

CHAPTER 5

5. GENERAL DISCUSSION

In the first part of the general discussion the choice and considerations of methodologies used in this research are discussed. In the second part the major findings on marama bean seed coats and condensed tannin sorghum bran as possible sources of natural dietary antioxidants with a potential to reduce oxidative stress which is implicated in the development of a number of chronic diseases such as cardiovascular and neurodegenerative diseases as well as cancer are discussed.

5.1. Discussion of methods used

Phenolic compounds are commonly extracted with organic solvents such as the alcohols methanol and ethanol, acetone, ethyl acetate or their aqueous mixtures (Krygier *et al.*, 1982). Organic solvents have limited use in the food industry and some of these solvents can be toxic to human health such as ethyl acetate therefore undesirable for extracting antioxidants for use in food (Tsuda *et al.*, 1995). In this study seed coats of marama beans and bran of condensed tannin sorghum were extracted under aqueous conditions because the aim of the study was to obtain an extract that is natural and possibly safe to use in food products. Kobue-Lekalake, Taylor and de Kock (2007) used water as an extraction solvent to prepare food compatible and safe aqueous infusions from sorghum bran for use in sensory evaluation studies. The other consideration was that water is cheaper and pollution free and does not require special storage and handling like organic solvents such as ethanol (Llorach, Espín, Tomás-Barberán & Ferreres, 2003). Extraction under aqueous acidic condition was also considered as a method to increase the antioxidant content of extracts by enhancing the extractability of free and esterified phenolic compounds and by liberating phenolic compounds bound to cell wall polysaccharides by acid hydrolysis (Liyana-Pathirana & Shahidi, 2005). Acidification may help disintegrate cell walls thus facilitating solubilisation and diffusion of phenolic compounds from plant material (Campos, Chirinos & Pedreschi, 2008). The disadvantage of extraction with water is the low recovery of phenolic compounds and the extraction of soluble polysaccharide and proteins which may impact on the purity of the extracts. Recovery of phenolic compounds can be improved by extracting at higher

temperature, increasing contact time, size reduction of material to finer particles and also using sub-critical water extraction (high pressure and temperature).

Due to the poor phenolic extraction rate of water and the possibility of extracting other compounds, methanol was used as an extraction solvent for determination of condensed tannin content of the seed coats and bran using the vanillin-HCl method (Price *et al.*, 1978). Acetone extracts were only prepared for the characterization of condensed tannins because acetone is the commonly used solvent for this purpose (Lazarus *et al.*, 1999; Prior *et al.*, 2001; Gu *et al.*, 2002).

The Folin-Ciocalteu method (Singleton & Rossi, 1965) for the determination of total phenolics was chosen because it is a simple and reproducible assay which is widely used for studying phenolic antioxidants (Macdonald *et al.*, 2006). It is based on the reducing power of phenolic hydroxyl groups (Sun, Ricardo da Silva & Spranger, 1998b). In this assay phenolic compounds react with the Folin-Ciocalteu reagent under basic conditions, through the dissociation of a proton from the phenolic hydroxyl group which leads to the formation of a phenolate anion (Macdonald *et al.*, 2006). The phenolate ion reduces the Folin-Ciocalteu reagent (a yellow acidic solution containing complex polymeric ions formed from phosphomolybdic and phosphotungstic heteropoly acids) to form a blue molybdenum-tungsten complex (Abu Bakar, Mohamed, Rahmat & Fry, 2009) that has an absorption maxima at 760 nm (Macdonald *et al.*, 2006). The disadvantage of this assay is that it is not very specific for phenolic compounds since it detects all phenols with varying sensitivity (Sun *et al.*, 1998b). Folin-Ciocalteu reagent can be reduced by non-phenolic compounds, including those in extractable proteins (Naczki & Shahidi, 2004; Macdonald *et al.*, 2006), aromatic amines and ascorbic acid (Alvarez-Suarez *et al.*, 2009). Therefore the total phenolic content of the extracts may be overestimated if the sample contains significant amount of proteins composed of phenolic amino acid with reducing properties. However, proteins are low in seed coats of legumes (Rodriguez & Mendoza, 1990). The protein content of maramba bean seed coats was found to be approximately 2.4%, while that of sorghum bran was approximately 9.2% as determined in our lab. Proteins of maramba bean cotyledons have been shown contain high levels of tyrosine (11.4 g/100 g) (Amonsou *et al.*, 2012) and this amino acid has been reported to have antioxidant properties (Nimalaratne, Lopes-Lutz, Schieber & Wu, 2011). However, the effect of protein on the total phenolic content of extracts from maramba beans seed coats was not a concern because of the low protein content.

