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Full Length Research Paper

Comparisons of antioxidant activity and total phenolics of *Camellia oleifera* Abel fruit hull from different regions of China

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The tea oil camellia, *Camellia oleifera* Abel, is used extensively in China as cooking oil. The antioxidant and antiradical activities of five *C. oleifera* fruit hull extracts from the major producing regions of China were examined. The content of total phenolics and extractable condensed tannins (ETC) in the extracts was calculated. Remarkable high phenolic content (gallic acid equivalent, GAE > 144 mg/g), extractable condensed tannin content (ETC > 96 mg/g), strong reducing ability (ascorbic acid equivalent, AAE > 5.5 mg/g) and antiradical activity (IC_{50} < 2.1 mg/ml) were found in samples from Guangxi, Jiangxi, Hunan and Zhejiang. The best results were obtained for the samples from Guangxi (phenolic content as GAE = 234.90 mg/g, extractable condensed tannin content as ETC = 166.98 mg/g, reducing power as AAE = 12.91 mg/g and DPPH radical-scavenging ability as IC_{50} = 899.25 µg/ml). A significant correlation was observed between reducing ability and the content of phenolic compounds of respective extracts. Phenolic compounds of extracts were analyzed by HPLC, and the gallic acid was found to be the predominant phenolic acids constitute. This result suggests the potential of *C. oleifera* fruit hull for the utilization as the source of natural antioxidant.

Key words: *Camellia oleifera* Abel, total phenolics, reducing power, radical-scavenging ability.

INTRODUCTION

Reactive oxygen species (ROS), including superoxide radicals, hydroxyl radicals, singlet oxygen and hydrogen peroxide, are often generated as by-products of biological reactions or from exogenous factors (Cerutti, 1991; Talaz et al., 2009). *In vivo*, ROS may be very damaging as they can induce oxidation of lipids and DNA, causing membrane damage, decreasing membrane fluidity, and leading to cancer via DNA mutation (Cerutti, 1994; Pietta, 2000). A potent scavenger of these ROS may serve as a possible preventive intervention of free radical mediated diseases (Ames et al., 1995; Gülçin, 2009).

A variety of plant secondary metabolites have been reported to act as antioxidants and amongst them phenolic compounds form a major group. There are several reports on the contribution of phenolic compounds to the antioxidant potential of different plant species. Cai et al. (2004), for example, reported a positive linear correlation between the total content of phenolic compounds and the antioxidant activities for aqueous and methanolic extracts of Chinese medicinal plants. Similarly, a positive correlation was reported for aqueous and methanolic extracts of different Jordanian plant species (Tawaha et al., 2007).

Camellia oleifera Abel is a species of important woody oil crop. The tea oil camellia, *C. oleifera*, is used extensively in China for cooking oil. It was also traditionally applied as a medicine for stomachache and burning injury in China (Yu et al., 1999). Approximately 1/7th of the Chinese population uses camellia oil as their primary cooking oil. The oil is similar to olive oil, having a high

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percent of oleic acid (75-80%). High oleic acid oils are healthy in that they help lower chlolesterol and triglycerides in the blood. However its fruit hull, containing abundant phenolic compounds, is still not effectively utilized and usually discarded to pollute environment. *C. oleifera* fruit hull is accounted for more than 60 percent of total weight of whole fruit. The fruit hull consists of multiple components, such as saponin, cellulose, hemicellu lose, lignin and phenolic compounds (Lee and Yen, 2006).

Available literature indicates that no previous antioxidant property studies have been done on *C. oleifera* fruit hull and there are no reports on their chemical constituents. This is the first time that the antioxidant properties of *C. oleifera* fruit hull from five major producing regions of China (Guangxi, Jiangxi, Hunan, Zhejiang and Fujian) are described. The aim of the present study was to examine the total phenolic con-tent and radical-scavenging capacity related to antioxidant potential in five *C. oleifera* fruit hull samples collected in different major producing regions of China. The infusions are prepared with regard to efficient extractable phenolics. Antioxidant potential has been determined as the free radical-scavenging ability using a stable radical, DPPH (1,1-diphenyl-2-picrylhydrazyl) and ascertained by measuring reducing power.

