JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

NMR, HPLC-ESI-MS, and MALDI-TOF MS Analysis of Condensed Tannins from *Delonix regia* (Bojer ex Hook.) Raf. and Their Bioactivities

Wei-Ming Chai,[†,§] Yan Shi,[†,§] Hui-Ling Feng,[†] Ling Qiu,[‡] Hai-Chao Zhou,[†] Zi-Wei Deng,[†] Chong-Ling Yan,*[,†] and Qing-Xi Chen*[,†]

[†]School of Life Sciences, Key Laboratory of the Ministry of Education for Coastal and Wetland Ecosystems, Xiamen University, Xiamen 361005, China

[‡]Key Lab of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, China

ABSTRACT: The structures of the condensed tannins isolated from leaf, fruit, and stem bark of *Delonix regia* (Bojer ex Hook.) Raf. have been investigated with ^{13}C nuclear magnetic resonance (^{13}C NMR) and high performance liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS) coupled with thiolysis and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyses. The results showed that these condensed tannins from *D. regia* possessed structural heterogeneity in monomer units and degree of polymerization. Propelargonidin (PP) and procyanidin (PC) were found in the leaf, fruit, and stem bark of *D. regia*, while prodelphinidin (PD) was found only in the leaves. The polymer chain lengths of condensed tannins from leaf and fruit organs were detected to be trimers to hexadecamers but from trimers to tridecamers for stem bark. B-type linkages were present in all these compounds. Condensed tannins from different parts of *D. regia* can be explored as tyrosinase inhibitors and food antioxidants because of their potent antityrosinase and antioxidant activities. The inhibitor concentration leading to 50% enzyme activity (IC_{50}) was estimated to be 38 ± 1, 73 ± 2, and 54 ± 1.5 μg/mL for the condensed tannins of leaf, fruit, and stem bark. Condensed tannins extracted from stem bark exhibited the highest antioxidant activity; the DPPH scavenging activity (IC_{50}) and the FRAP values were 90 ± 2 μg/mL and 5.42 ± 0.09 mmol AAE/g, respectively.

KEYWORDS: *D. regia, condensed tannins, NMR, HPLC-ESI-MS, MALDI-TOF MS, antityrosinase activity, antioxidant activities*

INTRODUCTION

Tyrosinase is an enzyme that plays a crucial role in the enzymatic browning in plants, and it may cause undesirable changes in color, flavor, and nutritive value of plant-derived foods and beverages.[1] Tyrosinase is also responsible for biosynthesis of melanin in melanocytes of human skin, and epidermal hyperpigmentation might cause various dermatological disorders, such as melasma, freckles, and age spots.[2] It catalyzes the key step of the formation of melanin, the oxidation of diphenol to quinones.[3] Therefore, safe and effective substances that inhibit tyrosinase activity are expected to prevent pigmentation disorders in humans and improve food quality. Furthermore, tyrosinase inhibitors are important in cosmetic applications for skin-whitening effects.[4] There is an increasing interest in using bioactive chemicals extracted from plants as a rich source of tyrosinase inhibitors.[5,6]

Reactive oxygen species (ROS) including, e.g., superoxide anion radical ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$), and hydrogen peroxide (H_2O_2), are generated by normal metabolic processes or exogenous factors and agents, and these can lead to oxidative damage of human cells, causing diseases such as cancer, cardiovascular disease, osteoporosis, and degenerative diseases.[7] A potent scavenger of these ROS may serve as a possible preventive intervention of free radical mediated diseases.[8] There are several reports that condensed tannins have good antioxidant properties.[9,10]

Condensed tannins, polyphenolic compounds, were found in different tissues of plants. They are considered as functional ingredients in botanical, nutritional supplements; therefore, they are attracting more and more attention. However, the bioactivity capacity of plant condensed tannins is generally recognized to be largely dependent on their structure.[10,11] Therefore, it is important to study structures of condensed tannins. These compounds are formed of flavan-3-ol monomer units (Figure 1), which are linked together through C4−C6 or C4−C8 bonds to oligomers and high molecular weight polymers.[12] The diversity of B-type condensed tannins, the most common type, derives for the structural variability and composition of the monomer units, polymer length (degree of polymerization). Because of the complexity and diversity, the characterization of highly polymerized condensed tannins remains very challenging.[9,13] Various techniques including NMR, HPLC-ESI-MS, and MALDI-TOF MS have also been used to characterize condensed tannins.[9,14] It was demonstrated that the determination of the ratio of the 2,3-cis to 2,3-trans stereochemistries could be achieved through ^{13}C NMR by virtue of the distinct differences in their respective C2 chemical shifts.[15] Thiolytic degradation coupled with HPLC-ESI-MS

Received: February 22, 2012
Revised: April 19, 2012
Accepted: April 20, 2012
Published: April 20, 2012

dx.doi.org/10.1021/jf300740d | J. Agric. Food Chem. 2012, 60, 5013−5022

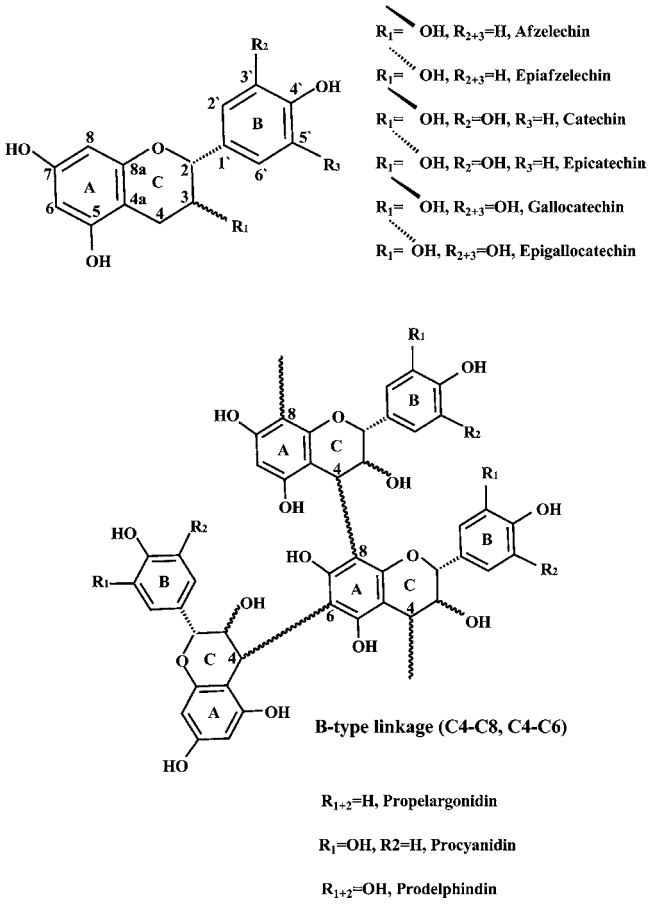

Figure 1. Chemical structures of flavan-3-ol monomer units and condensed tannins.

analysis was used in structure elucidation of the condensed tannins,[16] yielding information on the monomer composition and mean degree of polymerization. However, only the average composition of the sample is determined in this way. An alternative technique, MALDI-TOF MS, is accepted as a powerful tool for the characterization of condensed tannins.[17] It allows the determination of the polymer chain length and the chemical constitution of individual chains. Moreover, the sequential succession of monomer units in individual chains can be elucidated.[14] MALDI-TOF MS produces only a singly charged molecular ion for each parent molecule and allows detection of high mass with precision.[18]

D. regia is a kind of legume plant widely grown in tropical and subtropical regions. Tannins were found in the methanolic crude extracts of *D. regia* and showed strong free radical scavenging activity.[19] However, antityrosinase, antioxidant activities, and detailed information on the purified condensed tannins' profiles, including polymer chain length, chemical constitution of individual chains, and the sequential succession of monomer units in individual chains, has not been reported. This study therefore aims to study antityrosinase, antioxidant activities, and characterize the structures of the condensed tannins extracted from fruit, leaf, and stem bark of *D. regia* using ^{13}C NMR and reverse phase HPLC-ESI-MS coupled with MALDI-TOF MS analysis. These techniques are used for the first time on *D. regia* condensed tannins to elucidate the monomer units, nature of the interflavan linkage, distribution of polymerization degree, and their bioactivities.

