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Identification of phenolic compounds and appraisal of antioxidant and antityrosinase activities from litchi (*Litchi sinensis* Sonn.) seeds

K. Nagendra Prasad [a], Bao Yang [a], Shaoyu Yang [a], Yulong Chen [a], Mouming Zhao [b], Muhammad Ashraf [c], Yueming Jiang [a,*]

[a] South China Botanical Garden, Chinese Academy of Sciences, Guangzhou, Guangdong 510650, China
[b] College of Light Industry and Food Science, South China University of Technology, Guangzhou 510650, China
[c] Department of Botany, Faculty of Sciences, University of Agriculture, Faisalabad 38040, Pakistan

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ABSTRACT

Antioxidant and antityrosinase compounds from *Litchi sinensis* Sonn. seeds were extracted with five different types of polar solvents. The five extracts, namely ethanol extract (EE), 50% ethanol extract (50% EE), methanol extract (ME), 50% methanol extract (50% ME), and water extract (WE), were used for the evaluations of total phenolic content, antioxidant capabilities and antityrosinase activity. The 50% EE showed the highest total antioxidant capacity, scavenging the 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical and inhibitory activity against lipid peroxidation, and it was comparable to the activity of the synthetic antioxidant, butylated hydroxyl toluene. Fifty percent EE showed a better antityrosinase activity compared to the other extracts. After application of reverse phase high performance liquid chromatography, coupled to a diode array detector and electrospray ionisation mass spectra, five phenolic compounds, namely, gallic acid, procyanidin B2, (−)-gallocatechin, (−)-epicatechin and (−)-epicatechin-3-gallate were identified from 50% EE. This study suggests that litchi seed can potentially be used as a readily accessible source of natural antioxidants.

1. Introduction

Artificial antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butylhydroquinone (TBHQ), have been widely used in foods for preventing lipid peroxidation, but the usage of these artificial antioxidants is being gradually restricted in the food industry as they are suspected to be toxic and carcinogenic (Namiki, 1990). There has been an increasing demand for antioxidants from plant origins. Epidemiological studies have shown that the consumption of fruits and vegetables with high phenolic content can reduce the risk of cardio- and cerebro-vascular diseases and cancer mortality (Amin & Yazdanparast, 2007). The protective effect of fruits and vegetables against diseases has been attributed to various phenolic compounds possessing antioxidant activity (Jayaprakasha, Girennavar, & Patil, 2008; Prasad, Divakar, Shivamurthy, & Aradhya, 2005).

Tyrosinase (EC1.14.18.1) is involved in melanin production and the melanin production might be responsible for some of the histopathological features exclusive to malignant cancer. Therefore, tyrosinase inhibitors may be clinically helpful in dealing with skin cancer. In recent years, more attention has been paid to use the natural plant extracts such as tyrosinase inhibitors in the cosmetic industry (Momtaza et al., 2008).

The exploration of green technology and of low cost raw materials is important features for the food industry in making improved use of plant resources. As population increases, food production is more intense and a great quantity of waste is generated.

Litchi (*Litchi chinensis* Sonn.) is a subtropical fruit originating from south-east Asia. The fruit is accepted by consumers because of its delicious taste and attractive colour. Litchi by-products consist mainly of litchi pericarp and litchi seeds which are discarded as a waste. The previous studies on biochemical activities of litchi fruit are mainly focussed on its pericarp, because it has been found to be a rich source of a multitude of potential antioxidants (Duan, Wu, & Jiang, 2007; Zhang, Quantick, & Grigor, 2000; Zhao, Yang, Wang, Li, & Jiang, 2006). However, there is no information on litchi seeds, which are commonly used in Chinese Traditional Medicine to relieve neuralgic pain. In the present study, the possibility of litchi seeds being used as a natural antioxidant and tyrosinase inhibitor was investigated, while the major phenolic compounds are identified for the first time. This study could help better utilise litchi seeds, which is not only economical but also environmentally friendly, because they could be recycled in the food industry in the form of value-added products.