MATERIALS AND METHODS

Plant materials and extraction

The *C. oleifera* fruit hull was sampled respectively from Guangxi, Jiangxi, Hunan, Zhejiang, and Anhui province in China, during October - November, 2009. The samples were shed-dried, pulverized and stored in airtight containers for further extraction.

In all experiments infusions of the fruit hull samples were prepared according to a standard protocol. To 1 g of sample was added 20 ml of aqueous acetone (70%, v/v) for 18 h at room temperature. The extracts were filtered and diluted to 50 ml and aliquot of that extract were analyzed for their total phenolic content, reducing power and their free radical-scavenging capacity (Köksal and Gülçin, 2008; Gülçin et al., 2008).

Extraction and purification of condensed tannins

The condensed tannins were isolated and purified from *C. oleifera* fruit hull by column chromatography over Sephadex LH-20 (GE Healthcare, Sweden) as previously described by Zhang and Lin (2008).

Determination of total phenolics

The amount of total phenolics in extracts was determined according to the method of Jayaprakasha et al. (2001). The extracts were dissolved in water. Aliquots of 0.5 ml samples were mixed with 2.5 ml of 10-fold-diluted Folin-Ciocalteu reagent and 2 ml of 7.5% sodium carbonate. The mixture was allowed to stand for 30 min at room temperature before the absorbance was measured spectrophotometrically at 760 nm. A mixture of water and reagents was used as a blank. The total phenolic content was expressed as gallic acid equivalents (GAE) in milligram per gram of dry material.

Acid butanol assay

Extractable condensed tannins in the extracts were analyzed as previously described (Ossipova et al., 2001). A 1.0 ml sample of *C. oleifera* fruit hull extracts were added to 6.0 ml of a solution of 1-butanol/ydrochloric acid (95:5, v/v). The reaction mixture was heated at 95°C for 75 min and then cooled to room temperature. The absorbance was measured at 550 nm on a UV751GD UV/Vis spectrophotometer (Shanghai Xinyi, China). The content of extractable condensed tannins was quantified against purified condensed tannins as the standards.

Antiradical activity

The antiradical activity of different extracts and butylated hydroxyl toluene (BHT) as positive control was determined using the stable radical DPPH (Banerjee et al., 2008; Gülçin, 2010). Aliquots (20-100 µl) of the tested sample were placed in test tubes and 3.9 ml of freshly prepared DPPH solution (25 mg/l) in methanol was added in each test tube and mixed. 30 min later, the absorbance was measured at 517 nm. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenged (\%)} = \{(A_0 - A_t)/A_0\} \times 100$$

Where A_0 is the absorbance of the control reaction and A_t is the absorbance in presence of the sample of the extracts. The antioxidant activity of the extract was expressed as IC_{50}. The IC_{50} value was defined as the concentration in mg of dry material per ml that inhibits the formation of DPPH radicals by 50%. Each value was determined from regression equation.

Ferric-reducing antioxidant power (FRAP) assay

The ferric-reducing antioxidant power of the extracts was estimated according to the method described by Benzie and Strain (1996). 3 ml of FRAP reagent, prepared freshly, was mixed with 0.1 ml of test sample, or methanol (for the reagent blank). The FRAP reagent contained 10 mM TPTZ solution (2.5 ml) in 40 mM HCl plus 20 mM $FeCl_3$ (2.5 ml) and 0.3 M acetate buffer (pH 3.6, 25 ml). The absorbance of reaction mixture was measured spectrophotometrically at 593 nm after incubation at 25°C for 5 min. FRAP assay records the change in absorbance at 593 nm owing to the formation of a blue colored Fe^{II}-tripyridyltriazine compound from colorless oxidized Fe^{III} form by the action of electron donating antioxidants. All solutions were used on the day of preparation. Reducing power is given in ascorbic acid equivalent (AAE) in milligram per gram of dry material.

