

**Characterization of phenolic acids, flavonoids, proanthocyanidins and antioxidant activity of water extracts from seed coats of marama bean [*Tylosema esculentum*] - An underutilized food legume**

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## Summary

Freeze dried aqueous phenolic extracts with possible application as natural antioxidant functional food ingredients were prepared from marama bean seed coats by extracting with water. Phenolic acids, flavonoids and proanthocyanidins in the extracts were characterized by HPLC/MS. The major flavonoids were methyl (epi)afzelechin-3-O-gallate (40%), methyl (epi)catechin-3-O-gallate (28%) and the major phenolic acid was gallic acid (10%). Proanthocyanidins in the extracts were predominantly prodelphinidins composed of epicatechin-3-O-gallate and epigallocatechin present as major terminal and extension units and epigallocatechin-3-O-gallate and epicatechin present as minor extension unit constituents. The polymer structure was found to be unique compared to other legumes because of the high percentage of galloylated units. Extracts showed a high DPPH free radical scavenging activity (707  $\mu\text{mol TE g}^{-1}$ ), protective effect against AAPH-induced human red blood cell hemolysis and

copper-catalyzed human LDL oxidation suggesting that the extracts may have potential health benefits.

### **Keywords**

Marama bean; phenolics, LDL oxidation, antioxidant activity, proanthocyanidin, prodelphinidin, HPLC/MS; thiolysis.

### **Introduction**

The marama bean [*Tylosema esculentum* (Burchell. A. Shreiber)] is a long lived perennial tuberous legume plant growing wild in the arid regions of Southern Africa, in Botswana, Namibia and South Africa but also occurs in Angola, Zambia and Mozambique (Holse *et al.*, 2010). Although the bean has been an important food source for the people of the Kalahari Desert for centuries and it is widely consumed by the local people (Bower *et al.*, 1988), it is largely an underutilized crop (Jackson, *et al.*, 2011). The marama bean plant is drought resistant and has a potential for cultivation as a food crop (Bower *et al.*, 1988). The seeds are high in protein (28.8-38.4%) and fat (32.0-41.9%) (Holse *et al.*, 2010) and their proteins are comparable to soybean in essential amino acid content (Bower *et al.*, 1988). The bean is a potential source of value added processed products similar to those from soy bean (Muruatona *et al.*, 2010).

During processing the seed coats are removed and discarded and they make up 50% of the total weight of the seed (Holse *et al.*, 2010). The seed coats have been found to contain phenolic acid and flavonoid compounds (Chingwaru, *et al.*, 2011). Therefore, the seed coats may be used as raw material for the extraction of value added natural antioxidants for exogenous use as functional food ingredients with potential health benefits. This could diversify the utilization of

marama bean which in turn could encourage its cultivation as a food crop and thus contribute towards food security.

Phenolic acid and flavonoid compounds are absorbed across the intestinal epithelium into circulation mainly in conjugate forms such as glucuronides and/or sulphates and/or aglycone forms (Spencer *et al.*, 1999). Flavonoid compounds are also extensively metabolised into phenolic acids by colonic bacteria and these metabolites are also absorbed into circulation (Gao *et al.*, 2006). Therefore the compounds or their metabolites may protect inner tissue against oxidative stress. Proanthocyanidins which are major compounds with significant antioxidant activity in extracts from legumes with dark seed coats (Dueñas *et al.*, 2006; Xu *et al.*, 2007a) are not absorbed because of their high molecular weight. However, they are important because they may protect the integrity of the intestinal barrier against oxidative stress, through various mechanisms including antioxidant effect (Erlejman *et al.*, 2008) and they are also thought to play a unique role in sparing other antioxidants in the gastrointestinal tract and thus increasing antioxidant levels in other tissues (Hagerman *et al.*, 1998).

Phenolic compounds from plant material are commonly extracted with organic solvents however these solvents may pose potential problems of toxicity (Tsuda *et al.*, 1995). Aqueous extraction of bioactive compounds may represent a safer and preferred option for pharmaceutical and food grade commercial processes (Oomah *et al.*, 2010). The objective of this study was to identify and characterize phenolic acids, flavonoids and proanthocyanidins in water extracts from the seed coats of marama beans and to evaluate their antioxidant capacity in terms of their protective effect against 2,2'-azo-bis(2-amidinopropane) dihydrochloride (AAPH)-induced red blood cell hemolysis and copper-catalyzed human low density lipoprotein (hLDL) oxidation which may have a bearing on their potential health benefits.

