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Structural Composition and Free Radical Scavenging Activity of Proanthocyanidins Extracted from *Grevillea robusta*

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Abstract: The aim of this study was to investigate polymeric proanthocyanidin (PA) composition and free radical scavenging activity of leaf, stem bark and fine root of *Grevillea robusta*. The spectra obtained through MALDI-TOF MS analysis revealed that the examined PAs were built up a mixture of procyanidins and prodelphinidins. Acid-catalyzed cleavage of the PAs coupled with reversed-phase HPLC-ESI-MS showed that the main constituents of cleavage products were (epi)gallocatechin benzylthioether and (epi)catechin benzylthioether for leaf and stem bark, and was (epi)gallocatechin benzylthioether for fine root, respectively. The mean degrees of polymerization (mDP) of PAs of leaf, stem bark and fine root were 9.6, 19.0 and 10.1, respectively. The PAs extracted from leaf, stem bark and fine root exhibited higher antioxidant activity than those of ascorbic acid and synthetic antioxidant butylated hydroxyanisole (BHA), as measured by 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radical scavenging method.

Keywords: *Grevillea robusta*; proanthocyanidins; MALDI-TOF MS; thiolysis; ABTS.

1. Introduction

Grevillea robusta A. Cunn. (Proteaceae), widely known as “silky oak”, is native to eastern Australia [1]. Timbers from these trees are commercially valuable for making furniture [1]. Several phenolic glucosides, cytotoxic 5-alkylresorcinol metabolites, 5-alkylresorcinol glucosides derivatives have been isolated from *G. robusta* [2-5]. In addition, a methanol extract of its timber exhibit potent leishmanicidal activity [6]. *G. robusta* is rich in proanthocyanidins (PAs), but to the best of our knowledge the information regarding its concentration, structural composition and biological activity is limited. The unexplored PAs from this plant may be potential resources for novel bioactive compounds.

PAs also termed as condensed tannins, are a structurally complex subclass of polyphenolic compounds that are widely distributed in the plant kingdom [7]. PAs can be divided into different types depending on the substitution pattern of their monomeric flavan-3-ol units. The most widely

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distributed PAs in plants are propelargonidins, procyanidins and prodelphinidins, which consist of (epi)afzelechin, (epi)catechin and (epi)gallocatechin units, respectively (Figure 1). PAs have attracted considerable attention due essentially to their potential beneficial health effects, related to their protective action towards cardiovascular disease and the oxygen free radical scavenger capacity [8]. The physical, chemical, and biological activities of PAs depend largely on their chemical structure and particularly on their degree of polymerization [9, 10]. Due to the diversity and structural complexity of oligomeric and polymeric PAs, the analysis and characterization of PAs is a difficult task [11, 12]. PAs are considered to be a final frontier of flavonoid research [8].