HPLC analysis of phenolic compounds

The contents of phenolic compounds in the extracts of *C. oleifera* fruit hull were determined by HPLC, performed with a Shimadzu LC-20AB instrument equipped with a binary pump (Shimadzu, Japan). The analyses were carried out on an Inertex C18 column (5 µm, 250 mm × 4.6 mm, Scienhome, China). Extracts were filtered through a 0.45 µm filter before use. The mobile phase was composed of solvent (A) water (0.1% TFA, v/v) and solvent (B) CH_3CN. The gradient condition was: 0 - 5^{th} min 5% B, 5 - 10^{th} min 5 - 10% B. Other chromatographic conditions were as follows: flow rate at 1 ml/min; volume injected 20 µl; room temperature; detection at 280 nm. Retention times and spectra were compared with those of pure standards. Each sample analysis was repeated three times.

Identification of anthocyanidin monomers

Identification of anthocyanidins after hydrolysis of *C. oleifera*

Table 1. Total phenolic content, extractable condensed tannins (ETC), reducing power and antiradical activity of five *C. oleifera* fruit hull extracts from different regions of China.

Production region	GAE[a] mg/g of dry material	ETC[b] value (mg/g of dry material)	AAE[c] mg/g of dry material	IC_{50} value[d] (µg dry material/ml)
Hunan	201.22 ± 6.92 d	150.19 ± 9.81 c	12.03 ± 0.29 d	926.90 ± 29.17 a
Zhejiang	170.63 ± 5.43 c	96.68 ± 7.58 b	7.32 ± 0.107 c	1536.89 ± 79.33 b
Jiangxi	144.44 ± 6.89 b	103.29 ± 3.84 b	5.59 ± 0.53 b	2044.49 ± 61.57 c
Guangxi	234.90 ± 7.61 e	166.98 ± 13.03 d	12.91 ± 0.39 e	899.25 ± 14.58 a
Anhui	42.52 ± 1.67 a	20.17 ± 2.45 a	1.03 ± 0.12 a	7086.18 ± 376.16 d

Data presented as mean ± SD, Statistical analysis was done by Duncan's multiple range test. [a]GAE means mg gallic acid equivalent/g of dry material, r^2 = 0.9901. [b]ETC was expressed as mg condensed tannins equivalent purified from studied materials/g of dry material, r^2 = 0.9994. [c]AAE means mg ascorbic acid equivalent/g of dry material, r^2 = 0.9987. [d]BHA was used as positive control, r^2 = 0.9938.

condensed tannins in 1-butanol/hydrochloric acid mixture was performed using HPLC-electrospray ionization (ESI)-MS analysis. The HPLC system consisted of a Shimadzu LC-20AB binary pump and SPD 20A photodiode array detector. Separation was achieved using a reversed-phase Inertex C18 column (5 µm, 250 mm × 4.6 mm). The mobile phase consisted of solvent (A) water (0.1% TFA, v/v) and solvent (B) CH_3CN. Samples were eluted at a flow rate of 1 ml/min with a gradient condition: 0-6th min 15 - 22% B, 6 - 35th min 22 - 30% B, 35 - 60th min 30 - 48% B. The detection wavelength was 524 nm and the injection volume was 20 µl. Before ESI, the flow was split and only a minor part of the eluent was introduced into the ion source.

The MS data were determined with an Agilent technologies 6310 ion trap mass spectrometer (Agilent technologies, USA). The MS was operated in the positive ion modes with a scan range from *m/z* 100 - 1000 using a 0.4 atom mass unit (amu) step size. The following conditions were used for ESI-MS: capillary voltage, 3500 V; skimmer voltage, 40 V; the nebulizer and dry gases were set at positions 5.0 psi and 12.0 l/min, respectively; nitrogen gas temperature, 320°C.

Statistical analysis

Data were generated for each assay from three separate extracts of each sample in triplicate. A one-way ANOVA test was performed on the antioxidant activity results to investigate significant differences between the extracts. The method used to discriminate among the means was Duncan's multiple range tests. Simple regression analysis was performed to look for relationships between GAE, FRAP and AAE for different extracts. The computer program employed was SPSS for Windows, version 11.0.

RESULTS AND DISCUSSION

In this study, five different *C. oleifera* fruit hull samples from China were investigated for their total phenolic compounds, extractable condensed tannins and their antioxidant capacity, using two different methods. The *C. oleifera* seeds are used extensively in China for extraction of camellia oil. However, there are no data available on the total phenolic compounds and their antioxidant activity of these fruit hull.