MATERIALS AND METHODS

Chemicals and Materials. Mushroom tyrosinase (with specific activity 6680 U/mg), 3,4-dihydroxyphenylalanine (L-DOPA), dimethyl sulfoxide (DMSO), HPLC standards, Sephadex LH-20, benzyl mercaptan, Amberlite IRP-64 cation-exchange resin, cesium chloride, and 2,5-dihydroxybenzoic acid (DHB) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl-d_6 sulfoxide (DMSO-d_6) was purchased from Armar (Dŏttingen, Switzerland). 2,4,6-Tripyridyl-*S*-triazine (TPTZ), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and ascorbic acid were purchased from Sigma-Aldrich. Water used in this experiment was purified on a Millipore Milli-Q apparatus (TGI Pure Water Systems, USA). HPLC grade CH_3CN and trifluoroacetic acid (TFA) for analytical HPLC-ESI-MS were obtained from Sinopharm (Shanghai, China). All other solvents used were of analytical reagent (AR) purity grade. Leaf, fruit, and stem bark of *D. regia* were collected at the campus of Xiamen University (Xiamen, Fujian Province, China) and immediately freeze-dried and ground. The plant is identified by Professor Li (School of Life Sciences, Xiamen University).

Extraction and Purification of the Condensed Tannins. Freeze-dried leaf, fruit, and stem bark powders (30 g of each) were extracted thrice with 7:3 (v/v) acetone−water solution (3 × 250 mL) at room temperature. Each extract was filtered and pooled, and the solvent was removed under reduced pressure by using a rotary evaporator at 38 °C. The remaining aqueous fraction was extracted thrice with petroleum ether (3 × 150 mL) in order to remove chlorophyll and lipophilic compounds. The remaining crude tannin fraction was chromatographed on an LH-20 column (Pharmacia Biotech, Uppsala, Sweden), which was first eluted with methanol−water (50:50, v/v) and then with acetone−water (7:3, v/v). The last fraction of purified condensed tannins was freezed-dried and stored at −20 °C before analysis by MALDI-TOF mass spectrometry.

^{13}C NMR Analysis. The ^{13}C NMR spectra of condensed tannins were recorded on a Varian Mercury-600 spectrometer (Palo Alto, CA, USA) at 150 MHz. The samples for recording NMR spectra were prepared by dissolving them in DMSO-d_6.

Thiolysis of Condensed Tannins with Benzyl Mercaptan. Condensed tannin (500 μg, dissolved in methanol) was mixed with 3.3% hydrochloric acid methanol solution and 5% benzyl mercaptan methanol solution. The mixed solution was heated for 30 min at 40 °C, and then cooled to room temperature. The solution was filtered through a membrane filter with an aperture size of 0.45 μm, and 20 μL of sample solution was analyzed by RP-HPLC-ESI-MS.

Reversed-Phase HPLC-ESI-MS Analysis. The high performance liquid chromatograph was an Agilent 1100 system (Agilent, Santa Clara, CA, USA) equipped with a diode array detector and a quaternary pump. The thiolysis medium was further analyzed using LC/MS (QTRAP 3200, USA) with a Hypersil ODS column (4.6 × 250 mm) (Elite, Dalian, Liaoning Province, China). Two solvents, 0.5% TFA and CH_3CN, were used for elution. The elution system was 0−45 min, 12−80% CH_3CN (linear gradient); 45−50 min, 80−12% CH_3CN (linear gradient). The column temperature was controlled at 25 °C, and the flow rate was set at 1 mL/min. Detection was at 280 nm and the UV spectra were acquired between 200 and 600 nm. Degradation products were identified on chromatograms according to their retention times and LC/MS.

MALDI-TOF MS Analysis. The MALDI-TOF MS spectra were recorded on a Bruker Reflex III instrument (Germany). 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. 2,5-Dihydroxybenzoic acid was used as the matrix. The sample solutions were mixed with the matrix solution at a volumetric ratio of 1:3. The mixture (1 μL) was spotted 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 chloride was mixed with the analyte−matrix solution to promote the formation of a single type of ion adduct ($[M + Cs]^+$).[20]

Enzyme Assay. The assay of the enzyme activity was performed as described by Lin et al.[4] with minor modification. In this investigation, L-DOPA was used as a substrate for the enzyme activity assay. The reaction medium (3 mL) contained 0.5 mM L-DOPA in 50 mM sodium phosphate buffer (pH 6.8). The final concentration of mushroom tyrosinase was 6.67 μg/mL. The inhibitor was first dissolved in DMSO. The final concentration of DMSO in the test solution was 3.3%. The enzyme activity was monitored by dopachrome formation at 475 nm accompanying the oxidation of the substrate. Absorption was recorded using a Beckman UD-800 spectrophotometer (California, USA). The extent of inhibition by the addition of the sample was expressed as the percentage necessary for 50% inhibition (IC_{50}) for enzyme activity assay. Controls, without inhibitor but containing 3.3% DMSO, were routinely carried out. All measurements were carried out at 30 °C.

Ferric Reducing Antioxidant Power (FRAP). FRAP assay was performed according to the established procedure of Benzie and Strain[21] with minor modification. In brief, 3 mL of FRAP reagent (10 mM TPTZ, 20 mM ferric chloride, and 300 mM sodium acetate buffer (pH 3.6), mixed at a ratio of 1:1:10) was mixed with 0.1 mL of the test sample or methanol (for the reagent blank). The absorbance of reaction mixture at 593 nm was measured spectrophotometrically after incubation at 25 °C for 10 min. The FRAP values, expressed in millimoles of ascorbic acid equivalents (AAE)/g fraction, were derived from a standard curve. All samples were analyzed in three replicates.

DPPH Radical Scavenging Capacity. The effect of purified condensed tannins against DPPH radical was determined according to the method of Brand-Williams et al.[22] with some modifications. In brief, 0.1 mL of the sample at different concentrations (15.63, 31.25, 62.5, and 125 μg/mL dissolved in methanol) was added to 3 mL of DPPH solution (25 mg/L in methanol). An equal amount of methanol and DPPH served as the control. After the mixture was shaken and left at room temperature for 30 min, the absorbance at 517 nm was measured. The scavenging percentage of DPPH was calculated according to the following equation: DPPH% inhibition = $[(A_1 - A_2)/A_1] \times 100$, where A_1 = the absorbance of the control reaction; A_2 = the absorbance in the presence of the sample. The IC_{50} value (μg/mL) is the concentration that led to a 50% decrease in absorbance.

