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Identification of Two Polyphenolic Compounds with Antioxidant Activities in Longan Pericarp Tissues

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Longan fruits contain a significant amount of polyphenols. In the present study, polyphenols were extracted from longan pericarp tissues, and then two representative polyphenols were separated and purified by polyamide column chromatography, Sephadex LH-20 column chromatography, and silica gel column chromatography. On the basis of ^{1}H NMR, ^{13}C NMR, and electrospray ionization mass spectrometric (ESI-MS) data, the two compounds were identified as 4-*O*-methylgallic acid and (−)-epicatechin, respectively. In terms of reaction with longan polyphenol oxidase (PPO), (−)-epicatechin was further identified as the PPO substrate that caused longan fruit to brown. The results of antioxidant activity showed that 4-*O*-methylgallic acid had higher reducing power and 2,2-diphenyl-1-picrylhydrazyl- (DPPH-), hydroxyl radical-, and superoxide radical-scavenging activities than (−)-epicatechin.

KEYWORDS: Polyphenols; antioxidant; longan; 4-*O*-methylgallic acid; (−)-epicatechin

INTRODUCTION

Polyphenols are a large class of phytochemicals containing more than one phenol group per molecule. Thousands of known polyphenols in plants exist in nature, and some of them are widely distributed in different fruits. The derivatives of cinnamic acid, for example, chlorogenic acid, are found in pears, apples, apricots, plums, etc. (*1*−*4*). Flavonoids are identified from many fruits such as litchi, strawberry, citrus, mulberry, grape, etc. (*5*−*9*). Moreover, the derivatives of benzoic acid, for example, gallic acid, are also detected in grapes, mangoes, guavas, etc. (*10*, *11*). The plant polyphenols are the most important group of natural antioxidants because of their diversity and extensive distribution. They possess the ability to scavenge both active oxygen species and electrophiles (*12*). Recent investigations have showed that many phenolic compounds, including flavonoids, tannins, and phenolic acids, exhibited strong antioxidant properties (*13*−*15*). In some fruits, polyphenols with antioxidant properties, such as flavonoids, procyanidins, anthocyanins, and so on, have been identified by HPLC, NMR, or MS methods (*16*−*20*).

Longan, also known as lungan or dragon's eye, belongs to the family Sapindaceae (*21*, *22*). Because of its delicate and sweet flesh, longan fruit possesses a high commercial value (*23*). Previous researches have identified some polyphenolic compounds from different tissues of longan, including gallic acid, corilagin (an ellagitannin), ellagic acid, procyanidin A-type dimer, procyanidin B2, quercetin 3-*O*-rhamnoside, etc (*24*, *25*). The antioxidant activities of polyphenols in longan fruits are also reported in some papers. Soong and Barlow (*26*) found that total antioxidant capacity was related to phenolic contents in longan seeds and flesh. They further identified gallic acid and ellagic acid from longan seeds by reverse-phase (RP) HPLC coupled with photodiode array detection and confirmed that both compounds contributed to the potent antioxidant activity (*27*). Rangkadilok et al. (*28*) examined the antioxidant activities of longan seed and pulp extracts by using the scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH), scavenging activity of superoxide radicals, and oxygen radical absorbance capacity (ORAC) assay. Their results showed the polyphenols in longan seed extracts, such as corilagin, gallic acid, and ellagic acid, exhibited the same scavenging effect as DPPH and superoxide radicals in Japanese green tea extracts. These compounds also had the activity of ORAC. However, up to now, there have been few detailed reports on the antioxidant activities of polyphenols from longan pericarp tissues. Generally, the pericarps are discarded when people consume longan fruits, which results in not only degradation of the environment but also the waste of a resource. Therefore, it is important to investigate the functional components, especially polyphenols, in longan pericarp tissues, so that they can be utilized as health-beneficial bioactive compounds rather than just be discarded as waste.

The objective of this study was to extract and isolate the representative polyphenols with antioxidant activity in longan pericarp tissues. Structure elucidation of the polyphenolic

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compounds was conducted by nuclear magnetic resonance (NMR) and electrospray ionization mass spectrometry (ESI-MS). This research was to determine the chemical structure and antioxidant capability of phenolic components of longan fruit pericarp. These results would be useful in effectively developing and utilizing agriculture waste materials or converting them into valuable functional ingredients.