## **Materials and methods**

### **Materials and preparation of water extract**

Marama beans from 2008 season obtained from Botswana, Ghanzi province were cracked using a DF sample cracker (WMC Metal Sheet Works, Tzaneen, South Africa) and the seed coats were separated from the cotyledons manually. The seed coats were ground into a powder using a Waring blender, sieved through a 500  $\mu\text{m}$  aperture test sieve and packed under vacuum. Ten grams of sample was added to 150 mL distilled water and stirred for 30 min using a magnetic stirrer and then transferred to a shaker and shaken for a further 60 min (Liyana-Pathirana & Shahidi, 2005). Extracts were centrifuged at 7500 g, 25 °C for 15 min. The supernatant was decanted and freeze dried. Extracts were weighed and stored at -20°C in sealed zip-lock bags until analyzed.

### **Determination of condensed tannin content**

The freeze dried extracts were dissolved in methanol at a concentration of 0.35 mg mL<sup>-1</sup> and filtered through a Whatman no. 4 filter paper. The condensed tannin content was determined using the vanillin-HCl assay (Price *et al.*, 1978). Results were reported as mg catechin equivalent g<sup>-1</sup> (mg CE g<sup>-1</sup>) of dry sample weight.

### **Identification of phenolic acid and flavonoid compounds by HPLC-DAD/ESI-MS**

Glycosylated phenolics were converted to their aglycone forms by acid hydrolysis using the method of Hahn *et al.* (1983) with some modification. Extract (6 mg) was dissolved in 5 ml of 2 M HCl and heated in a shaking water bath at 98 °C for 1 h. Solution was allowed to cool and phenolic compounds extracted twice with 5 ml ethyl acetate using a separating funnel. Fractions

were combined and evaporated to dryness in a rotary vacuum evaporator model Rotavapor RE 120 (Büchi Labortechnik AG, Flawil, Switzerland) at 30 °C. The residue was re-dissolved in 2 ml methanol and filtered through a 0.2 µm PTFE filter.

*HPLC conditions:* The HPLC system consisted of a model 1525 binary pump, model 2487 dual wavelength absorbance detector and a YMC-Pack ODS AM-303 (250 x 4.6 mm i.d., 5 µm particle size) reverse phase column (Waters Associates, Milford, MA). Running conditions were according to Kim *et al.* (2007). Mobile phase A was 0.1% acetic acid in water and B was 0.1% acetic acid in acetonitrile. Solvents were delivered in a linear gradient as follows: 8-10% B (2 min), 10-30% B (25 min), 30-90% B (23 min), 90-100% B (2 min), 100-8% B (2 min) and 8% B (7 min). Total run time was 61 min, flow rate was 0.8 mL min<sup>-1</sup> and detector was set at 280 nm. Injection volume was 20 µL. Calibration curves were prepared from serial dilutions of phenolic standards dissolved in methanol.

*HPLC-DAD/ESI-MS conditions:* The system was an Agilent 1100 series HPLC system with degasser, auto sampler, binary pump, column heater and photo diode array detector (DAD) (Agilent Technologies, Santa Clara, CA, USA) coupled to a triple quadrupole / linear ion trap mass spectrometer, model 4000 Q TRAP LC/MS/MS system (AB SCIEX, Ontario, Canada). A YMC-Pack ODS AM-303 (250 x 4.6 mm i.d., 5 µm particle size) reverse phase column (Waters Associates, Milford, MA) was used. Injection volume was 20 µL, column temperature was 40 °C, detector wavelength was set at 190 – 600 nm and solvent running conditions were the same as outlined under HPLC conditions. Operating conditions for the mass spectrometer were set as follows: The source temperature was 400 °C, ion spray voltage -4000 V. Nitrogen at a pressure of 35 psi was used as a nebulizer gas (GS1) and at 40 psi as a heating gas (GS2) and at 23 psi as a curtain gas (CUR). Declustering (DP) and entrance potential (EP) were set at -70 and -10 V,

respectively. The mass of compounds was scanned from  $m/z$  70 Da to  $m/z$  1200 Da in 0.75 s and the data acquired and monitored by Analyst software, version 1.5 (Applied Biosystems / MDS Analytical Technologies Instruments, Ontario, Canada). Results were reported as  $\text{mg g}^{-1}$  sample dry weight. Phenolic standards, gallic acid, *p*-hydroxybenzoic acid, (+)-catechin, (-)-epicatechin, (-)-epicatechin-3-O-gallate, (-)-epigallocatechin and (-)-epigallocatechin-3-O-gallate were obtained from Sigma Aldrich (Johannesburg, South Africa) and analytical grade reagents and HPLC grade solvents were obtained from Merck (Johannesburg, South Africa).

