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Antioxidant activities of mangrove *Rhizophora apiculata* bark extracts

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Abstract

Depolymerisation of mangrove *Rhizophora apiculata* bark extracts in the presence of phloroglucinol nucleophiles in ethanol was carried out. The flavan-3-ols and their phloroglucinol adducts were separated using reversed phase liquid chromatography (HPLC). The HPLC analysis of mangrove *R. apiculata* showed that catechin was the most common component of the flavanoid monomers. The antioxidant activities of these mangrove tannins were evaluated and compared with several commercial tannins by using reducing power, DPPH and ABTS assays with butylated hydroxytoluene, BHT and L-(+)-ascorbic acid as standards. All tannins had reducing power and percentage scavenging activities similar to the (+)-catechin and L-(+)-ascorbic acid standards. In the DPPH assay, >90% of the maximum scavenging activity was attained at 30 μg ml^{-1}. Mangrove tannins had stronger antioxidant activity than the BHT standard in the DPPH assay. The results of the ABTS assay were correlated with the DPPH assay. Scavenging activity in the ABTS assay increased as the tannin concentration increased, up to a plateau at 50 μg ml^{-1}.

Keywords: *Rhizophora apiculata*; Mangrove tannins; Condensed tannins; Flavanoids; ABTS; DPPH

1. Introduction

Rhizophora species are medicinal plants of eastern and southeast Asia. The most common representatives are *Rhizophora mucronata*, *Rhizophora mangle* and *Rhizophora apiculata*. *R. mucronata* is an astringent, a folk remedy for angina and haemorrhage; its old leaves or roots are used for childbirth (Perry, 1980), while a polysaccharide extracted from the leaves of *R. apiculata* may have anti-HIV activity (Premanathan et al., 1999). *R. mangle* extract has a beneficial effect as an antiseptic and as an injury-healing promoter (Fernandez, Capdevila, Dalla, & Melchor, 2002), and the anti-hyperglycaemic effect of *R. mangle* has also been assessed in diabetic control (Alarcon-Aguilara et al., 1998). Tannins are known to exist in *R. apiculata* (Afidah et al., 2007; Sukardjo, 1987) and *R. mangle* (Wilson & Hatcher, 1988) but their chemical, biological and pharmacological properties have not yet been determined.

Condensed tannins are a group of phenolic polymers which are widely distributed in the plant kingdom particularly with woody growth habit. These compounds consist of flavan-3-ol units linked together through C4–C6 or C4–C8 bonds. These polyflavonoids contain phloroglucinol or resorcinol A-rings and catechol or pyrogallol B-rings. The C4–C6 interflavonoid linkage predominating in tannins is mostly composed of fisetinidin (resorcinol A-ring, catechol B-ring) and robinetidin (resorcinol A-ring, pyrogallol B-ring) repeating units. The C4–C8 interflavonoid linkage on the other hand predominating in tannins, is composed of catechin (phloroglucinol A-ring; catechol B-ring) and gallocatechin (phloroglucinol A-ring; pyrogallol B-ring) repeating units. When the polymeric tannins are composed of fisetinidin or robinetidin units the polymers are respectively called profisetinidin or probinetidin; when

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they are composed of catechin or gallocatechin the polymers are called procyanidin or prodelphinidin, respectively (Porter, 1992).

Mangrove (*R. apiculata*) tannins consist primarily of condensed tannins or proanthocyanidins (Afidah Abdul Rahim, 2005). Depolymerisation reactions in the presence of nucleophiles are often used in the structural analysis of condensed tannins. The nucleophile forms an adduct with the extender units in the polymer, which are purified or analysed by chromatography. Their structure, once established, is then used to determine the nature of the monomer units in the polymer. Numerous nucleophiles have been used in such analyses, including benzylthiol (Foo & Karchesy, 1991; Guyot, Doco, Jean-Marc, Moutounet, & Jean-Francois, 1997; Matthews et al., 1997) or phloroglucinol (Achmadi, Shahbirin, Choong, & Hemingway, 1994; Brandon, Foo, Porter, & Meredith, 1989; Koupai-Abrayani, McCallum, & Bohm, 1992). Depolymerisation in the presence of a nucleophile has several advantages and when combined with quantitative analysis of the products by chromatography, it may offer information on the nature of the condensed tannins. In contrast to colorimetric methods, interference by other plant constituents is avoided through the unambiguous identification of the proanthocyanidin-derived products. In comparison with butanol/HCl depolymerisation, the reaction preserves the stereochemistry of the C2–C3 positions of the polymer units. The use of a nucleophile also limits the side reactions that could reduce recovery yields of the products (Matthews et al., 1997).

