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Full Length Research Paper

Antioxidant activity of longan (*Dimocarpus longan*) barks and leaves

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In this paper, the barks and leaves of longan (*Dimocarpus longan* Lour.) were extracted with 80% methanol. The antioxidant activity and the contents of ellagic acid (EA) in the extracts were investigated. For the evaluation of antioxidant activities, the extracts possess almost the same 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity and reducing power. Besides, the antioxidant activity was concomitant with the development of the reducing power with high correlation coefficients. The contents of EA in the extracts were 0.91 and 3.723 mg/g dry samples, respectively. After hydrolysis, the EA contents increased almost three and four times. Therefore, the ellagic acid in longan barks and leaves exist mostly in the form of ellagitannins. The research showed that longan barks and leaves not only were excellent sources of free-radical inhibitors, but also had potential use in the production of ellagic acid.

Key words: Antioxidant activity, ellagic acid, longan, barks, leaves.

INTRODUCTION

Antioxidants are the compounds that can increase the shelf-life of lipids and lipid-containing foods when added, by retarding the process of lipid peroxidation, which is one of the major reasons for the deterioration of food products during processing and storage and in the pomological features, nutritional quality, polyphenol content analysis and antioxidant properties of domesticated and three wild ecotype forms of raspberries (*Rubus idaeus* L.) (Gülçin et al., 2011; Koksal1 et al., 2011). In the past decades, commercial available antioxidants were most synthetic antioxidants, such as 2-3-tert-butyl-4-methoxyphenol (BHA), 2,6-di-ter-butyl-4-methylphenol (BHT) and so on. However, those compounds had been suspected of possessing certain toxicity and may be responsible for the damage of human organs (Pan et al., 2004, 2007). Therefore, the research of isolation of natural antioxidants from natural plant, which have no such side effects, has been the focus of researchers in recent years.

Polyphenols are of great importance because of their effects on sensory properties, including astringency and colour, and possible health benefits. The use of plants as spices and herbs indicates that the antioxidative and antimicrobial constituents are present in all parts of the plants, including tree barks, stalks, leaves, fruits, roots and so on (Gülçin, 2011; Gülçin et al., 2010). Since all the phenolic classes have the structural requirements of free radical scavengers and have potential as food antioxidants (Bandoniene and Murkovic, 2002), phenolic compounds have been recognized as natural antioxidants that possess beneficial effects against free radicals in biological systems (Prasad et al., 2005) and considerable interest have been focused on the field of vegetables and fruits in recent years (Hollman and Katan, 1999; Li and Jiang, 2007).

Ellagic acid (EA) is one of the important polyphenols, which mainly exists in nuts and berries, such as strawberry, raspberry and blackberry (Amakura et al., 2000; Vekiari et al., 2008). This compound can exist as free form, glycoside or linked as ellagitannins esterified with glucose (Bate-Smith, 1972 ; Haddock et al., 1982;

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Abbreviations: DPPH, 2 ,2 - Diphenyl -1- picrylhydrazyl; **EA,** ellagic acid.

Maas and Galletta, 1991). In the1960s, EA was mainly studied for its effects on blood clotting, whitening of the skin and its hemostatic activity. Meanwhile, there has been increasing interest in its antioxidant, antimutagenic, anti-inflammatory and cardioprotective activity and possible antimutagenic, antiviral and anticarcinogenic effects during the past few years. Most of these works have been proved in laboratory animals, while a few works are even reported in humans (Akagi et al., 1995; Hakkinen et al., 2000; Sigman et al., 1984; Priyadarsini et al., 2002). The hepatoprotective property of ellagic acid has been reported both *in vitro* and *in vivo* (Singh et al., 1999). Besides, there is a profusion of pomegranate nutraceutical products called "standardized to 40% ellagic acid" in the market (Lansky, 2006). Ellagitannins are the primary source of ellagic acid, and some of them have also been shown to possess anti-tumor-promoting activity, antibacterial and antiviral properties, as well as host-mediated antitumor effects (Okuda, 2005).