The total flavonoid assay is based on the reaction between flavonoids and aluminium ions to form a coloured flavonoid-aluminium complex that can be monitored spectrophotometrically (Popova, Bankova, Butovska, Petkov, Nikolova-Damyanova, Sabatini, Marcazzan & Bogdanov, 2004; Abu Bakar *et al.*, 2009). Aluminium ions react with the C-4 keto group and either the C3 or C5 hydroxyl group of flavones and flavonols to form an acid stable complex and also with the dihydroxy groups in the A- and B-ring of flavonoids to form an acid labile complex (Chang, Yang, Wen & Chern, 2002). However, according to the authors this assay is specific for flavones and flavonols because some flavonoid compounds, such as the flavanones naringenin and hesperetin react with aluminium chloride to form complexes that have an absorption λ_{\max} that is different from that of flavone and flavonol complexes. Therefore the assay may underestimate the total flavonoid content of extracts. Sorghum bran aqueous extracts had three flavanone compounds taxifolin, eriodictyol and naringenin and the last compound may have low absorbance as it lacks the ortho dihydroxyl group on B-ring. The flavonoid compounds identified in marama bean seed coats were galloylated flavanols which lack the C-4 keto group and as a result may exhibit low absorbance values. However, the galloyl groups with dihydroxyl groups may complex with metals such as aluminium to form complexes and the use of catechin (a flavanol) as a standard may reduce the error.

The use of spectrophotometric methods gives good estimate of total phenol content but these methods do not give quantitative amounts of each compound in each class (Adamsom, Lazarus, Mitchell, Prior, Cao, Jocaobs, Kremers, Hammerstone, Rucker, Ritter & Schmitz, 1999). Therefore phenolic acids and flavonoid compounds in the extracts were hydrolyzed to their aglycone forms and analyzed by HPLC-MS. Acid hydrolysis as described by Hahn *et al.* (1983) and Svensson, Sekwati-Manang, Lutz, Schieber & Ganzle (2010) was used as a method of cleavage to remove sugar from glycosylated phenolics (Dueñas *et al.*, 2003). This was necessary because analysis of phenolic glucosides in plant material is often difficult because most reference compounds are not commercially available. Therefore to reduce the complexity of analysis the compounds were identified and quantified as aglycones after hydrolysis (Haghi & Hatamin, 2010) using HCl which has become standard practice (Mattila, Astola & Kumpulainen, 2000).

The phenolic aglycones were analyzed with reversed-phase HPLC coupled to UV-VIS detection and mass spectrometry. This method was chosen because it is the most widely used method for separation, identification and quantification of phenolic compounds (Rodríguez-

Medina, Segura-Carretero & Fernández-Gutiérrez, 2009). It provides UV and MS spectra for each peak which makes identification of each peak possible sometimes without the need for a reference compound and by comparing with literature data (He, Lian & Lin, 1997). Electrospray ionization (ESI) (for mass spectrometry) was chosen because it is a gentle ionization technique at atmospheric pressure. It generates mainly the deprotonated molecules or pseudomolecular ion $[M-H]^-$ in negative mode (Gioacchini, Roda, Galletti, Bocchini, Manetta & Baraldini, 1996) for rapid determination of molecular mass (Soong & Barlow, 2005). It is suitable for the analysis of thermally labile, non-volatile, polar compounds with high sensitivity (Friedrich, Eberhardt & Galensa, 2000). Negative mode was chosen because deprotonation of phenolic compounds is easier as these compounds are weakly acidic (Friedrich *et al.*, 2000). Positive ion mode was not used because it is reported to generate higher background signal noise (Sun & Miller, 2003) and also results in complex adduct formation (Soong & Barlow, 2005). MS detection has a great advantage of being able to distinguish between compounds that co-elute (Shui, Leong & Wong, 2005) or have overlapping chromatographic peaks (Bocchi, Careri, Groppi, Mangia, Manini & Mori, 1996). However, it cannot distinguish between isomers having the same molecular weight.

