Full Paper

Evaluation of the Antioxidant Properties of Litchi Fruit Phenolics in Relation to Pericarp Browning Prevention

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Abstract: Phenolics were extracted from litchi fruit pericarp (LFP) tissues, purified and their antioxidant properties analyzed. LFP phenolics strongly inhibited linoleic acid oxidation and exhibited a dose-dependent free-radical scavenging activity against α,α-diphenyl-β-picrylhydrazyl (DPPH·) and hydroxyl radicals and superoxide anions. The degradation of deoxyribose by hydroxyl radicals was inhibited by phenolics acting mainly as iron ion chelators, rather than by directly scavenging the radicals. Phenolics from litchi fruit pericarp were found to display similar reducing power activity as ascorbic acid. The effect of phenolic compound treatment on pericarp browning and electrolyte leakage of litchi fruit was also evaluated and it was observed that application of exogenous litchi phenolics to harvested litchi fruit significantly prevented pericarp browning and delayed increases in electrolyte leakage. These results suggest that litchi pericarp tissue phenolics could be beneficial in scavenging free radicals, maintaining membrane integrity and, thereby inhibiting pericarp browning of litchi fruit.

Introduction

Oxidation is essential to many living organisms for the production of energy necessary for biological processes. However, reactive oxygen species (ROS) are produced *in vivo* during some oxidative reactions [1] and these species are not only strongly associated with lipid peroxidation, leading to food deterioration, but also involved in development of a variety of diseases including cellular aging, mutagenesis, carcinogenesis, coronary heart disease, diabetes and neurodegeneration [2,3]. Although almost all organisms possess antioxidant defense and repair systems to protect them from oxidative damage, in some cases these systems are insufficient to entirely prevent such damage [4]. In the case of foods, over time the decreased protective ability of the antioxidative defenses can be overcome by the ROS, leading, for example, to cellular damage during postharvest storage of fruit [5]. Currently, there is a trend towards replacement of the widely used synthetic antioxidants, such as butylhydroxyanisole (BHA) and butylated hydroxytoluene (BHT), with antioxidants from natural sources to extend the shelf life of foods [6].

Fruits and vegetables are known to contain a variety of different antioxidant compounds such as ascorbic acid, tocopherol, glutathione and carotenoids, which may all contribute to protection against oxidative damage [1]. It has been recently shown that phenolics from edible fruits are effective *in vitro* antioxidants [7,8]. The antioxidative properties of phenolics arise from their high reactivity as hydrogen or electron donors and from the ability of polyphenol-derived radicals to stabilize and delocalize the unpaired electron or from their ability to chelate transition metal ions (*i.e.* cause termination of the Fenton reaction) [9]. Some reducing compounds or antioxidants, such as ascorbic acid and glutathione, are very efficient in controlling enzymatic browning [10,11]. Thus, endogenous phenolics may also play a role in inhibiting fruit browning processes.

Litchi (*Litchi chinensis* Sonn.) is a tropical to subtropical fruit native to China, Vietnam, Indonesia and the Philippines, but now also grown elsewhere in the world. The fruit has a bright red pericarp surrounding a white aril [5]. Litchi pericarp contains large amounts of phenolics [12]. In our preliminary study, the phenolics content of harvested litchi fruit pericarp tissues was 13.9 mg/g on a fresh weight basis (Duan and Jiang, unpublished data). The major phenolics in litchi fruit pericarp tissues were identified as epicatechin, procyanidin B4 and procyanidin B2 (Figure 1). Furthermore, although a number of phenolics have been identified in different litchi cultivars [12,13], little information is available about the antioxidant activity of these phenolics from litchi fruit peel. The objective of the current study was to evaluate the antioxidant property of phenolics from litchi fruit pericarp tissues and then investigate the effects of exogenous phenolic treatments on the pericarp browning of the fruit.