The Folin-Ciocalteu method measures the reduction of the reagent by phenolic compounds with the formation of a blue complex that can be measured at 750 nm against gallic acid as a standard (Imeh and Khokhar, 2002; Gülçin et al., 2007). There was a wide variation in the amount of total phenolics in *C. oleifera* fruit hull ranging from 42.52 - 234.90 mg GAE/g dry material (Table 1). Among samples, the highest found in Guangxi (234.90 mg GAE/g dry material) and lowest in Anhui (42.52 mg GAE/g dry material). The amount of total phenolic content of the fruit hull under investigation can be arranged in descending order: Guangxi > Hunan > Zhejiang > Jiangxi > Anhui. Extractable condensed tannin contents ranged from 20.17 - 166.98 mg/g dry material with the highest found in Guangxi (166.98 mg/g dry material) and lowest in Anhui (20.17 mg/g dry material) (Table 1). This difference can be attributed to different plant ecology. Our results demonstrate that the quantities of total phenolic compounds in the *C. oleifera* fruit hull are different amongst the different regions of China. Consequently, a comparative study of free radical-scavenging activity and antioxidant activity of these fruit hull was undertaken.

In order to evaluate the free radical-scavenging activity of the fruit hull extracts, we used a method based on the reduction of DPPH, a stable free radical. Because activities are expressed as the sample concentration required to achieving a 50% decrease in absorbance at 517 nm (IC_{50}), the smaller sample concentration indicates the higher DPPH radical-scavenging activity (Gülçin et al., 2007). Table 1 summarizes the IC_{50} value of DPPH of extracts from different fruit hull samples. All samples studied showed free radical-scavenging activity. The highest activity was shown by samples from Guangxi (IC_{50} value is 899.25 µg dry material/ml). Sample from Anhui, appears less active than the other samples (IC_{50} value is 7086.18 µg dry material/ml).

The FRAP assay is based on the ability of antioxidants to reduce Fe^{3+} to Fe^{2+} in the presence of TPTZ, forming an intense blue Fe^{2+}-TPTZ complex with an absorption maximum at 593 nm. The absorbance increase is proportional to the antioxidant content (Benzie and Strain, 1996). The reducing power of different fruit hull was evaluated as mg AAE/g dry material as shown in Table 1. In