* Corresponding author. Tel.: +86 20 37252525; fax: +86 20 37253821.
E-mail address: ymjiang@scbg.ac.cn (Y. Jiang).

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2. Materials and methods

2.1. Plant materials

Fresh fruits of litchi (*Litchi sinensis* Sonn.) at the mature stage were collected from an orchard in Guangzhou, China. Fruits were chosen for uniformity in shape and colour. The fruits were carefully washed in potable water. Litchi seeds were manually separated, then dried for 24 h, using a hot air oven at 60 °C, and powdered, using a blender. The moisture content was determined to be 62%.

2.2. Chemicals and reagents

1,1-diphenyl-2-picryl hydrazyl (DPPH), ascorbic acid, L-tyrosine, tyrosinase solution (1000 units/ml), gallic acid, procyanidin B2, (−)-gallocatechin, (−)-epicatechin, (−)-epicatechin-3-gallate and thiobarbituric acid (TBA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Butylated hydroxyly toluene (BHT) was obtained from Sinopharm Chemical Reagent Co. (Shanghai, China). All other chemicals and solvents used in this study were of analytical grade and obtained from Tianjin Reagent Company (Tianjin, China).

2.3. Extraction

The dried powder (10 g) of litchi seeds was extracted for 12 h in a rotary shaker with 100 ml of five different types of polar solvents (ethanol, 50% ethanol, methanol, 50% methanol and water) separately in a conical flask at 30 °C. The extracts were filtered and then evaporated to dryness using a rotary evaporator (RE-52A, Shanghai Woshi Co., Shanghai, China) and lyophilised using a freeze-dryer (Savant, Vapornet VN 100, Labequip Ltd., Markham, Ontario, Canada) to obtain five different freeze-dried extracts, namely ethanol extract (EE), 50% ethanol extract (50% EE), methanol extract (ME), 50% methanol extract (50% ME), and water extract (WE).

2.4. Determination of total phenolic content

Total phenolic contents of litchi seed extracts were determined by the method of Singleton and Rossi (1965) and then expressed as gallic acid equivalents.

2.5. Antityrosinase activity

Inhibition of tyrosinase activity was analysed according to the method of Kobayashi, Kayahara, Tadasa, Nakamura, and Tanaka (1995), with some modifications. L-Tyrosine solution (4 ml) at 0.5 mg/ml was dissolved in 20 mM phosphate buffer (pH 6.8) and then added to 1 ml of litchi seed extract (dissolved in 50% ethanol). After 10 min of incubation at 37 °C, 1 ml of mushroom tyrosinase (50 units/ml, dissolved in 0.2 M phosphate buffer, pH 6.8) was added to the mixture solution. The absorbance was recorded after 10 min at 475 nm using a spectrophotometer (UV-2802, Unico Co. Ltd., Shanghai, China). Fifty percent ethanol solution was used as a blank, while 1 ml of distilled water was used as the control. Percent tyrosinase inhibitory activity was calculated using the following formula: (Control OD − Sample OD/Control OD) × 100.

2.6. 1,1-Diphenyl-2-picryl hydrazyl radical (DPPH?) radical-scavenging activity

The DPPH radical-scavenging activities of litchi seed extracts were appraised by the method of Blois (1958) with some modification. Different concentrations (25, 50, 75 and 100 μg/ml, dissolved in 50% ethanol) of the samples were taken in different test tubes. The volume was adjusted to 100 μl by adding 50% ethanol and mixed with 0.9 ml of 0.2 mM DPPH (dissolved in methanol). The reaction mixture was incubated for 20 min at 28 °C in the dark. The control contained all reagents except the extract sample while methanol was used as blank. The DPPH radical-scavenging activity was determined by measuring the absorbance at 517 nm using a spectrophotometer. The antioxidant activity was expressed as a percentage of scavenging activity of DPPH radicals and calculated using the following formula: (Control OD − Sample OD/Control OD) × 100. The DPPH radical-scavenging activity of BHT was also assayed for comparison.