RESULTS AND DISCUSSION

^{13}C NMR Analysis of Condensed Tannins. The liquid-state ^{13}C NMR spectrum of the purified condensed tannin from leaf (A), fruit (B), and stem bark (C) of *D. regia* is depicted in Figure 2. The signal assignment was interpreted according to relevant literature reports.[23,24] The ^{13}C NMR spectrum of the *D. regia* condensed tannins in DMSO-d_6 shows the presence of PC together with PP for the fruit and stem bark and PP along with PC and PD for the leaf, respectively. Especially, the spectrum shows distinct signals at 157 ppm, which are assignable to C4′ in PP units or C5, C7, and C8a carbons of PC (C5, C7, and C8a carbons of PC appear at 157 to 150 ppm). Peaks at 145 and 145.1 ppm belong to C3′ and C4′ of PC units. The signals at 114.8, 115.1, and 118.1 ppm are assignable to the C2′, C5′, and C6′ of PC units. Indeed, C3′ and C5′ of PD units showed a typical resonance at 146 ppm for the leaf, which was not found in the fruit and stem bark. The absence of a clear signal with such a chemical shift in the spectra of the condensed tannins from fruit and stem bark revealed that they are composed of PP and PC units, and leaf condensed tannins contained PP, PC, and PD. PD is also detected by its C4′ signal, which appears at 131 ppm in the leaf condensed tannins, overlapping with the chemical shifts of C1. The cluster of peaks between 110 and 90 ppm is assigned to C8, C4a, and C6 of PC, and C6′ and C2′ of PD is also appearing in this area for the leaf. The region between 70 and 90 ppm is sensitive to the stereochemistry of the C ring. The

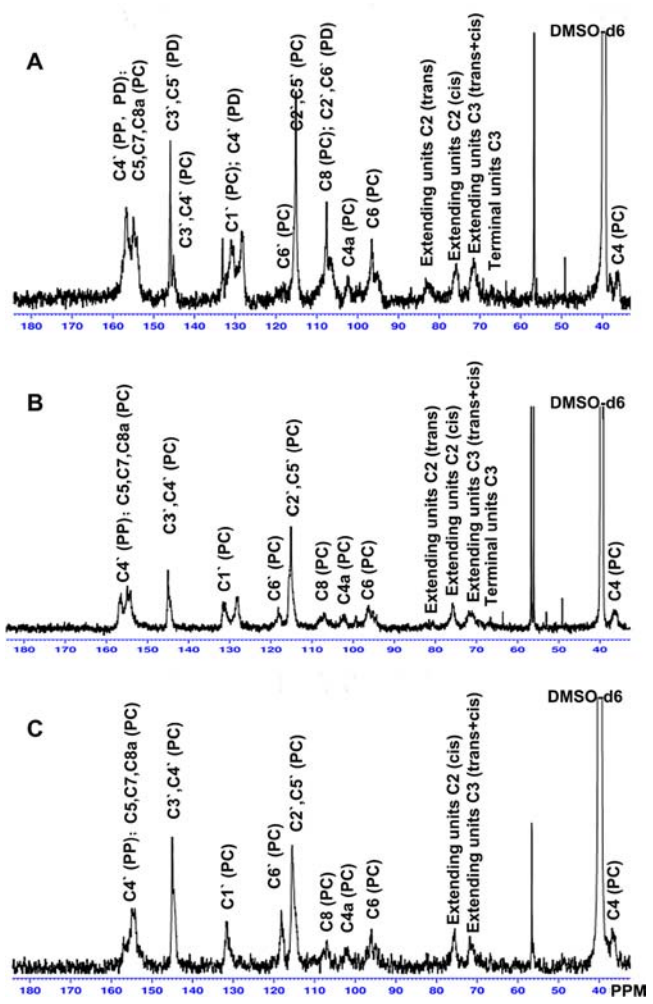

Figure 2. ^{13}C NMR (150 MHz) spectrum of condensed tannins from leaf (A), fruit (B), and stem bark (C) of *D. regia* in DMSO-d_6; PP, propelargonidin; PC, procyanidin; PD, prodelphinidin; DMSO-d_6, dimethyl-d_6 sulfoxide.

ratio of the 2,3-cis to 2,3-trans isomers could be determined through the distinct differences in their respective C2 chemical shifts. C2 gives a resonance at 76 ppm for the cis form and 83 ppm for the trans form. From the peak areas, it is estimated that the cis isomer is dominant. The percentage of cis form was estimated to be 68%, 97.5%, and 100% for the condensed tannins from leaf, fruit, and stem bark. C3 of both cis and trans isomers occurs at 71.5 ppm. A sharp line at 64 ppm is due to C3 of the terminal unit. The terminal to extender ratio were evaluated to be 15.23 and 7.67 for the leaf and fruit condensed tannins, but the terminal signal of stem bark condensed tannins was not detected in the ^{13}C NMR spectrum. The C4 atoms of the extension units showed a broad peak at 36.2 ppm.

Thiolysis with Benzyl Mercaptan Followed by Reversed-Phase HPLC-ESI-MS. To obtain more detailed information on the chemical composition of condensed tannins from leaf, fruit, and stem bark of *D. regia*, reversed-phase HPLC-ESI-MS analysis was performed. The constituent flavanoid units in condensed tannins from leaf, fruit, and stem bark of *D. regia* were identified by degradation of these compounds using acid hydrolysis in the presence of benzyl mercaptan. Degradation of these compounds with acids in the presence of various nucleophiles is a well-known method since