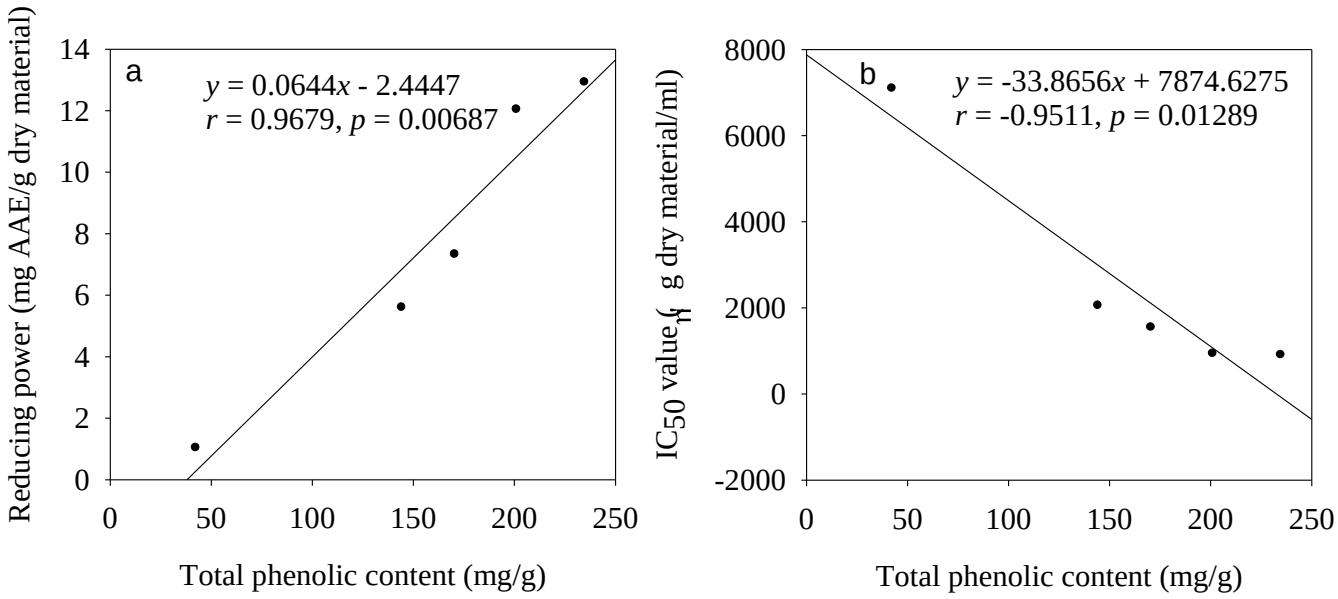

Figure 1. Linear correlation between the amount of total phenolics (GAE) and reducing power (AAE) (Correlation coefficient r = 0.9679, p = 0.00687) (a); Linear correlation between the amount of total phenolics (GAE) and the antiradical activity (Correlation coefficient r = -0.9511, p = 0.01289) (b). The two tailed $p < 0.01$ considered significant. Values are mean of three determinations ± SD. GAE, gallic acid equivalent; AAE, ascorbic acid equivalent.

accordance with finding from the DPPH assay, the FRAP values ranged from 1.03 - 12.91 mg AAE/g of dry material. A higher absorbance corresponds to a higher ferric reducing power. In brief, the reducing power of *C. oleifera* fruit hull followed the order: Guangxi (12.91 mg AAE/g of dry material) > Hunan (12.03 mg AAE/g of dry material) > Zhejiang (7.32 mg AAE/g of dry material) > Jiangxi (5.59 mg AAE/g of dry material) > Anhui (1.03 mg AAE/g of dry material). We suppose that the diversity of antiradical activity and antioxidant activity may contribute to the difference of total phenolic content in samples. Consequently, the relationship between antioxidant activity and total phenolics was investigated.

A significant linear correlation (correlation coefficient r = 0.9679, $p < 0.01$) was established between total phenolics (measured as mg GAE/g dry material) and corresponding reducing ability (measured as mg AAE/g dry material) of *C. oleifera* fruit hull extracts (Figure 1a). The sample from Guangxi exhibited the highest total phenolic content and showed maximum reducing ability. In contrast, the correlation between total phenolic content and scavenging activity is not significant ($p > 0.01$), although there is a good linear response (r = -0.9511) (Figure 1b). Obviously, other factors are involved. These might be different phenolic composition or the presence of non-phenolic scavengers. Our results also show that all *C. oleifera* fruit hull analyzed possess free radical-scavenging activity which justify their use as a source of natural antioxidants.

In this report we found a linear correlation (r = 0.9679, $p < 0.01$) between the phenolic content (GAE) and ferric reducing capacity (AAE) (Figure 1) of five different extracts from five *C. oleifera* fruit hull samples available in China. In another report, a significant correlation (r^2 = 0.9653, $p < 0.0001$) was observed and authors use the same assay (ferric reducing power, FRAP) to determine antioxidant power (Katalinic et al., 2006). That was in fair agreement to our observation. Another group reported no linear response between total phenolics and antioxidant activity, but the authors used different assay methods e.g. oxygen radical absorbance capacity (ORAC), and inhibition of methyl linoleate oxidation to ascertain antioxidant response (Ou et al., 2003; Kahkonen et al., 1999). This fact may add further insight between the chemical nature of phenolic compounds and their antioxidant response.

Many plants have been investigated for the antioxidant activities and the search is gradually increased in recent times since ROS were the salient features behind many dreadful diseases. Several anti-inflammatory, digestive, antinecrotic, neuroprotective and hepatoprotective (Ropetto and Llesuy, 2002; Perry et al., 1999) drugs have recently been shown to have an antioxidant and/or radical-scavenging mechanism as part of their activity. Antioxidant activity of tea seed oil camellia, *C. oleifera*, extracts from various solvents was investigated by Lee and Yen (2006), and found the methanol extract exhibited the highest yield and the strongest antioxidant activity. But there are no studies available including antioxidant activity of *C. oleifera* fruit hull. This report could be useful to exploit their use as a source of natural antioxidants.