### **Characterization of proanthocyanidins by HPLC/ESI-MS**

Proanthocyanidins in the extracts were fractionated into monomer (FI), oligomer (FII) and polymer (FIII) fractions using  $\text{C}_{18}$  and  $\text{tC}_{18}$  Sep Pak cartridges (Waters, Milford, MA, USA) connected in series ( $\text{C}_{18}$  on top) under vacuum, according to the method of Sun *et al.* (1998). Oligomer (FII) and polymer (FIII) fractions were subjected to thiolysis degradation according to the method by Gu *et al.* (2003). Extract fraction (50  $\mu\text{L}$ ), 50  $\mu\text{L}$  methanol acidified with concentrated HCl (3.3% v/v) and 100  $\mu\text{L}$  benzyl mercaptan (5% v/v in methanol) were placed into safety lock eppendorf tubes. The tubes were sealed and heated at 40 °C for 30 min in a water bath and then allowed to stand at room temperature for 10 h to complete the reaction. The reaction mixture was kept at -20 °C until analysis.

*HPLC conditions:* Twenty microlitres of thiolysis degradation reaction mixture was injected into the Waters HPLC system as described above and running conditions were according to Gu *et al.* (2003) with some modification. Mobile phase A was 2% aqueous acetic acid and B was methanol delivered in a linear gradient of B from 15 – 80% in 45 min, 80% - 15% in 10 min and 15% for 10 min. Flow rate was 0.5  $\text{mL min}^{-1}$  and detector was set at 280 nm. Relative

percentage composition of flavan-3-ol and the mean degree of polymerization (mDP) were calculated from the peak areas. The mDP was calculated according to the following formula (Gu *et al.*, 2002):

$$\text{mDP} = 1 + \left\{ \frac{\text{Sum of peak areas of benzylthioether derivative extension units}}{\text{sum of peak areas of free flavan 3-ol terminal units}} \right\}$$

*HPLC/ESI-MS conditions:* Thiolysis degradation products of oligomer (FII) and polymer (FIII) fractions were analyzed with the Agilent 1100 series HPLC system coupled to the triple quadrupole / linear ion trap mass spectrometer, as described above. Solvent system running conditions were as outlined under HPLC conditions. Column temperature was set at 40 °C, detector wavelength at 275 - 285 nm and injection volume was 20 µL. The operating conditions for the mass spectrometer were set as follows: The source temperature was 400 °C, ion spray voltage was -4000 V. Nitrogen at a pressure of 35 psi was used as a nebulizer gas (GS1) and at 40 psi as a heating gas (GS2) and at 23 psi as a curtain gas (CUR). Declustering (DP) and entrance potential (EP) were set at -60 and -10 V, respectively. The mass of compounds were scanned from  $m/z$  70 to  $m/z$  1200 in 0.75 s. Analytical grade reagents and HPLC grade solvents were obtained from Merck (Johannesburg, South Africa).

### **Antioxidant activity**

The extracts were analyzed for their 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging capacity using the method of Brand-Williams *et al.* (1995) with some modification. One hundred and fifty microlitres of sample extract in methanol ( $0.1 \text{ mg mL}^{-1}$ ) or 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) standard solution and 2850 µL of 0.57 mM DPPH working solution were added into tubes, sealed and covered with foil. Tubes were

shaken for 60 min and absorbance measured at 515 nm using a T80+ UV-VIS spectrophotometer (PG Instruments, Leicestershire, UK). Results were expressed as  $\mu\text{mol}$  trolox equivalent per gram ( $\mu\text{mol TE g}^{-1}$ ) on dry weight basis. DPPH and trolox were obtained from Sigma Aldrich (Johannesburg, South Africa) and all other reagents from Merck (Johannesburg, South Africa)

### **Effect of extract on AAPH-induced red blood cell hemolysis**

Human red blood cells (RBC) were obtained from the Department of Pharmacology of the University of Pretoria. Hemolysis experiment was performed according to the procedure of Tang & Liu (2008) with some modification. The RBC were washed 3 times by mixing with phosphate buffer saline (PBS) pH 7.4 (8.1 mM  $\text{Na}_2\text{HPO}_4$ , 1.9 mM  $\text{NaHPO}_4$ , 3 mM KCl and 0.137 M NaCl) solution in a ratio of 1:1 and centrifuged at 2750 rpm for 3 min and supernatant discarded. Extracts were dissolved in distilled water in three concentrations 10, 40, and 80  $\mu\text{g mL}^{-1}$ . For treatments 100  $\mu\text{L}$  PBS, 10  $\mu\text{L}$  RBC, 40  $\mu\text{L}$  extract and 40  $\mu\text{L}$  of 110 mM AAPH solution prepared in PBS were added into 1.5 ml eppendorf tube. Sample blanks were prepared without AAPH solution. Positive controls (140  $\mu\text{L}$  PBS, 10  $\mu\text{L}$  RBC, and 40  $\mu\text{L}$  of 110 mM AAPH solution) and negative controls (180  $\mu\text{L}$  PBS and 10  $\mu\text{L}$  RBC) were prepared in tubes, sealed, mixed and incubated at 37 °C for 16 h. After incubation tubes were mixed and centrifuged at 2750 rpm for 3 min. Supernatant (50  $\mu\text{L}$ ) was transferred into a 96 well plate and absorbance read at 405 nm on a 96 well plate reader Bio Tek ELx 800 (Biotek instruments Inc, Winooski, VT). AAPH was obtained from Sigma Aldrich (Johannesburg, South Africa) and all other reagents from Merck (Johannesburg, South Africa).