2.7. Inhibitory activity against lipid peroxidation

Lipid peroxidation was assessed, following the method used by Tsuda et al. (1994) with some modifications. In brief, liposome sample (egg lecithin, 6 mg/ml in 0.2 M phosphate buffer, pH 7.4) was sonicated using an Ultrasonicator (SB-5200DTD, Xinzhi Biotech Co., Ningbo, China, 40 kHz) for 1 h. Aliquots (100 μl) of samples were dissolved in 50% ethanol to obtain different concentrations of 25, 50, 75 and 100 μg/ml and then added to 0.5 ml of the liposome mixture. Lipid peroxidation was induced by adding 10 μl of 0.2 M $FeCl_3$ and 10 μl of 0.2 M L-ascorbic acid. After incubation for 1 h at 37 °C, the reaction was stopped by adding 2 ml of 0.25 M HCl containing 15% trichloroacetic acid (TCA) and 0.375% TBA. The reaction mixture was subsequently boiled for 15 min, cooled and centrifuged, and the absorbance of the supernatant read at 532 nm. The blank consisted of all the reagents without the lipid, while the control contained all the reagents except the test sample. Percent lipid peroxidation inhibitory activity of the sample was calculated as: (1−absorbance of sample/absorbance of control) × 100. The lipid peroxidation inhibitory activity of BHT was also assayed for comparison.

2.8. Total antioxidant activity

The total antioxidant capacity of litchi seed extracts was investigated according to the method of Prieto, Pineda, and Aguilar (1999). In brief, the samples (0.1 ml, dissolved in 50% ethanol) at 25, 50, 75 and 100 μg/ml concentrations were mixed with 0.3 ml of the reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate solutions). The tubes were capped and the reaction mixture then incubated for 90 min at 95 °C. The absorbance of the cooled mixture was measured at 695 nm against a blank. The blank contained the reagent solution and solvent. The total antioxidant activity was expressed as the absorbance of the sample. The higher absorbance value indicated higher antioxidant activity. The antioxidant activity of BHT was also assayed for comparison.

2.9. Identification of phenolic compounds

Reverse phase high performance liquid chromatography was used to analyse phenolic compounds present in the 50% EE sample, using the separation module (LC-20 AT, Shimadzu Corporation, Japan) equipped with a C_{18} column (Vydac, 218 TP, 250 × 4.6 mm, 5 μm particle size, Sigma–Aldrich, St. Louis, MO, USA) and a diode array detector (Rheodyne, USA). The samples were eluted with a gradient system consisting of solvent A (2% acetic acid, v/v) and solvent B (acetonitrile: methanol, 10:15, v/v), used as the mobile phase, with a flow rate of 1 ml/min. The temperature of the column was maintained at 25 °C and the injection volume was 10 μl. The gradient system started from 90% A at 0 min, to 80% A at 10 min, 70% A at 15 min, 60% A at 25 min, 50% A at 30–40 min, 75% A at 42 min, and 90% A at 44 min. The peaks of the phenolic compounds

were monitored at 270 nm. UV–Vis absorption spectra were recorded on-line from 200 to 600 nm during the HPLC analysis.

Electrospray ionisation mass spectroscopic (ESI–MS) analysis of phenolic compounds in 50% EE sample was performed using an Applied Biosystems (API2000 LC/MS/MS System, ABI, Foster city, CA). Mass spectra were achieved by electrospray ionisation in both positive and negative modes. The capillaries 4500 V (negative) and 5500 V (positive) were used in this study. The electrospray probe-flow was adjusted to 20 ml/min. Continuous mass spectra were obtained by scanning from 100 to 800 m/z. Identification of the phenolic compounds of the 50% EE sample from litchi seeds was achieved by comparison with retention times of standards and their UV–Vis absorption spectra and ESI–MS spectra comparisons with literature reports or with reference standards available.

2.10. Statistical analysis

Data were expressed as means ± standard deviations (SD) of three replicate determinations and then analysed by SPSS V.13 (SPSS Inc., Chicago, USA). One way analysis of variance (ANOVA) and Duncan's New Multiple-range test were used to determine the differences among the means. P values < 0.05 were regarded as significant. Relationship between total phenolic content and antioxidant activity was assessed through correlation and regression, worked out using the Microsoft office Excel programme.