MATERIALS AND METHODS

Plant Materials. The common longan cultivar in markets of South China, Shixia (*Dimocarpus longan* Lour. cv. Shixia), was selected as plant material. Fruits at a mature stage were obtained from a commercial orchard in Guangzhou in August 2005. Uniform fruits without disease symptoms were selected and then peeled. The fresh pericarp tissues were collected, lyophilized in liquid nitrogen, and stored at −20 °C until extraction and analysis.

Extraction of Polyphenolic Compounds. Polyphenols in longan percarp tissues were extracted according to the modified methods of Kujala et al. (*29*), Sarni-Manchado et al. (*30*), and Zhang et al. (*31*). Lyophilized pericarp tissues (600 g) were ground in liquid nitrogen with a mortar and pestle. The ground powder was extracted twice for 20 min at 4 °C with 2000 mL of methanol−acetone−water (4.5:4.5:1 v/v/v) containing 6 g of ascorbic acid by continuous stirring. These extracts were combined and filtered under vacuum. The collected filtrate was centrifuged for 15 min at 7000*g*. The supernatant was extracted twice in a separatory funnel with petroleum ether (petroleum ether: supernatant = 2:1 v/v) to remove the fat-soluble pigments. The organic phase was discarded. The aqueous phase was collected, concentrated, and then dried under vacuum at 50−55 °C. The obtained extracts (23.7 g) were stored at −20 °C prior to further purification.

Purification of Two Polyphenolic Compounds. The extracts (6 g randomly taken from a 23.7 g sample) were purified by polyamide, Sephadex LH-20, and silica gel column chromatography. Aliquots (6 g) of the extracts were subjected to a 450 × 25 mm column of polyamide (245 g, 60−80 mesh, Taizhou Luqiao Biochemical Corp., Taizhou, China), and eluted with 550 mL of water, with 20%, 40%, 60%, and 80% aqueous methanol, and then with methanol. The eluents were collected into seven fractions. Each fraction reacted with 0.5% $FeCl_3$ solution to detect the presence of polyphenols (*5*). Among them, the fraction containing polyphenols, which turned positive with $FeCl_3$ (i.e., the eluted fraction turned blue after addition of $FeCl_3$ solution), was concentrated under vacuum to obtain a yellowish syrup (2805 mg). An aliquot (290 mg) of this syrup was rechromatographed on a 1500 × 15 mm Sephadex LH-20 column (Amersham Biosciences, Uppsala, Sweden), with 600 mL of methanol as an eluent. A major fraction (119 mg) eluted from the Sephadex LH-20 column then was further separated on a 400 × 6 mm silica gel (4 g, 200−300 mesh, Qingdao Marine Chemical Factory, Qingdao, China) column, with 650 mL of chloroform/methanol/formic acid (90:10:0.4 v/v/v) as an eluent. The major fractions eluted from silica gel column were combined, concentrated, and then reacted with 0.5% $FeCl_3$ solution to identify the presence of representative polyphenols.

Enzymatic Reaction with PPO. Longan pericarp polyphenols (2.5 mL) prepared in 0.01 M sodium phosphate buffer (pH 6.8) were mixed with 0.1 mL of longan PPO (protein content about 75.6 μg/mL, total activity 181 × 10^3 units) extracted from pericarp tissues by the method of Jiang (*32*). The absorbance variations of reaction solutions were measured at the absorption peak of enzyme-catalyzed products on a UV-2802 spectrophotometer (Unic, Shanghai, China).

^{1}H and ^{13}C NMR Analysis. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra of the representative polyphenols were recorded on a Bruker DRX-400 NMR spectrometer (Bruker Co., Rheinstetten, Germany), with deuterated methanol (methanol-d_4) as the solvent and tetramethylsilane (TMS) as the internal standard. Chemical shifts (δ) are given in parts per million (ppm) relative to TMS.

ESI-MS Analysis. Electrospray ionization mass spectroscopic (ESI-MS) analysis of longan polyphenols was performed on a PE Sciex API 2000 LC/MS/MS system (ABI, Foster City, CA) in negative- and positive-ion modes. The representative polyphenols were dissolved in chromatographically pure methanol (Merck, Darmstadt, Germany) and injected directly into the instrument at a rate of 5 μL/min. The samples were analyzed by use of a turbo ionspray ionization source with a voltage of 5500 V maintained on the ESI interface. Mass spectrometric data were acquired by accumulation of 10 MCA (multiple channel acquisition) scans over mass ranges of *m*/*z* 50−400 and *m*/*z* 50−700 for different samples.

