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Tannins from *Canarium album* with potent antioxidant activity*

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Abstract: The contents of total phenolics and extractable condensed tannins in the leaves, twigs and stem bark of *Canarium album* were determined. The structural heterogeneity of condensed tannins from stem bark was characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and nuclear magnetic resonance (NMR) analyses. The results show the predominance of signals representative of procyanidins and prodelphinidins. In addition, epicatechin and epigallocatechin polymers with galloylated procyanidin or prodelphinidin were also observed. The tannins were screened for their potential antioxidant activities using 1,1-diphenyl-2-picrylhydrazyl (DPPH) and ferric reducing/antioxidant power (FRAP) model systems. Tannins extracted from leaves, twigs and stem bark all showed a very good DPPH radical scavenging activity and ferric reducing power.

Key words: *Canarium album*, Tannins, Antioxidant capacity, Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS)

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INTRODUCTION

Tannins known as the group of phenolic compounds are the significant plant secondary metabolites. Tannins in vascular plants occur as two types, the condensed and the hydrolysable (Hernes *et al*., 2001). Condensed tannins are also known as proanthocyanidins (PAs), the oligomeric and polymeric flavan-3-ols, which are linked through C4-C8 or C4-C6 linkages. The diversity of condensed tannins is given by the structural variability of the monomer units (different hydroxylation patterns of the aromatic rings A and B, and different configurations at the chiral centers C2 and C3) (Fig.1). The size of PA molecules can be described by their degrees of polymerization (DPs). The molecules are water-soluble and can form complexes with proteins and polysaccharides (Haslam, 1998). PAs are of great interest in nutrition and medicine because of their potent antioxidant capacity and possible protective effects on human health (Santos-Buelga and Scalbert, 2000). They have antioxidant properties related to their radical scavenging capacity (Ricarda Da Silva *et al*., 1991), and these properties have been used against heart disease through reducing lipid oxidation. It was hypothesized that the free radical scavenging properties of PAs may reduce the risk of cardiovascular diseases, cancer (Bagchi *et al*., 2000) and blood clotting, and certain types of trimeric PAs may protect against urinary tract infections (Santos-Buelga and Scalbert, 2000). However, tannins are diverse compounds with great variation in structure and concentration within and among plant species. Therefore, biomedical researches on the health benefits and risks of increased tannins consumption are severely limited by lack of methods for rapid characterization and standardization.

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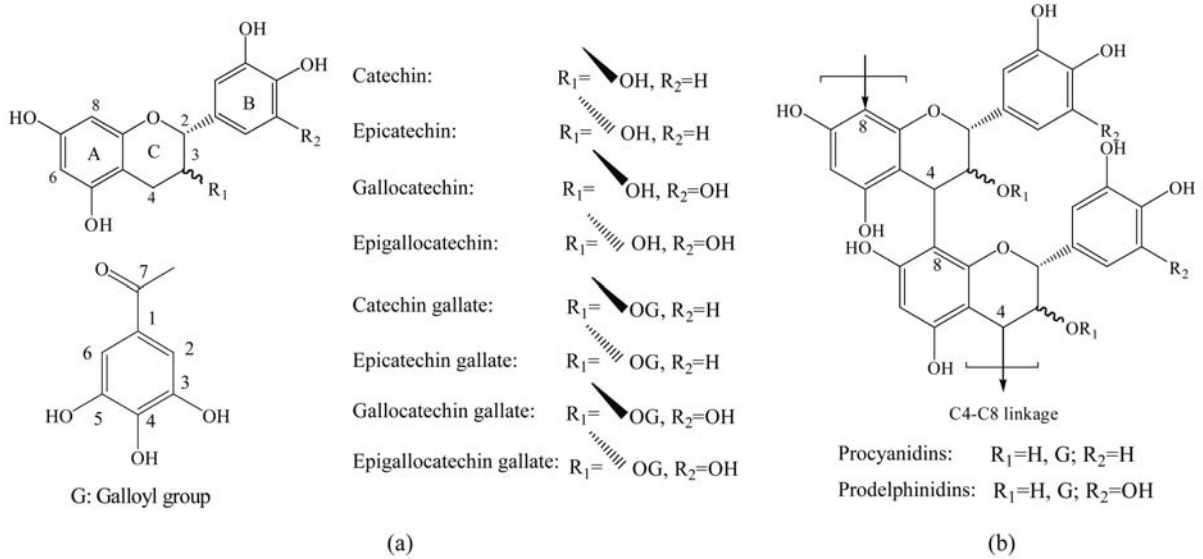