3. Results and discussion

3.1. Extraction yield and total phenolic content

Extraction yield and total phenolic content of each extract of litchi seed are given in Table 1. Fifty percent EE gave the highest percentage of extraction yield (26.8 ± 0.24), whereas WE gave the lowest (23.5 ± 0.07). For the total phenolics, ME exhibited the highest value, and WE the lowest. However, there were no significant differences (P < 0.05) in the total phenolic content among all the extracts, except for WE. In our previous work, application of methanol or ethanol gave a higher extraction yield from litchi fruit pericarp tissues when compared to other solvents, e.g. ethyl acetate and water (Prasad et al., 2009). Thus, it is necessary to extract polyphenolic compounds effectively from litchi seed prior to further evaluation of antioxidant activity. A combination of ethanol/methanol with water (50:50 v/v) was reported to be effective in extraction of polar compounds, such as flavonoids, phenolic acids and sugars, depending upon their polarity (Jayaprakasha et al., 2008; Markham, 1982).

3.2. Inhibitory activity of tyrosinase

The litchi seed extracts showed inhibitory activity of tyrosinase in a concentration-dependent manner (Table 1). As the concentration increased, the inhibitory activity by litchi seed extract was enhanced. Fifty percent EE showed the highest antityrosinase activity, at 100 μg/ml, compared with the other extracts.

3.3. DPPH radical-scavenging activity

The DPPH radical-scavenging activities of all the extracts from litchi seed increased with increase in concentration (Fig. 1). At 100 μg/ml, percent scavenging ability of 50% EE was significantly higher (48.9 ± 0.25) than that of BHT (42 ± 1.5). Therefore, the DPPH radical-scavenging activity, in increasing order, was; 50% EE > EE > ME > 50%ME > BHT > WE. The data obtained in this study reveal that the extracts of litchi are free radical scavengers which react with DPPH radical by their electron-donating ability.

Table 1
Comparative analysis of extraction yield, total phenolic content and antityrosinase activity of litchi seed using different polar solvents.

Extraction solvent	Extraction yield (%)	TPC*	Tyrosinase inhibitory activity (%)		
			50 μg/ml	75 μg/ml	100 μg/ml
Ethanol	23.3 ± 0.03^{c}	241 ± 5^{a}	3.7 ± 0.08^{a}	5.8 ± 0.2^{a}	8.3 ± 0.08^{a}
50% Ethanol	26.8 ± 0.24^{a}	239 ± 3^{a}	2.8 ± 0.4^{b}	5.1 ± 0.1^{a}	8.8 ± 0.8^{a}
Methanol	26.2 ± 0.25^{b}	244 ± 1.4^{a}	1.3 ± 0.2^{c}	3.5 ± 0.2^{b}	5.8 ± 0.2b
50% Methanol	25.8 ± 0.02^{b}	239 ± 2^{a}	0.4 ± 0.1^{d}	3.2 ± 0.6^{b}	4.8 ± .08^{c}
Water	23.5 ± 0.07^{c}	158 ± 5^{b}	NA	1.3 ± 0.2^{c}	2.3 ± 0.3^{d}

For each treatment, means within the column followed by different letters are significantly different at P < 0.05. NA – No activity.
* Total phenolic content expressed as gallic acid equivalent (μg/g DW).