Longan (*Dimocarpus longan* Lour.) is a member of the Sapindaceae family. It is a highly attractive fruit extensively distributed in China and South East Asia including Thailand, Vietnam and the Philippines. For the past decade, the antioxidant activity and content of EA in longan mostly concentrated on its fruits, fruit pericarp and seeds (Hsieh et al., 2008; Prasad et al., 2009, 2010; Nuchanart et al., 2007; Richard and Jutamaad, 2005). However, there have been few reports on the extracts of longan barks and leave. Agricultural by-products are quite potential and attractive sources of antioxidant components (Moure et al., 2001). The primary waste fractions, which are peels, barks, leaves and seed residues, contain high amounts of bioactive components that can be potentially exploited as antioxidant agents and nutraceutical.

In this paper, the antioxidant activity and content of ellagic acid in the methanol extracts of longan barks and leaves were investigated. The results show that these wastes not only were excellent sources of free-radical inhibitors, but also had potential use in the production of ellagic acid. This investigation provides a new way for the reuse of agricultural by-products.

MATERIALS AND METHODS

The materials used in this paper were collected just from the longan trees planted in the institute. Mature leaves and barks were first cleared and dried at 60°C, and then were ground using a stainless-steel grinder. They were stored in vacuum-packaged polyethylene pouches at -20°C until required for analysis.

Chemicals

High performance liquid chromatography (HPLC) grade methanol was from Thermo Fisher Co. Ellagic acid (EA), Folin–Ciocalteu's (FC) phenol reagent and gallic acid (GA) were purchased from Fluka. The 2,2'-diphenyl-2-picrylhydrazyl (DPPH) radical was received from Sigma. All the other chemicals were of analytical grade and used without further purification. Ultra-pure water was used for the preparation of solutions.

Sample preparation

Approximately 200 mg of dried longan barks and leaves were separately weighed and refluxed with 30 ml of 80% methanol at 70°C for 3 h under magnetic stirring, respectively. The above extracts were passed through Whatman filter paper (no. 4) and subsequently used for various assays.

Hydrolysis

An amount of 8.74 ml of the extracts received above were accurately taken and 1.26 ml concentrated hydrochloric acid (HCl) was added by careful mixing (the final HCl concentration was 1.5 M). The solutions were stirred using a magnetic stirrer and refluxed at 85°C for 2 h by the method similar to that previously reported by Hertog et al., 1992). The final solutions were evaporated to dryness under vacuum below 40°C. The residue was dissolved in 5 ml of HPLC grade methanol and filtered through a 0.22 µM filter prior to injection (10 µL) into the HPLC system.

Determination of total phenol content (TPC)

TPC was determined using the FC assay described before (Singleton and Rossi, 1965). Typically, samples (0.2 ml) were introduced into test tubes and followed by the addition of 1.0 ml of Folin-Ciocalteu's reagent (diluted 10 times with water in advance) and 0.8 ml of water. The solutions were allowed to stand 5 min at room temperature before the addition 0.8 ml of sodium carbonate solution (7.5% w/v). After reacting in dark for 30 min, the absorbance of the solutions were measured at 765 nm on a UV–vis spectrophotometer (Shimadzu UV-1700, Japan). The test was run in triplicate, and then a calibration curve was prepared using a standard solution of gallic acid. The results were expressed as mg gallic acid equivalents (GAE)/g dry samples.

DPPH free radical-scavenging assay

The free radical scavenging activity of the extracts was performed by measuring the decrease in absorbance of DPPH solution at 517 nm in the presence of the extracts by the method proposed by Şerbetçi et al. (2010) with some modification. The solution of 5 mM was prepared by dissolving DPPH in methanol. For the evaluation of free radical scavenging activity, 1 ml of DPPH was added into 1 ml of the extracts with different concentrations. The mixture was then allowed to stand at room temperature for 30 min in dark before the absorbance at 517 nm was read. The control was prepared as above without extract. The antioxidant activity could be expressed as the following equation:

$$\text{Scavenging activity} = \frac{A_0 - A_s}{A_0} \times 100\%$$

Where A_0 and A_s are the absorbance at 517 nm of the control and sample solution, respectively. The inhibition concentration IC_{50} was defined as the amount of sample extracted into 1 ml solution necessary to decrease 50% of the initial DPPH concentration. The value of IC_{50} was derived from the percentage disappearance vs. concentration plot. Here, concentration means mg of samples extracted into 1 ml solution.