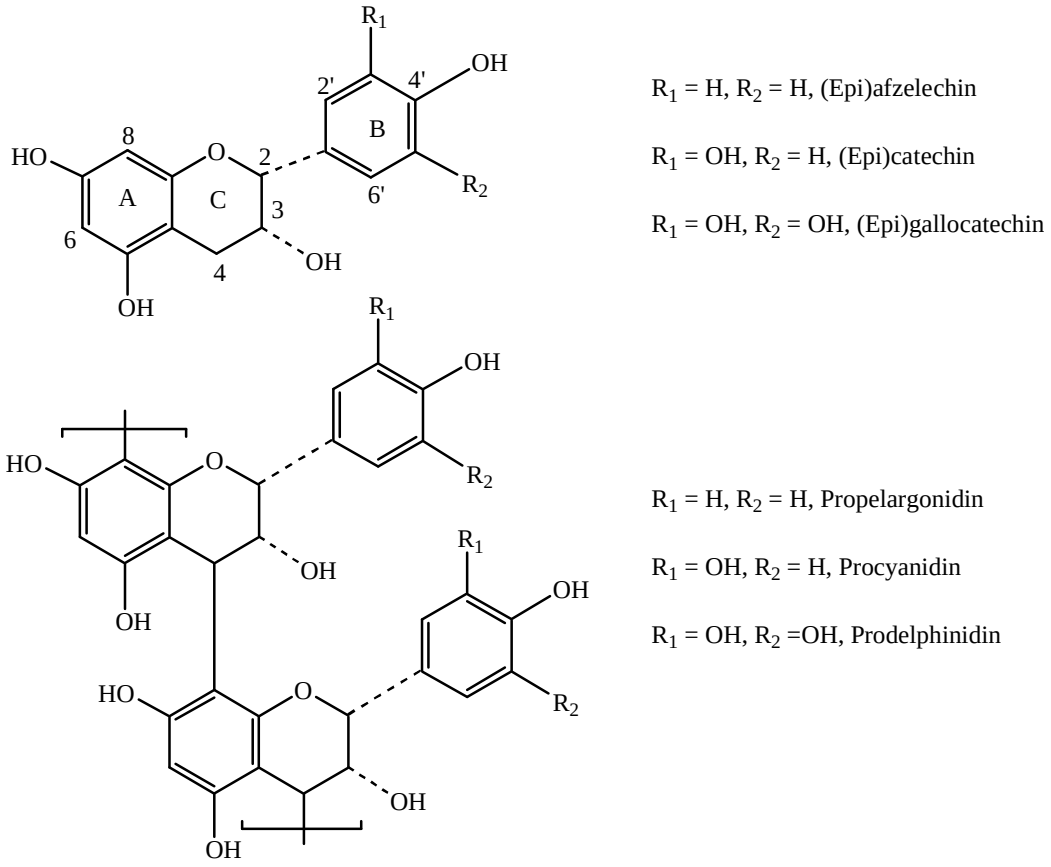

Figure 1. Chemical structure of flavan-3-ol monomer units and proanthocyanidin polymers.

In this study, concentrations of total phenolics and extractable condensed tannins in leaf, stem bark and fine root of *G. robusta* were determined. The structural compositions of their PAs were investigated by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and thiolytic degradation coupled with reversed-phase high performance liquid chromatography-electrospray ionization mass spectrometry (HPLC-ESI-MS) analysis. Furthermore, the ability of these PA extracts to scavenge 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) radicals was also evaluated.

2. Materials and Methods

2.1. Chemicals and Plant Materials

The solvents methanol, ethanol, acetone, hexane and dichloromethane were of analytical reagent (AR) purity grade. Trifluoroacetic acid (TFA) and acetonitrile used for the analysis were of HPLC grade. Folin-Ciocalteu reagent, gallic acid, 2,5-dihydroxybenzoic acid (DHB), cesium chloride, benzyl mercaptan, 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), butylated hydroxyanisole (BHA), and ascorbic acid were purchased from Sigma (St. Louis, MO, USA). Sephadex LH-20 was purchased from Amersham (USA) and HPLC standards were purchased from Sigma (St. Louis, MO, USA). The leaf, stem bark and fine root of *G. robusta* were collected at the campus of Xiamen University (Xiamen, P.R. China), freeze dried, ground finely, and stored at -20 °C until required.

2.2. Extraction and Purification of the Polymeric PAs

The finely ground leaf, stem bark and fine root powders (25 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 use a rotary evaporator at 38 °C. The remaining aqueous fraction (150 mL) was extracted thrice with hexane (3 × 150 mL) and then with dichloromethane (3 × 150 mL) in order to remove pigments, lipids, and other nonpolar materials. The remaining crude tannin fraction was chromatographed on a 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 polymeric PAs was freezed-dried and stored at -20 °C until analysis.

2.3. Determination of the Concentrations of Total Phenolics and Extractable Condensed Tannins

The established procedures were used [13]. The concentration of total phenolics was determined using the Folin-Ciocalteu method [14]. Briefly, 0.2 mL aliquot of extract was added to a test tube containing 0.3 mL of distilled H_2O. 0.5 mL of Folin-Ciocalteu reagent and 2.5 mL 20% Na_2CO_3 solution were added to the mixture and shaken. After incubation for 40 min at room temperature, the absorbance versus a blank was determined at 725 nm. The total phenolic concentrations of extracts were expressed as mg gallic acid equivalents/g dry weight (DW).

