting of 0.5 M HCl at 25 °C. Extract was filtered through Whatman No. 1 paper, and the filter residue was re-extracted until the absorbance of the extract at 510 nm was $\leqslant$0.01. Filtrates were combined and concentrated to a small volume at 40 °C by a rotary evaporator. The concentrated filtrates were then loaded onto an Amberlite XAD-7 resin column (1.5×40 cm) (Sigma), and washed with distilled water, followed by an elution with 0.1% HCl in methanol. The fractions with the highest absorbance at 510 nm were pooled and evaporated to remove methanol and dried under vacuum at 35 °C. The residue was re-dissolved in 20 ml of methanol for the analysis of antioxidant properties.

2.3. Antioxidant activity in a linoleic acid system

The antioxidant activity of anthocyanins against lipid peroxidation was measured through ammonium thiocyanate assay, as described by Takao, Kitatani, Watanabe, Yagi, and Sakata (1994) with some modifications. The reaction solution, containing 0.2 ml of 100 μg/ml anthocyanin extract, 0.2 ml of linoleic acid emulsion (25 mg/ml in 99% ethanol) and 0.4 ml of 50 mM phosphate buffer (pH 7.4), was incubated in the dark at 40 °C. A 0.1 ml aliquot of the reaction solution was then added to 3 ml of 70% (v/v) ethanol and 0.1 ml of 30% (w/v) ammonium thiocyanate. Precisely 3 min after the addition of 0.1 ml of 20 mM ferrous chloride in 3.5% (v/v) hydrochloric acid to the reaction mixture, the absorbance of the resulting red colour was measured at 500 nm. Aliquots were assayed every 24 h until the day after the absorbance of the control solution (without anthocyanin extract) reached maximum value. Butylated hydroxytoluene (BHT) was used as positive control.

2.4. Scavenging activity of DPPH radical

Scavenging activity of anthocyanins against DPPH radicals was assessed according to the method of Larrauri, Sanchez-Moreno, and Saura-Calixto (1998) with some modifications. Briefly, 0.1 ml of various concentrations of anthocyanin was mixed with 2.9 ml of 0.1 mM DPPH-methanol solution. After the solution was incubated for 30 min at 25 °C in dark, the decrease in the absorbance at 517 nm was measured. Control contained methanol instead of the antioxidant solution while blanks contained methanol instead of DPPH solution. In the experiment, L-ascorbic acid and BHT were used as positive controls. The inhibition of DPPH radicals by the samples was calculated according to the following equation: DPPH-scavenging activity (%) = [1 − (absorbance of sample − absorbance of blank)/absorbance of control] × 100.

2.5. Determination of superoxide radical-scavenging activity

Superoxide radicals were generated by the method of Giannopolites and Ries (1977), described by Siddhurajua, Mohanb, and Beckera (2002), with some modifications. All solutions were prepared in 0.05 M phosphate buffer (pH 7.8). The photo-induced reactions were performed in an aluminium foil-lined box with two 30 W fluorescent lamps. The distance between the reaction solution and the lamp was adjusted until the intensity of illumination reached about 4000 lux. A 30 μl aliquot of various concentrations of anthocyanins was mixed with 3 ml of reaction buffer solution (1.3 μM riboflavin, 13 mM methionine, 63 μM nitro blue tetrazolium and 100 μM EDTA, pH 7.8). The reaction solution was illuminated for 15 min at

25 °C. The reaction mixture, without sample, was used as a control. The scavenging activity was calculated as follows: scavenging activity (%) = (1 − absorbance of sample/absorbance of control) × 100.

2.6. Determination of reducing power

The reducing power was determined according to the method of Oyaizu (1986). A 0.25 ml aliquot of various concentrations of anthocyanins was mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50 °C for 20 min. After 2.5 ml of 10% trichloroacetic acid (w/v) were added, the mixture was centrifuged at 650*g* for 10 min. A 5 ml aliquot of the upper layer was mixed with 5 ml of distilled water and 1 ml of 0.1% ferric chloride, and the absorbance at 700 nm was measured. A higher absorbance indicated a higher reducing power.