Ferric reducing power

The antioxidant capacity of the extracts was also tested by the ferric reducing power. This assay was performed according to a modified method described by Juntachote and Berghofer (2005). Sample extracts of 1 ml with different concentrations were added into 0.5 ml of 0.2 M phosphate buffer (pH 6.6) and 1.5 ml of potassium ferricyanide (0.3%). The mixtures were incubated at 50°C for 30 min, and then 1 ml of trichloroacetic acid (10%) was added. After centrifuging at 5000 rpm for 10 min, 1 ml of ferric trichloride (0.3%) was added into the mixture. The absorbance of the solution at 700 nm was measured after standing for 30 min. The assay was run in triplicate and the increase in absorbance of the reaction indicated the reducing power of the samples.

HPLC analysis

HPLC analysis was performed on a Shimadzu HPLC (Model LC-20A) equipped with a UV-vis detector (Model SPD-20A). The separation was conducted on a Shim-Pack column (GCP-ODS, 250 × 4.6 mm i.d., GL Sciences Inc., Japan) and the data were collected on a HP personal computer with the software of LC solution. The temperature of the column was 35°C. EA was eluted at a flow rate of 1.0 ml/min, using a mobile phase consisting of 50% methanol and 50% acetic acid solution (1%). The mobile phase was vacuum filtered through a 0.22 µM membrane filter before used. The appearance of EA was tested by comparison of the peak areas obtained at wavelength 254 nm.

Construction of reference standards calibration curve

The stock solution (400 mg/L) was prepared by dissolving EA in HPLC-grade methanol, and the working curve was prepared by diluting the stock solution with the same solvent.

RESULTS AND DISCUSSION

Total phenol content

Polyphenolic compounds are quite important constitutes for fruits and their trees. Since polyphenols are sensitive to heat (Soong and Barlow, 2004), the temperature of 60°C was chosen for the treatment of materials in oven. The total phenol content (TPC) expressed as gallic acid equivalents (GAE) achieved by FC method in the leaves and barks were 132.47 and 140 mg/g dry weight, respectively. The results show that the TPC in mature longan barks and leaves were quite high and close. Moreover, it is well known that plant phenolics are highly effective free radical scavengers and antioxidants, and the activity is derived largely from the phenolic and polyphenolic compounds. Therefore, the investigation on the antioxidant activity of longan barks and leaves was of great importance.

DPPH assay and reducing power

Due to its operating simplicity, DPPH is one of the most popular methods employed for the evaluation of antioxidant ability. In the radical form, the molecule of DPPH has an absorbance at 517 nm, which will disappear after the acceptance of an electron or hydrogen radical from an antioxidant in the solution to become a stable diamagnetic molecule (Matthäus, 2002). Besides, DPPH has the advantage of being unaffected by certain side reactions of polyphenols, such as metal ion chelation and enzyme inhibition.

In this paper, different concentrations of extracts were used. The relationship between the scavenging activity and concentrations are shown in Figure 1. It can be clearly seen that the scavenging activity of longan barks and leaves extracts were concentration-dependent. With the increase of the amount extracted into solution, the scavenging activity of the extracts of both the leaves and the barks increased accordingly. At each concentration, the scavenging activity of the bark extract was almost the same as that of the leaf extract. IC_{50} was defined as the concentration of the methanol extracts to quench 50% of DPPH in the solution under the chosen experimental conditions. The values of IC_{50} for barks and leaves of longan extracts derived from the figure of scavenging activity and concentrations were 0.057 and 0.058 mg/ml, respectively. The data obtained here reveal that the methanol extracts are quite good free-radical inhibitors and have potential use in the termination of free radical reactions.