The extractable condensed tannin concentration was assayed by the butanol-HCl method [15], using the respective purified polymeric PAs as the standards. All samples were analyzed in three replications.

2.4. 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 (DHB, 10 mg/mL 30% acetone solution) was used as the matrix. The sample solutions (10 mg/mL 30% acetone solution) 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 (1.52 mg/mL) was mixed with the analyte-matrix solution (1:3, v/v) to promote the formation of a single type of ion adduct ($[M + Cs]^+$) [16].

2.5. Thiolysis of PAs with Benzyl Mercaptan

Thiolysis was carried out by a method based on that described by Gu et al. [17] with slight modifications. Briefly, the PAs extracted from leaf, stem bark and fine root of *G. robusta* (5 mg/mL in methanol, 50 µL) were placed in a vial and to this was added hydrochloric acid in methanol (3.3:96.7, v/v; 50 µL) and benzyl mercaptan in methanol (5:95, v/v; 100 µL). The solution was heated at 40 °C for 30 min, and cooled to room temperature. The thiolysis reaction medium (20 µL) filtrated through a membrane filter with an aperture size of 0.45 µm was analyzed by reversed-phase HPLC.

The high performance liquid chromatograph was an Agilent 1200 system (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 mm × 250 mm, 5 µm) (China). Two solvents, namely A = 0.5% (v/v) TFA in aqueous and B = CH_3CN, were used. The gradient condition was: 0-45 min, 12%-80% B (linear gradient); 45-50 min, 80%-12% B (linear gradient). The column temperature was 25 °C and the flow-rate was set at 1 mL/min. Detection was at a wavelength of 280 nm and the UV spectra were acquired between 200-600 nm. Degradation products were identified on chromatograms according to their relative retention times and LC/MS. The mean degree of polymerization (mDP) of the condensed tannins was calculated by comparing the peak areas, based on the following equation:

$$\text{mDP} = 1 + \frac{\text{area under the curve of benzyl thioether derivative of flavan-3-ol units}}{\text{area under the curve of flavan-3-ol units}}$$

2.6. Free Radical Scavenging Activity

The free radical scavenging activity of PAs of leaf, stem bark and fine root was determined according to the method described by Re et al. [18]. $ABTS^{\cdot+}$ radical cation was generated by reacting 7 mM ABTS and 2.45 mM potassium persulfate after incubation at room temperature in dark for 16 hr until reaching a stable oxidative state. On the day of analysis, the $ABTS^{\cdot+}$ solution was diluted with 80% ethanol to an absorbance of 0.700 ± 0.050 at a wavelength of 734 nm. 0.1 mL of the respective PAs (12.5, 25, 50, 100 and 200 µg/mL dissolved in 80% ethanol) was added to $ABTS^{\cdot+}$ solution (3.9 mL; absorbance of 0.700 ± 0.050) and mixed thoroughly. The reactive mixture was allowed to stand at room temperature for 6 min and the absorbance was immediately recorded at 734 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 $ABTS^{\cdot+}$ concentration by 50%, was calculated from the results and used for comparison. The capability to scavenge the $ABTS^{\cdot+}$ radical was calculated by the following equation:

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

Where A_1 = the absorbance of the control reaction; A_2 = the absorbance in the presence of the sample. BHA and ascorbic acid were used as standards.

2.7. Statistical Analysis

All data were expressed as means ± standard deviation of three independent determinations. 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 13.0 for windows.

3. Results and Discussion

3.1. Total Phenolic and Extractable Condensed Tannin Concentrations

Stem bark had the highest concentrations of total phenolics (125.06 ± 4.62 mg/g) and extractable condensed tannins (175.85 ± 9.27 mg/g) compared to those of leaf and fine root (Table 1). Plant phenolics constitute one of the major groups of compounds acting as primary antioxidants or free radical scavenging terminators [19]. The phenolic concentration may contribute directly to the antioxidative action [20-22]. The results suggested that phenolics were important compounds in leaf, stem bark and fine root of *G. robusta*, and some of their pharmacological effects could be attributed to the presence of these valuable constituents.