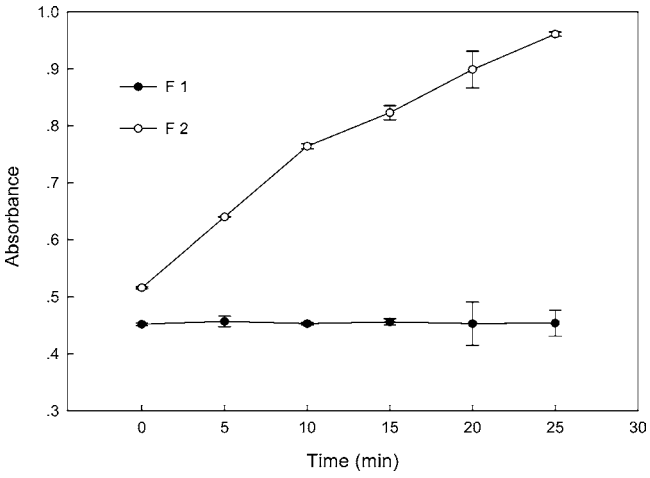

Figure 1. Absorbance of two polyphenolic compounds catalyzed by longan PPO at absorption peak.

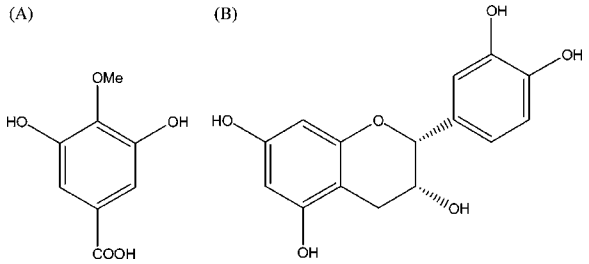

Figure 2. Chemical structures of (**A**) F1, 4-*O*-methylgallic acid, and (**B**) F2, (−)-epicatechin.

Assay for Antioxidant Activity: *Determination of Reducing Power.* The ferric-reducing antioxidant power (FRAP) assay was determined according to the modified method of Oyaizu (*33*). Polyphenols isolated from longan pericarp tissues were prepared into 20, 40, and 100 μg/mL solutions in ethanol. Aliquots (0.5 mL) of 0 (control; i.e., 0.5 mL of ethanol instead of polyphenol solution), 20, 40 and 100 μg/mL polyphenol solutions was mixed with 2.5 mL of 0.2 M 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) was added into each sample, 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. The absorbance at 700 nm was measured on the UV-2802 spectrophotometer. Higher absorbance indicated higher reducing power.

Determination of DPPH-Scavenging Activity. The DPPH-scavenging activity was measured by the method of Yang et al. (*34*) with some modifications. Aliquots (0.5 mL) of 0 (control), 20, 40, and 100 μg/mL longan polyphenols dissolved in ethanol were added into 2.5 mL of 0.2 mM DPPH solution in ethanol. The absorbance at 517 nm of samples was measured after 30 min of incubation at 25 °C. DPPH-scavenging activity (%) = $1 - [(A - B)/A_0] \times 100$, where A_0 = absorbance of control, A = absorbance of sample, and B = absorbance of 0.5 mL of polyphenolic compound + 2.5 mL of ethanol.

Determination of Hydroxyl Radical-Scavenging Activity. The hydroxyl radical-scavenging activity was measured according to the method of Lee et al. (*35*) with some modifications. Aliquots (0.5 mL) of 0 (control), 20, 40, and 100 μg/mL longan polyphenols dissolved in ethanol were mixed with 1 mL of reaction buffer [containing 100 μM