Furthermore, since the reducing capacity of compounds may serve as a significant indicator of their potential antioxidant activity, the antioxidant activity of the methanol extracts were also investigated by reducing power. During the reducing power assay, the presence of reductants (antioxidants) in the samples would result in the reduce of Fe^{3+}/ferricyanide complex to the ferrous form (Fe^{2+}), which can be then monitored by measuring the formation of Prussian blue at 700 nm (Chung et al., 2002). Figure 2 shows the reducing power of methanol extracts with different concentrations. Similar to that of the DPPH assay, the reducing power of the longan methanol extracts were also concentration-dependent. At same concentration levels, the extract of longan leaves showed a little higher reducing power than that of the longan barks.

It had been reported that the antioxidant activity may be concomitant with the development of the reducing power, and it can be seen from Figures 1 and 2 that both the scavenging activity and the reducing power increased with the increase of extract concentration. The correlation coefficients between antioxidant activity and reducing power for the extracts were quite high (R^2 = 0.987 for longan barks and 0.983 for longan leaves) (Figure 3), indicating that antioxidant properties were concomitant with the development of reducing power. This result is in agreement with that of Juntachote and Berghofer (2005), who reported that the reducing power of Holy basil and Galangal extracts correlated well with the extent of antioxidant activity.

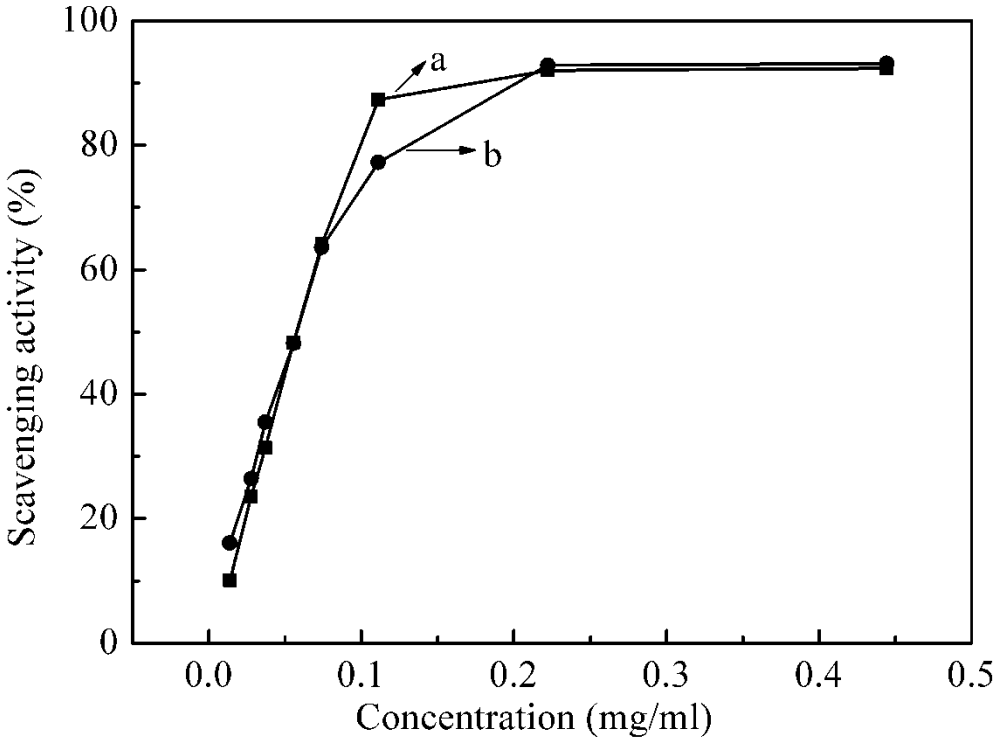

Figure 1. Scavenging activity of the methanol extracts of (a) longan barks and (b) longan leaves.