Table 1. Concentrations of total phenolics and extractable condensed tannins in leaf, stem bark and fine root of *G. robusta*.

Samples	Total phenolics (mg/g) [a]	Extractable condensed tannins (mg/g) [b]
Leaf	101.04 ± 3.55 b	82.97 ± 3.03 b
Stem bark	125.06 ± 4.62 a	175.85 ± 9.27 a
Fine root	93.77 ± 5.50 b	83.95 ± 3.05 b

[a] Using gallic acid as the standard; [b] Using respective purified PAs from leaf, stem bark and fine root as the standards. Different letters in the same column show significant differences from each other at $P < 0.05$ level.

3.2. MALDI-TOF MS Analysis

MALDI-TOF MS is very sensitive to molecular weight, and nowadays is considered a powerful method of choice for characterization of the synthetic and natural polymers such as PAs [23-25]. MALDI-TOF mass spectra of the PAs extracted from leaf, stem bark and fine root of *G. robusta*, recorded as CS^+ adducts in the positive ion reflectron mode, are shown in Figure 2. The displayed magnification demonstrated the good resolution of the spectra. Leaf PAs was characterized by mass spectrum with a series of peaks with distances of 288 Da (e.g., *m/z* 1015, 1303, 1591, 1879, etc.), corresponding to one catechin/epicatechin monomer unit. Another strongly repeated pattern within each main set of peaks was signals separated by Δ16 Da difference (Figure 2a, Table 2). These masses might be produced by prodelphinidin-type flavan-3-ol units, where the third hydroxyl group introduces difference of 16 Da [26]. The leaf PAs was built up of a mixture of procyanidin and prodelphinidin units.

In the case of the PAs from stem bark and fine root, the masses of the highest peaks among the polyflavonoid tannin polymers with identical degree of polymerization (DP) increased by Δ304 Da (e.g., *m/z* 1047, 1351, 1655, 1959, 2263, etc.), which corresponds to a mass difference of one gallocatechin/epigallocatechin (Figure 2b and c). Prolongation of the PAs in stem bark and fine root was due to the addition of gallocatechin/epigallocatechin monomers. 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 and fine root (Table 2). These masses could be produced by procyanidin-type flavan-3-ol units, which is lack of one hydroxyl group (16 Da) at the 5′ position of the B-ring compared to prodelphinidin-type flavan-3-ol units. Given the absolute masses corresponding to each peak, it was further suggested that PAs from stem bark and fine root contained prodelphinidins and procyanidins, with the prodelphinidins dominating.