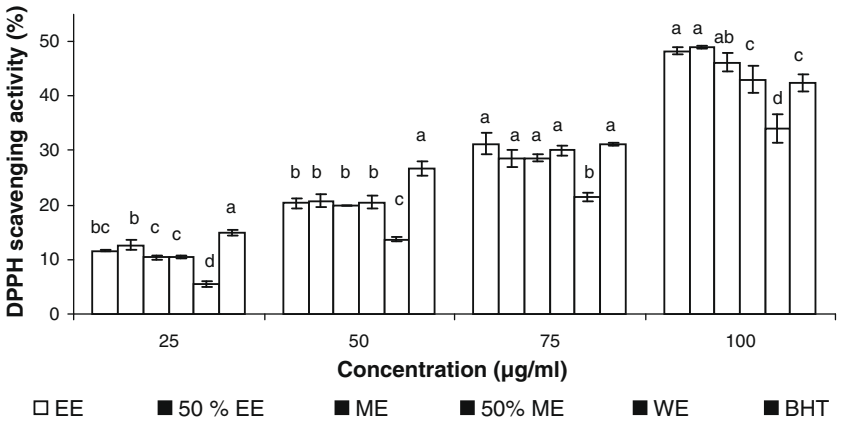

Fig. 1. DPPH radical-scavenging activity of ethanol extract (EE), 50% ethanol extract (50% EE), methanol extract (ME), 50% methanol extract (50% ME) and water extract (WE) from litchi seeds. For each treatment, means within the same set followed by different letters are significantly different at P < 0.05.

3.4. Lipid peroxidation inhibitory activity

Lipid peroxidation is an oxidative alteration of polyunsaturated fatty acids (in the cellular membranes) that generates several types of free radicals. The level of TBA-reactive substances, the products of lipid peroxidation, is often used to assess the extent of oxidation that occurs in biological systems. TBA reacts with malondialdehyde to form a pink chromogen which can be detected spectrophotometrically. The degree of discoloration indicates the inhibitory activity of the antioxidants which could be determined by measuring a decrease in absorbance at 532 nm (Tsuda et al., 1994). To evaluate the antioxidant activity of litchi seed extracts against lipid peroxidation, a liposome model system was used. The inhibitory activity of lipid peroxidation by all the extracts from litchi seed, except WE, was remarkable and comparable to that of BHT (Fig. 2). The activities of all the extracts were dose-dependent. At 100 g/ml, the inhibitory activities of lipid peroxidation were 61.7 ± 0.4, 61.7 ± 2, 60 ± 0.8, 52 ± 0.7, 50 ± 2.9 and 57.6 ± 1.4 for EE, 50% EE, ME, 50% ME, WE, and BHT, respectively. However, no significant differences ($P < 0.05$) were observed in any extracts tested except WE. Kulkarni, Aradhya, and Divakar (2004) reported that inhibition of lipid peroxidation of pomegranate membrane was by chain termination, by scavenging peroxyl radicals. The lipid peroxidation inhibitory activities of all the extracts from litchi seed in this study might have been due to chain termination of free radicals.

3.5. Total antioxidant capacity

Total antioxidant capacities of the extracts from litchi seed were analysed by the phosphomolybdenum method. A high absorbance value of the sample indicates high antioxidant activity. Fig. 3 shows the total antioxidant activities of all the litchi extracts and BHT. In view of these results, all the extracts, except WE, showed a significant total antioxidant activity and were concentration-dependent. The total antioxidant activity of 50% EE, at 100 μg/ml, was 0.82 ± 0.02, while that of BHT was 0.76. The total antioxidant activities of all the extracts, except WE, were higher than those of BHT at 75–100 μg/ml. The total antioxidant capacity of the extracts from litchi seeds may be attributed to their chemical composition and phenolic acid content. Recent studies by Jayaprakasha et al. (2008) showed that some bioactive compounds from citrus had strong total antioxidant activity, which was probably due to the presence of flavonoids, carotenoids and ascorbic acid.

3.6. Identification of phenolic compounds

In this study, as 50% EE exhibited good antioxidant ability and inhibitory activity against tyrosinase, it was chosen for identification of phenolic compounds. Fig. 4 shows the HPLC chromatogram of 50% EE from litchi seed. Peak identification was performed by comparing retention times (t_R), UV–Vis spectra and mass spectra