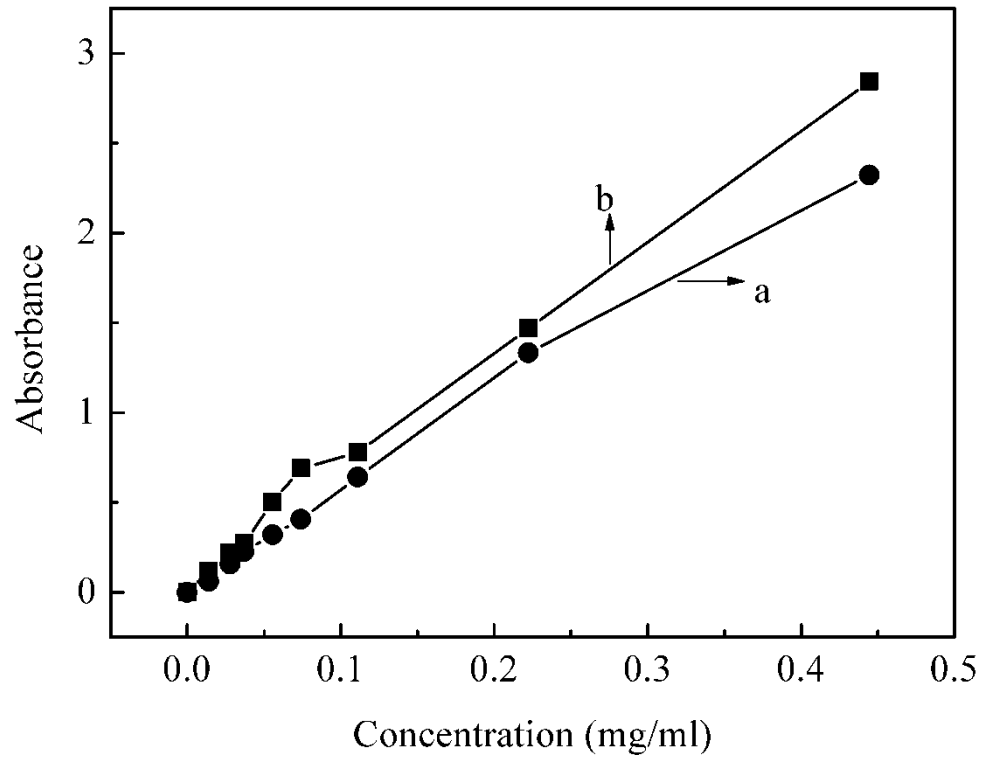

Figure 2. Reducing power of the methanol extracts of (a) longan barks and (b) longan leaves.