Results and Discussion

Extraction and purification of phenolics from litchi fruit pericarp

Litchi pericarp contains a large amount of phenolics [5]. In this study, the measured content of phenolics in litchi pericarp tissues after purification was about 11.8 mg/g fresh weight. In a previous study, we extracted and purified phenolics from the pericarp tissue of Huaizhi cv. litchi and found that the major flavonoids present were epicatechin, procyanidin B2 and procyanidin B4 (Figure 1) [14].

Zhang *et al*. [12] have reported that the phenolic constituents of litchi fruit pericarp are mainly epicatechin, epicatechin 3-gallate and procyanidin B2, while Sarni-Manchado *et al*. [13] have mentioned condensed tannins, epicatechin and procyanidin A2 as the major phenolic compounds found in litchi fruit pericarp. We attribute the observed differences to the use of different cultivars in these studies.

Figure 1. Three major flavonoids from pericarp tissues of Huaizhi cv. litchi [14].

Epicatechin

Procyanidin B4   Procyanidin B2

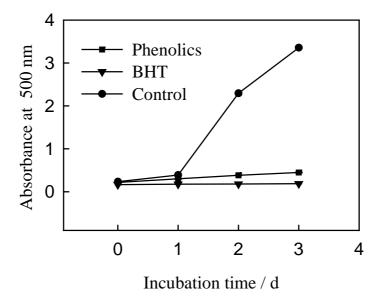*Antioxidant activity in a linoleic acid system*

As shown in Figure 2, phenolics from litchi pericarp tissues can significantly inhibit peroxidation of linoleic acid and reduce the formation of hydroperoxides, implying that these phenolics are powerful natural antioxidants. The measured antioxidant activity of the litchi phenolics was nevertheless slightly lower than that of BHT, a widely used commercial antioxidant.
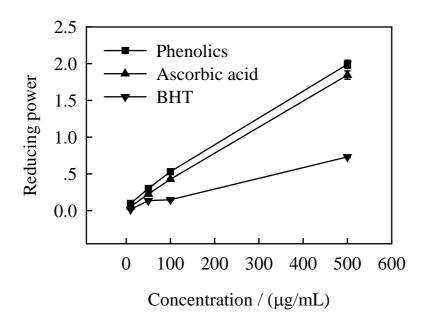
Reducing power

Reducing power is widely used to evaluate the antioxidant activity of polyphenols [8,15,16]. As shown in Figure 3, phenolics from litchi pericarp tissues exhibited higher reducing power than BHT and similar reducing power to ascorbic acid, suggesting that the litchi phenolics possess strong electron donating capacity. Furthermore, a linear relationship existed between the concentration and reducing power of ascorbic acid, BHT and litchi phenolics, with correlation coefficients of 0.9997 (y = 0.0036 x + 0.0412), 0.9958 (y = 0.0014 x + 0.025) and 0.9990 (y = 0.0038 x + 0.1039) for ascorbic acid, BHT and the litchi phenolics, respectively.

Figure 2. Antioxidant activity of phenolics from litchi pericarp tissues, measured by the ferric thiocyanate method.

Luximon-Ramma *et al.* [16] have reported the existence of a similar apparent linear relationship between antioxidant capacity (assessed as ferric ion reducing power) and total phenol content in *Cassia fistula* extracts. Other similar findings have been obtained in assessments of the antioxidant activities of crude extracts of *Ribes*, *Rubus* and *Vaccinium* [17], fruit juices [18] and red wine [19].

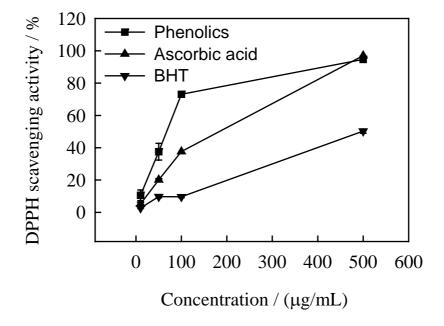**Figure 3**. Reducing power of phenolics from litchi pericarp tissues.