Phenolic compounds, such as quercetin, rutin, narigin, catechins, caffeic acid, gallic acid and chlorogenic acid,

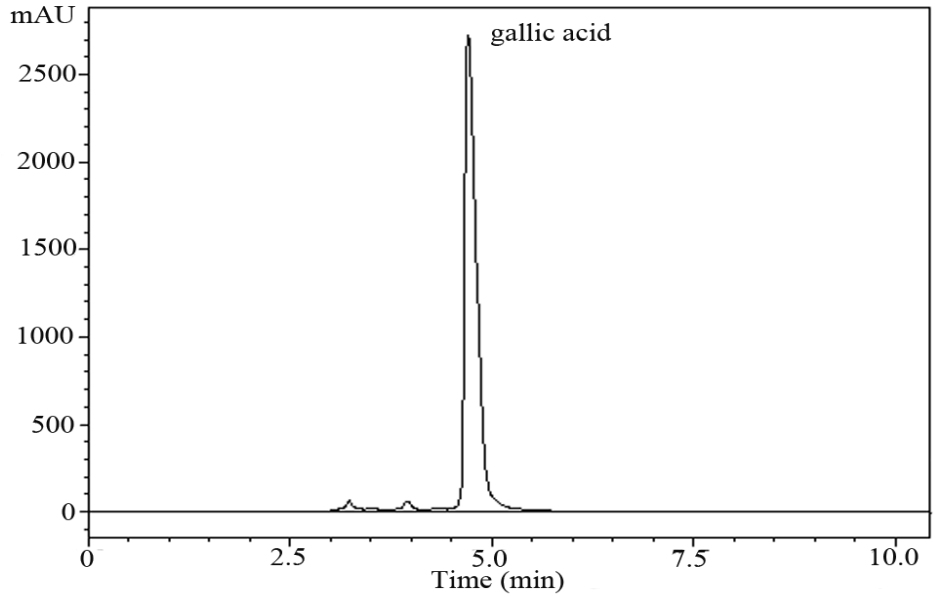

Figure 2. HPLC chromatogram (280 nm) of the *C. oleifera* fruit hull extracts.

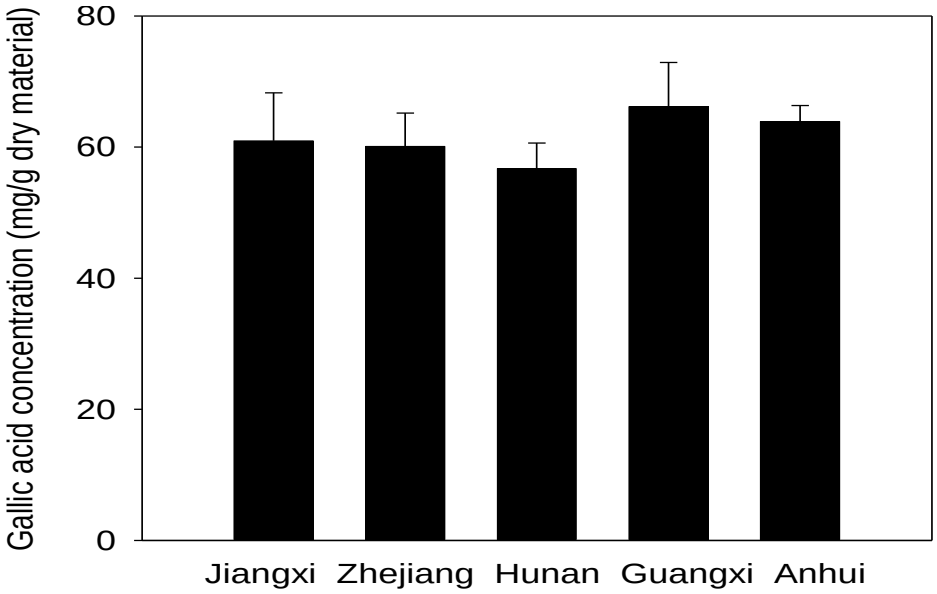

Figure 3. Concentrations of gallic acid recovered from the *C. oleifera* fruit hull extracts.