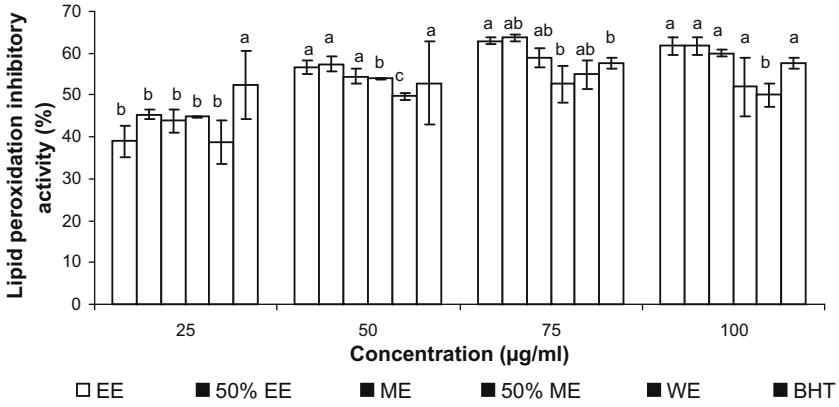

Fig. 2. Lipid peroxidation inhibitory activity of ethanol extract (EE), 50% ethanol extract (50% EE), methanol extract (ME), 50% methanol extract (50% ME) and water extract (WE) from litchi seeds. For each treatment, means within the same set followed by different letters are significantly different at $P < 0.05$.

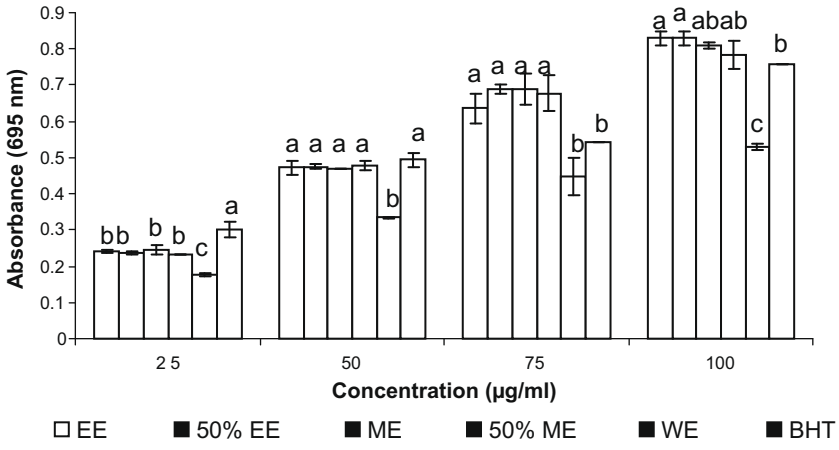

Fig. 3. Total antioxidant activity, using phosphomolybdenum method, of ethanol extract (EE), 50% ethanol extract (50% EE), methanol extract (ME), 50% methanol extract (50% ME) and water extract (WE) from litchi seeds. Higher absorbance value indicates higher antioxidant activity. For each treatment, means within the same set followed by different letters are significantly different at $P < 0.05$.

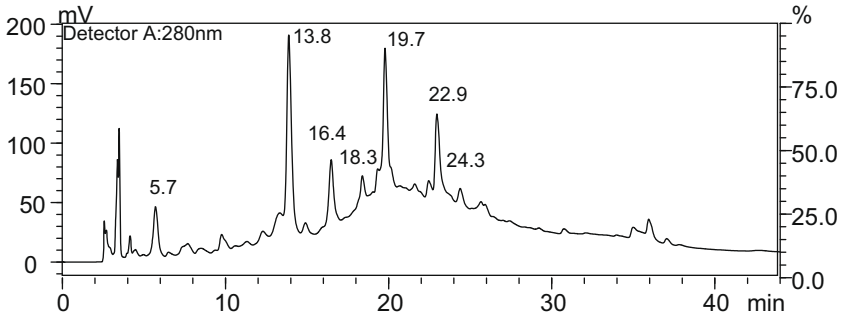

Fig. 4. HPLC profile of 50% ethanol extract from litchi seeds. Retention time (min) (t_R): gallic acid = 5.7, (−)-gallocatechin = 16.4, procyanidin B2 = 18.3, (−)-epicatechin = 19.7 and (−)-epicatechin-3-gallate = 24.3.