Tannins are antioxidants often characterised by reducing power (Gulcin, Oktay, Kirecci, & Kufrevioglu, 2003; Mi-Yea, Tae-Hun, & Nak-ju, 2003) and scavenging activities (Minussi et al., 2003; Peng & Jay-Allemand, 1999). The antioxidant capabilities of tannins depend on (1) the extent of their colloidal state, (2) the ease of interflavonoid bond cleavage or its stereochemical structure, (3) the ease of pyran ring (C-ring) opening, and (4) the relative numbers of –OH groups on A and B rings (Noferi, Masson, Merlin, Pizzi, & Deglise, 1997). Compounds with a trihydroxyl structure in the B-ring have the greatest antioxidant activity (Rice-Evans, Miller, & Paganga, 1996; Salah et al., 1995).

In this study, the characterisation of phenolic compounds from mangrove tannins using reversed phase high performance liquid chromatography (HPLC) is discussed. The reducing power of mangrove tannins and the scavenging activities of mangrove tannins using ABTS and DPPH assays were undertaken and compared with several commercial tannins.

2. Materials and methods

2.1. Tannin isolation

Mangrove (*R. apiculata*) bark samples from 15 year old trees were obtained from the Matang Forest, Malaysia. The barks were dried and ground to ~250 mesh followed by further drying, until a constant weight was obtained. Tannins from mangrove barks were extracted by total immersion of finely ground barks (50 g) in 70% aqueous acetone (500 ml) for 72 h at room temperature (30–31 °C) according to the procedure of Ohara and Ohmura (1998), with a slight modification. The acetone was removed under pressure and the resulting aqueous fraction freezed–dried. The 70% aqueous acetone extract of the mangrove barks yielded 27–29% (by weight) dark brown tannin powder. The tannin powder (1.5 g) was defatted with hexane (50 ml), followed by extraction with ethyl acetate (50 ml). A fraction of the aqueous phase (1.0 g) was dissolved in methanol/water 1:1 and loaded onto a Sephadex LH 20 column; elution with acetone/water 1:1 produced condensed tannins. Commercial mimosa and quebracho tannin powder from bark extracts were obtained from Silvachimica, Italy. AR grade solvents were used for the extraction.

2.2. Identification of condensed tannins

2.2.1. Phloroglucinol degradation

Method 1. Condensed tannins (25 mg), phloroglucinol (Fluka) (8 mg) and dioxane: 0.2 M aqueous HCl, (1:1; 0.5 ml) were added to a tube that was sealed and heated at 80 °C for 20 min. An aliquot was then diluted with the same volume of HPLC grade methanol:water (1:1). (Matthews et al., 1997).

Method 2. Using the modified method of Foo and Karchesy (1991), condensed tannin (10 mg) was dissolved in 1.5 ml of phloroglucinol solution (5 mg ml^{-1} phloroglucinol in ethanol) and allowed to react at room temperature (30–31 °C) overnight. The solvent was then evaporated under nitrogen, and the residue dissolved in 0.5 ml distilled water. This solution was extracted three times with ethyl acetate (1.5 ml per extraction). The three ethyl acetate fractions were combined and evaporated under nitrogen. The residue was dissolved in 1.0 ml of 70% aqueous HPLC grade methanol. The procedure was repeated using flavanoid standards (+)-catechin hydrate, (−)-epicatechin (Fluka), (−)-catechin, (−)-gallocatechin, (−)-epigallocatechin, (−)-catechin gallate, (−)-epicatechin gallate, (−)-gallocatechin gallate, (−)-epigallocatechin gallate (Sigma) and methanol–water fraction samples.

2.2.2. HPLC analysis

Degradation products were analysed on a Shimadzu AD-VP HPLC system, with a Crestpak C18S column (4.6 mm I.D. × 150 mm l), at a flow-rate of 1.0 ml min^{-1} and detected at 280 nm with a UV detector. All solvents were filtered with a 0.45 μm Millipore filter paper and degassed ultrasonically for 15–20 min. From each sample 20 μl were injected. The elution conditions for method 1 were: Solvent A, water:phosphoric acid 999:1; solvent B, methanol; linear gradient 0–90% B in 30 min.

Two elution conditions were used for method 2: (i) solvent A, 1% aqueous acetic acid; solvent B, methanol;

0–30 min, 0–15% B in A (linear gradient); 30–45 min 15–60% B in A (linear gradient); 45–50 min, 60% B in A (isocratic) (Koupai-Abrayani et al., 1992). (ii) Solvent A, 1% aqueous acetic acid; solvent B, methanol: solvent A, 60:40 (v/v); $t = 0$, 100% solvent A; $t = 60$ min, 40% solvent A, 60% solvent B; $t = 65$ min, 100% solvent B (modified from Koupai-Abrayani et al., 1992).