### **Effect of extracts on TBARS formation in copper-catalyzed LDL oxidation**

The effect of extracts against copper-catalyzed human low density lipoprotein (hLDL) oxidation was evaluated using the thiobarbituric acid assay (TBA assay) (Xu *et al.*, 2007b). Freeze dried extracts were dissolved in distilled water in three different concentrations 10, 40 and 80  $\mu\text{g mL}^{-1}$ . Two microliter hLDL solution ( $5.5 \text{ mg mL}^{-1}$ ), 168  $\mu\text{L}$  0.02 M phosphate buffer saline solution pH 7.4 and 10  $\mu\text{L}$  sample extract or trolox solution were added into eppendorf tubes and oxidation was initiated by adding 20  $\mu\text{L}$  of 100 mM copper sulphate solution. Positive controls were prepared by adding 2  $\mu\text{L}$  hLDL solution, 170  $\mu\text{L}$  PBS and 20  $\mu\text{L}$  copper sulphate solution. Negative controls were prepared by adding 2  $\mu\text{L}$  hLDL solution, 198  $\mu\text{L}$  PBS solution. Tubes were sealed and incubated in a water bath at 37 °C for 3 h. After incubation 200  $\mu\text{L}$  of 10 mM EDTA solution, 200  $\mu\text{L}$  of 20% (w/v) trichloroacetic acid solution and 200  $\mu\text{L}$  of 0.67% (w/v) thiobarbituric acid solution in 0.2 M NaOH were added. Tubes were sealed, heated at 80 °C for 30 min in a water bath and after cooling they were centrifuged at 1500 g for 15 min and supernatant transferred into 1 mL cuvette. Absorbance was measured at 532 nm. Trolox and hLDL were obtained from Sigma Aldrich (Johannesburg, South Africa) and all other reagents from Merck (Johannesburg, South Africa).

### **Statistical analyses**

Results from two independent experiments performed in duplicate were expressed as means  $\pm$  standard deviations. Data were analyzed using t-test to compare two means and one-way analysis of variance (ANOVA) and post hoc testing using Fishers LSD test for multiple comparisons of means. Statistica 8.0 program (StatSoft, Inc, Tulsa, OK) was used for statistical data analysis and significance was accepted at  $p < 0.05$ .

## Results and discussion

### Condensed tannin content

The condensed tannin content of the extracts (Table 1) was comparable to levels reported in methanolic extracts from seed coats of dark coloured common beans which ranged from 216 to 449 mg CE g<sup>-1</sup> of seed coat sample depending on cultivar (Ranilla *et al.*, 2007). Water is a poor extraction solvent for phenolic compounds (Al-Farsi & Lee, 2008). The levels in the water extracts observed in this work was an indication that marama bean seed coats have high levels of condensed tannins or proanthocyanidins compared to other seed coats of brown beans. Levels of condensed tannins determined in our lab in 70% aqueous acetone extracts from marama bean seed coats were found to be 1226 mg CE g<sup>-1</sup> on dry weight basis (data not shown).

### Identification of phenolic acids and flavonoids by HPLC-DAD/ESI-MS

The chromatographic trace of the phenolic acid and flavonoid compounds in the extracts is shown in Figure 1A and the identity of the compounds is shown in Table 2. Gallic acid (MW = 170.12) was the predominant phenolic acid accounting for 28% of total phenolic acid and flavonoid compounds (Table 2). The other phenolic acids were homogentisic acid (MW = 168.14) and *p*-hydroxybenzoic acid (MW = 138.12). Both gallic acid and *p*-hydroxybenzoic acid have been reported in acidified methanol extracts from marama bean seed coats and the latter compound was the most abundant phenolic acid (Chingwaru *et al.*, 2011). These phenolic acids have also been reported in other legumes. Homogentisic acid has been reported in lentils (Zadernowski *et al.*, 1992) while gallic acid and *p*-hydroxybenzoic acid have been reported in alkaline hydrolysates of seed coats of mung beans, lentils, faba beans, pigeon and cowpeas (Sosulski & Dabrowski, 1984).