with those of reference standards or literature data. Peak 1 with the t_R of 5.7 was identified as gallic acid (λ_{max} 271.74, similar to that of standard gallic acid, λ_{max} 272) and the $[M-H]^+$ peak of gallic acid was at *m/z* 169. These results are in agreement with those reported in the literature (Pelillo et al., 2004; Tumbas, Mandic, Cetkovic, Dilas, & Canadanovic-Brunet, 2004). The second peak, with a t_R of 16.4, λ_{max} 280, and $[M-H]^+$ of 304, was identified as (−)-gallocatechin, as reported by Pelillo et al. (2004). Peak 3, with the t_R of 18.3, was identified as procyanidin B2 (λ_{max} 281.48) and the $[M-H]^+$ peak of procyanidin B2 was observed at *m/z* 577.3. Its characteristic fragment ions, such as *m/z* 424 and 396, were also identical with those reported in the literature (Osman & Wong, 2007; Wollgast, Pallaroni, Agazzi, & Anklam, 2001; Zhao et al., 2006). The fourth peak was identified as (−)-epicatechin (λ_{max} 280.50) with a t_R of 19.7. The $[M-H]^-$ peak of 289.2 (along with the fragment ions at 245.1, 179.1 and 129.1) was similar to those reported by Amarowicz, Shahidi, and Wiczkowski (2003), Zhao et al. (2006) and Sun, Shi, Jiang, Xue, and Wei (2007). The fifth peak was identified as (−)-epicatechin-3-gallate with the t_R of 24.3 at λ_{max} 280. However, the $[M-H]^-$ peak of 441 could not be found, but its fragment ions at 289 and 165 were found, similar to those reported elsewhere (Amarowicz et al., 2003; Wang, Lu, Miao, Xie, & Yang, 2008). Peaks at t_R of 13.8 (λ_{max} 311.53) and at 22.9 (λ_{max} 280) need to be identified further. Gallic acid, procyanidin B2,