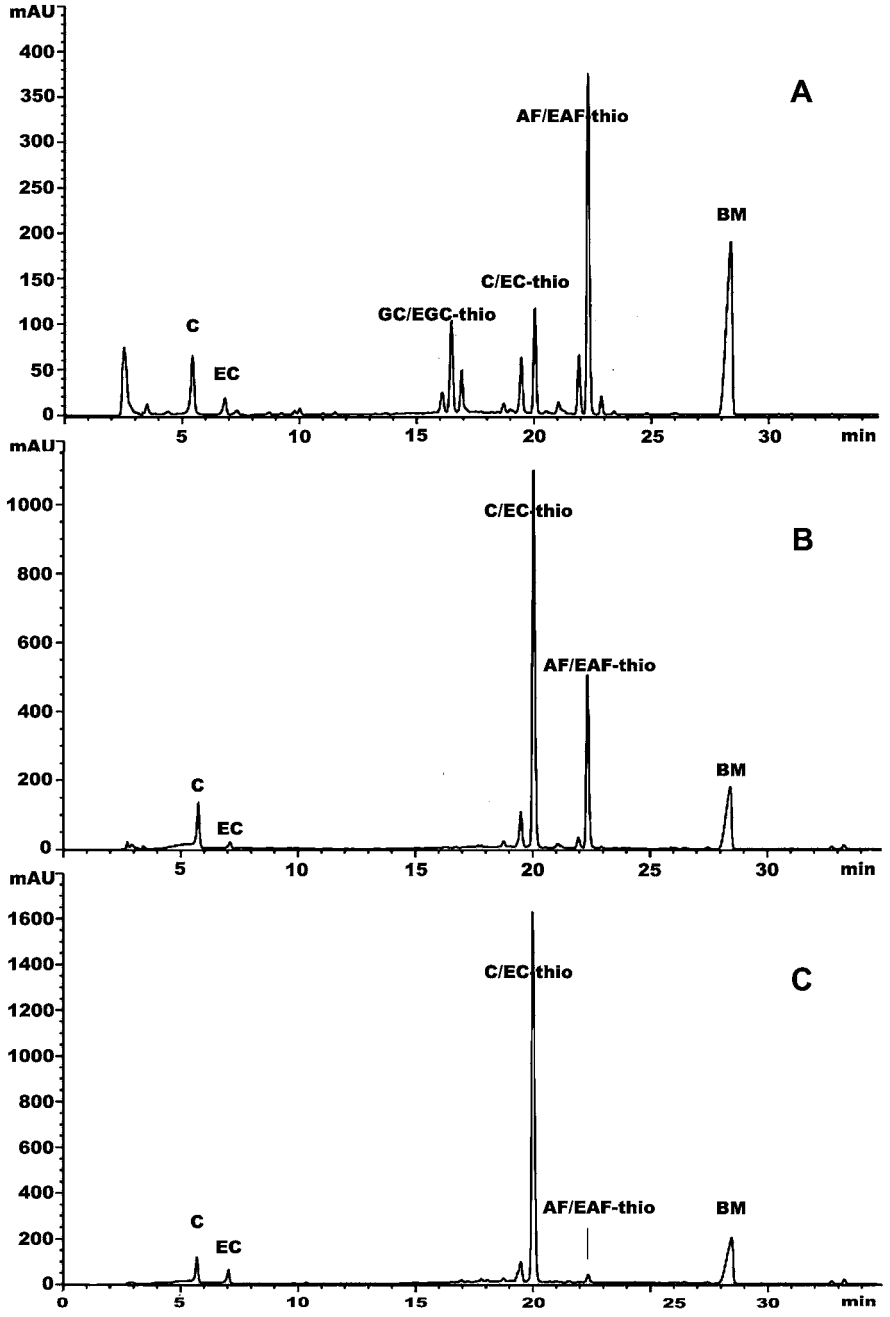

Figure 3. Reversed-phase HPLC chromatograms of condensed tannins from leaf (A), fruit (B), and stem bark (C) of the *D. regia*. Terminal units: catechin (C), epicatechin (EC). Extender units: afzelechin (AF-thio), epiafzelechin (EAF-thio), catechin (C-thio), epicatechin (EC-thio), gallocatechin (GC-thio), epigallocatechin (EGC-thio), catechin gallate (CG-thio), and epicatechin gallate (ECG-thio) benzylthioethers; BM, benzyl mercaptan.

the stereochemistry at C2 and C3 positions is preserved. In current studies, acid-catalyzed thiolysis has been used by many researchers[25,26] and the use of benzyl mercaptan as the nucleophile has been proved a good way for separation of the degradation products when using different chromatographic systems. The identity of the individual units that make up a condensed tannins polymer and the length of the polymer chain can be estimated by subjecting the polymer to strong acid-catalyzed cleavage in the presence of benzyl mercaptan. These reactions result in the release of terminal units as free flavan-3-ols, whereas extender units are distinguished as benzylthioether adducts, which are formed by nucleophilic capture of the carbocations generated under the acid conditions of the reaction.[27] The depolymerization products were then separated on reversed-phase HPLC-ESI-MS.

The chromatograms of thiolytic degraded condensed tannins from leaf (A), fruit (B), and stem bark (C) of *D. regia* were shown in Figure 3. Condensed tannins from different parts of *D. regia* possessed large structural heterogeneity. Analysis of the condensed tannins degradation products by reversed-phase HPLC-ESI-MS showed that the extension units in condensed tannins from the leaf of *D. regia* contain afzelechin/epiafzelechin, catechin/epicatechin, and gallocatechin/epigallocatechin, with the afzelechin/epiafzelechin dominating, and the fruit and stem bark both containing afzelechin/epi-afzelechin and catechin/epicatechin, both with the catechin/epicatechin

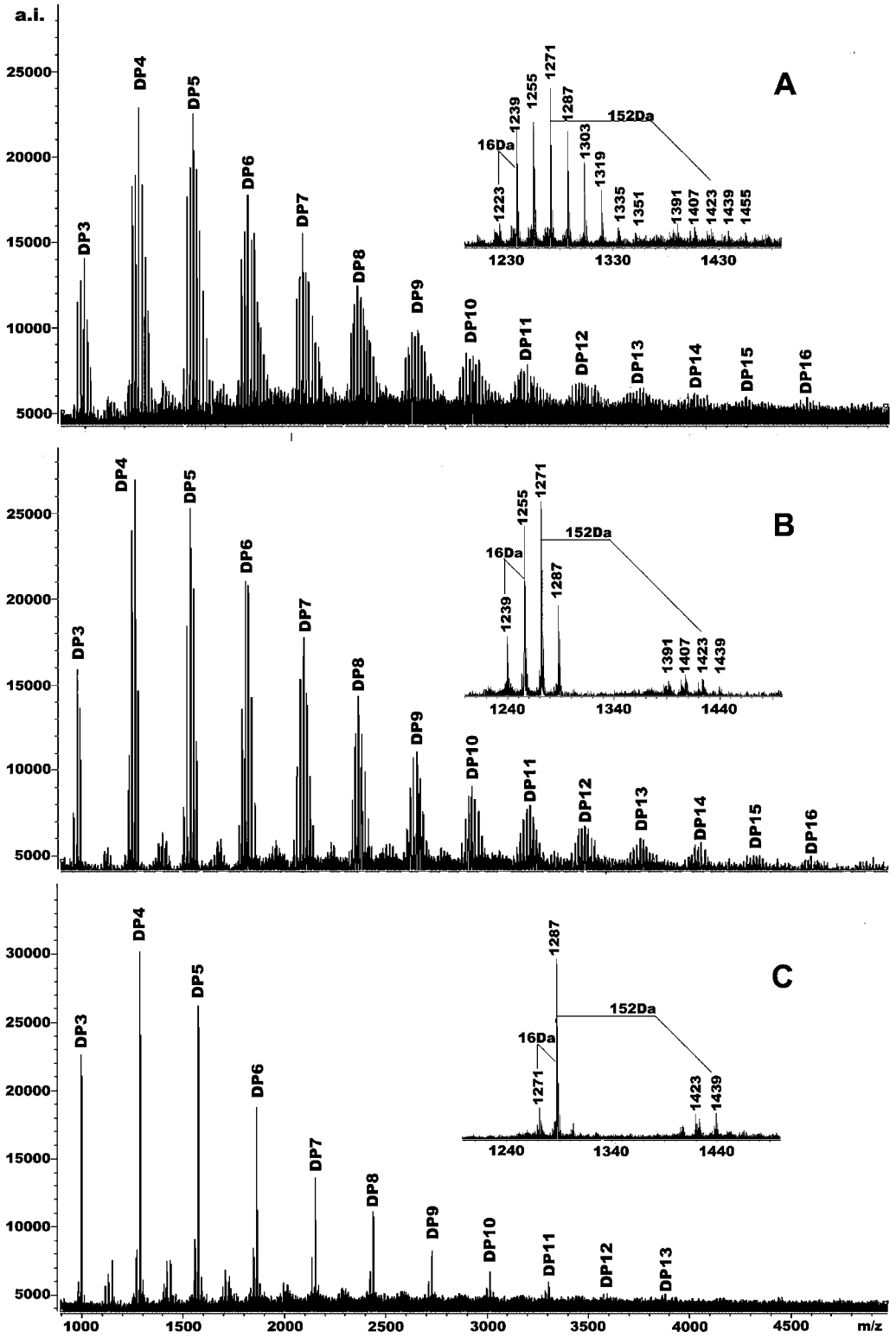

Figure 4. MALDI-TOF positive reflectron mode mass spectra of the condensed tannins from different parts of *D. regia:* leaf (A), fruit (B), and stem bark (C). DP, degree of polymerization.

dominating, and the stem bark have only rare afzelechin/epiafzelechin benzylthioethers compared with leaf and fruit. The terminal units in condensed tannins from leaf, fruit, and stem bark of *D. regia* are all catechin and epicatechin. The catechin-type to epicatechin-type ratio in the terminal unit were estimated to be 4.41, 4.94, and 1.64 for condensed tannins from leaf, fruit, and stem bark, respectively. Therefore, the chemical composition of condensed tannins from different parts of *D. regia* analyzed by reversed-phase HPLC-ESI-MS was consistent with the NMR results.