are very important plant constituents because of their antioxidant activities (Paganga et al., 1999). RP-HPLC coupled with UV-Vis SPD detector was employed to separate, identify and quantify phenolic compounds in the extracts of *C. oleifera* fruit hull. The concentrations were determined by calculating the HPLC peak areas which are proportional to the amount of analyte in a peak and presented as the mean of three determinations which were highly repeatable. Figure 2 shows the chromatogram of *C. oleifera* fruit hull extracts. Phenolic compounds in the extracts have been identified as gallic acid according to their retention times and spectral characteristics of their peaks against the standard, as well as by spiking the samples with standards.

The result from the chromatograms indicated that the extracts of Guangxi contained the highest content of gallic acid (Figure 3). By comparing the different extracts, the content of gallic acid decreased in order of Guangxi (66.18 mg/g dry material), Anhui (63.91 mg/g dry material), Jiangxi (60.94 mg/g dry material), Zhejiang

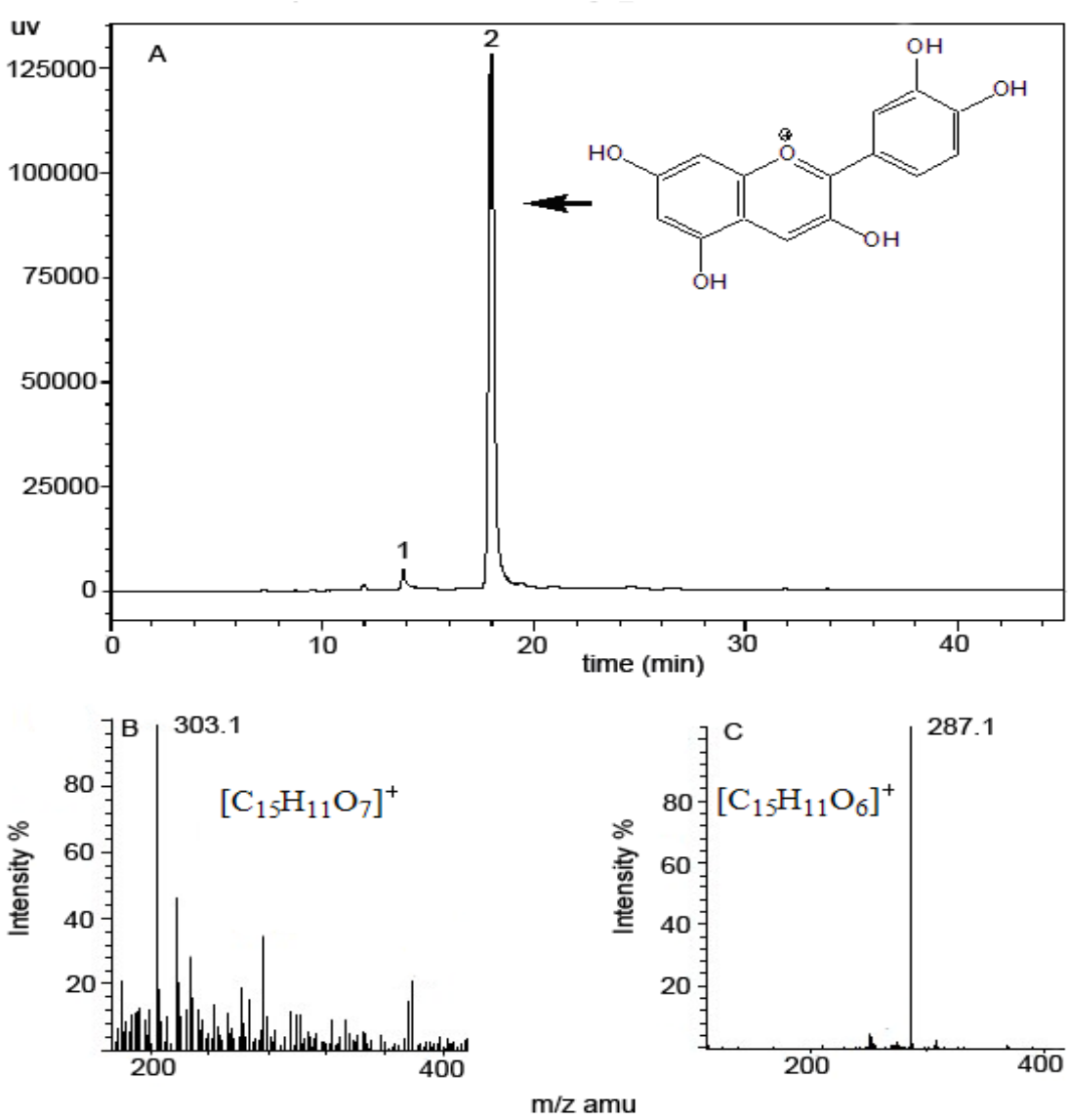

Figure 4. (A) HPLC chromatogram of anthocyanidin monomers obtained following hydrolysis of condensed tannins from *C. oleifera* fruit hull in 1-butanol/hydrochloric acid. (Absorbance at 524 nm. 1, delphinidin; 2, cyanidin). Low panels show the positive ion ESI mass spectra of these anthocyanidins (B, delphinidin; C, cyanidin).

(60.10 mg/g dry material) and Hunan (56.72 mg/g dry material), and the rank order was different according to their antioxidant potency and free radical-scavenging ability. This result indicates that, besides phenolic acids, the other complex phenolic compounds in *C. oleifera* fruit hull extracts, such as condensed tannins can be also responsible for the antioxidant activity. Sanchez-Moreno et al. (1999) indicated that the inhibition of lipid oxidation of the phenolic compounds and antioxidant standards followed the order: rutin, ferulic acid > gallic acid, tannic acid, resveratrol > BHA, quercetin > tocopherol > caffeic acid, in a linoleic acid system. Meanwhile, the free radical-scavenging activity was in the order: gallic acid > tannic acid, caffeic acid, quercetin, BHA, rutin > ferulic acid, tocopherol, resveratrol. According to our observation, gallic acid may have important roles in the antioxidant activity and free radical-scavenging ability of *C. oleifera* fruit hull extracts.