The compound in peak 4 was tentatively identified as methyl (epi)catechin-3-O-gallate (MW = 456) and peaks 5 and 6 were tentatively identified as isomers of methyl (epi)afzelechin-3-O-gallate (MW = 440). They accounted for 28% and 40% of total phenolic acid and flavonoid compounds, respectively. These compounds have not been reported in legumes, however, similar mass spectral data of methyl (epi)catechin-3-O-gallate has been reported in extracts from green tea (*Camellia sinensis*) and the molecular ion at  $m/z$  439 (methyl (epi)afzelechin-3-O-gallate) also appeared in the mass spectra of ethanolic extracts from green tea (Bastos *et al.*, 2007). Peaks 7 and 8 were identified as isomers of (epi)catechin-3-O-gallate (MW = 442). Similar mass spectral data for (epi)catechin-3-O-gallate has been reported in green tea (Miketova *et al.*, 1998) and oolong extracts (Dou *et al.*, 2007).

### **Characterization of proanthocyanidin constitutive units**

HPLC compound profile of thiolysis degradation products of proanthocyanidins in the polymer fraction (FIII) is shown in Figure 1B and the identity of the compounds and their percentage composition is shown in Table 3. Terminal units released as free flavan-3-ol units are shown as peak 1 and 2 and they were (epi)gallocatechin-3-O-gallate (MW = 458) and (epi)catechin-3-O-gallate (MW = 442) respectively. The latter was the major compound. Similar mass spectral data for (-)-epigallocatechin-3-O-gallate and (-)-epicatechin-3-O-gallate have been reported in green tea (Miketova *et al.*, 1998) and oolong tea (Dou *et al.*, 2007) extracts. Terminal units of proanthocyanidins in seed coats of pinto beans, small red beans and red kidney beans were reported to be composed of (+)-catechin (7.3 – 14.5%) and (-)-epicatechin (0.4 – 1.1%) (Gu *et al.*, 2003). In lentils they were composed of (+)-catechin (8.6 – 11.9%), (-)-epicatechin (1.3-1.7%) and traces of (-)-epicatechin-3-O-gallate (Dueñas *et al.*, 2003).

Extension units released as benzylthioether derivatives are shown as peaks 3 to 6. Peak 3 was identified as (epi)gallocatechin benzylthioether (MW = 428) and peak 4 was identified as (epi)gallocatechin-3-O-gallate benzylthioether (MW = 580). Similar mass spectra data for these compounds were reported in proanthocyanidins from persimmon fruit (Li *et al.*, 2010). The compound eluting in peak 5 was identified as (epi)catechin benzylthioether (MW = 412). Similar mass spectra data for (epi)catechin benzylthioether was reported by Gu *et al.* (2002). Peak 6 was identified as (epi)catechin-3-O-gallate benzylthioether (MW = 564). Gu *et al.* (2003) identified (-)-epicatechin-3-O-gallate benzylthioether by the molecular ion  $m/z$  563 and fragment ion  $m/z$  439. Extension units in proanthocyanidins from seed coats of pinto beans, small red beans and red kidney beans were (-)-epicatechin (43.1 – 73.9%), (+)-catechin (5.4-33.2%) and (epi)afzelechin (8.7 – 14.6%) (Gu *et al.*, 2003). In lentils they were catechin (49.5 – 61.9%), (-)-epicatechin (20.8 – 33.8), (-)-epigallocatechin (3.8-6.7%) and traces of (-)-epigallocatechin-3-O-gallate (Dueñas *et al.*, 2003).

The flavan-3-ol composition suggests that marama bean seed coat proanthocyanidins have a heteropolyflavan polymer structure (Figure 2). This structure seems to be unique in that it contains a higher percentage of flavan-3-O-gallate units compared to other legumes such as the common bean and lentils which are mainly composed of flavan-3-ol units. The mDP of marama bean seed coat proanthocyanidin polymer and oligomer fractions were 9.2 and 5.1 respectively. These mDP values were comparable to those of unfractionated aqueous acetone extracts from pinto beans, small red beans and red kidney beans which ranged from 6.3 – 12.0, depending on variety (Gu *et al.*, 2003). That of lentils ranged from 6.9 – 9.4 and 3.8 – 4.7 for polymeric and oligomeric fractions, respectively (Dueñas *et al.*, 2003).