MALDI-TOF MS Analysis. Although the ^{13}C NMR spectrum and reversed-phase HPLC-ESI-MS reveals complex structural characteristics of condensed tannins from leaf, fruit, and stem bark of *D. regia*, quantitative data regarding the high

dx.doi.org/10.1021/jf300740d | *J. Agric. Food Chem.* 2012, 60, 5013−5022

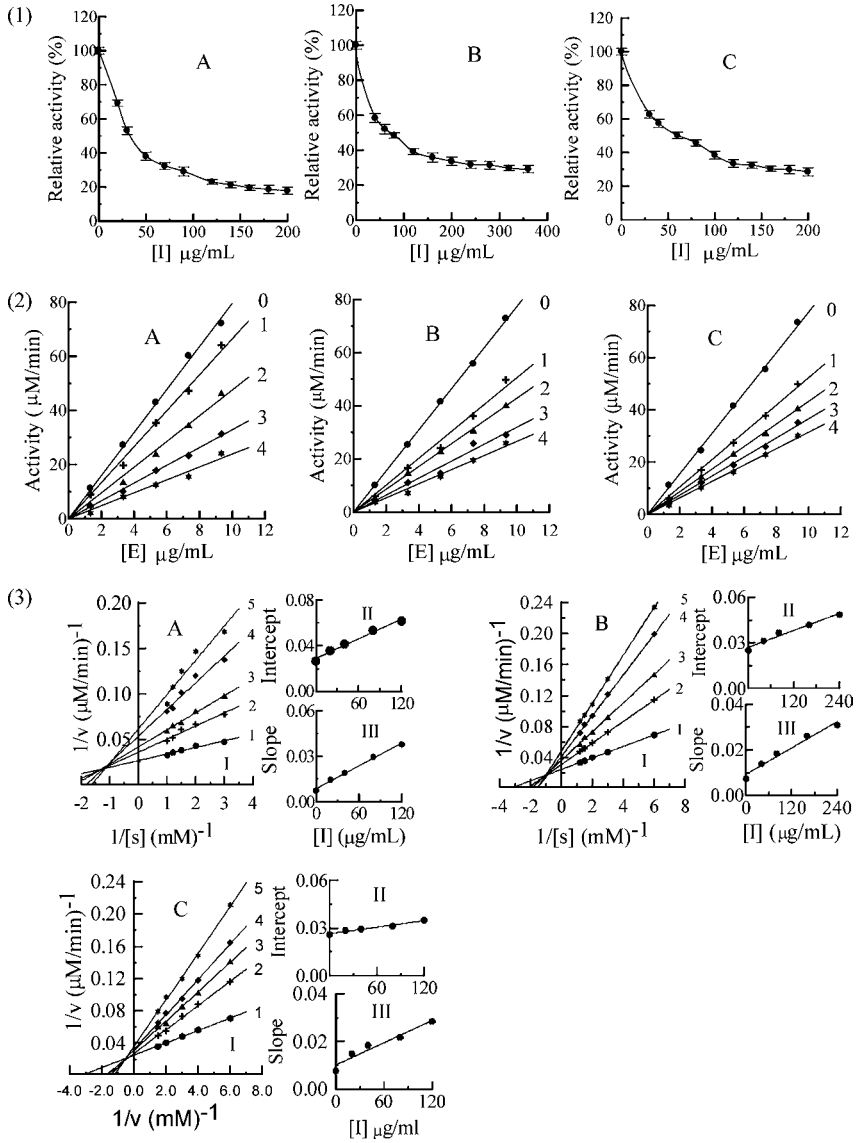

Figure 5. Determination of the inhibitory effects (1), inhibitory mechanism (2), and inhibitory type and inhibition constants (3) of condensed tannins extracted from leaf (A), fruit (B), and stem bark (C) of *D. regia* on mushroom tyrosinase for the catalysis of L-DOPA at 30 °C. Assay conditions: 3 mL reaction system containing 50 mM phosphate sodium buffer pH 6.8 and 3.3% DMSO.

degree of polymerization cannot be reliably obtained. Further characterization was achieved by MALDI-TOF MS. MALDI-TOF MS is a sensitive and powerful tool for the analysis of nonvolatile molecules, and now is considered as a powerful method for analysis of tannins exhibiting high structural heterogeneity. When this technique is used, fragmentation of the analyte molecules upon laser irradiation can be substantially reduced by embedding them in a light absorbing matrix. Thus, intact analyte molecules are desorbed and ionized along with the matrix and can be analyzed in a mass spectrometer.[28] Several factors including the selection of an appropriate matrix, drying of matrix and sample, optimal mixing, adjustment of laser strength, and optimal selection of cationization reagent must be optimized to develop MALDI-TOF MS techniques.

MALDI-TOF mass spectrum of the polymeric tannin mixtures isolated from leaf, fruit, and stem bark of *D. regia* was shown in Figure 4, recorded as Cs^+ adducts in the positive ion reflectron mode and showing repeating condensed tannin polymers. The polydisperse condensed tannin is reflected by the periodic occurrence of peak series representing different chain lengths. The results obtained indicated that condensed tannins isolated from the different parts of *D. regia* are heteropolymers. The masses of the highest peaks among the condensed tannin polymers with identical DP increased at the different distance of 272, 288, or 304 Da in leaf, corresponding to afzelechin/epiafzelechin, catechin/epicatechin, and gallocatechin/epigallocatechin monomers. Similar distances of 272 and 288 Da were observed in fruit tannins, corresponding to afzelechin/epiafzelechin and catechin/epicatechin monomers, and 288 Da in stem bark, corresponding to catechin/epicatechin monomers. The mass spectrum of leaf condensed tannins is complicated; each DP had a subset of masses 16 Da lower, and the subset of masses 16 Da higher were also detected, which can be explained by heteropolymers of repeating flavan-3-ol units containing an additional hydroxyl group at the position 5′ of the B-ring. Nine peaks were detected when the tetramer spectrum was enlarged (Figure 4). In contrast, the mass spectrum of stem bark condensed tannins is

simpler. The compounds are characterized by mass spectra with a series of peaks with distances of 288 Da, corresponding to PC; therefore, prolongation of condensed tannins is due to the addition of PC monomers (Figure 4). In addition to the predicted homopolyflavan-3-ol mass series mentioned above, each DP had a subset of masses 16 Da lower in the spectra of stem bark (Figure 4). These masses indicated the polymer chains contained monomers with only one hydroxyl group (16 Da) on the aromatic ring B. It was further suggested that the condensed tannins from stem bark and fruit contain PP and PC and that the condensed tannins from leaf contain PP, PC, and PD when the absolute masses corresponding to each peak were obtained. For the condensed tannins indicated above, 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) (Figure 4) in spectra of all three parts of *D. regia*.