2.7. Determination of inhibitory effect on deoxyribose degradation

Inhibitory effect of anthocyanins on deoxyribose degradation was determined by measuring the reaction activity between either antioxidants and hydroxyl radicals (referred to as non-site-specific scavenging assay) or antioxidants and iron ions (referred to as site-specific scavenging assay), described by Lee, Kim, Kim, and Jang (2002). For the non-site-specific scavenging assay, a 0.1 ml aliquot of different concentrations of anthocyanin was mixed with 1 ml of reaction buffer (100 μM $FeCl_3$, 104 μM EDTA, 1.5 mM H_2O_2, 2.5 mM deoxyribose, and 100 μM L-ascorbic acid, pH 7.4) and incubated for 1 h at 37 °C. A 1 ml aliquot of 0.5% 2-thiobarbituric acid in 0.025 M NaOH and 1 ml of 2.8% trichloroacetic acid were added to the mixture and it was heated for 30 min at 80 °C. The mixture was cooled on ice and the absorbance was measured at 532 nm. Site-specific scavenging activity, which represented the ability of anthocyanins to chelate iron ions and interfere with hydroxyl radical generation, was measured using the same reaction buffer without EDTA. Percent inhibition of deoxyribose degradation was calculated as (1 − absorbance of sample/absorbance of control) × 100.

2.8. Determination of total anthocyanin content

Anthocyanin content in methanolic extracts from litchi pericarp tissues was determined using the pH differential method described by Zhang et al. (2004). Results were expressed as milligrammes of cyanidin-3-glucoside equivalents per ml of extract.

2.9. Anthocyanin treatments

Anthocyanin, extracted and purified from litchi pericarp tissues, was used for litchi fruit treatment. Infiltration was done by immersing 40 fruits in a 3 l of anthocyanin solutions at 50 mg/l in a desiccator under an air pressure of 53 kPa for 3 min, with three replicates per treatment. The fruit were dried for 1 h at 28 °C, packed in polyethylene bags (0.03 mm thick), sealed with a rubber band and stored at 28 °C. The fruit treated with water was used as control. The fruit from each replicate of each treatment, removed after being held for 2, 4, or 6 days, were sampled for measurements of pericarp browning index and membrane permeability.

2.10. Pericarp browning assessment

Pericarp appearance was assessed by measuring the extent of the total browned area on each fruit pericarp of 30 fruit using the following scale: 1 = no browning (excellent quality); 2 = slight browning; 3 = <1/4 browning; 4 = 1/4–1/2 browning; 5 = >1/2 browning (poor quality). The browning index was calculated as ∑(browning scale × percentage of corresponding fruit within each class). The subjective evaluation of pericarp browning index correlated well with the objective determination of the value of the absorbance at 410 nm of the pericarp extract (Jiang, 2000).

2.11. Measurement of membrane permeability

Membrane permeability, expressed by relative leakage rate, was determined according to the method of Jiang and Chen (1995). Discs were removed with a cork borer (10 mm in diameter) from the equatorial region of 30 fruit. Thirty discs (about 2 g) were rinsed twice and then incubated in 25 ml of 0.3 M mannitol in distilled water at 25 °C, and shaken for 30 min. Electrolyte leakage was determined with a conductivity meter (Model DDS-11A, Shanghai Scientific Instruments). Another batch of discs was boiled for 15 min in 25 ml distilled water and then cooled to 25 °C to assess total electrolytes. The relative leakage was expressed as a percentage of the total electrolytes.

2.12. Statistical analyses

All analyses were performed in triplicate. The data were expressed as means ± standard error (SE) and analysed by SPSS (version 10.0). One-way analysis of variance (ANOVA) and Tukey multiple comparisons were carried out to test any significant differences between the means. Differences between means at the 5% level were considered significant.

3. Results and discussion

3.1. Extraction and purification of anthocyanin from litchi fruit pericarp

Anthocyanins were extracted from litchi pericarp tissues with 0.1% HCl and purified by Amberlite XAD-7

chromatography. This extraction method was used previously by Zhang et al. (2004) to recover high levels of litchi anthocyanins. The Amberlite XAD-7 resin column was also used previously and shown to have high affinity for the anthocyanins (Baublis, Spomer, & Berber-Jimenez, 1994). The total content of anthocyanins from litchi pericarp tissues was about 18.6 mg/100 g fresh weigh after purification. Our previous work revealed that litchi (*var.* Huaizhi) pericarp tissues contained mainly cyanidin-3-rutinside, representing 94.3% of the total anthocyanins. Three other anthocyanins were present but only in small amounts (Zhang et al., 2004).