The absorption curve of the anthocyanidin solution after hydrolysis of *C. oleifera* condensed tannins in 1-butanol/hydrochloric acid reagent showed a maximum at 524 nm that is characteristic for cyanidin-type condensed tannins. HPLC-ESI-MS analysis of the products of condensed tannins hydrolysis showed the presence of two individual anthocyanidins (Figure 4). Two peaks were deter mined according to their positive ion ESI-MS and the commercially available anthocyanidins delphinidin and cyanidin. In the positive ion ESI-MS of these anthoc

- yanidins, abundant $[M]^+$ ion peaks at *m/z* 303.1 $[C_{15}H_{11}O_7]^+$ and 287.1 $[C_{15}H_{11}O_6]^+$ (Figure 4) were present. On the basis of these results, two anthocyanidin monomers were identified as delphinidin and cyanidin, repectively. This result suggested that the condensed tannins of *C. oleifera* fruit hull are mainly composed of procyanidins with some of prodelphinidins.

In the present study, the antioxidant activity and total phenolic content of five *C. oleifera* fruit hull extracts from different regions of China was examined. The active extracts were identified according to their phenolic content between 42.52 - 234.90 mg GAE/g dry material, extractable condensed tannin contents ranged from 20.17 - 166.98 mg/g dry material and reducing power 1.03 - 12.91 mg AAE/g of dry material as well as DPPH radical-scavenging activity having IC_{50} value 899.25 - 7086.18 µg dry material/ml. The HPLC data indicated that *C. oleifera* fruit hull extracts contained phenolic acids, such as gallic acid. The antioxidant activity of fruit hull extracts may be related to their phenolic substances. A significant linear relationship between antioxidant potency and the content of phenolic compounds of extracts supported this observation.

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