All flavanoid standards (1.0 mg ml^{-1}) were eluted at a flow-rate of 0.5 ml min^{-1} and 1.0 ml min^{-1} using elution condition (i). The phloroglucinol and flavanoid standards (1 mg ml^{-1}), prepared in 70% aqueous HPLC grade methanol, were used to identify the peaks. Quantification of monomers was carried out on six degraded samples of condensed tannins by injecting duplicate samples. Calibration curves for (+)-catechin, (−)-epicatechin, (−)-epigallocatechin and (−)-epicatechin gallate were made from standard solutions of various concentrations.

2.3. Antioxidant activities

2.3.1. Reducing power

The reducing power of samples was determined as previously described (Gulcin et al., 2003) with slight modifications. Standard L-(+)-ascorbic acid solutions of concentrations 20 μg ml^{-1} to 0.1 mg ml^{-1} were prepared. To 1 ml of the standard solution, 2.5 ml of 0.2 M phosphate buffer (pH 6.6) and 2.5 ml of 1% (w/v) potassium fericyanide solution were added. The mixture was incubated at 50 °C for 20 min, after which 2.5 ml 10% (w/v) trichloroacetic acid was added. The resultant mixture was centrifuged for 20 min at 2500 rpm. The upper layer (2.5 ml) was discarded and 2.5 ml distilled water and 0.5 ml of 0.1% (w/v) ferric chloride hexahydrate solution were added. Absorbance was measured at 700 nm. The procedure was repeated for (+)-catechin hydrate standard solutions, mangrove tannins, mimosa tannins, quebracho tannins and chestnut tannins. In this procedure, $Fe(CN)_6^{3-}$ ions are reduced to $Fe(CN)_6^{4-}$ ions when an antioxidant is added. The ferric chloride solution reacts with these ions to form a $Fe_4[Fe(CN)_6]_3$ complex. The presence of Fe^{2+} in the complex is detected at 700 nm and an increased absorbance indicates increased reducing power.

2.3.2. DPPH free radical-scavenging activity

The free radical-scavenging capability was determined as described previously (Gulcin et al., 2003) with slight modifications. 1,1-Diphenyl-2-picrylhydrazil (DPPH) radical solution was prepared by dissolving an appropriate amount of DPPH in methanol to a concentration of 1 mM. To 4 ml standard solutions of L(+)-ascorbic acid ranging between 5 μg ml^{-1} to 0.1 mg ml^{-1}, 0.5 ml of DPPH solution was added. The absorbance of the mixture was measured after 30 min at 517 nm. The procedure was repeated with standard solutions of butylated hydroxytoluene, (BHT) and (+)-catechin hydrate, mangrove tannins, mimosa tannins, quebracho tannins and chestnut tannins. A blank sample (without antioxidants), containing the same amount of methanol and the DPPH radical was prepared and measured daily. The DPPH radical standard solution was prepared fresh daily, covered with aluminium foil and stored at 4 °C between measurements. The scavenging ability of antioxidants was calculated as:

$$\text{DPPH scavenging activity}(\%) = [(A_0 - A/A_0)] \times 100$$

where A_0 is the absorbance of the control reaction and A is the absorbance in the presence of samples.

2.3.3. ABTS free radical-scavenging activity

2,2′-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) scavenging activity was measured, as previously described by Goh, Barlow, and Yong (2003) with slight modifications. ABTS diammonium salt (75 mM) and potassium persulfate (1.225 mM) were mixed overnight. The mixture was diluted 10-fold with 99.5% ethanol before use. To 1 ml L-(+)-ascorbic acid standards (5 μg ml^{-1} to 0.1 mg ml^{-1}), 3 ml of the diluted ABTS radical was added. The absorbance of the resultant mixture was measured after 60 min at 414 nm. The procedure was repeated with (+)-catechin hydrate standard solution, followed by mangrove tannins, mimosa tannins, quebracho tannins and chestnut tannins. A blank sample (without antioxidants), containing the same amount of ethanol and ABTS radical was prepared and measured daily. The scavenging ability of antioxidants was calculated as:

$$\text{ABTS scavenging activity}(\%) = [(A_0 - A/A_0)] \times 100$$

where A_0 is the absorbance of the control reaction and A is the absorbance in the presence of samples.

3. Results and discussion

3.1. Identification of condensed tannins

In an HPLC chromatogram (Fig. 1) of a mixture of all the standards eluted at a flow-rate of 1 ml min^{-1} and 0.5 ml min^{-1} of elution condition (i), peaks 4 and 5 were very close. Hence, these peaks were identified by spiking the condensed tannin sample with the appropriate standard.