3.2. Antioxidant activity in linoleic acid system

In the present study, the antioxidant activity of anthocyanins from litchi pericarp tissues was determined by peroxidation of linoleic acid using the ferric thiocyanate method (FTC). During linoleic acid peroxidation, peroxides were formed and these compounds oxidized Fe^{2+} to Fe^{3+}. The Fe^{3+} ion formed a complex with SCN^{-}, which had a maximum absorbance at 500 nm (Takao et al., 1994). Thus, a high absorbance value was an indication of high peroxide formation during the emulsion incubation. As shown in Fig. 1, the absorbance of the control at 500 nm increased to a maximal value of 3.36 after 72 h, while the anthocyanin sample increased to 0.867. These results indicate that anthocyanins can significantly inhibit peroxidation of linoleic acid and reduce formation of hydroperoxide, thus implying that the anthocyanins are powerful natural antioxidants. However, the antioxidant activity of litchi anthocyanins was slightly less effective than that of BHT, a widely used commercial antioxidant.

3.3. Reducing power

It has been reported that reducing power is associated with antioxidant activity and may serve as a significant reflection of the antioxidant activity (Chang, Yen, Huang, & Duh, 2002; Yen & Duh, 1993). As shown in Fig. 2, anthocyanins from litchi pericarp exhibited a higher reducing power than BHT and ascorbic acid, suggesting that the anthocyanins had strong electron-donating capacity. The reducing power of litchi anthocyanins, ascorbic acid and BHT, at 100 μg/ml, were 3.70, 0.427 and 0.148, respectively. Furthermore, a linear relationship existed between concentration (⩽100 μg/ml) and reducing power of ascorbic acid, BHT and anthocyanins. The corresponding correlation coefficients were 0.9997 ($y = 0.0036x + 0.0412$), 0.9958 ($y = 0.0014x + 0.025$) and 0.9036 ($y = 0.0291x + 1.072$) for ascorbic acid, BHT and anthocyanins, respectively.

3.4. DPPH radical-scavenging activity

Free radical-scavenging is one of the known mechanisms by which antioxidants inhibit lipid peroxidation (Bloknina et al., 2003; Rice-Evans et al., 1997). The DPPH radical-scavenging activity has been extensively used for screening antioxidants from fruit and vegetable juices or extract (Robards, Prenzler, Tucker, Swatsitang, & Glover, 1999; Sanchez-Moreno, 2002). Fig. 3 shows the DPPH radical-scavenging activity of litchi anthocyanins, ascorbic acid and BHT. The anthocyanins significantly inhibited the activity of DPPH radicals in a dose-dependent manner. Anthocyanins had the highest scavenging activity, followed by ascorbic acid and BHT. At 50 μg/ml, the scavenging effects were 91.3%, 20.1% and 9.73% for anthocyanins, ascorbic acid and BHT, respectively, while almost complete inhibition of DPPH radical activity was observed when the anthocyanins were used at 100 μg/ml. It appears that litchi anthocyanins have a strong hydrogen-donating capacity and can efficiently scavenge DPPH radicals. Gracia-Alonso, de Pascual-Teresa, Santos-Buelga, and Rivas-Gonzalo (2004) investigated antioxidant properties of 28 different fruits using TBARS and ABTS methods, and found that fruits with high antioxidant activity were all rich in anthocyanins. Einbond et al. (2004) reported that the DPPH radical-scavenging activity of *Surinam cherry*,

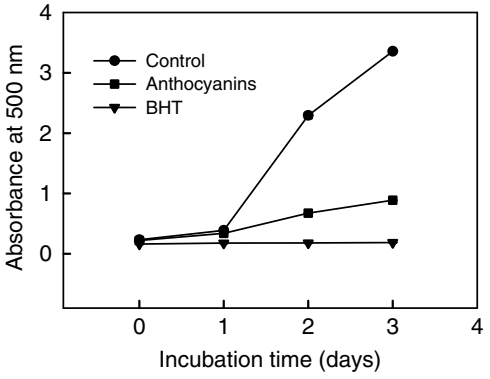

Fig. 1. Antioxidant activity of anthocyanins from litchi pericarp tissues, measured by ferric thiocyanate method (FTC). Each value is presented as mean ± standard error ($n = 3$). The vertical bars indicate standard errors where they exceeded the symbol size.