DPPH· radical scavenging activity

Free radical scavenging is one of the known mechanisms whereby antioxidants inhibit lipid peroxidation [1,9]. The DPPH radical scavenging activity assay has been used extensively for screening antioxidants from fruit and vegetable juices or extracts [20]. Figure 4 compares the DPPH radical
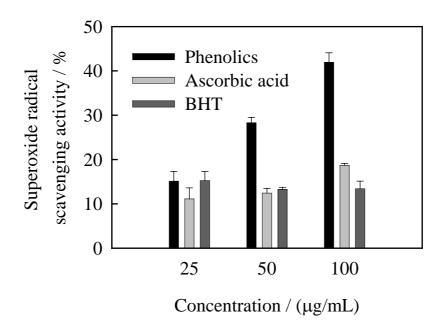
scavenging activity of litchi phenolics, ascorbic acid and BHT. The litchi phenolics exhibited a dose-dependent DPPH radical scavenging activity. Furthermore, these litchi phenolics had the highest such scavenging activity, followed by ascorbic acid and BHT, respectively.

Figure 4. Scavenging effect of phenolics from litchi pericarp tissues on DPPH radicals.

At 100 µg/mL, the DPPH radical scavenging activities of litchi phenolics, ascorbic acid and BHT were 73.09 %, 37.64 % and 9.65 %, respectively. Sun *et al.* [21] investigated antioxidant properties of 11 different fruits (including cranberry, apple, red grape, strawberry, pineapple, banana, peach, lemon, orange, pear, and grapefruit) using a total oxyradical scavenging capacity (TOSC) assay, and also found that there was a direct relationship between total phenolic content and total antioxidant activity in extracts of these fruits.

Figure 5. Superoxide radical scavenging activity of phenolics from litchi pericarp tissues.
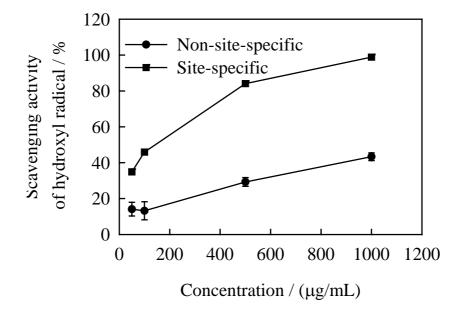
Superoxide anion scavenging activity

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 [1]. The relative scavenging effects of litchi phenolics towards superoxide radicals are compared with those of ascorbic acid and BHT in Figure 5. At 50 µg/mL the litchi phenolics exhibited the strongest superoxide anion scavenging activity, compared to ascorbic acid and BHT, with values of 28.30 %, 12.42 % and 13.25 %, respectively.

Inhibitory effects of deoxyribose degradation

Hydroxyl radicals 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, capable of damaging almost every molecule found in living cells [22]. These radicals have the capacity to join with the nucleotides in DNA and cause strand breakage, which contributes to carcinogenesis, mutagenesis and cytotoxicity [3].

In addition, this species is considered to be one of the quick initiators of the lipid peroxidation process, due to abstraction of hydrogen atoms from unsaturated fatty acids [22]. To determine whether litchi phenolics could reduce hydroxyl radical generation either by chelating metal ions or by directly scavenging hydroxyl radicals, the effects of the litchi phenolics on hydroxyl radicals generated by Fe^{3+} ions were analyzed by determining the degree of deoxyribose degradation. Figure 6 shows the concentration-dependent inhibition of hydroxyl radicals by litchi phenolics in both the site-specific and non-site-specific assays.