Fig.1 Chemical structures of flavan-3-ol (a) monomers and (b) polymers

Canarium album or Chinese olive (Burseraceae), is the native species in the southeast of China. It is a good fruit species because of its tolerance to poor soils (e.g., rocky hillsides, saline or alkaline soils) (Wei *et al*., 1999). The dried fruits are a traditional medicine material that has some pharmacological functions such as anti-bacterium, anti-virus, anti-inflammation and detoxification (Ding, 1999). The fresh fruits are widely used in food industry, while the leaves and stem bark of the plants are discarded as waste products.

Polyphenols make a major contribution to free radical scavenging capacities of Mediterranean olives species (Visioli *et al*., 1998). Previous studies indicated that there was a direct relationship between antioxidant activity and total phenolics content in selected herbs, vegetables and fruits (Oszmianski *et al*., 2007; Wu, 2007). In this study, contents of total phenolics and extractable condensed tannins of leaves, twigs and stem bark of *C. album* were determined, and the structural heterogeneity of condensed tannins from stem bark was characterized by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and nuclear magnetic resonance (NMR) analyses. Meanwhile, the free radical scavenging capacities and ferric reducing power of condensed tannins from leaves, twigs and stem bark were also discussed.

MATERIALS AND METHODS

Samples

Leaves, twigs and stem bark samples of *C. album* were collected in Fuzhou in the southeast of China. 1,1-Diphenyl-2-picrylhydrazyl (DPPH), 2,4,6-tripyridyl-*S*-triazine (TPTZ), ascorbic acid (AA), butylated hydroxyanisole (BHA), gallic acid and ellagic acid were purchased from Sigma (USA). Sephadex LH-20 was purchased from Amersham (USA). All chemicals were of analytical reagent (AR) purity grade.

Methods

1. Solvent extraction

Ten grams of fresh materials were weighed and extracted with 100 ml acetone-water solution (70:30, v/v) twice at room temperature. The extract was filtered and pooled, and the solvent was removed under reduced pressure by using a rotary vacuum-evaporator at 40 °C. The remaining aqueous fraction was extracted thrice with hexane in order to remove chlorophyll and lipophilic compounds. The remaining crude tannins fraction was chromatographed on an Sephadex LH-20 column (3 cm×22 cm) which was first eluted with methanol:water (50:50, v/v) to remove oligomeric tannins and then followed by acetone:water (70:30, v/v). The last fraction, containing

the polymeric tannins, was freeze-dried and stored at −20 °C until required.

2. Determination of total phenolics and condensed tannins (PAs)

Established procedures (Lin *et al*., 2006) were used to determine total phenolics and condensed tannins. Total phenolics were measured with the Prussian blue method (Graham, 1992), and condensed tannins were assayed by the butanol-HCl method (Terrill *et al*., 1992), using purified tannins from *C. album* and tannic acid as the standards.

3. NMR analysis

^{13}C NMR spectra were recorded in CD_3COCD_3-D_2O mixture with a Varian Metcury-600 spectrometer at 150 MHz (proton decoupling mode for carbon).