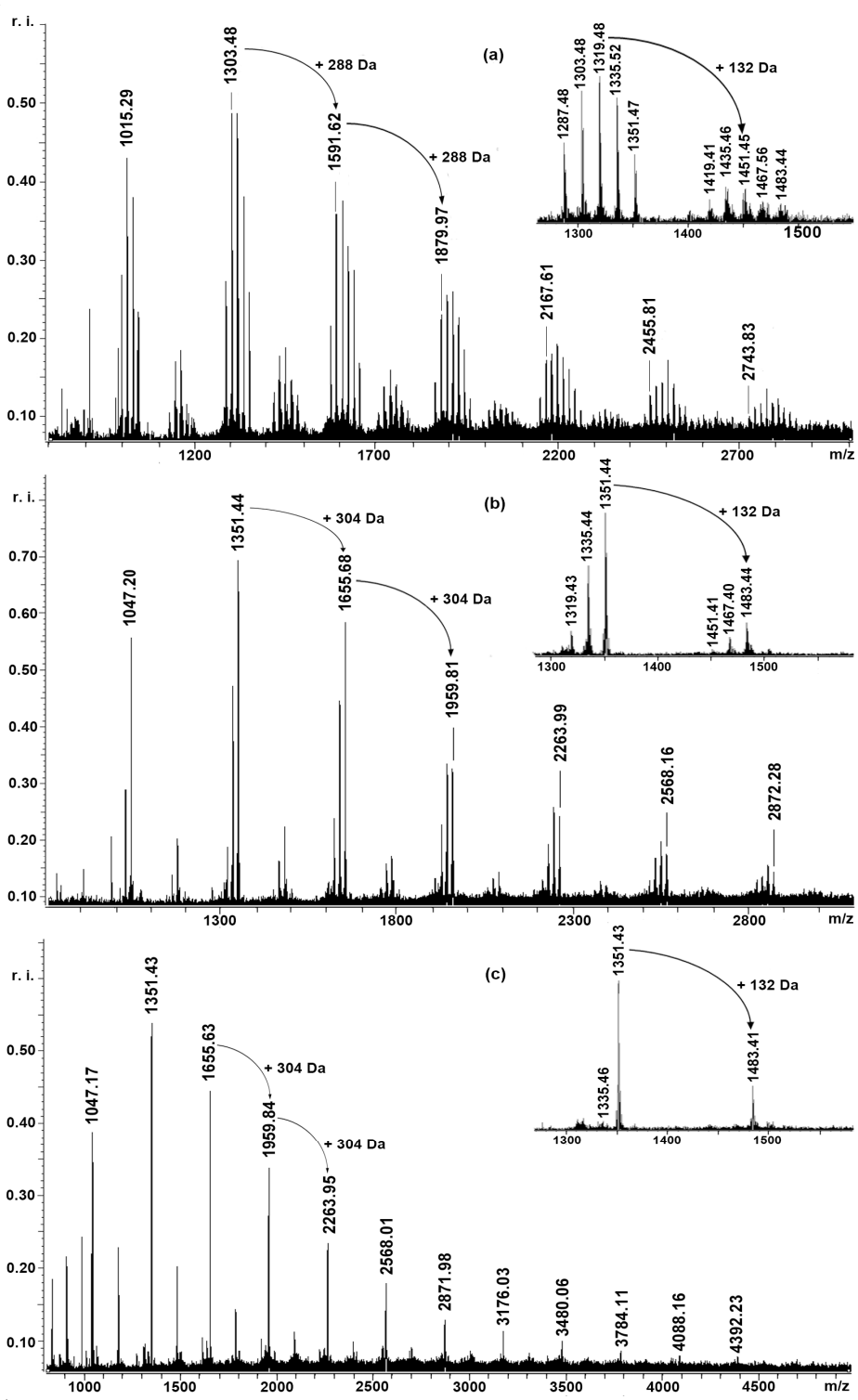

Figure 2. MALDI-TOF positive reflectron mode mass spectra of the PAs extracted from leaf (a), stem bark (b) and fine root (c) of *G. robusta*.

Table 2. MALDI-TOF mass spectra of polymeric PAs extracted from leaf, stem bark and fine root of *G. robusta*.