Figure 6. Inhibitory effect of phenolics from litchi pericarp tissues on deoxyribose degradation in non-site-specific and site-specific assay.
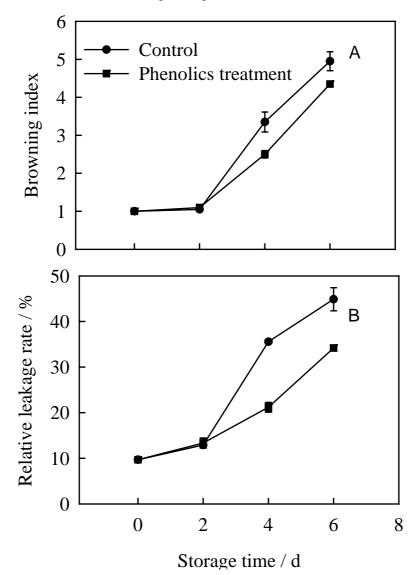
Using the same concentrations, relatively greater antioxidant activity was observed in the site-specific assay than in the non-site-specific one, implying that the litchi phenolics inhibited deoxyribose degradation mainly by chelating metal ions rather than by directly scavenging hydroxyl radicals. Similar results were reported for the crude extracts of *Opuntia ficus-indica* var. Saboten [23] and *Hypericum perforatum* L. [24].

Effect of litchi phenolics treatment on pericarp browning and relative electrolyte leakage rate of litchi fruit

As shown in Figure 7A, the pericarp browning index of litchi fruit increased rapidly with storage time. Application of litchi phenolics reduced the pericarp browning index. After 6 days of storage, the browning index for control fruit was 4.95, while fruit treated with litchi phenolics had a browning index of 4.35.

Figure 7. Effect of exogenous phenolics on pericarp tissues browning (A) and relative leakage rate (B) of litchi fruit during storage at 28 °C.

Browning is associated with loss of membrane integrity which occurs during tissue deterioration and senescence [25,26]. Electrolyte leakage, as an indicator of membrane integrity, gradually increased during storage. However, fruit treated with litchi phenolics had a significantly lower relative leakage rate than control fruit (Figure 7B), indicating that membrane integrity was partially 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 [5,10]. Thus, treatment with litchi phenolics increased free radical scavenging activity, which was possibly beneficial in inhibiting lipid peroxidation and maintaining membrane integrity of pericarp tissues of harvested litchi fruit, which in turn may be responsible for reduced browning.

Conclusions

Litchi phenolics exhibited excellent antioxidant activity. They significantly inhibited the peroxidation of linoleic acid, and acted as a strong electron-donating agent in the Fe^{3+} to Fe^{2+} assay and a hydrogen-donating agent in the DPPH˙ assay. Furthermore, the phenolics from litchi pericarp tissues were effective in scavenging superoxide anion radicals and inhibiting deoxyribose degradation induced by hydroxyl radicals, mainly via chelation of iron ion. In addition, exogenous treatment with litchi phenolics significantly delayed increase in electrolyte leakage and prevented pericarp browning of the fruit, which may be attributed to a strong antioxidant activity.

Experimental

Plant materials

Litchi (*Litchi chinensis* Sonn.) Huaizhi cv. fruits at the commercial mature stage were obtained from an orchard in Guangzhou, China. Fruits were selected for uniformity of shape and color, while blemished or diseased fruit were discarded. The fruits were dipped in a 0.1 % Sportak® (prochloraz, Bayer) fungicide solution for 3 min. and air-dried for 2 h. at 28 °C, prior to the treatment with the solutions containing the litchi phenolic compounds.

Extraction and purification of phenolics

Phenolic compounds were extracted and purified according to Zhang *et al.* [12]. Fresh litchi pericarp (50 g) was cut into small pieces, then ground under liquid nitrogen with a pestle and mortar, and finally extracted for 30 min. by stirring at 4 °C with cold aqueous ethanol (65 %, 500 mL) containing 0.5 % sodium metabisulphite. The homogenate was filtered through four layers of cheesecloth, and the residue was then extracted with two additional portions (250 mL each) of the same extraction solution as described above. The combined filtrate was centrifuged at 7,000 ×g for 15 min. at 4 °C and residue was discarded. Ethanol was removed from the supernatant under vacuum on a rotary evaporator at 35 °C, and the pigments were eliminated by two successive extractions with petroleum ether. After addition of 20 % ammonium sulphate and 2 % metaphosphoric acid to the aqueous phase, phenolic compounds were

extracted three times with ethyl acetate. The extracts were combined, evaporated and then dried under vacuum at 35 °C. The residue was re-dissolved in methanol (20 mL) for analyses of antioxidant properties.