The elution order of some flavanoids is influenced by the substitution pattern of the B-ring (Fig. 2) and the stereochemistry of the components (Koupai-Abrayani et al., 1992). Thus reported elution order is: (+)-gallocatechin, (−)-epigallocatechin, (+)-catechin, (−)-epicatechin. In this study the elution order was:

(−)-gallocatechin, (+)-catechin/(−)-catechin, (−)-epigallocatechin, (−)-epigallocatechin gallate, (−)-epicatechin, (−)-gallocatechin gallate, (−)-epicatechin gallate, (−)-catechin gallate, which is similar to that reported by Pelillo et al. (2002) for green tea catechins. Unlike either Koupai-Abrayani et al., 1992 or Pelillo et al., 2002, it was found that (+)-catechin eluted before (−)-epigallocatechin.

The stereochemical position of the OH group at C-3 of the C-ring (Fig. 2) altered the elution order, as evidenced by the shorter retention time of (+)-catechin than (−)

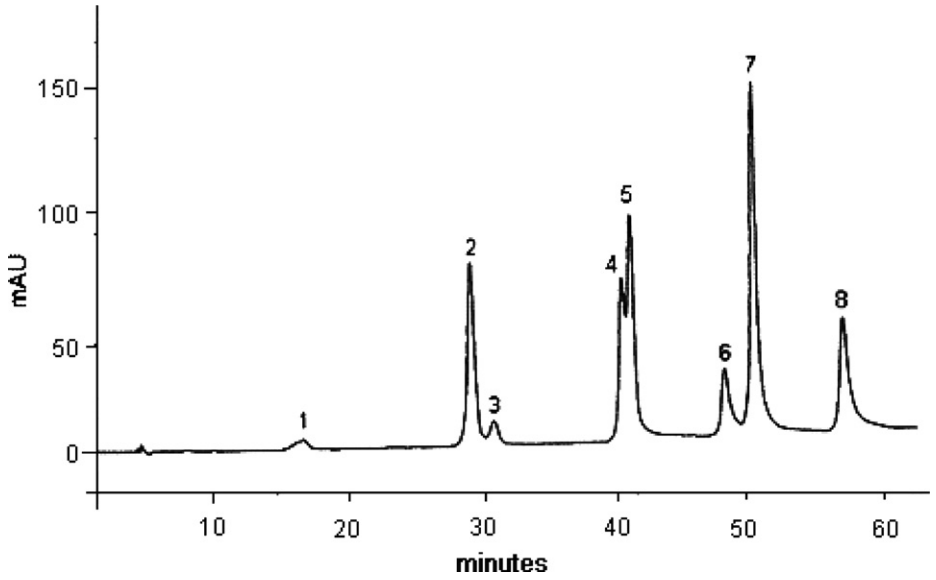

Fig. 1. HPLC chromatogram of flavan-3-ol standards eluted at a flow-rate of 0.5 ml min^{-1} of elution condition (i). Peaks: 1 = (−)-gallocatechin, 2 = (+)-catechin/(−)-catechin, 3 = (−)-epigallocatechin, 4 = (−)-epigallocatechin gallate, 5 = (−)-epicatechin, 6 = (−)-gallocatechin gallate, 7 = (−)-epicatechin gallate, 8 = (−)-catechin gallate.