4. MALDI-TOF MS analysis

The MALDI-TOF MS spectra were recorded on a Bruker Reflex III. The irradiation source was a pulsed nitrogen laser with a wavelength of 337 nm, and the duration of the laser pulse was 3 ns. In the positive reflectron mode, an accelerating voltage of 20.0 kV and a reflectron voltage of 23.0 kV were used. The spectra of condensed tannins were obtained from a sum of 100~150 shots and calibrated using Angiotensin II (1046.5 MW), Bombesin (1619.8 MW), ACTHclip1839 (2465.2 MW), and Somatostatin28 (3147.47 MW) as external standards. 2,5-Dihydroxy benzoic acid (DHB, 10 mg/ml aqueous solution) was used as the matrix. The sample solutions (7.5 mg/ml aqueous) were mixed with the matrix solution at a volumetric ratio of 1:3. The mixture (1 μl) was applied to the steel target. Amberlite IRP-64 cation-exchange resin (Sigma-Aldrich, USA), equilibrated in deionized water, was used to deionize the analyte-matrix solution thrice. Cesium trifluoroacetate (1 mg/ml) was mixed with the analyte-matrix solution (1:3, v/v) to promote the formation of a single type of ion adduct ($[M+Cs]^+$) (Xiang *et al*., 2006).

5. Hydrolysis of the tannins

The tannins from leaves and twigs (0.25 g) were hydrolyzed with 2 ml of 2 mol/L HCl in a boiling water bath for 1 h. After cooling, 2 ml of 2 mol/L NaOH and then 6 ml methanol were added to the vial. The slurry was sonicated for 20 min with occasional shaking. Further, the slurry was centrifuged at 19000×*g* and the supernatant was used for HPLC analysis. The HPLC apparatus consisted of an Aglient 1100 diode array detector and a quaternary pump.

The samples were previously dissolved in mobile phase and then filtrated through membrane filter with an aperture size of 0.45 μm. Ten milliliters of the clear supernatant after centrifugation at 19000×*g* for 5 min was injected. Separation was performed on a Hypersil ODS column (4.6 mm×250 mm, 5 μm) thermostatted at 30 °C. The mobile phase was composed of solvent A (0.1% (v/v) trifluoroacetic acid (TFA) in water) and solvent B (0.1% (v/v) TFA in acetonitrile). The gradient condition was: 0~2nd minutes 100% A, 2nd~6th minutes 100%~95% A, 6th~10th minutes 95% A, 10th~15th minutes 95%~80% A, 15th~20th minutes 80% A, and 20th~25th minutes 70% A. Other chromatographic conditions were as follows: flow rate at 1 ml/min, detection at 280 nm, and scanning performed between 200 and 600 nm.

6. Free-radical scavenging activity

The free-radical scavenging activity was measured according to the method of Braca *et al*.(2001). A 100 μl of sample at different concentration (15~250 μg/ml) was added to 3 ml of DPPH solution (0.004% (w/w) methanolic solution). Thirty minutes later, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The IC_{50} value, defined as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%, was calculated from the results and used for comparison. The capability to scavenge the DPPH radical was calculated by using the following equation:

$$\text{DPPH scavenging effect (\%)}=[(A_1-A_2)/A_1]\times 100,$$

where A_1 is the absorbance of the control reaction, and A_2 is the absorbance in the presence of the sample. BHA and ascorbic acid were used as the controls.

7. Ferric reducing/antioxidant power (FRAP) assay

FRAP assay is a simple and reliable colorimetric method commonly used for measuring the total antioxidant capacity (Benzie and Strain, 1996). Briefly, 3 ml of FRAP reagent prepared freshly was mixed with 100 μl of test sample or methanol (for the reagent blank). The FRAP reagent was prepared from 300 mmol/L acetate buffer (pH 3.6), 20 mmol/L ferric chloride and 10 mmol/L TPTZ made up in 40 mmol/L hydrochloric acid. All the above three solutions were mixed together in the ratio of 25:2.5:2.5 (v/v/v). The

absorbance of reaction mixture at 593 nm was measured spectrophotometrically after incubation at 25 °C for 10 min. The FRAP values, expressed in mmol ascorbic acid equivalents (AAE)/g dried tannins, were derived from a standard curve.