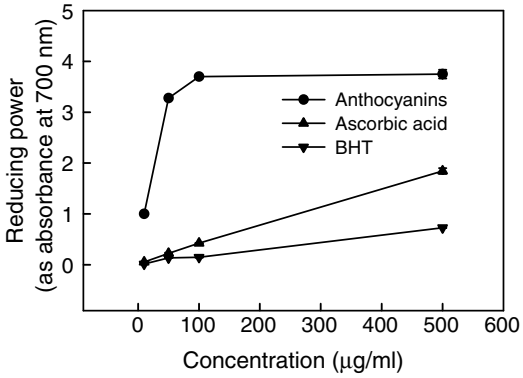

Fig. 2. Reducing power of anthocyanins from litchi pericarp tissues. Each value is presented as mean ± standard error ($n = 3$). The vertical bars indicate standard errors where they exceeded the symbol size.

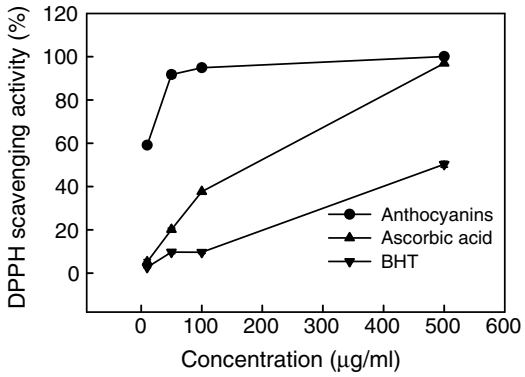

Fig. 3. Scavenging effects of anthocyanins from litchi pericarp tissues on DPPH radicals. Each value is presented as mean ± standard error ($n = 3$). The vertical bars indicate standard errors where they exceeded the symbol size.

Jamuica cherry, and *Salal*, *Jaboticalea*, were due to the presence of large amount of anthocyanins.

3.5. Superoxide anion-scavenging activity

Superoxide anion radicals are produced by a number of cellular reactions, including various enzyme systems, such as lipoxygenases, peroxidase, NADPH oxidase and xanthine oxidase. Superoxide anion plays an important role in plant tissues and is involved in the formation of other cell-damaging free radicals (Bloknina et al., 2003). In the present study, superoxide radical was generated by illuminating a solution containing riboflavin. The relative scavenging effects of litchi anthocyanins on superoxide radical are shown in Fig. 4. As with the reducing power, anthocyanins exhibited an excellent superoxide anion-scavenging activity, which was much higher than those of ascorbic acid and BHT. At 50 μg/ml, the scavenging activity of anthocyanins, ascorbic acid and BHT were 91.4%, 12.4% and 13.3%, respectively.

3.6. Inhibitory effects of deoxyribose degradation

Hydroxyl radical can be formed by the Fenton reaction in the presence of reduced transition metals, such as Fe^{2+}, and H_2O_2, which is known to be the most reactive of all the reduced forms of dioxygen and is thought to initiate cell damage in vivo (Rollet-Labelle et al., 1998). To determine whether litchi anthocyanins reduce hydroxyl radical generation by chelating metal ions or by directly scavenging hydroxyl radicals, the effects of the anthocyanins on hydroxyl radical generated by Fe^{3+} ions were analyzed by determining the degree of deoxyribose degradation. Fig. 5 shows the concentration-dependent inhibition of hydroxyl radical induced deoxyribose degradation by litchi anthocyanins in both the site-specific and non-site-specific assays. Using the same concentrations, relatively greater antioxidant activity was observed in the site-specific assay than in the non-site-specific assay (Fig. 5), implying that the anthocyanins inhibited deoxyribose degradation mainly by chelating metal ions rather than by scavenging hydroxyl radical directly. Similar results were reported for extracts of *Opuntia ficus-indica* var. Saboten (Lee et al., 2002) and *Hypericum perforatum* L. (Zhou, Lu, & Wei, 2004).

3.7. Effect of exogenous anthocyanin treatment on pericarp browning and relative leakage rate of litchi fruit

As shown in Fig. 6A, litchi pericarp browning index increased rapidly with storage time. Application of anthocyanins reduced litchi pericarp browning index. After 6 days of storage, the browning index for control fruit was 4.95 while fruit treated with anthocyanins had a browning index of 3.54.