### **Antioxidant activity**

The DPPH free radical scavenging activity of the extract ( $707 \mu\text{mol TE g}^{-1}$ ) (Table 1) was higher than that reported for 70% aqueous methanol extracts from the seed coats of dark colored common beans which ranged from 147 to  $518 \mu\text{mol TE g}^{-1}$  on fresh weight basis (Ranilla *et al.*, 2007). The extracts exhibited a protective effect against AAPH-induced human red blood cell hemolysis in a dose-dependent manner (Figure 3A). The reduction in hemolysis was as much as 70% at the lowest extract concentration ( $10 \mu\text{g ml}^{-1}$ ). Phenolic compounds protected the RBC membrane by scavenging peroxy radicals generated by AAPH. They may also protect by preventing access of deleterious molecules to the hydrophobic region of the bilayer by interacting with membrane phospholipids through hydrogen bonding and accumulate at the membrane surface (Verstraeten *et al.*, 2003). High molecular weight proanthocyanidins in the extract may potentially protect cells such as those lining the gastrointestinal tract against free radical oxidative damage, which is an important factor in gastrointestinal cancers and inflammatory conditions (Erlejman *et al.*, 2008). The extracts at  $80 \mu\text{g ml}^{-1}$  concentration inhibited TBARS formation in copper-catalyzed human LDL oxidation (Figure 3B). The protection was higher than that of 100  $\mu\text{M}$  trolox solution. However at low concentrations ( $10$  and  $40 \mu\text{g ml}^{-1}$ ) TBARS formation could not be inhibited. Phenolic compounds in the water extracts delayed or inhibited the initiation phase of LDL oxidation by chelating  $\text{Cu}^{2+}$  and by scavenging free radicals (Rüfer & Kulling, 2006). Overall, these results indicate that at appropriate concentrations the phenolic compounds in water extracts may provide protection against free radical LDL oxidation which is a risk factor in cardiovascular disease.

The phenolic acids, galloylated flavonoid compounds and the highly galloylated condensed tannins in the extracts could be responsible for the observed antioxidant effects, especially the

proanthocyanidins because of their high molecular weight and close proximity of many aromatic rings and hydroxyl groups in the polymer structure (Hagerman *et al.*, 1998).

## **Conclusion**

In conclusion, the major low molecular weight phenolic compounds in the water extracts are gallic acid, methyl (epi)catechin-3-O-gallate and methyl (epi)afzelechin-3-O-gallate. High molecular weight proanthocyanidins in the extracts are composed of (epi)galocatechin-3-O-gallate and (epi)catechin-3-O-gallate as major constituents and epigallocatechin and epicatechin as minor components. Therefore marama bean proanthocyanidins are predominantly prodelphinidins with a minor proportion of procyanidins. The highly galloylated proanthocyanidins structure is unique when compared to that of other legumes such as the common bean and lentils. Both low and high molecular weight phenolic compounds in the water extracts from marama bean seed coats could contribute to the observed antioxidant activity and protective effects on biological molecules against free radical and copper-catalyzed oxidative damage. Therefore marama bean seed coats are potentially a rich source of flavan-3-O-gallates for use as functional food ingredient with potential health benefits.

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**Table 1** Condensed tannin content and free radical scavenging activity of water extracts from marama bean seed coats<sup>a</sup>

	<b>Water extract</b>
Condensed tannin content <sup>b</sup>	200.0 ± 1.1
DPPH antioxidant activity <sup>c</sup>	707.0 ± 10.6

<sup>a</sup>Values are means ± standard deviations of four determinations (on dry weight basis) from two independent experiments.

<sup>b</sup>Vanillin-HCl method ( mg CE g<sup>-1</sup>).

<sup>c</sup>DPPH assay (μmol TE g<sup>-1</sup>).

**Table 2** Phenolic acid and flavonoid compounds identified in water extracts prepared from marama bean seed coats

Peak No.	t <sub>R</sub> (min)	m/z [M-H] <sup>-</sup> (% Intensity)	m/z MS <sup>n</sup> (% Intensity)	λ <sub>max</sub> (nm)	Compound identity	Concentration <sup>a</sup> (mg g <sup>-1</sup> )
1	7.2	169 (100)	125 (63)	214, 270	Gallic acid <sup>b</sup>	1.98 ± 0.18
2	11.8	167 (100)	123 (88)	208	Homogentisic acid <sup>b</sup>	0.49 ± 0.04
3	13.9	137 (100)	-	208, 278	<i>p</i> -Hydroxybenzoic acid	1.76 ± 0.15
4	14.6	455 (100)	169 (10)	214, 280	Methyl (epi)catechin-3-O-gallate <sup>c</sup>	5.84 ± 0.42
5	16.7	439 (100)	169 (91)	210, 280	Methyl (epi)afzelechin-3-O-gallate <sup>c</sup>	-
6	20.5	439 (100)	169 (13)	208, 280	Methyl (epi)afzelechin-3-O-gallate <sup>c</sup>	8.34 ± 0.33
7	22.6	441 (100)	289 (7), 169 (59)	208, 278	(Epi)catechin 3-O-gallate <sup>c</sup>	-
8	22.9	441 (100)	289 (5), 169 (48)	208, 278	(Epi)catechin 3-O-gallate <sup>c</sup>	1.74 ± 0.16
					Total	20.15 ± 1.06