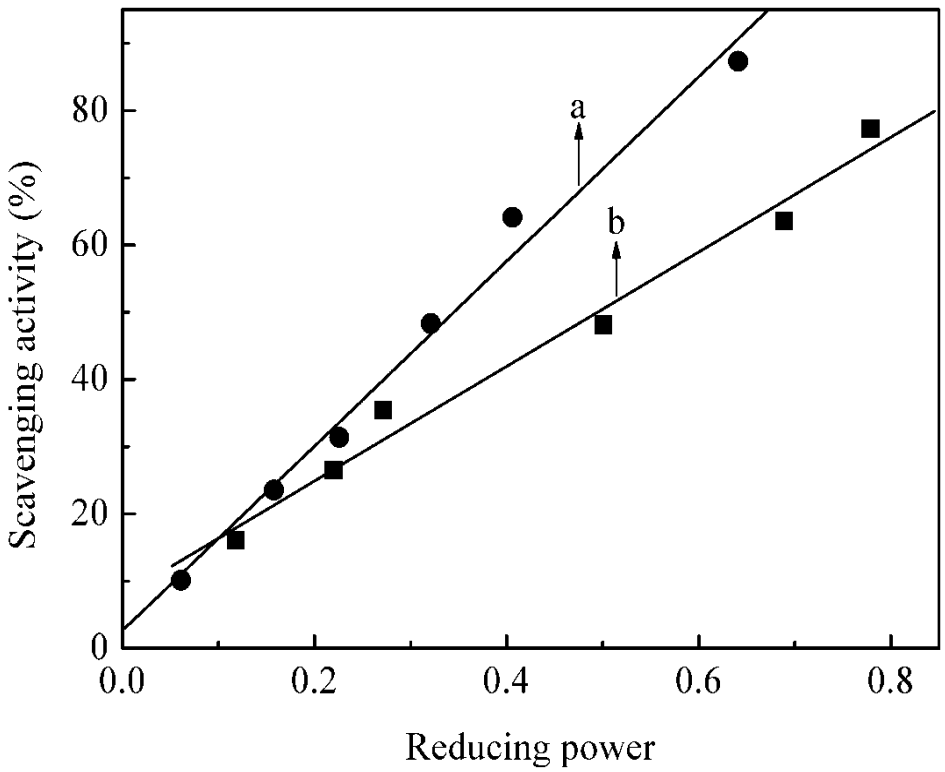

Figure 3. Linear correlation between reducing power and the percentage of DPPH radical-scavenging activity of the methanol extracts of (a) longan barks and (b) longan leaves. DPPH, 2,2-Diphenyl-1-picrylhydrazyl.

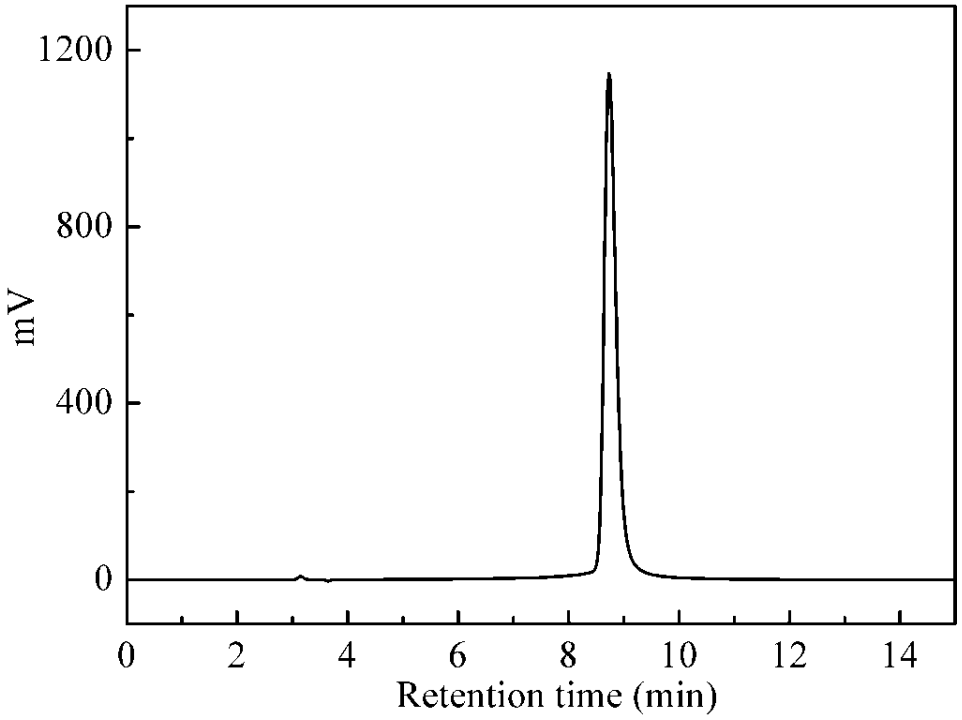

Figure 4. A typical HPLC chromatogram of ellagic acid. HPLC, High performance liquid chromatography.