8. Statistical analysis

All measurements were replicated three times. One-way analysis of variance (ANOVA) was used, and the differences were considered to be significant at $P<0.05$. All statistical analyses were performed with SPSS 11.0.

RESULTS AND DISCUSSION

Content of total phenolics and extractable condensed tannins

Total phenolic content of the leaves was the highest, followed by twigs, and then stem bark for *C. album* (Table 1). The leaves of *C. album* had higher extractable condensed tannin content than twigs and stem bark.

Table 1 Contents of total phenolics and extractable condensed tannins in leaves, twigs and stem bark of *C. album*

Samples	Total phenolics (mg/g)[a]	Extractable condensed tannins (mg/g)[b]
Leaves	164.39±14.67	117.70±9.39
Twigs	161.99±13.07	48.99±2.40
Stem bark	54.96±3.12	39.98±3.05

[a] Using tannic acid as the standard; [b] Using purified leaf tannins as the standard

NMR analysis

The purified condensed tannins from stem bark were analyzed by ^{13}C NMR spectroscopy. The ^{13}C NMR spectra showed distinct signals at 145.2×10^{-6} and 145.4×10^{-6}, which are assignable to C3′ and C4′ in procyanidin units (catechin/epicatechin) (Fig.2). The dominance of the procyanidin unit of the polymeric sample was further corroborated by the presence of strong peaks at 114.0×10^{-6}~115.5×10^{-6} consistent with the C2′ and C5′ chemical shifts of the catechol B-ring, and at 118.1×10^{-6}~120.2×10^{-6} consistent with the C6′ chemical shift of the catechol B-ring. In addition, a strong signal at 145×10^{-6}~146×10^{-6} was also obtained. Prodelphinidin units (gallocatechin/epigallocatechin) generally showed a typical resonance at 146×10^{-6} (Behrens *et al*., 2003). The presence of a clear signal with such a chemical shift in the spectra of the condensed tannins from stem bark revealed that they are mostly composed of procyanidin and prodelphinidin units.

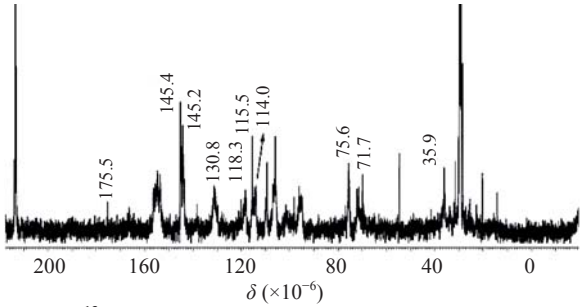

Fig.2 ^{13}C NMR spectrum of condensed tannins from stem bark of *C. album*

The region between 70×10^{-6} and 90×10^{-6} is sensitive to the stereochemistry of the C-ring. The determination of the ratio of the 2,3-*cis* to 2,3-*trans* stereochemistries could thus be achieved through the distinct differences in their respective C2 chemical shifts (Es-Safi *et al*., 2006). Whereas C3 of both *cis* and *trans* isomers occurs at 73×10^{-6} (in the presence of galloyl groups), C2 gives a resonance at 76×10^{-6} for the *cis* form and at 84×10^{-6} for the *trans* form. The absence of the latter signal peak in the spectrum of the condensed tannins fraction indicates the only presence of epicatechin and epigallocatechin subunits. The presence of a signal at 35.9×10^{-6} was consistent with a C4 being shifted upfield by the presence of a 3-O-gallate unit. This was further confirmed by the observation of signals for ester carbonyl carbons at 175.5×10^{-6} (Gal-C7) and galloyl ring carbons at 114.0×10^{-6} (Gal-C2, Gal-C6), 130.8×10^{-6} (Gal-C1) and 143×10^{-6}~145×10^{-6} (Gal-C4). These results thus show that the polymeric PA fraction of the stem bark is predominantly constituted of procyanidin and prodelphinidin with (−)-epicatechin and (−)-epigallocatechin as main constitutive monomers, some with galloyl groups attached (Spencer *et al*., 2007).