Polymer	Number of (epi)catechin units	Number of (epi)gallocatechin units	Calculated $[M + Cs]^+$	Observed $[M + Cs]^+$		
				Leaf	Stem bark	Fine root
Trimer	3	0	999	999.32	ND	ND
	2	1	1015	1015.29	ND	ND
	1	2	1031	1031.31	1031.19	1031.16
	0	3	1047	1047.30	1047.20	1047.17
Tetramer	4	0	1287	1287.48	ND	ND
	3	1	1303	1303.48	ND	ND
	2	2	1319	1319.48	1319.43	ND
	1	3	1335	1335.52	1335.44	1335.46
	0	4	1351	1351.47	1351.44	1351.43
Pentamer	5	0	1575	1575.63	ND	ND
	4	1	1591	1591.62	ND	ND
	3	2	1607	1607.64	1607.58	ND
	2	3	1623	1623.23	1623.70	ND
	1	4	1639	1639.67	1639.66	1639.52
	0	5	1655	1655.67	1655.68	1655.63
Hexamer	6	0	1863	1863.67	ND	ND
	5	1	1879	1879.97	ND	ND
	4	2	1895	1895.72	ND	ND
	3	3	1911	1911.71	1911.82	ND
	2	4	1927	1927.71	1927.86	ND
	1	5	1943	1943.78	1943.86	1943.72
	0	6	1959	1959.66	1959.81	1959.84
Heptamer	7	0	2151	2151.66	ND	ND
	6	1	2167	2167.61	ND	ND
	5	2	2183	2183.74	ND	ND
	4	3	2199	2199.80	ND	ND
	3	4	2215	2215.83	2215.91	ND
	2	5	2231	2231.78	2231.96	ND
	1	6	2247	2247.65	2248.04	2247.87
	0	7	2263	2263.72	2263.99	2263.95
Octamer	8	0	2439	2439.94	ND	ND
	7	1	2455	2455.81	ND	ND
	6	2	2471	2472.57	ND	ND
	5	3	2487	2488.71	ND	ND
	4	4	2503	2504.78	ND	ND
	3	5	2519	2520.93	2520.11	ND
	2	6	2535	2536.65	2536.16	ND
	1	7	2551	2552.78	2552.10	2552.06
	0	8	2567	2568.62	2568.16	2568.01
Nonamer	9	0	2727	2727.92	ND	ND
	8	1	2743	2743.83	ND	ND
	7	2	2759	2760.56	ND	ND
	6	3	2775	2776.68	ND	ND
	5	4	2791	2792.81	ND	ND
	4	5	2807	2808.76	ND	ND
	3	6	2823	2824.83	2824.21	ND
	2	7	2839	2840.67	2840.14	ND

	1	8	2855	2856.71	2856.29	ND
	0	9	2871	2872.81	2872.28	2871.98
Decamer	0	10	3175	ND	ND	3176.03
Undecamer	0	11	3479	ND	ND	3480.06
Dodecamer	0	12	3783	ND	ND	3784.11
Tridecamer	0	13	4087	ND	ND	4088.16
Tetradecamer	0	14	4391	ND	ND	4392.23

ND means no observed peaks corresponding to the calculated ones.

Furthermore, each peak was always followed by mass signals at a distance of 132 Da in the spectra of leaf, stem bark and fine root (Figure 2), which might be quasimolecular ions $[M + 2Cs - H]^+$ generated by simultaneous attachment of two Cs^+ and loss of a proton [27]. 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 does not occur between adjacent flavan-3-ol subunits for leaf, stem bark and fine root. To our knowledge, the chemical composition of PAs in different parts of *G. robusta* was well resolved by MALDI-TOF MS for the first time.

3.3. Thiolysis of PAs and Identification of the Cleavage Products

The thiolysis reaction has been used frequently for the characterization of PAs [17, 28]. The reaction occurs when PAs are heated in the presence of acid and benzyl mercaptan and corresponds to the acidic cleavage of the inter-flavan linkage of PAs. Terminal units are liberated as the free flavan-3-ols, whereas extension subunits are liberated as a flavanyl carbocation immediately converted into the corresponding benzylthioether adduct [29]. The chromatograms of thiolytic PAs extracted from leaf, stem bark, and fine root of *G. robusta* are shown in Figure 3.

Gallocatechin (peak 1), epigallocatechin (peak 2), catechin (peak 3) and epicatechin (peak 4) were found as terminal units, and in the negative ion mode the *m/z* values of the ions of these terminal units were 305, 305, 289 and 289, respectively. The extension units were identified to be (epi)gallocatechin benzylthioether (peak 5) and (epi)catechin benzylthioether (peak 6) as they exhibited $[M - H]^-$ ions at *m/z* 427 and 411, respectively. Due to lack of authentic standards, the stereochemistry of these compounds could not be confirmed based on mass spectra. The results after thiolysis of PAs showed that the main constituents of cleavage products were (epi)gallocatechin benzylthioether and (epi)catechin benzylthioether for leaf and stem bark, and was (epi)gallocatechin benzylthioether for fine root, respectively. PAs present in leaf, stem bark, and fine root consisted of a mixture of procyanidins and prodelphinidins, which was in agreement with the findings obtained by MALDI-TOF MS. In addition, the calculated mean degrees of polymerizations (mDP) of PAs were 9.6, 19.0 and 10.1 for leaf, stem bark and fine root, respectively.