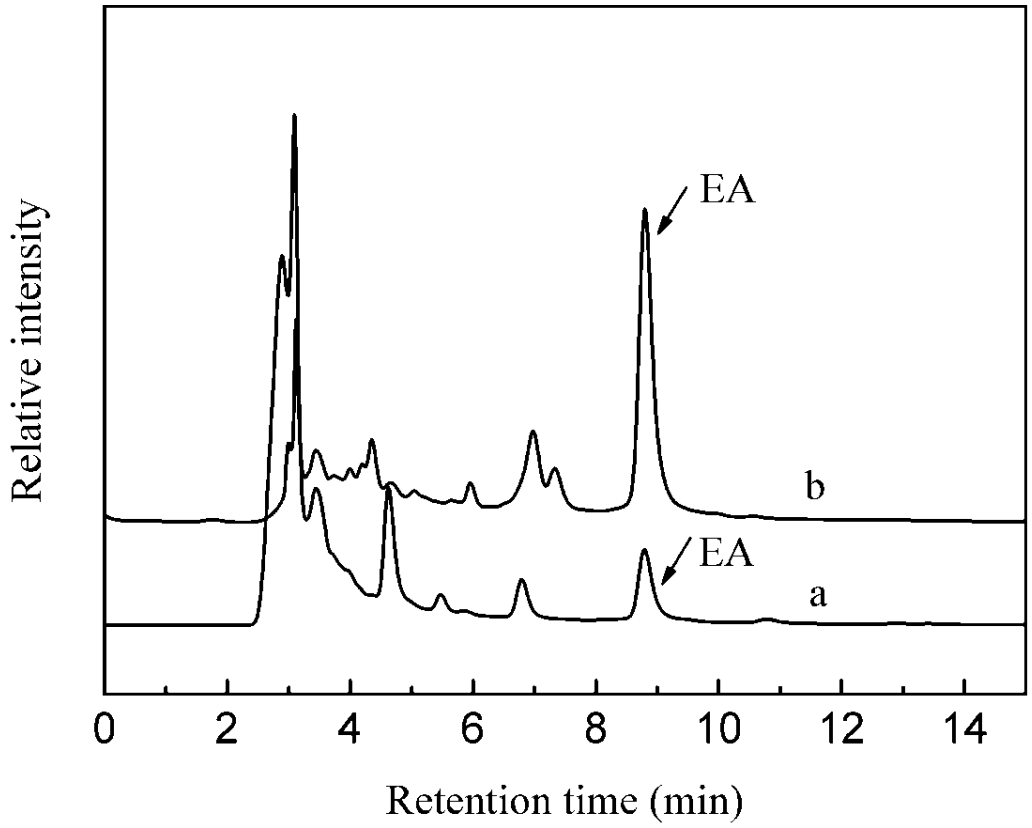

Figure 5. HPLC chromatograms of (a) unhydrolyzed and (b) hydrolyzed barks. HPLC, High performance liquid chromatography.

Identification and quantification of EA by HPLC assay

Reverse phase (RP)-HPLC was considered as an effective tool for the determination of ellagic acid in plants. Shown in Figure 4 is the chromatogram of authentic standard of this compound. The retention time of EA was 8.73 min under the chosen conditions. The relative standard deviation (RSD) of reproducibility in the retention time obtained with 10 successive determinations and intra-day (n=3) were 0.1 and 0.2%, respectively. The linear range for the determination of EA was from 0.1 to 200 ppm, with a regression coefficient of 0.9998. All these showed that the method used herein was quite stable and widely applicable.

Determination of free EA in the barks and leaves of longan

The chromatograms for the methanol extracts of longan barks and leaves were given in Figures 5a and 6a. The chromatograms showed clear peaks with a retention time of 8.76 and 8.77 min, respectively. This indicated the appearance of EA in both barks and leaves of longan trees. Furthermore, the content of free EA in the two parts of longan tree was determined by HPLC. The results were 0.091 and 3.723 mg/g dry samples for the barks and leaves, respectively. Apparently, the contents of free EA in the barks and leaves of longan were quite high and so such waste of longan had potential use in the production of ellagic acid.