MALDI-TOF MS analysis

Individual oligomers of vegetable tannins are well resolved in MALDI-TOF MS spectra with their molecular weights determinated in the analysis of condensed tannins (Ohnishi-Kameyama *et al*., 1997; Yang and Chien, 2000; Ishida *et al*., 2005). When MALDI-TOF MS is used to characterize tannins, the

mass spectra tend to favor an association with naturally abundant Na^+ or K^+ over the formation of a protonated molecular ion $[M+H]^+$ (Ohnishi-Kameyama *et al*., 1997; Krueger *et al*., 2003). In order to promote the formation of single tannin-ion adducts, K^+, Na^+, Ag^+ or Cs^+ is usually added to the analyte/matrix and the adducts are detected in the positive ion mode. However, MALDI-TOF mass spectra of condensed tannins are notably affected by various added and naturally abundant ions, except for the over-evaluation of hydroxyl substitution in flavan-3-ol oligomers due to the formation of both $[M+Na]^+$ and $[M+K]^+$ from one species (Krueger *et al*., 2003). Using MALDI-TOF with deionization and selection of Cs^+ as the cationization reagent rather than selection of Na^+, condensed tannin polymers of higher DP were observed. Meanwhile, the polymer with the highest intensity ion peak changed with the ion adducts used (Xiang *et al*., 2007).

Fig.3 shows the MALDI-TOF mass spectrum of the polymeric mixture, recorded as Cs^+ adducts in the positive ion reflectron mode and showing a series of repeating procyanidin polymers. The polymeric character is reflected by the periodic peak series representing different chain lengths. The results indicate that condensed tannins from stem bark are characterized by mass spectra with a series of peaks with distances of 288 Da corresponding to a mass difference of one catechin/epicatechin between each polymer. Therefore, prolongation of condensed tannins is due to the addition of catechin/epicatechin monomers. The spectrum showed a series polyflavan-3-ols extending from the dimer (*m*/*z* 711) to the octamer (*m*/*z* 2439) that did not contain ions with 2 Da lower than that predicted in positive ion reflectron mode (Table 2).

Table 2 Observed and calculated masses[a] of heteropolyflavan-3-ols by MALDI-TOF MS

Polymer	n_1	n_2	n_3	C_1	C_2	Polymer	n_1	n_2	n_3	C_1	C_2
Dimer	2	0	0	711	711.04	Pentamer	2	3	0	1623	1622.73
	2	0	1	863	862.91		2	3	1	1775	1775.64
	1	1	0	727	727.09	Hexamer	6	0	0	1863	1862.77
	1	1	1	879	878.88		6	0	1	2015	2014.76
	0	2	0	743	742.88		5	1	0	1879	1878.85
	0	2	1	895	894.93		5	1	1	2031	2030.82
Trimer	3	0	0	999	998.92		4	2	0	1895	1894.80
	3	0	1	1151	1150.86		4	2	1	2047	2046.78
	2	1	0	1015	1014.85		3	3	0	1911	1910.80
	2	1	1	1167	1166.82		3	3	1	2063	2063.80
	1	2	0	1031	1030.84		2	4	0	1927	1926.81
	1	2	1	1183	1182.80		2	4	1	2079	2078.80
	0	3	0	1047	1046.75	Heptamer	7	0	0	2151	2150.84
	0	3	1	1199	1198.69		7	0	1	2303	2302.81
Tetramer	4	0	0	1287	1286.84		6	1	0	2167	2167.78
	4	0	1	1439	1438.80		6	1	1	2319	2319.82
	3	1	0	1303	1302.79		5	2	0	2183	2182.83
	3	1	1	1455	1454.79		5	2	1	2335	2335.69
	2	2	0	1319	1318.80		4	3	0	2199	2198.84
	2	2	1	1471	1470.77		4	3	1	2351	2350.76
	1	3	0	1335	1334.78	Octamer	8	0	0	2439	2438.67
	1	3	1	1487	1486.77		8	0	1	2591	2591.70
Pentamer	5	0	0	1575	1574.76		7	1	0	2455	2455.74
	5	0	1	1727	1726.80		7	1	1	2607	2606.81
	4	1	0	1591	1590.79		6	2	0	2471	2471.74
	4	1	1	1743	1742.78		6	2	1	2623	2623.77
	3	2	0	1607	1606.78		5	3	0	2487	2487.81
	3	2	1	1759	1758.76		5	3	1	2639	2639.78