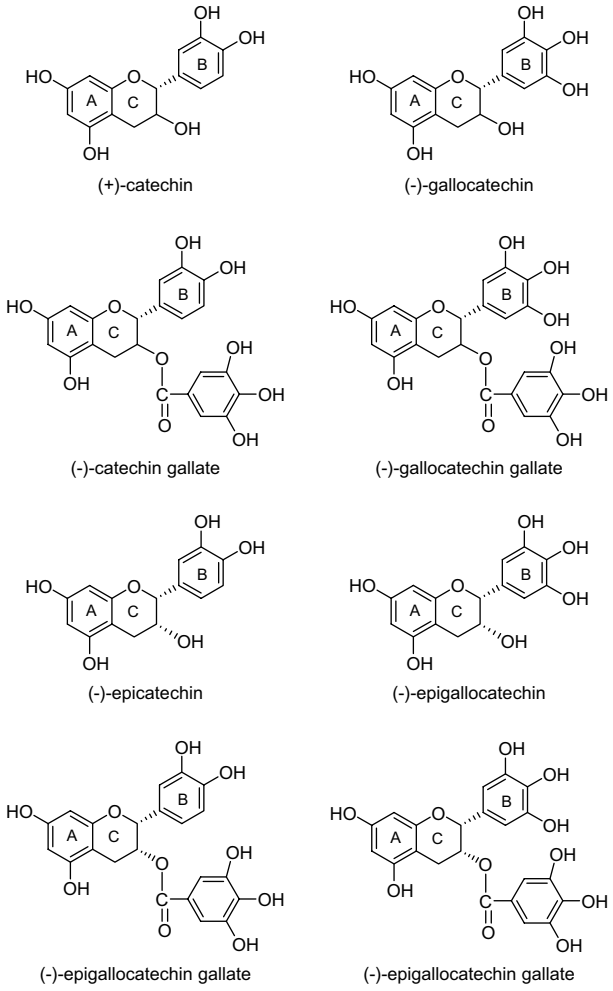

Fig. 2. Chemical structure of flavanoid monomer standards.

-epicatechin, and of (−)-gallocatechin than (−)-epigallocatechin. Introduction of a gallate group onto the B-ring however resulted in the elution of (−)-epicatechin gallate, followed by (−)-catechin gallate. The retention times of compounds with three hydroxyl groups on the B-ring also were shorter than those with two hydroxyl groups. The elution conditions used could not separate peaks of optically different isomers e.g. the peaks for (+)-catechin and (−)-catechin overlap (Fig. 1).

During acid depolymerisation in the presence of phloroglucinol, the interflavan bonds are protonated and broken, leaving the terminal unit intact and the extender unit as a carbocation (Schofield, Mbugua, & Pell, 2001). The carbocation is then captured either α or β to the C-ring, producing a monomer phloroglucinol adduct. The depolymerisation products were then separated on reversed-phase HPLC.

When the condensed tannin was subjected to acid degradation in the presence of phloroglucinol and dioxane, two main narrow peaks and one large broad peak were observed (Fig. 3). The two narrow peaks were identified as phloroglucinol and phloroglucinol adduct (Matthews et al., 1997). The use of phloroglucinol in dioxane in the depolymerisation reaction was thus unfavourable. In addition to producing a large unresolvable peak associated with the fraction resistant to the depolymerisation process (Matthews et al., 1997) dioxane is toxic. Hence, the use of phloroglucinol in ethanol was preferred. When the condensed tannin was degraded in the presence of phloroglucinol and ethanol (method 2) using elution condition (i), five peaks followed by a large unresolvable peak were observed (Fig. 4). However when elution condition (ii) was adopted, well resolved peaks were observed (Fig. 5). Thus this elution condition was used for subsequent evaluations.

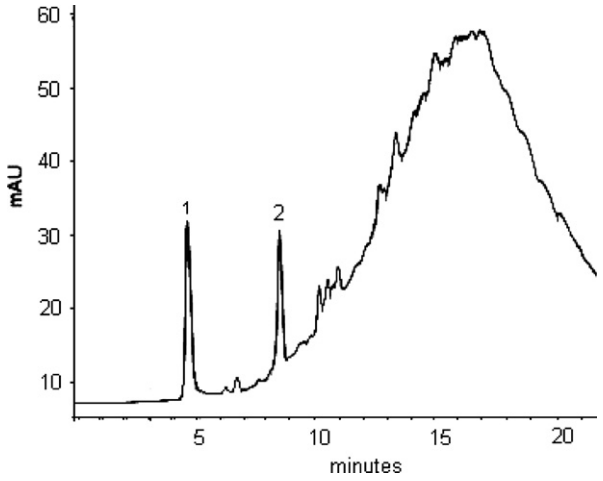

Fig. 3. HPLC chromatogram of condensed tannins, degraded in the presence of acidic dioxane and phloroglucinol.

Spiking the samples with flavanoid standards ensured the correct identification of the individual monomers. Four monomers, namely catechin, epigallocatechin, epicatechin and epicatechin gallate as terminal units were identified from the condensed tannins.

Due to the commercial unavailability of phloroglucinol adducts, these adducts were synthesised. When the sample was spiked with the synthesised catechin adduct, a pair of adduct peaks appeared (Fig. 6), indicating the absence of the catechin monomer as the extender unit. Similarly for all of the other adducts synthesised, additional peaks were again observed, indicating the absence of all other monomer adducts.