Hydrolysis of the extracts

Ellagitannins are known as the primary source of ellagic acid. These precursor molecules yield ellagic acid when undergoing hydrolysis with acid or base. In the past decade, an acid solution was mostly employed for the hydrolysis of ellagitannins (Rommel and Wrolstad, 1993; Michael and Augustin, 2000). Here in this paper, an acid solution of 1.5 M HCl was used for the hydrolysis of the methanol extracts. It can be clearly seen that the hydrolyzed samples had a quite larger area as compared with those unhydrolyzed (Figures 5b and 6b). The contents of EA in longan barks and leaves after hydrolysis were 3.6 and 18.196 mg/g dry samples, respectively. The data show that the content in hydrolyzed barks and leaves were almost three and four times more than that of the unhydrolyzed ones. This indicates that most of EA exists the form of

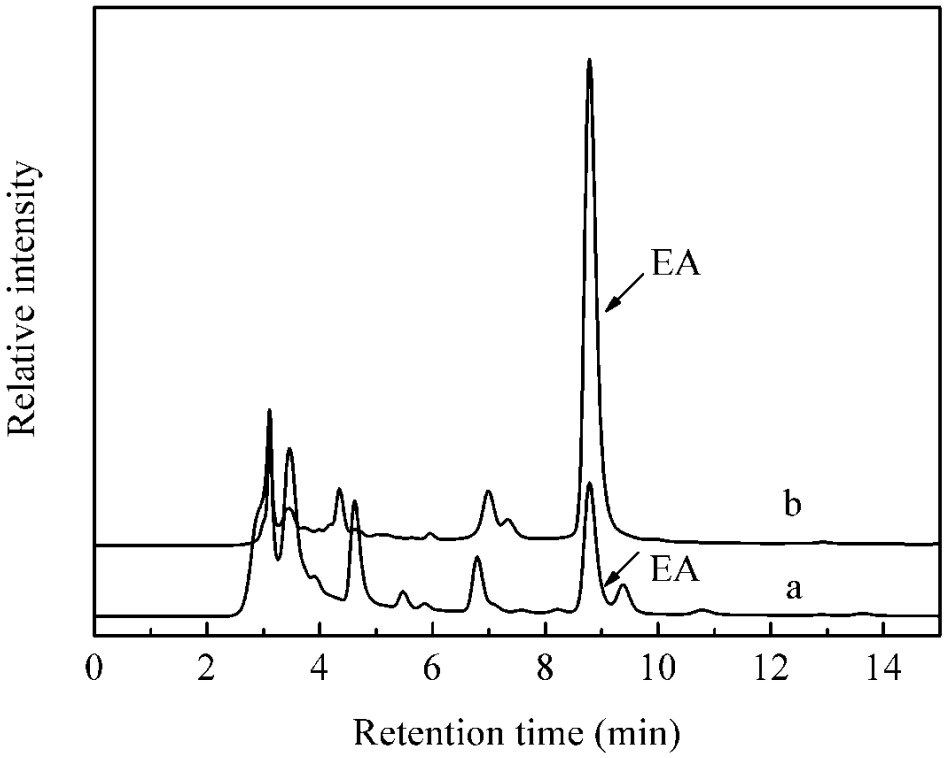

Figure 6. HPLC chromatograms of (a) unhydrolyzed and (b) hydrolyzed leaves. HPLC, High performance liquid chromatography.

ellagitannins in longan barks and leaves and hydrolysis by acid is an effective way of converting ellagitannins into free ellagic acid.

Conclusion

In the present research, the extracts of longan barks and leaves were achieved by refluxing the plant materials in 80% methanol solution. Furthermore, the antioxidant activities and the contents of ellagic acid in the extracts were investigated and results show that the extracts were excellent sources of free-radical inhibitors, and also possessed almost same DPPH radical scavenging activity and reducing power. Besides, the antioxidant activities were concomitant with the development of the reducing power with high correlation coefficients. The antioxidant activities were also dependent on total phenolic content in the solution. For the determination of ellagic acid, the contents in the barks and leaves were quite high. After hydrolysis, the contents were almost four and five times of the unhydrolyzed ones, respectively, which indicates that the ellagic acid in longan barks and leaves exist mostly in the form of ellagitannins. This research therefore demonstrates the potential use of longan barks and leaves not only as antioxidants, but also in the production of ellagic acid. This study has also opened a new window for the reuse of agricultural and food by-products.

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