The condensed tannins from the three different parts of *D. regia* had different polymer chain length varying from trimers to hexadecamers for fruit and leaf and to tridecamers for stem bark. No series of compounds that are 2 Da multiples lower than those described peaks for heteropolyflavan-3-ols were detected, so A-type interflavan ether linkage appears not to exist between adjacent flavan-3-ol subunits for leaf, fruit, and stem bark. All compounds are linked by B-type bonds. Structures of condensed tannins from different parts of *D. regia* were thus successfully characterized using MALDI-TOF MS for the first time.

Concentration Effects of Condensed Tannins on Mushroom Tyrosinase Activity. The effects of condensed tannins from leaf (A), fruit (B), and stem bark (C) of *D. regia* on the oxidation of L-DOPA catalyzed by mushroom tyrosinase was first studied. When the activity of mushroom tyrosinase was assayed by using L-DOPA as substrate, the reaction course achieved a steady-state rate quickly. With increasing the concentration of the compounds, the activity of mushroom tyrosinase was observably reduced in a concentration-dependent pattern as shown in Figure 5-1. The quality of the tyrosinase inhibitor about the condensed tannins from different parts of *D. regia* was determined by the IC_{50} values (the concentration with inhibitory activity of 50%). A lower value of IC_{50} indicates greater antityrosinase activity.

Wang et al.[29] studied tyrosinase-inhibiting activities of extracts from sorghum distillery residue; their result showed that methanol extracts were the strongest tyrosinase inhibitor with IC_{50} of 580 μg/mL. Wu et al.[30] found that red koji extracts (containing a high percentage of condensed tannins) showed an inhibitory effect with an IC_{50} value of 5570 μg/mL on mushroom tyrosinase activity. In this present study, the inhibitor concentration leading to 50% tyrosinase enzyme activity (IC_{50}) was estimated to be 38 ± 1, 73 ± 2, and 54 ± 1.5 μg/mL for the condensed tannins extracted from leaf, fruit, and stem bark of *D. regia*, respectively. Therefore, the aromatic compounds in this study were potent inhibitors on tyrosinase.

There are two copper ions in the active center of tyrosinase.[31] According to the 3D structure of tyrosinase, the copper ions were of great importance for tyrosinase activity, and a slight change in the dicopper center may lead to activity loss.[2] The strong inhibitory effects of condensed tannins from *D. regia* on the tyrosinase might be due to binding with copper ions with their −OH group. The relationship between the phenolic hydroxylation pattern and tyrosinase inhibition suggested that the condensed tannins with a 5,7-dihydroxyphenyl structure in the A-ring and a 3,4,5-trihydroxyphenyl structure in the B-ring (PD) have potent tyrosinase inhibitory activity.[32] Our results revealed that condensed tannins from leaf of *D. regia* possessed the best inhibitory effect on the tyrosinase. This can be explained by PD, which existed only in the leaves, but correlation was not observed between the degree of polymerization of condensed tannins and tyrosinase inhibition. We supposed the effect of the degree of polymerization on the tyrosinase is less than phenolic hydroxylation pattern. Our results showed that condensed tannins from leaf, fruit, and stem bark of *Delonix regia* might be potential sources of natural tyrosinase inhibitors.

Inhibition Mechanism of Condensed Tannins on the Mushroom Tyrosinase. Plots containing a family of straight lines were obtained when the remaining enzyme activity versus the concentrations of enzyme in the presence of different concentrations. If all straight lines were parallel lines, the inhibition was irreversible, but if they passed through origo, the inhibition was reversible. Figure 5-2 shows the relationship of enzyme activity to the enzyme concentration, lines 0−4 represent different concentrations of condensed tannins. The plots of the remaining enzyme activity versus the concentrations of enzyme at different inhibitor concentrations gave a family of straight lines, which all passed through the origo. Therefore, the inhibition was reversible. The slope of the line descended with an increase of inhibitor concentration, indicating that the presence of inhibitor did not bring down the amount of enzyme but just lead to a decrease in activity of the enzyme for oxidation of L-DOPA. Therefore, the mechanism of inhibition of the mushroom tyrosinase by condensed tannins from leaf (A), fruit (B), and stem bark (C) of *D. regia* during the oxidation of L-DOPA was successfully first studied.

Inhibition Type and Inhibition Constants of Condensed Tannins on the Enzyme. The kinetic behavior of the oxidation of L-DOPA, catalyzed by mushroom tyrosinase at different concentrations of condensed tannins from leaf (A), fruit (B), and stem bark (C) of *D. regia*, was studied. Figure 5-3 shows the double-reciprocal plots of the enzyme inhibited by these compounds. The plots of $1/v$ versus $1/[S]$ give a family of lines with different slope and intercept, which intersected in the second quadrant, indicating that condensed tannins from different parts of *D. regia* are mixed-type inhibitors (Figure 5-3I). The result revealed that the inhibitors bind with free enzymes as well as enzyme−substrate complexes. From these results, we suppose that these tannin compounds might compete for attachment on the active site of the enzyme with L-DOPA due to its −OH group. The inhibitor constant (K_I) was obtained from the plots of the slope versus the concentration of these compounds, and the enzyme−substrate complex (K_{IS}) was obtained from the vertical intercept versus the concentration of these compounds (Figure 5-3II,III). The values of K_I and K_{IS} were determined to be 340 and 104, 973 and 289, and 64 and 381 μg/mL for condensed tannins from leaf, fruit, and stem bark of *D. regia*, respectively. The values of K_I were higher than that of K_{IS} for condensed tannins from leaf and fruit, indicating that the affinity of the inhibitor for enzyme−substrate complexes was stronger than that of the free enzyme. In contrast, the condensed tannins obtained from stem bark have lower values of K_I than that of K_{IS}, indicating that the affinity of the inhibitor for free enzyme was stronger than that for the enzyme−substrate complexes. Tight binding of the

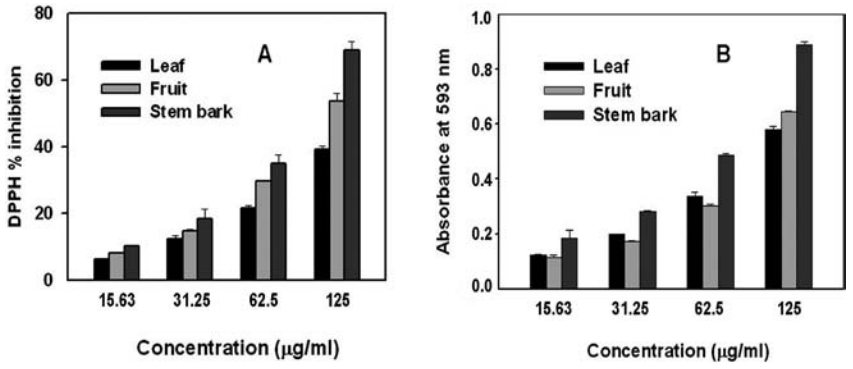

Figure 6. DPPH radical scavenging capacity (A) and ferric reducing antioxidant power (FRAP) (B) of condensed tannins extracted from different parts of *D. regia*.

inhibitor to the enzyme or enzyme−substrate may be responsible for the low value.