3.2. Quantification of condensed tannins via HPLC

The total content of condensed tannins from mangrove barks using the vanillin assay was $36 \pm 1.2\%$ in (+)-catechin equivalent (Afidah Abdul Rahim, 2005). Among the weaknesses of the vanillin assay is a lack of specificity for condensed tannins. Any appropriately substituted monomeric flavanol reacts in the assay. The major problem with the vanillin assay is the variable reactivity of the subunits of the tannin polymer. The structural variations in proanthocyanidins also affect the colour yield with vanillin (Schofield et al., 2001). Thus the HPLC analysis developed is an alternative method for quantifying condensed tannins. The increasing % (wt) of flavanoid monomers was: epigallocatechin < epicatechin gallate < epicatechin < catechin (Table 1). Thus catechin was used in antioxidant studies to represent the mangrove tannin monomers and as a reference standard. The results from HPLC analyses and vanillin assays were not correlated (Afidah Abdul Rahim, 2005), with the condensed tannins measured by the vanillin assay much higher than the amount determined by HPLC. This discrepancy could be explained if the vanillin reacts with all units of the polymer, but only the terminal flavanoid monomers were measured in the HPLC analysis. Similar observations have been reported previously (Matthews et al., 1997) for low condensed tannins content and later supported by Ferreira and Nogueira (2000), in a study of the vanillin-condensed tannin reaction using flow injection spectrophotometry.

3.3. Antioxidant property

3.3.1. Reducing power of tannins

The reducing power of mangrove tannins was comparable with that of commercial tannins investigated (Fig. 7). The reducing power of mangrove tannins increased slightly as the concentration increased from 20 μg ml^{-1} to 60 μg ml^{-1}, after which it reached a plateau. Both mimosa and quebracho tannins behaved similarly to the mangrove tannins. All tannins had almost the same reducing power at all the concentrations investigated. According to this assay the L-(+)-ascorbic acid standard showed a greater reducing

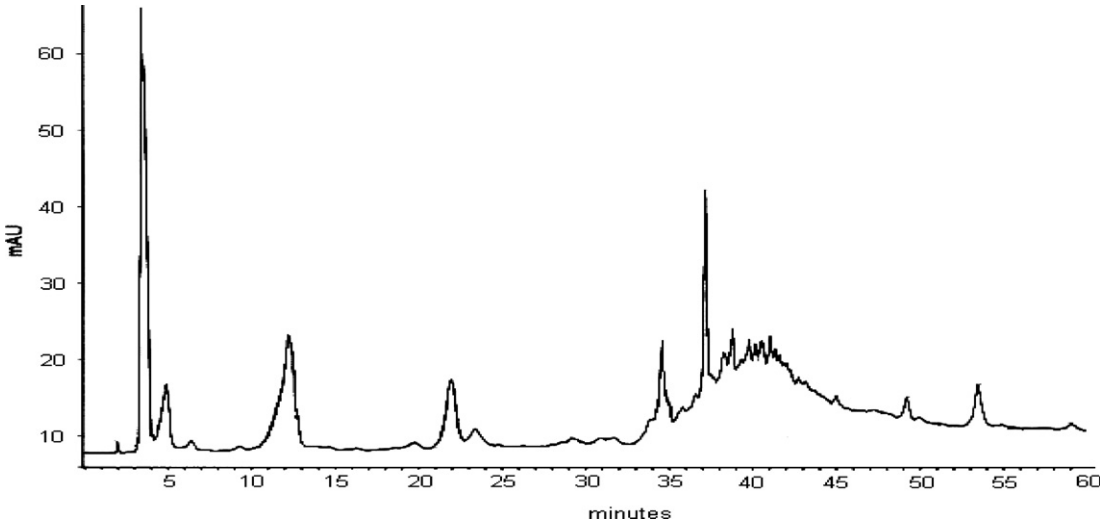

Fig. 4. HPLC chromatogram of condensed tannins, degraded in the presence of acidic ethanol and phloroglucinol, using elution condition (i).