<sup>a</sup>Results are means ± standard deviation of four determinations from two independent experiments on dry weight basis

<sup>b</sup>Values expressed as gallic acid equivalent

<sup>c</sup>Values expressed as (-)-epicatechin-3-O-gallate equivalent

- Concentration of isomer compounds were combined

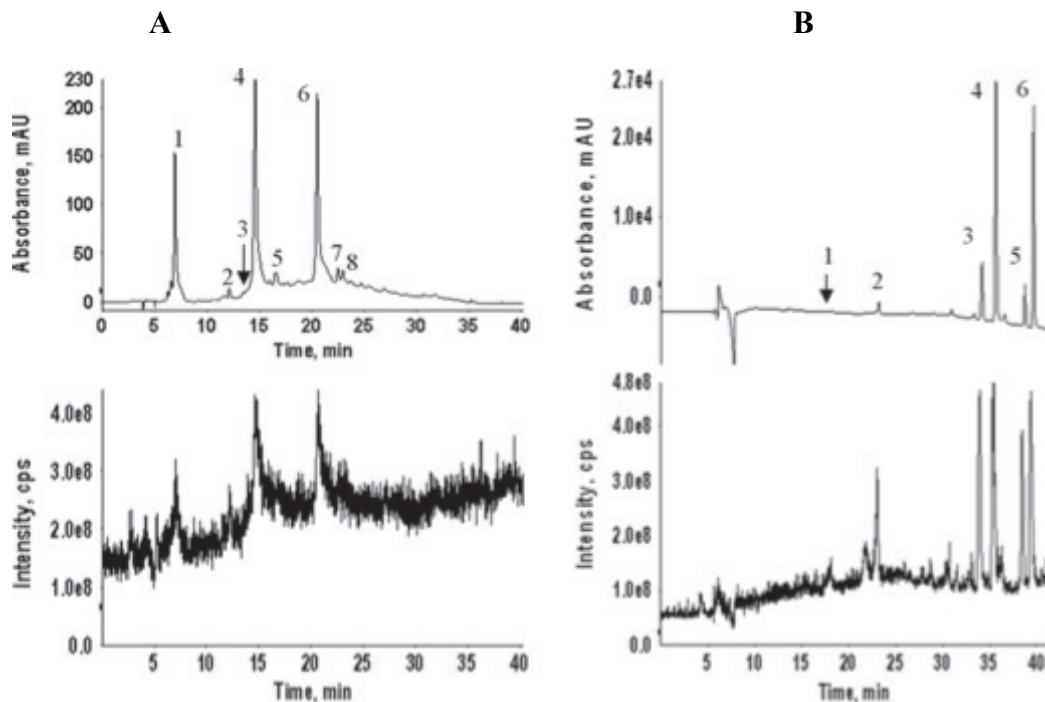
**Table 3** Mass spectral data of thiolysis degradation products and constitutive unit composition (relative percentage by peak area) of proanthocyanidins in water extracts from marama bean seed coats

Peak No.	$t_R$ (min)	$m/z$ [M-H] <sup>-</sup> (% intensity)	$m/z$ MS <sup>n</sup> (% intensity)	Identification	% Composition <sup>a</sup>	
					Oligomer (FII)	Polymer (FIII)
Terminal units released as flavan 3-ol units						
1	18.1	457.7 (100)	305.9 (14), 169.6 (32)	EGCG <sup>b</sup>	2.7 ± 0.2a <sup>c</sup>	5.9 ± 0.8b
2	23.0	441.8 (100)	289.5 (10), 169.3 (40)	ECG	8.2 ± 0.7a	13.6 ± 0.8b
Extension units released as benzylthioether derivatives						
3	33.9	427.7 (100)	303.5 (80)	EGC benzylthioether	6.6 ± 1.2a	8.2 ± 2.1a
4	35.3	579.8 (100)	455.6 (14), 303.6 (20), 169.6 (11)	EGCG benzylthioether	43.3 ± 0.7a	35.1 ± 0.8b
5	38.4	411.7 (90)	287.7 (100)	EC benzylthioether	5.7 ± 0.4a	7.0 ± 0.5a
6	39.3	563.5 (100)	439.8 (17), 287.7 (53), 169.1 (38)	ECG benzylthioether	33.5 ± 1.5a	30.1 ± 2.1b