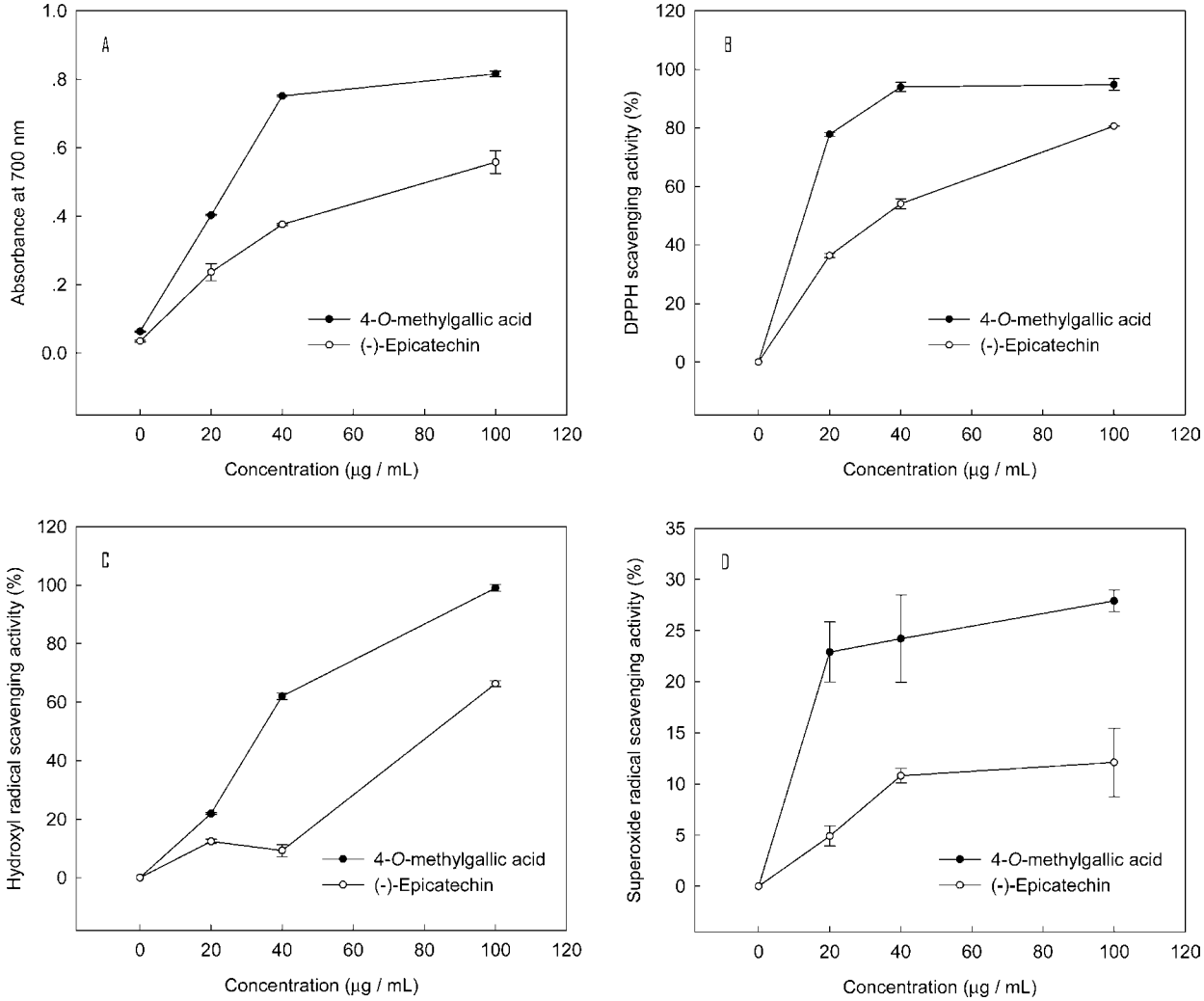

Figure 3. Comparison of (**A**) reducing power, (**B**) DPPH-scavenging activity (%), (**C**) hydroxyl radical-scavenging activity (%), and (**D**) superoxide radical-scavenging activity (%) of 4-*O*-methylgallic acid and (–)-epicatechin.

$FeCl_3$, 104 μM ethylenediaminetetraacetic acid (EDTA), 2.5 mM H_2O_2, 2.5 mM deoxyribose, and 100 μM ascorbic acid]. The reaction solutions were incubated for 1 h at 37 °C, and then 1 mL of 0.5% thiobarbituric acid, dissolved in 0.025 M NaOH, and 1 mL of 2.8% trichloroacetic acid were added into the mixtures. The mixtures were incubated for 30 min at 80 °C and cooled rapidly in an ice bath. The absorbance of samples was measured at 532 nm. Hydroxyl radical-scavenging activity (%) = (1 − absorbance of sample/absorbance of control) × 100.