Antioxidant activity in linoleic acid system

The antioxidant activity of phenolics against lipid peroxidation was measured by studying the peroxidation of linoleic acid using the ferric thiocyanate method (FTC), as described by Takao *et al*. [27] albeit with some modifications. During linoleic acid peroxidation, peroxides are formed and these compounds can oxidize Fe^{2+} to Fe^{3+}. The Fe^{3+} ions form a complex with SCN^-, which has a maximum absorbance at 500 nm. Thus, a high absorbance value indicated a high peroxide formation during the emulsion incubation. A reaction mixture containing phenolic extract (100 μg/mL, 0.2 mL), linoleic acid emulsion (25 mg/mL in 99 % ethanol, 0.2 mL) and 50 mM phosphate buffer (pH=7.4, 0.4 mL) was incubated in the dark at 40 °C. An aliquot (0.1 mL) of the reaction solution was then added to 70 % (v/v) ethanol (3 mL) and 30 % (w/v) ammonium thiocyanate (0.1 mL). Three min. after of the addition of 20 mmol/L ferrous chloride in 3.5 % (v/v) hydrochloric acid (0.1 mL) to the reaction mixture, the absorbance of the resulting solution was measured at 500 nm using a Unic UV-2802 spectrophotometer. Aliquots were assayed every 24 h. until the absorbance of the control solution (without phenolic extract) reached maximum value. Butylated hydroxytoluene (BHT) was used as a positive control. Three replicates were performed for the assessment of antioxidant activity of phenolics against lipid peroxidation in linoleic acid system.

Scavenging activity of α,α-diphenyl-β-picrylhydrazy (DPPH·) radicals

The scavenging activity of phenolics against DPPH· radicals was assessed according to the method of Larrauri *et al*. [28] with some modifications. Briefly, litchi phenolics at various concentrations (0.1 mL) were mixed with 0.1 mM DPPH-methanol (2.9 mL) solution. After the solution was incubated for 30 min. at 25 °C in dark, the decrease in absorbance was measured at 517 nm. The control contained methanol instead of the phenolic extract solution while the blank 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 these samples was calculated by the following equation: DPPH· radicals scavenging activity/%= [1−(A_{sample}−A_{blank})/$A_{control}$]·100, which *A* is the absorbance using sample, blank or control. Ascorbic acid and BHT were used as positive controls to compare their scavenging capacity with litchi phenolic compounds. Three replicates were carried out to determine scavenging activity of litchi phenolics against DPPH· radicals.

Superoxide radical scavenging activity

Determination of superoxide radical scavenging activity was conducted according to the method described by Siddhurajua *et al*. [29]. All solutions were prepared in 0.05 mol/L 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 luminous

intensity reached about 4,000 lux. An aliquot of various concentrations of litchi phenolics (30 μL) was mixed with reaction buffer solution (1.3 μmol/L riboflavin, 13 mmol/L methionine, 63 μmol/L nitro blue tetrazolium and 100 μmol/L EDTA, pH=7.8, 3 mL). The reaction solution was illuminated for 15 min. at 25 °C. The reaction mixture without any sample was used as a control. The superoxide radical scavenging activity was calculated by the relation $(1-A_{sample}/A_{control})\cdot 100$, where A is the absorbance using sample or control. Three replicates were performed.

Reducing power

The reducing power was determined according to the method of Oyaizu [30]. An aliquot (0.25 mL) of litchi phenolics at various concentrations was mixed with 200 mmol/L sodium phosphate buffer (pH=6.6, 2.5 mL) and 1 % potassium ferricyanide (2.5 mL). The mixture was then incubated for 20 min at 50 °C. After 10 % trichloroacetic acid (w:v, 2.5 mL) were added, the mixture was centrifuged at 650 × *g* for 10 min. An aliquot (5 mL) of the upper layer was mixed with distilled water (5 mL) and 0.1 % ferric chloride (1 mL) before the absorbance was measured at 700 nm. The control contained methanol instead of litchi phenolic solution. A higher absorbance indicated a higher reducing power. Three replicates were conducted.