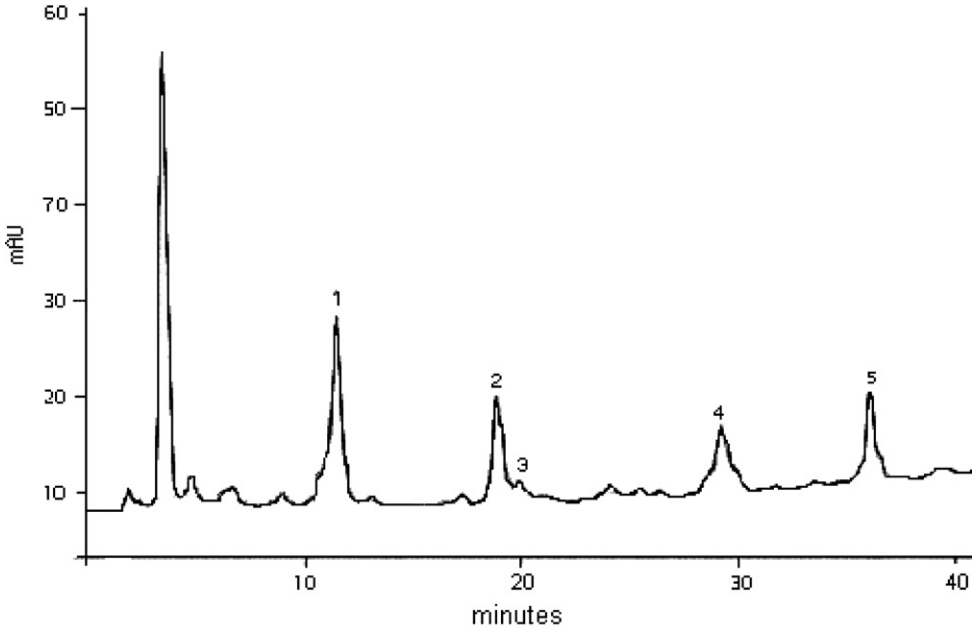

Fig. 5. HPLC chromatogram of condensed tannins degraded in the presence of acidic ethanol and phloroglucinol, using elution condition (ii). Peaks: 1 = phloroglucinol adduct, 2 = catechin, 3 = epigallocatechin, 4 = epicatechin, 5 = epicatechin gallate.

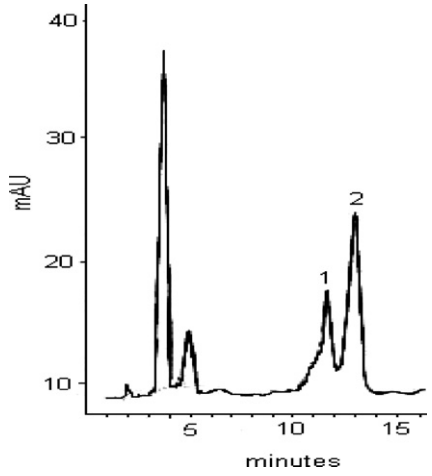

Fig. 6. HPLC chromatogram of condensed tannins when spiked with the synthesised adducts of (a) (+)-catechin. Peaks: 1 = phloroglucinol adduct of condensed sample, 2 = corresponding synthesised catechin adducts.

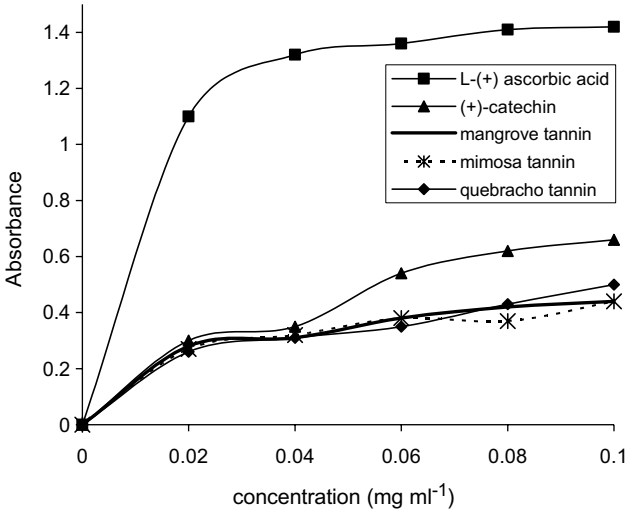

Fig. 7. The reducing power of tannins as compared to L-(+)-ascorbic and (+)-catechin standards.

Table 1
Quantification of flavanoid monomers as determined by HPLC

Monomers	Content (mg)	Content (%w/w)
(+)-catechin	4.6×10^{-2} to 6.8×10^{-2}	0.55 ± 0.09
(−)-epigallocatechin	5.0×10^{-3} to 1.0×10^{-2}	$(7.50 \pm 1.70) \times 10^{-2}$
(−)-epicatechin	4.0×10^{-2} to 4.2×10^{-2}	0.41 ± 0.01
(−)-epicatechin gallate	2.0×10^{-2} to 2.4×10^{-2}	0.22 ± 0.02

power than the (+)-catechin standard, with an optimum concentration of 40 μg ml^{-1}. The reducing power of mangrove tannin is far below that of the L-(+)-ascorbic acid standard and is comparable with that of (+)-catechin at the lower concentrations (20 μg ml^{-1} and 40 μg ml^{-1}). This result is not unexpected since the HPLC analysis found that catechin was common in mangrove tannins.