Determination of Superoxide Anion-Scavenging Activity. The superoxide anion-scavenging activity was measured by the method of Siddhurajua et al. (*36*) with minor modifications. Aliquots (0.2 mL) of 0 (control), 20, 40, and 100 μg/mL longan polyphenols dissolved in ethanol were mixed with 3 mL of reaction solution (containing 1.3 μM riboflavin, 13 mM methionine, 63 μM NBT, 100 μM EDTA, and 0.05 M sodium phosphate buffer, pH 7.8). The reaction solutions were incubated for 15 min under the 4000 lux illumination. The absorbance of samples was measured at 560 nm. Superoxide anion-scavenging activity (%) = (1 − absorbance of sample/absorbance of control) × 100.

Data Analysis. The enzymatic reaction and the determinations of antioxidant activity of two polyphenolic compounds were carried out in triplicate. The results represented mean ± standard error (SE) of three replicate determinations.

RESULTS AND DISCUSSION

Extraction and Purification of Two Polyphenolic Compounds. The polyphenolic compounds extracted from longan pericarp tissues were first purified by polyamide column and seven fractions were collected. Among them, fraction 2 turned blue after reacting with 0.5% $FeCl_3$ solution, which indicated that this fraction contained phenolic substances. Fraction 2 was then separated by a column of Sephadex LH-20 and a subfraction (119 mg) which turned blue after reacting with 0.5% $FeCl_3$ solution, was obtained. The subfraction was further applied onto a column of silica gel. After elution with chloroform/methanol/formic acid (90:10:0.4 v/v/v), two purified fractions (F1, 27 mg, and F2, 52 mg) were finally obtained, which turned blue after reacting with 0.5% $FeCl_3$ solution, indicating that they contained phenolic compounds.

Enzymatic Reaction with PPO. After 2.5 mL of F1 and F2 prepared in 0.01 M sodium phosphate buffer (pH 6.8) were mixed with 0.1 mL of longan PPO, the absorbance of F1 was invariable at absorption peak (435 nm) of enzyme-catalyzed products and the reaction solution did not turn brown. However, the absorbance of F2 continuously increased at the absorption peak (**Figure 1**) because of gradual accumulation of the enzyme-catalyzed products, and after 25 min, the enzyme-catalyzed reaction solution turned brown. The results showed that F2 but not F1, as the substrate, could react with longan PPO, which would induce the browning of longan pericarp tissues.

Structure Elucidation of Two Polyphenolic Compounds. F1 and F2 isolated from longan percarp tissues were identified as 4-*O*-methylgallic acid (**Figure 2A**) and (−)-epicatechin (**Figure 2B**) on the basis of ^1H, ^{13}C NMR, and ESI-MS analyses.

In our study, F1 was colorless needlelike crystals. Its structure was confirmed by comparison with the literature data (*37*). $[M - H]^-$ peak of F1 was observed at *m*/*z* 183.0 in the ESI-MS spectrum (scanning range *m*/*z* 50−400), which corresponded to the deprotonated molecule of 4-*O*-methylgallic acid ($C_8H_8O_5$), whose exact mass is 184.150, and the characteristic fragment ions of F1, such as *m*/*z* 169.1 and 123.8, were identical with those of 4-*O*-methylgallic acid reported in literature (*37*). In addition, two hydrogen signals and six carbon signals detected on ^1H and ^{13}C NMR spectra of F1 were also consistent with those reported in the literature for 4-*O*-methylgallic acid (*37*). Rangkadilok et al. (*24*) have found one of major polyphenols in longan fruit is gallic acid. It has a similar structure to 4-*O*-methylgallic acid, which is just the methyl ether derivative of gallic acid.

F2 was yellowish needlelike crystals. Its $[M - H]^-$ peak was detected at *m*/*z* 289.2 (scanning range *m*/*z* 50−700), corresponding to the deprotonated molecule of (−)-epicatechin ($C_{15}H_{14}O_6$), whose exact mass is 290.079 (*38*). The characteristic fragment ions of F2, such as *m*/*z* 245.1, 179.1, and 137.1, were also identical with those of (−)-epicatechin reported in the literature (*38*, *39*). Moreover, in ^1H and ^{13}C NMR spectra of F2, the characteristic signals of 9 hydrogens and 15 carbons were consistent with those reported in the literature for (−)-epicatechin (*40*−*44*).