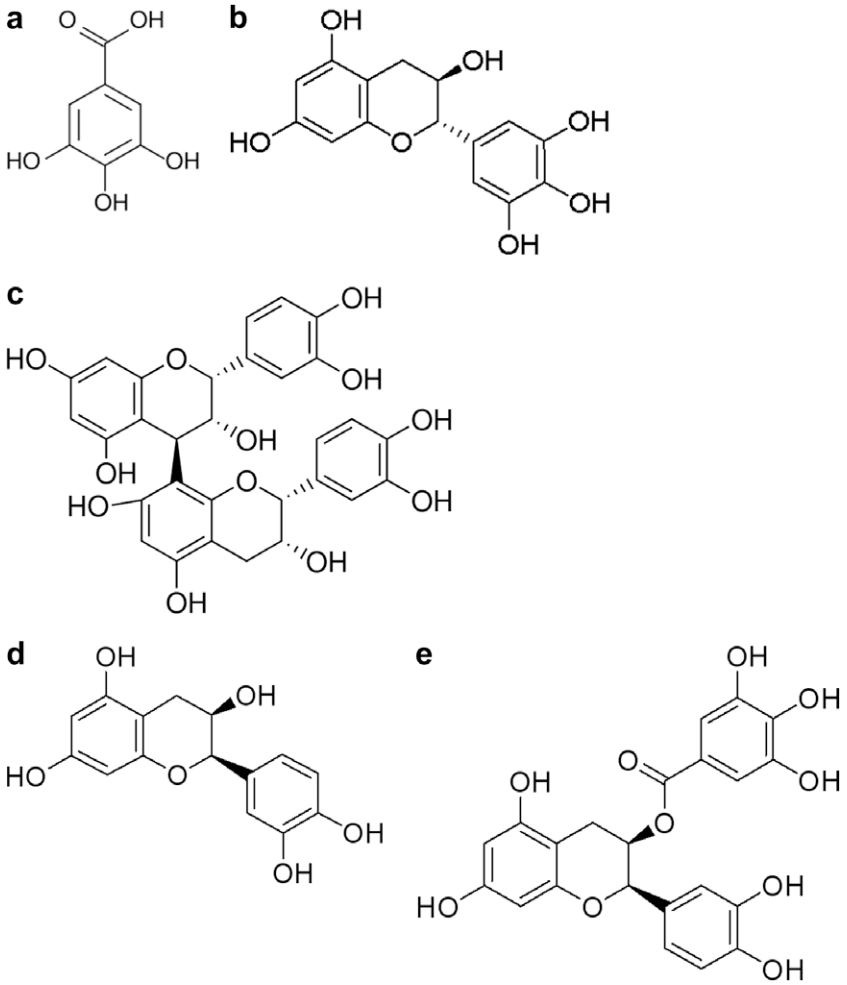

Fig. 5. Structures of (a) gallic acid, (b) (−)-gallocatechin, (c) procyanidin B2, (d) (−)-epicatechin, and (e) (−)-epicatechin-3-gallate.

(−)-gallocatechin, (−)-epicatechin, and (−)-epicatechin-3-gallate were previously reported from litchi pericarp (Duan et al., 2007; Zhang et al., 2000; Zhao et al., 2006). This study shows, for the first time, that gallic acid, procyanidin B2, (−)-gallocatechin, (−)-epicatechin and (−)-epicatechin-3-gallate compounds (Fig. 5) are in litchi seeds.

Antioxidant and antityrosinase activities from various seed sources, e.g. longan seeds (Rangkadilok et al., 2007), grape seeds (Poudel, Tamura, Kataoka, & Mochioka, 2008; Yamakoshi et al., 2006) and soya seeds (Chang, Ding, Tai, & Wu, 2007; Malencic, Maksimovic, Popovic, & Miladinovic, 2008) have previously been reported. The antioxidant activity of litchi seed extract was in agreement with the amount of phenolics found in the litchi seed extracts. Statistical correlations between total phenolic content and antioxidant activity were determined. Total phenolic content exhibited highest associations with total antioxidant capacity (R^2 = 0.9773), while correlations with lipid peroxidation assay and DPPH activity were 0.5169 and 0.8594, respectively. Thus, the content of phenolic compounds could be used as an important indicator of antioxidant capacity.

Several reports have convincingly shown a close relationship between antioxidant activity and total phenolic content (Duan et al., 2007; Pan et al., 2008; Zhao et al., 2006). Litchi seed extracts have high levels of phenolic compounds, which are composed of one (or more) aromatic rings bearing one or more hydroxyl groups, which can exhibit extensive free radical-scavenging activities as hydrogen donors or electron-donating agents, and metal ion-chelating properties. The greater the number of hydroxyl groups in the phenolics, the higher is the antioxidant activity (Prasad et al., 2005; Rangkadilok et al., 2007).

The inhibition of tyrosinase activity might depend on the hydroxyl groups of the phenolic compounds of the litchi seed extracts that could form a hydrogen bond to a site of the enzyme, leading to a lower enzymatic activity. Some tyrosinase inhibitors act through hydroxyl groups that bind to the active site on tyrosinase, resulting in steric hindrance or changed conformation (Baek et al., 2008; Kim, Kang, & Yokozawa, 2008). Gallic acid, (−)-epicatechin, procyanidin B2 and (−)-epicatechin-3-gallate, identified in litchi seed, proved to be effective inhibitors of tyrosinase activity, as reported by many other authors (Kubo, Chen, & Nihei, 2003; Momtaza et al., 2008; Yamakoshi et al., 2006). The antioxidant activity mechanism may also be one of the important reasons for tyrosinase inhibition activity (Kim et al., 2008).

4. Conclusions

In the present study, application of polar solvents, to extract antioxidant compounds from litchi seeds, was investigated. The study indicated that litchi seeds had high phenolic content, exhibited good antioxidant and antityrosinase activities, compared with that of BHT. Furthermore, five compounds were identified from litchi seed, namely gallic acid, procyanidin B2, (−)-gallocatechin, (−)-epicatechin, and (−)-epicatechin-3-gallate. The use of litchi seeds for obtaining natural antioxidant compounds appears to be an alternative to use of synthetic antioxidants. This study is the first report about antioxidant and antityrosinase activities and the identification of phenolic compounds of litchi seed. Further investigation is required to examine the cytotoxicity effects of litchi seed extract.

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