Determination of Antioxidant Property. Two methods were used for measuring the antioxidant properties of condensed tannin materials to better reflect their potential protective effects. The DPPH method is based on the capacity of a sample to scavenge the DPPH radical as compared to a standard antioxidant,[33] while FRAP assay was based on the redox reaction of ferric ion in the presence of a reductant.[21] The reduction capacity of a compound may serve as a significant indicator of its potential antioxidant activity.[34] The reduction capability of DPPH radical is determined by the decrease in absorbance at 517 nm induced by antioxidants. The percentages of DPPH inhibition in the presence of condensed tannins from different parts of *D. regia* at different concentrations are shown in Figure 6A. A dose−response relationship is found in the radical scavenging activity; the activity increased with the increasing concentration of condensed tannins. The quality of the antioxidants about the condensed tannins from different parts of *D. regia* was determined by the IC_{50} values (the concentration with scavenging activity of 50%). A lower value of IC_{50} indicates greater antioxidant activity. The results shows that all three parts of condensed tannins from *D. regia* have a good antioxidant activity. The IC_{50} values of stem bark (90 ± 2 μg/mL) were significantly higher than those of fruit (115 ± 3 μg/mL) and leaf (161 ± 9 μg/mL), indicating that the condensed tannins of stem bark exhibited the highest scavenging effect (Table 1). The scavenging effect on the DPPH radical decreased in the order stem bark > fruit > leaf.

The antioxidant activity of condensed tannins was also measured by FRAP assay, a higher absorbance corresponds to a higher ferric reducing power. At each concentration, the condensed tannins of stem bark exhibited higher reducing power than those of fruit and leaf (Figure 6B). The FRAP values, expressed in ascorbic acid equivalents, were listed in Table 1. The FRAP values for the condensed tannins of stem bark, fruit, and leaf were 5.42 ± 0.09, 3.39 ± 0.08, and 3.80 ± 0.15 mmol AAE/g, respectively.

Antioxidant should have broad applications, so much effort has been spent searching for feasible and effective antioxidant. In our previous paper, condensed tannins isolated from *Litchi chinensis* Sonn.[35] and *Acacia confusa*.[12] had been proved to have potent antioxidant activity. Cai et al.[11] confirmed that the number and position of hydroxyl groups and the related glycosylation and other substitutions largely determined radical scavenging activity of the phenolic compounds. Yokozawa et al.[36] thought an increase of galloyl groups, molecular weight, and *ortho*-hydroxyl structure enhanced the scavenging activity of tannins. In this study, the DPPH free radical scavenging assay and the FRAP assay both revealed that condensed tannins from stem bark of *D. regia* were potent antioxidants. It can be concluded that PC have better antioxidant activities than PP. Furthermore, we supposed the different bioactivities were mainly due to their different structure. Zhou et al.[35] found that the higher polymerization degree of polymeric proanthocyanidins from litchi fruit stones exhibited higher antioxidant activities than those from litchi pericarps. In contrast, the higher polymerization degree of polymeric condensed tannins from fruit and leaf exhibited lower antioxidant activities than those from stem bark in this study. The result can be explained that degree of polymerization played a modified role in enhancing or reducing the activity. In conclusion, this plant (especially the stem bark) might be a good resource for further development as an antioxidant.

Table 1. Antioxidant Activities of the Condensed Tannins from Different Parts of *D. regia* Using the (DPPH) Free Radical Scavenging Assay and the Ferric Reducing Antioxidant Power (FRAP) Assay[a]

	antioxidant activity	
samples	IC_{50}/DPPH (μg/mL)	FRAP (mmol AAE/g)
leaf	161 ± 9	3.80 ± 0.15
fruit	115 ± 3	3.39 ± 0.08
stem bark	90 ± 2	5.42 ± 0.09
ascorbic acid	96 ± 3	

[a]Values are expressed as mean of triplicate determinations ± standard deviation; all values in each column are different ($P < 0.05$).

AUTHOR INFORMATION

Corresponding Author

*E-mail: chenqx@xmu.edu.cn (Q.-X.C.); ycl@xmu.edu.cn (C.-L.Y).

Author Contributions

[§]These authors contributed equally to this work.

Funding

The present investigation was supported by the National Foundation for fostering talents of basic science (no. J1030626), the Natural Science Foundation of China (no. 31071611 and no. 31070522), and the Science and Technology Foundation of Fujian Province (no. 2010N5013).

Notes

The authors declare no competing financial interest.

■ ABBREVIATIONS USED

D. regia, *Delonix regia*; ^{13}C NMR, ^{13}C nuclear magnetic resonance; HPLC-ESI-MS, high performance liquid chromatography electrospray ionization mass spectrometry; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PP, propelargonidin; PC, procyanidin; PD, prodelphinidin; L-DOPA, 3,4-dihydroxyphenyl-alanine; DMSO, dimethyl sulfoxide; DHB, 2,5-dihydroxybenzoic acid; TFA, trifluoroacetic acid; DMSO-d_6, dimethyl-d_6 sulfoxide; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant power; TPTZ, 2,4,6-tripyridyl-*S*-triazine.

■ REFERENCES

(1) Kubo, I.; Kinst-Hori, I.; Kubo, Y.; Yamagiwa, Y.; Kamikawa, T.; Haraguchi, H. Molecular design of antibrowning agents. *J. Agric. Food Chem.* **2000**, *48*, 1393−1399.

(2) Li, Z. C.; Chen, L. H.; Yu, X. J.; Hu, Y. H.; Song, K. K.; Zhou, X. W.; Chen, Q. X. Inhibition kinetics of chlorobenzaldehyde thiosemicarbazones on mushroom tyrosinase. *J. Agric. Food Chem.* **2010**, *58*, 12537−12540.

(3) Pan, Z. Z.; Li, H. L.; Yu, X. J.; Zuo, Q. X.; Zheng, G. X.; Shi, Y.; Liu, X.; Lin, Y. M.; Liang, G.; Wang, Q.; Chen, Q. X. Synthesis and antityrosinase activities of alkyl 3,4-dihydroxybenzoates. *J. Agric. Food Chem.* **2011**, *59*, 6645−6649.

(4) Lin, M.; Ke, L. N.; Han, P.; Qiu, L.; Chen, Q. X.; Lin, H. T.; Wang, Q. Inhibitory effects of *p*-alkylbenzoic acids on the activity of polyphenol oxidase from potato (*Solanum tuberosum*). *Food Chem.* **2010**, *119*, 660−663.

(5) Sugimoto, K.; Nakagawa, K.; Hayashi, S.; Amakura, Y.; Yoshimura, M.; Yoshida, T.; Yamaji, R.; Nakano, Y.; Inui, H. Hydrolyzable tannins as antioxidants in the leaf extract of *Eucalyptus globulus* possessing tyrosinase and hyaluronidase inhibitory activities. *Food Sci. Technol. Res.* **2009**, *15*, 331−336.

(6) Zhuang, J. X.; Hu, Y. H.; Yang, M. H.; Liu, F. J.; Qiu, L.; Zhou, X. W.; Chen, Q. X. Irreversible competitive inhibitory kinetics of cardol triene on mushroom tyrosinase. *J. Agric. Food Chem.* **2010**, *58*, 12993−12998.

(7) Siswoyo, T. A.; Mardiana, E.; Lee, K. O.; Hosokawa, K. Isolation and characterization of antioxidant protein fractions from the Melinjo (*Gnetum gnemon*) seeds. *J. Agric. Food Chem.* **2011**, *59*, 5648−5656.

(8) Gülçin, I. Antioxidant activity of L-adrenaline: A structure-activity insight. *Chem.-Biol. Interact.* **2009**, *179*, 71−80.