Antioxidant Activity of Two Polyphenolic Compounds. Overall, 4-*O*-methylgallic acid and (−)-epicatechin isolated from longan pericarp tissues possessed antioxidant capabilities, on the basis of our studies. By comparison of reducing power, DPPH-scavenging activity, hydroxyl radical-scavenging activity, and superoxide radical-scavenging activity of the two polyphenols, it could be demonstrated that 4-*O*-methylgallic acid had higher antioxidant activity than (−)-epicatechin.

Yen and Duh (*45*) reported that reducing power was associated with antioxidant activity. High reducing power also indicates a high antioxidant activity. **Figure 3A** shows that the reducing powers of 4-*O*-methylgallic acid and (−)-epicatechin exhibit an increasing trend in conjunction with an increase in the two polyphenols' concentration. From 0 to 40 μg/mL, the reducing power of both increased markedly, but when the polyphenol concentrations exceeded 40 μg/mL, the increase in reducing power slowed down because the redox reaction about FRAP gradually tended to stabilize. These results indicate that, under our experimental conditions, the antioxidant activities of the two polyphenols were enhanced at increasing concentrations. When the concentration exceeded 40 μg/mL, the polyphenols remained at high antioxidant activities but varied little. It may also be seen from **Figure 3A** that, at the same concentration, 4-*O*-methylgallic acid exhibited a higher reducing power than (−)-epicatechin, suggesting that 4-*O*-methylgallic acid had a stronger electron-donating capacity (*46*), that is, a stronger antioxidant activity, than (−)-epicatechin.

In order to further compare antioxidant activities, the DPPH-scavenging activity (**Figure 3B**), hydroxyl radical-scavenging activity (**Figure 3C**), and superoxide radical-scavenging activity (**Figure 3D**) of the two polyphenols were analyzed. The results showed that 4-*O*-methylgallic acid exhibited a stronger radical scavenging effect than did (−)-epicatechin when the concentration of polyphenols was at 20, 40, or 100 μg/mL. From 0 to 20 μg/mL, the DPPH and superoxide radical-scavenging activities of 4-*O*-methylgallic acid increased obviously, but when its concentration exceeded 20 μg/mL, scavenging activities increased but slowly, because the reaction of the scavenging radicals gradually tended to stabilize from 20 to 100 μg/mL. A similar trend was also found in the DPPH and superoxide radical-scavenging activities of (−)-epicatechin. From 0 to 40 μg/mL, both scavenging activities of (−)-epicatechin increased markedly, but beyond 40 μg/mL the scavenging activities increased only slowly. In addition, at 100 μg/mL, the DPPH and hydroxyl radical-scavenging activities of 4-*O*-methylgallic acid exceeded 80% and those of (−)-epicatechin exceeded 60%. However, their superoxide radical-scavenging activities were under 30%, which indicates that both polyphenols showed higher DPPH and hydroxyl radical-scavenging activities than their superoxide radical-scavenging activity at this concentration.

Conclusions. In the present study, two representative polyphenolic compounds, 4-*O*-methylgallic acid and (−)-epicatechin, were identified by NMR and ESI-MS analyses. (−)-Epicatechin could react with longan PPO and resulted in pericarp browning after harvest because of its *o*-dihydroxyphenol structure (The *o*-dihydroxyphenol can specifically bind to PPO and then is catalyzed into *o*-quinones, which further react with other compounds to produce the brown-colored byproducts.). This result is helpful to determine the chemical mechanism of longan pericarp browning. By comparing reducing power and DPPH-, hydroxyl radical-, and superoxide radical-scavenging activities, it could be found that 4-*O*-methylgallic acid and (−)-epicatechin possessed antioxidant properties and that 4-*O*-methylgallic acid exhibited stronger antioxidant capability than (−)-epicatechin. These polyphenols potentially provide benefits for human health because of their antioxidant activities, so they can be derived from the discarded pericarp of longan. This provides a useful example of converting wastes into valuable functional ingredients.

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