Browning is associated with loss of membrane integrity which occurs during tissue deterioration and senescence (Duan et al., 2004; Liu, Jiang, Chen, Zhang, & Li, 1991). Membrane permeability, as an indicator of membrane integrity, gradually increased during storage. However, fruit treated with anthocyanins had significantly lower rel-

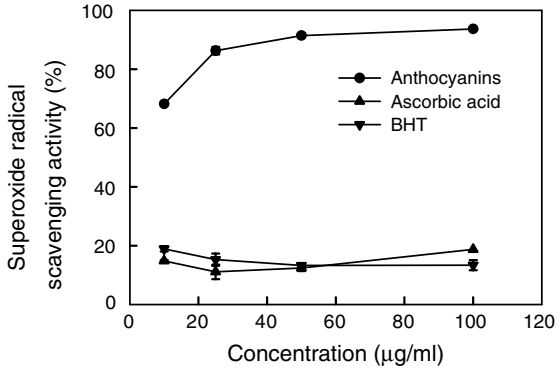

Fig. 4. Superoxide radical-scavenging activity of anthocyanins from litchi pericarp tissues. Each value is presented as mean ± standard error ($n = 3$). The vertical bars indicate standard errors, where they exceeded the symbol size.

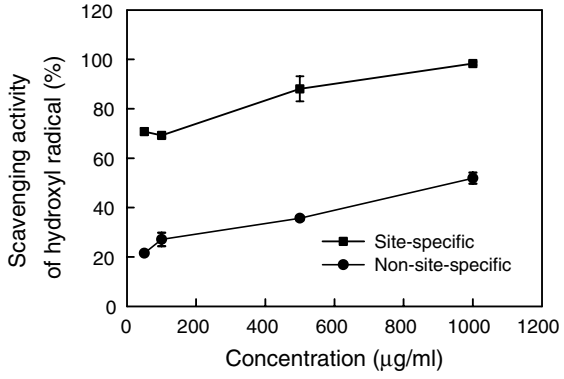

Fig. 5. Inhibitory effects of anthocyanins from litchi pericarp tissues on deoxyribose degradation in non-site-specific assay and site-specific assay. Each value is presented as mean ± standard error ($n = 3$). The vertical bars indicate standard errors, where they exceeded the symbol size.

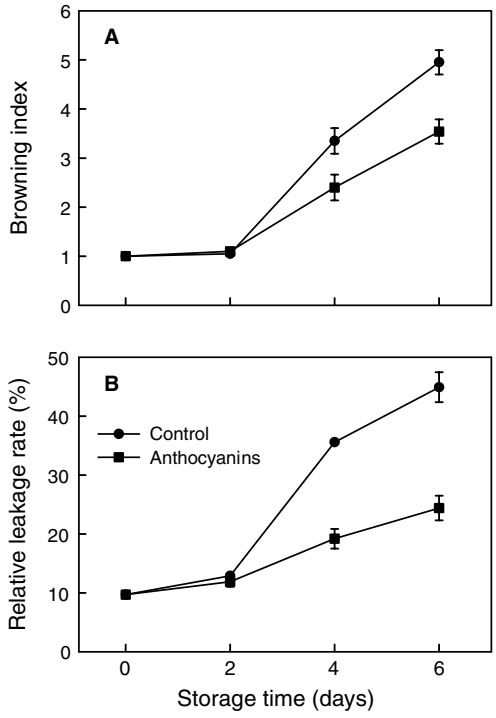

Fig. 6. Effect of exogenous anthocyanin treatment on pericarp tissues browning (A) and relative leakage rate (B) of litchi fruit during storage at 28 °C. Each value is presented as mean ± standard error ($n = 3$). The vertical bars indicate standard errors, where they exceeded the symbol size.

ative leakage rates (Fig. 6B) than control fruit, indicating that membrane integrity was maintained. There is a great deal of evidence showing that lipid peroxidation, which results from a decreased ability to scavenge reactive oxygen species, leads to loss of membrane integrity and pericarp browning of harvested litchi fruit (Jiang, Duan, Joyce, Zhang, & Li, 2004; Jiang & Fu, 1998; Lin et al., 1988). Thus, exogenous anthocyanin treatment enhanced free radical-scavenging activity, inhibited lipid peroxidation, and relatively maintained membrane integrity of litchi fruit pericarp tissues, which was responsible for reduced browning.

4. Conclusions

Litchi anthocyanins exhibited excellent antioxidant activity. They significantly inhibited the peroxidation of linoleic acid, and acted as strong electron-donating agents in the Fe^{3+} to Fe^{2+} assay and hydrogen-donating agents in the DPPH assay. Furthermore, the anthocyanins were effective in scavenging superoxide anion radical and inhibited deoxyribose degradation induced by hydroxyl radical, mainly via chelating iron ion rather than scavenging hydroxyl radical directly. In addition, exogenous anthocyanin treatment significantly prevented pericarp browning and delayed increase in membrane permeability of harvested litchi, which may be attributed to a strong free radical-scavenging activity.

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