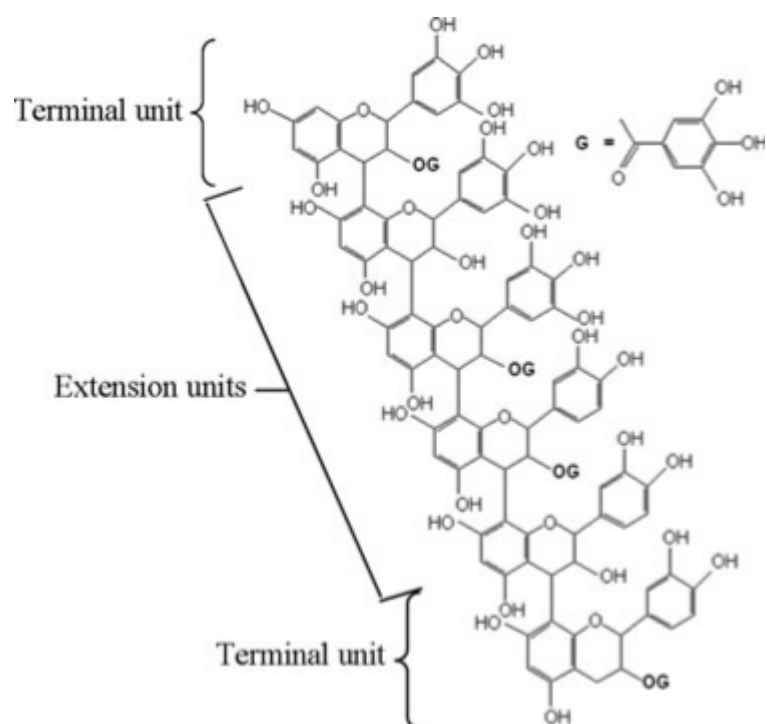
<sup>a</sup>Data are means ± standard deviations of four determinations from two independent experiments.

<sup>b</sup>Abbreviations: EGCG, (epi)gallocatechin-3-O-gallate; ECG, (epi)catechin-3-O-gallate; EGC, (epi)gallocatechin; EC, (epi)catechin.

<sup>c</sup>Means with the same letter in the same row are not significantly different ( $p \leq 0.05$ ).

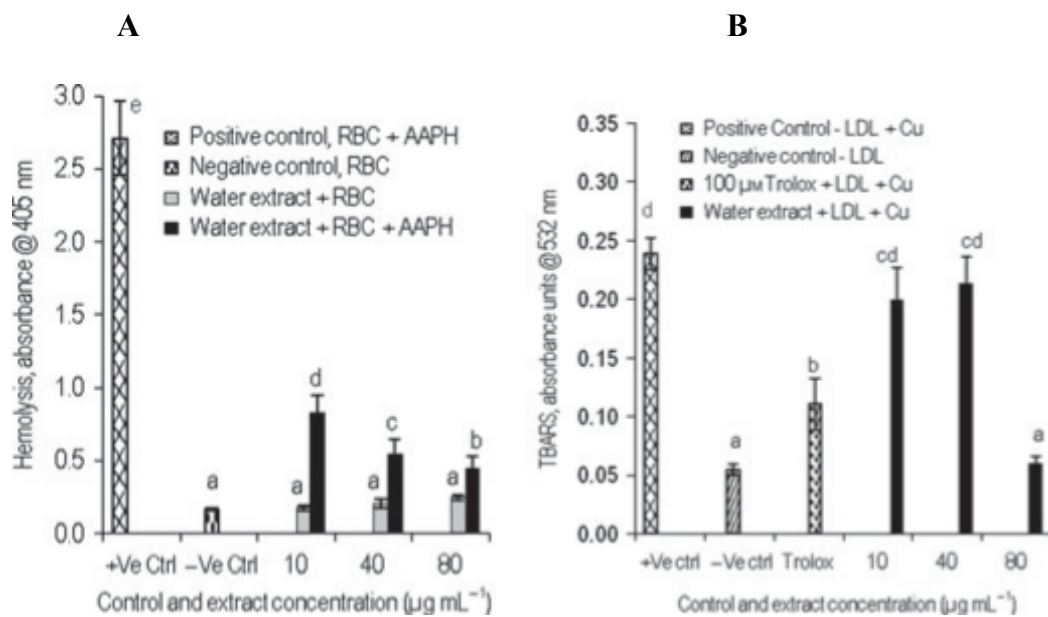


**Figure 1** HPLC chromatograms recorded at 280 nm (top) and total ion current chromatogram (bottom) of A) phenolic acid and flavonoid compounds (refer to Table 2 for identification of peaks) and B) thiolysis degradation products of proanthocyanidins in polymer fraction (FIII) (refer to Table 3 for identification of peaks) from water extracts prepared from marama bean seed coats.



**Figure 2** Polymer structure of proanthocyanidins in water extracts from marama bean seed coats.





**Figure 3** Effect of water extracts prepared from marama bean seed coats on A) AAPH-induced *in vitro* red blood cell hemolysis and B) copper-induced *in vitro* human LDL oxidation.