3.4. Free Radical Scavenging Activity

ABTS assay is based on the inhibition of the absorbance of the radical action $ABTS^+$, which has a characteristic long wavelength absorption spectrum [30]. The ABTS assay has been widely used as a tool for assessing the total radical scavenging activity of pure substances and aqueous mixtures [31, 32]. The PAs extracted from leaf, stem bark, and fine root of *G. robusta*, and reference compounds (ascorbic acid and BHA) showed a concentration-dependent ABTS radical scavenging activity (Figure 4). At a concentration of 100 μg/mL, the scavenging activity of the leaf (70.72%) was significantly higher than those of stem bark (68.02%), fine root (63.26%), BHA (55.59%), and ascorbic acid (45.04%).

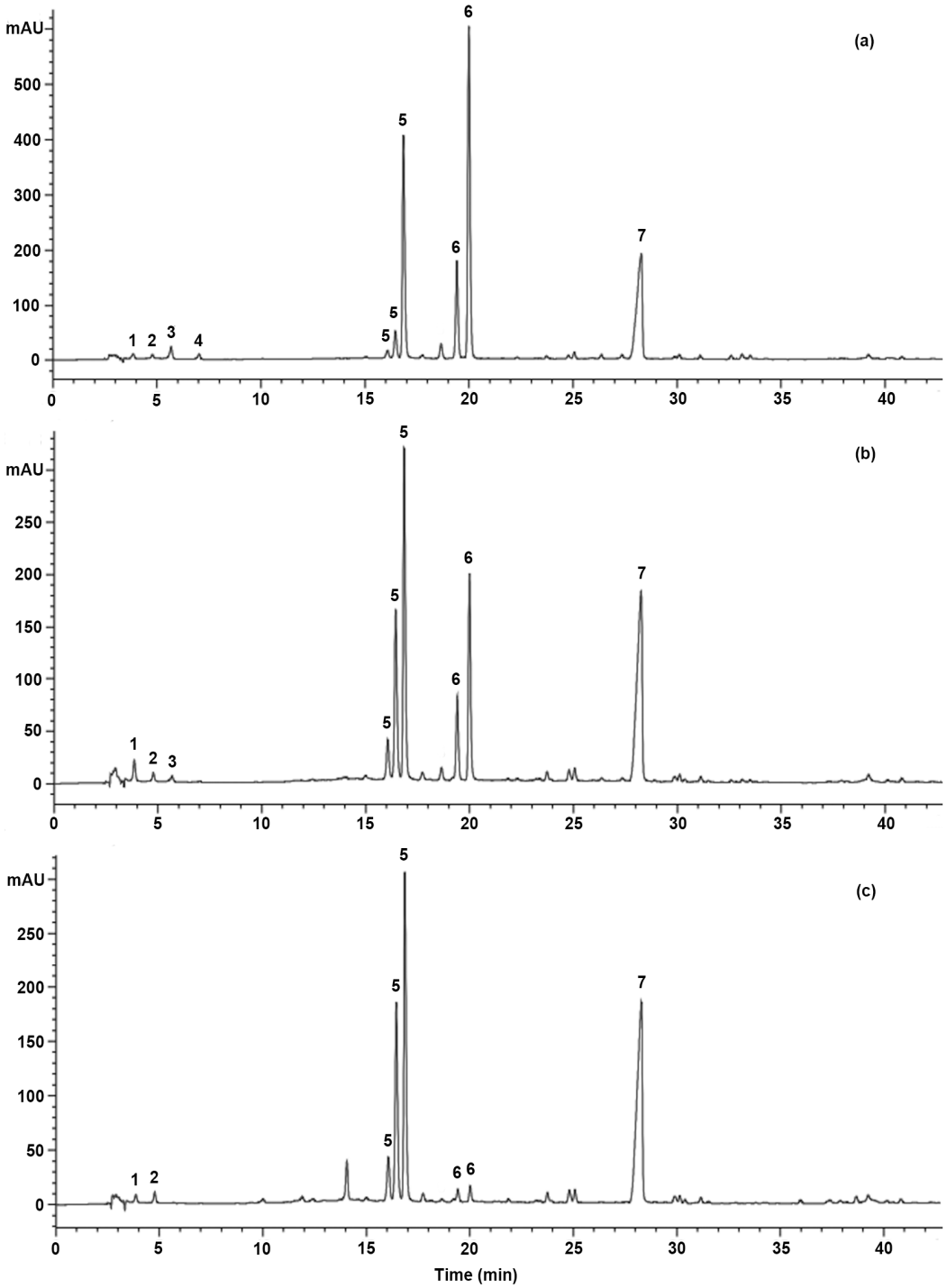

Figure 3. Reversed-phase HPLC chromatograms (detected at 280 nm) of the PAs extracted from leaf (a), stem bark (b) and fine root (c) of *G. robusta* after thiolysis. Peak numbering: 1, gallocatechin; 2, epigallocatechin; 3, catechin; 4, epicatechin; 5, (epi)gallocatechin benzylthioether; 6, (epi)catechin benzylthioether; 7, benzyl mercaptan.

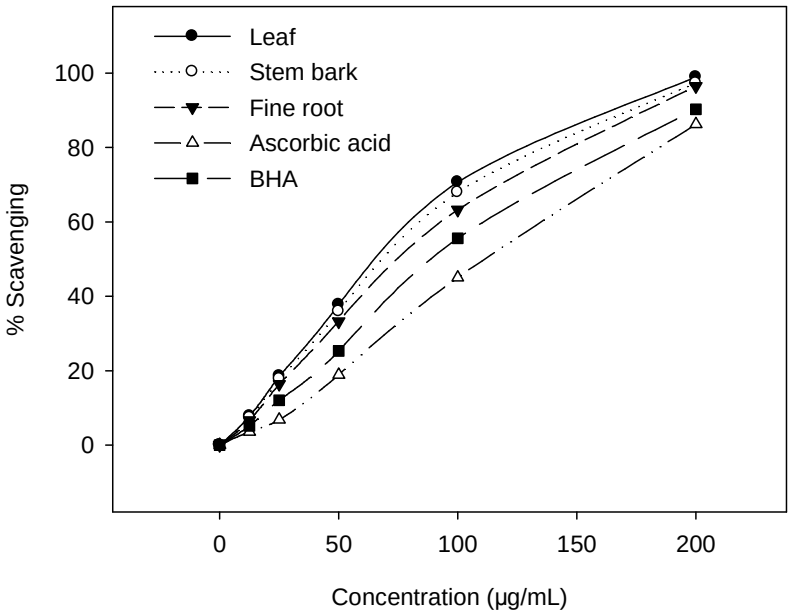

Figure 4. Percentage of free radical scavenging activity of PAs extracted from leaf, stem bark, and fine root of *G. robusta*, ascorbic acid and BHA.

The free radical scacenging activity can also be expressed by the antioxidant concentration required for a 50% ABTS reduction (IC_{50}). Lower IC_{50} value reflects better ABTS radical scavenging activity. By comparison of the corresponding IC_{50} values, the free radical scavenging activities of PAs of leaf (69.67 ± 0.74 µg/mL), stem bark (72.63 ± 0.77 µg/mL) and fine root (78.16 ± 0.24 µg/mL) were higher than those of BHA (91.00 ± 0.29 µg/mL) and ascorbic acid (117.10 ± 1.54 µg/mL), suggesting that these PA extracts had a significant free radical scavenging effect.

To summerize, we are the first to report the isolation and identification of PAs in the different parts of *G. robusta*. This study demonstrated that the PAs were built up a mixture of procyanidins and prodelphinidins, consisting mainly of (epi)gallocatechin and (epi)catechin units linked by B-type bonds for leaf and stem bark, and predominately of (epi)gallocatechin units linked by B-type linkages for fine root, respectively. The antioxidant activities of the PAs, investigated through reduction of the ABTS free radical, showed that the PAs extracted from leaf, stem bark and fine root exhibited a higher antioxidant power compared to those of ascorbic acid and BHA.

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