(9) Es-Safi, N. E.; Guyot, S.; Ducrot, P. H. NMR, ESI/MS, and MALDI-TOF/MS analysis of pear juice polymeric proanthocyanidins with potent free radical scavenging activity. *J. Agric. Food Chem.* **2006**, *54*, 6969−6977.

(10) Noferi, M.; Masson, E.; Merlin, A.; Pizzi, A.; Deglise, X. Antioxidant characteristics of hydrolysable and polyflavonoid tannins: An ESR kinetics study. *J. Appl. Polym. Sci.* **1997**, *63*, 475−482.

(11) Cai, Y. Z.; Sun, M.; Xing, J.; Luo, Q.; Corke, H. Structure−radical scavenging activity relationships of phenolic compounds from traditional Chinese medicinal plants. *Life Sci.* **2006**, *78*, 2872−2888.

(12) Wei, S. D.; Zhou, H. C.; Lin, Y. M.; Liao, M. M.; Chai, W. M. MALDI-TOF MS analysis of condensed tannins with potent antioxidant activity from the leaf, stem bark and root bark of *Acacia confusa*. *Molecules* **2010**, *15*, 4369−4381.

(13) Hümmer, W.; Schreier, P. Analysis of proanthocyanidins. *Mol. Nutr. Food Res.* **2008**, *52*, 1381−1398.

(14) Behrens, A.; Maie, N.; Knicker, H.; K gel-Knabner, I. MALDI-TOF mass spectrometry and PSD fragmentation as means for the analysis of condensed tannins in plant leaves and needles. *Phytochemistry* **2003**, *62*, 1159−1170.

(15) Eberhardt, T. L.; Young, R. A. Conifer seed cone proanthocyanidin polymers: characterization by ^{13}C NMR spectroscopy and determination of antifungal activities. *J. Agric. Food Chem.* **1994**, *42*, 1704−1708.

(16) Gu, L.; Kelm, M. A.; Hammerstone, J. F.; Beecher, G.; Holden, J.; Haytowitz, D.; Ronald, L. Screening of foods containing proanthocyanidins and their structural characterization using LC-MS/MS and thiolytic degradation. *J. Agric. Food Chem.* **2003**, *51*, 7513−7521.

(17) Krueger, C. G.; Vestling, M. M.; Reed, J. D. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry of heteropolyflavan-3-ols and glucosylated heteropolyflavans in sorghum [*Sorghum bicolor* (L.) Moench]. *J. Agric. Food Chem.* **2003**, *51*, 538−543.

(18) Xiang, P.; Lin, Y.; Lin, P.; Xiang, C.; Yang, Z.; Lu, Z. Effect of cationization reagents on the matrix-assisted laser desorption/ionization time-of-flight mass spectrum of Chinese gallotannins. *J. Appl. Polym. Sci.* **2007**, *105*, 859−864.

(19) Farrukh, A.; Iqbal, A.; Zafar, M. Antioxidant and free radical scavenging properties of twelve traditionally used Indian medicinal plants. *Turk. J. Biol.* **2006**, *30*, 177−83.

(20) Xiang, P.; Lin, Y.; Lin, P.; Xiang, C. Effects of adduct ions on matrix-assisted laser desorption/ionization time of flight mass spectrometry of condensed tannins: a prerequisite knowledge. *Chin. J. Anal. Chem.* **2006**, *34*, 1019−1022.

(21) Benzie, I. F. F.; Strain, J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. *Anal. Biochem.* **1996**, *239*, 70−76.

(22) Brand-Williams, W.; Cuvelier, M.; Berset, C. Use of a free radical method to evaluate antioxidant activity. *LWT—Food Sci. Technol.* **1995**, *28*, 25−30.

(23) Fu, C.; Loo, A. E. K.; Chia, F. P. P.; Huang, D. Oligomeric proanthocyanidins from mangosteen pericarps. *J. Agric. Food Chem.* **2007**, *55*, 7689−7694.

(24) Zhang, L L.; Lin, Y M. Antioxidant tannins from *Syzygium cumini* fruit. *Afr. J. Biotechnol.* **2009**, *8*, 2301−2309.

(25) Guyot, S.; Marnet, N.; Laraba, D.; Sanoner, P.; Drilleau, J. F. Reversed-phase HPLC following thiolysis for quantitative estimation and characterization of the four main classes of phenolic compounds in different tissue zones of a French cider apple variety (*Malus domestica* var. Kermerrien). *J. Agric. Food Chem.* **1998**, *46*, 1698−1705.

(26) Guyot, S.; Marnet, N.; Drilleau, J. F. Thiolysis-HPLC characterization of apple procyanidins covering a large range of polymerization states. *J. Agric. Food Chem.* **2001**, *49*, 14−20.

(27) Meagher, L.; Lane, G.; Sivakumaran, S.; Tavendale, M.; Fraser, K. Characterization of condensed tannins from Lotus species by thiolytic degradation and electrospray mass spectrometry. *Anim. Feed. Sci. Tech.* **2004**, *117*, 151−163.

(28) Oo, C.; Pizzi, A.; Pasch, H.; Kassim, M. Study on the structure of mangrove polyflavonoid tannins with MALDI-TOF mass spectrometry. *J. Appl. Polym. Sci.* **2008**, *109*, 963−967.

(29) Wang, C. Y.; Ng, C. C.; Lin, H. T.; Shyu, Y. T. Free radical-scavenging and tyrosinase-inhibiting activities of extracts from sorghum distillery residue. *J. Biosci. Bioeng.* **2011**, *111*, 554−556.

(30) Wu, L.; Chen, Y.; Ho, J. A.; Yang, C. Inhibitory effect of red koji extracts on mushroom tyrosinase. *J. Agric. Food Chem.* **2003**, *51*, 4240−4246.

(31) Matoba, Y.; Kumagai, T.; Yamamoto, A.; Yoshitsu, H.; Sugiyama, M. Crystallographic evidence that the dinuclear copper center of tyrosinase is flexible during catalysis. *J. Biol. Chem.* **2006**, *281*, 8981−8990.

(32) Takagi, K.; Mitsunaga, T. Tyrosinase inhibitory activity of proanthocyanidins from woody plants. *J. Wood Sci.* **2003**, *49*, 461−465.

(33) Soares, J.; Dinis, T.; Cunha, A.; Almeida, L. Antioxidant activities of some extracts of *Thymus zygis*. *Free Radical Res.* **1997**, *26*, 469.

(34) Meir, S.; Kanner, J.; Akiri, B.; Philosoph-Hadas, S. Determination and involvement of aqueous reducing compounds in oxidative defense systems of various senescing leaves. *J. Agric. Food Chem.* **1995**, *43*, 1813−1819.

(35) Zhou, H. C.; Lin, Y. M.; Li, Y. Y.; Li, M.; Wei, S. D.; Chai, W. M.; Tam, N. F. Antioxidant properties of polymeric proanthocyanidins from fruit stones and pericarps of *Litchi chinensis* Sonn. *Food Res. Int.* **2011**, *44*, 613−620.
(36) Yokozawa, T.; Chen, C. P.; Dong, E.; Tanaka, T.; Nonaka, G. I.; Nishioka, I. Study on the inhibitory effect of tannins and flavonoids against the 1,1-diphenyl-2-picrylhydrazyl radical. *Biochem. Pharmacol.* **1998**, *56*, 213−222.