Inhibitory effect on deoxyribose degradation

The inhibitory effect of litchi phenolics on deoxyribose degradation was determined by measuring the reaction activity between either antioxidants and hydroxyl radicals (referred as non-site-specific scavenging assay) or antioxidants and iron ions (referred as site-specific scavenging assay), described by Lee *et al*. [23]. For the non-site-specific scavenging assay, an aliquot of litchi phenolics at different concentrations (0.1-mL) was mixed with reaction buffer (100 μmol/L $FeCl_3$, 104 μmol/L EDTA, 1.5 mmol/L H_2O_2, 2.5 mmol/L deoxyribose, and 100 μmol/L L-ascorbic acid, pH=7.4, 1 mL) and then incubated for 1 h. at 37 °C. An aliquot of 0.5 % 2-thiobarbituric acid in 0.025 mol/L NaOH (1 mL) and 2.8 % trichloroacetic acid (1 mL) were added to the mixture, which was then heated for 30 min. at 80 °C. The mixture was cooled on ice and its absorbance was then measured at 532 nm. Site-specific scavenging activity, which corresponded to the ability of litchi phenolics to chelate iron ions and interfere with hydroxyl radical generation, was measured using the same reaction buffer without EDTA. The control contained methanol instead of litchi phenolic solution. Percent inhibition of deoxyribose degradation was calculated as $(1-A_{sample}/A_{control})\cdot 100$, where A is the aborbance using sample or control. Three replicates were used for determination of the inhibitory effect on deoxyribose degradation.

Total phenolic content

Total phenolic content in the above-mentioned litchi extract was determined according to the method of Folin-Ciocalteu reaction [31], using gallic acid as standard.

Fruit treatment with litchi phenolic compounds

Litchi phenolics were extracted from pericarp tissues, purified and then used for treatment of litchi fruit. The fruits were randomly distributed into six groups (90 fruits per group). For each treatment, three replicates were used. Fruits were immersed in 100 mg/L aqueous phenolic solution in a desiccator and then infiltrated under an air pressure of 53 kPa for 3 min. The fruit were air-dried for 1 h. at 28 °C, packed in polyethylene bag (0.03 mm thick) and then stored at 28 °C. The fruit treated with water was used as a control. The fruit from each replicate of each treatment after being held for 2, 4 or 6 days were sampled for measurements of pericarp browning index and electrolyte leakage.

Pericarp browning assessment

Pericarp appearance was assessed according to the method of Duan *et al.* [25] by measuring the extent of the total browned area on each pericarp of 30 fruits using the following scale: 1 = no browning (excellent quality); 2 = slight browning; 3 = <1/4 fruit surface showing browning symptoms; 4 = 1/4–1/2 surface showing browning symptoms and 5 = >1/2 surface showing browning symptoms (poor quality). The browning index was calculated as $\sum$ (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 [32].

Measurement of electrolyte leakage

Electrolyte leakage was determined according to the method of Jiang and Chen [33]. 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, then incubated in 0.3 mol/L mannitol in distilled water (25 mL) at 25 °C, and finally shaken for 30 min. Electrolyte leakage was determined with a conductivity meter (Model DDS-11A, Shanghai Scientific Instruments). The same batch of discs was boiled for 15 min. in 0.3 mol/L mannitol in distilled water (25 mL) and then cooled to 25 °C to assess total electrolytes. The relative leakage was expressed as a percentage of the total electrolytes.

Statistical analysis

All analyses were performed in triplicate. The data were expressed as means ± standard errors (SE) in six figures and then analyzed by SPSS (Version 10.0). One-way analysis of variance (ANOVA) and Tukey multiple comparisons were carried out to test any significant difference between the means. Differences between means at the 5 % level were considered to be significant.

Acknowledgements

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Sample Availability: Available from the authors.