3.3.2. DPPH and ABTS free radical-scavenging activity

Both the mangrove tannins and other tannins evaluated had substantial DPPH scavenging activity (Fig. 8). Scavenging activity increased as the tannin concentration increased, with a maximum scavenging activity of >90% at 30 μg ml^{-1}. At 10 μg ml^{-1}, the scavenging activity was between 40% and 60% and the order of scavenging activity of tannins was: quebracho < mangrove < mimosa tannins. Scavenging activity increased by almost 50% for mangrove tannins when the concentration increased from 10 μg ml^{-1}

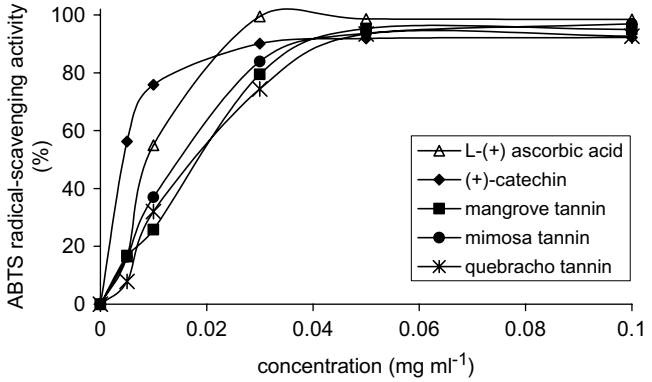

Fig. 8. Free radical-scavenging activities of tannins, measured using DPPH assay. L-(+)-ascorbic acid, BHT and (+)-catechin were used as reference compounds.

to 30 μg ml^{-1}. All the L-(+)-ascorbic acid, (+)-catechin and BHT standards used in this study gave the same percentage scavenging activity from 50 μg ml^{-1} onwards although they exhibited different optimum concentrations of 10 μg ml^{-1}, 30 μg ml^{-1} and 50 μg ml^{-1}, respectively. The scavenging ability of all tannins was comparable to that of (+)-catechin and L-(+)-ascorbic acid antioxidant standards and exceeded the DPPH radical-scavenging ability of the synthetic antioxidant BHT from 5 μg ml^{-1} to 50 μg ml^{-1}.

Similar scavenging activity patterns were seen in the ABTS assay (Fig. 9). Scavenging activity increased as the tannin concentration increased. At 30 μg ml^{-1}, the order of scavenging activity of tannins was: quebracho < mangrove < mimosa tannins. A plateau was reached at 50 μg ml^{-1} with a scavenging activity of >92% for all tannins. At the same dose all tannins had essentially the same percentage scavenging activity as the (+)-catechin and L-(+)-ascorbic acid antioxidant standards, indicating that all of the tannins, and particularly mangrove tannin are potent antioxidants.

Mimosa and quebracho tannins are condensed tannins and, according to a comparative ^{13}C NMR study of polyflavonoids, mimosa and quebracho tannins were found to be predominantly profisetinidin/prorobinetidin-type tannins. However the study also showed that mimosa tannins had a lower degree of polymerisation, lower number of C4–C8 interflavanoid linkages, lower number of catechin/gallocatechin units and a higher proportion of pyrogallol B-rings than quebracho tannins (Pizzi & Stephanou, 1993). Nevertheless all of the tannins examined had similar antioxidant activities. In view of the structurally similar properties and antioxidant behaviour of catechin and tannins, it is suggested that catechin is an appropriate standard for future tannin antioxidant analyses.

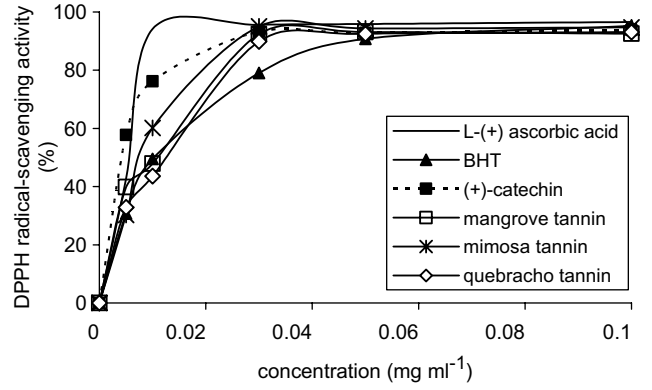

Fig. 9. Free radical-scavenging activities of tannins, measured using ABTS assay. L-(+)-ascorbic acid and (+)-catechin were used as reference compounds.

4. Conclusion

HPLC analyses of condensed tannins from the mangrove *R. apiculata*, following depolymerisation in phloroglucinol and ethanol, identified four terminal units, namely catechin, epicatechin, epigallocatechin and epicatechin gallate and one extender unit. A rapid reversed-phase HPLC method developed for the quantification of condensed tannins identified that catechin was the most predominant constituent of mangrove tannins. The mangrove tannins have substantial reducing power, DPPH as well as ABTS free radical-scavenging abilities, that are comparable to the synthetic standards and other commercial tannins evaluated. These results suggest that further studies of the use of mangrove tannins as new sources of medicinal plants are warranted.

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