n_1: Number of catechin unit; n_2: Number of gallocatechin unit; n_3: Number of galloylated esters; C_1: Calculated $[M+Cs]^+$ mass; C_2: Observed $[M+Cs]^+$ mass. [a]Mass calculations were based on the equation M=290+288a+304b+152c+133, where M is the calculated mass; 290, 288, 304 and 152 are the molecular weights of the terminal epicatechin unit, the epicatechin extending unit, the epigallocatechin extending unit and the galloyl ester, respectively; 133 is the atomic weight of cesium (Cs); a and b are the DPs contributed by the epicatechin extending unit and the epigallocatechin extending unit, respectively; c is the number of galloyl esters

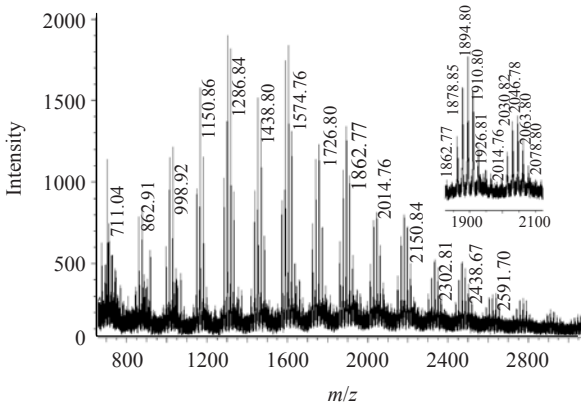

Fig.3 MALDI-TOF positive ion reflectron mode mass spectrum of the tannins from *C. album* stem bark and the enlarged spectrum of the polyflavan-3-ol hexamer

In addition to the predicted homopolyflavan-3-ol mass series mentioned above, each DP had a subset of masses 16, 32 and 64 Da higher (Fig.3 and Table 2). These masses can be explained by heteropolymers of repeating flavan-3-ol units containing an additional hydroxyl group (Δ16 Da) at the position 5 of the B-ring. Given the absolute masses corresponding to each peak, it was further suggested that they contain procyanidin and prodelphinidin, as have already been indicated in the ^{13}C NMR spectrum.

On the basis of the structures described by Krueger *et al*.(2003), an equation was formulated to predict heteropolyflavan-3-ols of a higher DP (Table 2). The equation is M=290+288a+304b+152c+133, where M is the calculated mass; 290, 288, 304 and 152 are the molecular weights of the terminal epicatechin unit, the epicatechin extending unit, the epigallocatechin extending unit and the galloyl ester, respectively; 133 is the atomic weight of cesium (Cs); a and b are the DPs contributed by the epicatechin extending unit and the epigallocatechin extending unit, respectively; c is the number of galloyl esters. Application of this equation to the experimentally obtained data revealed the presence of a series of condensed tannins consisting of well-resolved oligomers. The broad peaks in these spectra indicate that there is a large structural heterogeneity within DP.

Each peak of the condensed tannins was always followed by mass signals at a distance of 152 Da corresponding to the addition of one galloyl group at the heterocyclic C-ring. Thus, peak signals corresponding to monogalloylated derivatives of various procyanidin oligomers were easily attributed. No procyanidins with more than one galloyl group were detected. Therefore, MALDI-TOF MS indicated the simultaneous occurrence of a mixture of procyanidin polymers and monogalloylated derivatives of procyanidin polymers, showing that there was a mixture of galloylated procyanidin and procyanidin occurring in procyanidin oligomers of stem bark.

The series of compounds with 2 Da multiples lower than those described in the predictive equation for heteropolyflavan-3-ols were not detected, so A-type interflavan ether linkage does not occur between adjacent flavan-3-ol subunits.

Hydrolysis of the tannins

Gallic acid, as a constituent of gallotannins, was observed at high concentration in leaves ((56.02±0.51) mg/g) and twigs ((85.68±0.48) mg/g) (Fig.4).

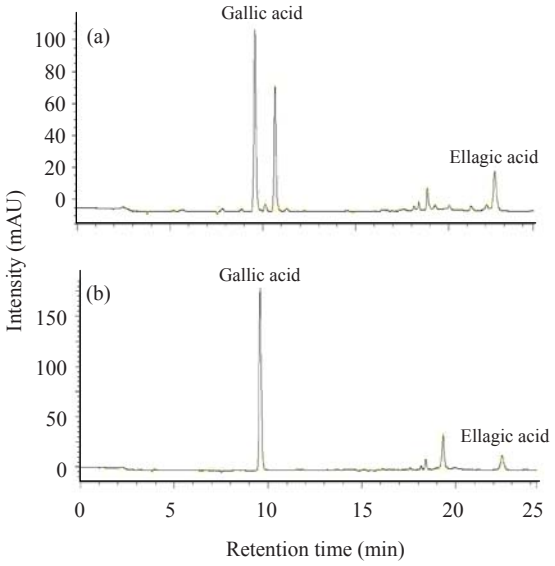

Fig.4 HPLC chromatogram (280 nm) of the tannins from *C. album* leaves (a) and twigs (b) after hydrolysis

Ellagic acid (ellagotannins constituent) was also found in leaves ((16.48±0.79) mg/g) and twigs ((9.13±0.30) mg/g), but lower than gallic acid. It was indicated that *C. album* had antimicrobial activity on some common bacteria, mildews and microzymes, and gallic acids might be an important composition for this function (Yuan *et al*., 2001).

Radical-scavenging activities on DPPH

Relatively stable organic radical DPPH has been widely used in the determination of antioxidant activity of single compounds as well as the different plant extracts (Brand-Williams *et al*., 1995). The free radical-scavenging activities of tannins from leaves, twigs and stem bark along with the reference standards of ascorbic acid and BHA were determined by the DPPH assay. Because activities are expressed as the tannins concentration required to achieve a 50% decrease in absorbance at 517 nm (IC_{50}), the smaller tannins concentration indicates the higher DPPH radical-scavenging activity. Tannins from leaves, twigs and stem bark all showed the significantly higher inhibition percent of DPPH radical compared to reference ascorbic acid. Great decreases in a concentration-dependent manner of remaining DPPH indicated that *C. album* possesses potent free radical-scavenging activity (Table 3 and Fig.5).

Table 3 Antioxidant activities of the tannins from *C. album* using the DPPH free radical-scavenging assay and ferric reducing antioxidant assay

Sample	IC_{50} (μg/ml)*	*FRAP***
Twigs	62.31±2.05b	3.74±0.07c
Stem bark	54.80±0.50c	4.49±0.11ab
Leaves	56.86±1.56c	4.28±0.02b
BHA	57.46±0.97c	4.66±0.10a
AA	78.25±1.41a	–

* The antioxidant activity was evaluated as the concentration of the test sample required to decrease the absorbance at 517 nm by 50% in comparison to the control; ** FRAP values are expressed in mmol ascorbic acid equivalent (AAE)/g sample in dry weight. BHA: Butylated hydroxyanisole; AA: Ascorbic acid. Data are presented as mean±*SD*; Means with different letters in the same column are significantly different at $P<0.05$ level

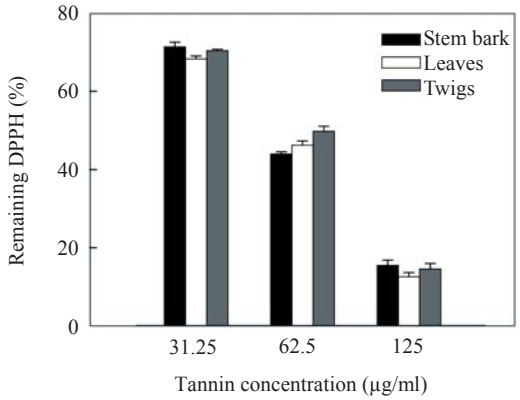

Fig.5 Remaining DPPH after addition of the tannins from *C. album* for 30 min

Ferric reducing antioxidant power

The reduction capacity of a compound may serve as a significant indicator of its potential antioxidant activity (Meir *et al*., 1995). Antioxidant potential of tannins from *C. album* was estimated from their ability to reduce TPTZ-Fe(III) complex to TPTZ-Fe(II) complex (Table 3 and Fig.6).

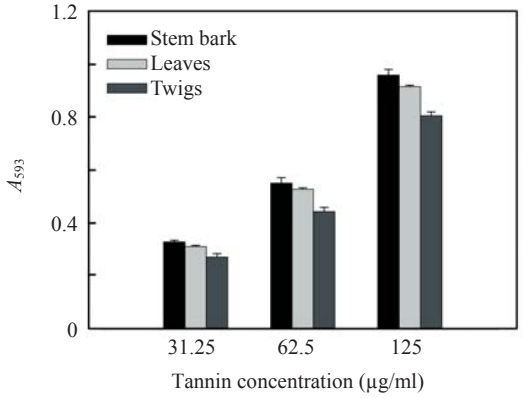

Fig.6 Ferric reducing power after addition of the tannins from *C. album*

A_{593}: Absorbance at 593 nm

In accordance with findings from the DPPH assay, the FRAP values ranged from 3.74 to 4.49 mmol AAE/g dried tannins. A higher absorbance corresponds to a higher ferric reducing power. All tannins showed increased ferric reducing power with the increasing concentration. At 125 μg/ml, the reducing power of tannins from stem bark (A_{593} (absorbance at 593 nm)=0.958) was superior to those of leaves (A_{593}=0.915) and twigs (A_{593}=0.804).

In brief, the reducing power of the tannins followed the order: stem bark (4.49 mmol AAE/g dried tannins)≈leaves (4.28 mmol AAE/g dried tannins)> twigs (3.74 mmol AAE/g dried tannins). But the reducing power of the tannins was less than that of BHA (4.66 mmol AAE/g dried sample). High FRAP and DPPH values of tannins from leaves, twigs and stem bark corresponded to structural heterogeneity of tannins. The polymeric procyanidins from stem bark showed significant radical-scavenging and antioxidant activities.

CONCLUSION

C. album leaves had a relatively high level of

total phenolics and extractable condensed tannins. Structure of condensed tannins from stem bark characterized by NMR and MALDI-TOF MS analyses showed that the condensed tannins consisted of predominantly procyanidins and prodelphinidins with 2,3-*cis* stereochemistry. The mean DP and the average molecular weight were 5.3 and 1578.25 Da, respectively. Tannins extracted from leaves, twigs and stem bark all showed very good DPPH radical scavenging activity (IC_{50} of 56.86, 62.31 and 54.80 μg/ml) and ferric reducing power (4.28, 3.74 and 4.49 mmol AAE/g dried tannins).

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