Legumes with dark seed coats have been shown to contain condensed tannins (Ranilla *et al.*, 2007). Therefore the seed coats of maramba beans were investigated for condensed tannins and bran from condensed tannin sorghum was included as a control as the tannins from sorghum have been characterized (Dykes & Rooney, 2006). The modified vanillin-HCl assay (Price, Van Scoyoc & Butler, 1978) is widely used for the quantitative determination of condensed tannins in plant materials (Butler, Price & Brotherton, 1982). This assay is based on the condensation of the aromatic aldehyde vanillin with monomeric flavanols and their oligomers to form a red adduct with absorbance maxima at 500 nm (Dykes & Rooney, 2006). The vanillin-HCl assay is specific for flavanols and dihydrochalcones which have a single bond at the 2,3 position and free meta oriented hydroxyl groups on the B-ring (Sarkar & Howarth, 1976). The disadvantage of the vanillin-HCl assay is that it does not measure tannin content accurately because of the heterogeneity of tannins in nature (Dykes & Rooney, 2006)). Catechin is often used as an external standard (Butler *et al.*, 1982; Adamson *et al.*, 1999). The disadvantage of using catechin as a standard is that it leads to an overestimation of tannin content (Price *et al.*, 1978). Anthocyanins and other natural pigments may interfere with the assay (Awika, McDonough & Rooney, 2005). To correct for this interference extract sample blanks; extracts subjected to the same reaction conditions but without the

vanillin reagent (Ranilla *et al.*, 2007) were used in this study and the absorbance values of these blanks were subtracted from those of the samples.

The presence of condensed tannins in the extracts from marama bean seed coats needed to be confirmed using another method as the vanillin-HCl method is not completely specific for condensed tannins as it also reacts with dihydrochalcones and anthocyanins (Sarkar & Howarth, 1976). The butanol-HCl method is more specific than the vanillin-HCl method (Makkar, Gamble & Becker, 1999). It is based on the cleavage of the interflavanoid bond of proanthocyanidins under hot acidic conditions followed by an autoxidation reaction that converts the released flavan-3-ol units to anthocyanidin (Porter *et al.*, 1986; Mathews *et al.*, 1997). The anthocyanidins formed assume a red colour with absorbance maxima around 550 nm (Sun *et al.*, 1998b). Incomplete transformation of proanthocyanidins to anthocyanidins and occurrence of side reactions leading to the formation of red-brown polymers has been reported to result in inconsistent results (Porter *et al.*, 1986). The authors reported that the presence of traces of transition metals such as iron salt in the reaction mixture gave consistent yield of anthocyanidins therefore in performing this assay in this study $\text{NH}_4\text{Fe}(\text{SO}_4)_2$ was added into the reaction mixture to achieve consistent results.

Tannins are characterized by their affinity for proteins (Hagerman & Butler, 1980). The protein precipitation capacity assay (Hagerman & Butler, 1978) was used for further confirmation of the presence of condensed tannins in extracts from the marama bean seed coats. In this assay, tannins in the extracts interact with bovine serum albumin (BSA) (added in excess) to form an insoluble tannin-protein complex that is isolated and dissolved in an alkaline sodium dodecyl sulphate-triethanolamine solution. Ferric chloride solution is added to react with the protein-tannin complex to form a violet complex that has an absorbance maxima at 510 nm.

In order to determine the constitutive unit composition of proanthocyanidins in extracts, the first step is to purify proanthocyanidins. A solid phase extraction method described by Sun, Leandro, Ricardo da Silva and Spranger (1998a) was used for the purification of condensed tannins in the extracts. This method utilizes C_{18} Sep-Pak cartridges and it was chosen because it is simple and it has been successfully used in the characterization of proanthocyanidins in grapes (Sun *et al.*, 1998a) and lentils (Dueñas *et al.*, 2003). In this method phenolic acids and sugars were first removed by eluting with water pH 7 and the

extracts fractionated into three fractions namely, monomers (FI), oligomer (FII) and polymer (FIII). Other workers have used Sephadex LH-20 to purify condensed tannin (Strumeyer & Malin, 1975; Prieur *et al.*, 1994).

Acid-catalyzed degradation of proanthocyanidins in the presence of toluene- α -thiol (Ricardo da Silva *et al.*, 1991) was chosen as a method to investigate the chemical structure of condensed tannins in the oligomeric (FII) and polymeric (FIII) fractions as it distinguishes between terminal and extension units. Extension units are released as benzylthioether derivatives and terminal units are released as free flavan-3-ol units and ester moieties of O-gallates are preserved (Prieur *et al.*, 1994) as shown in Fig. 5.1.1.

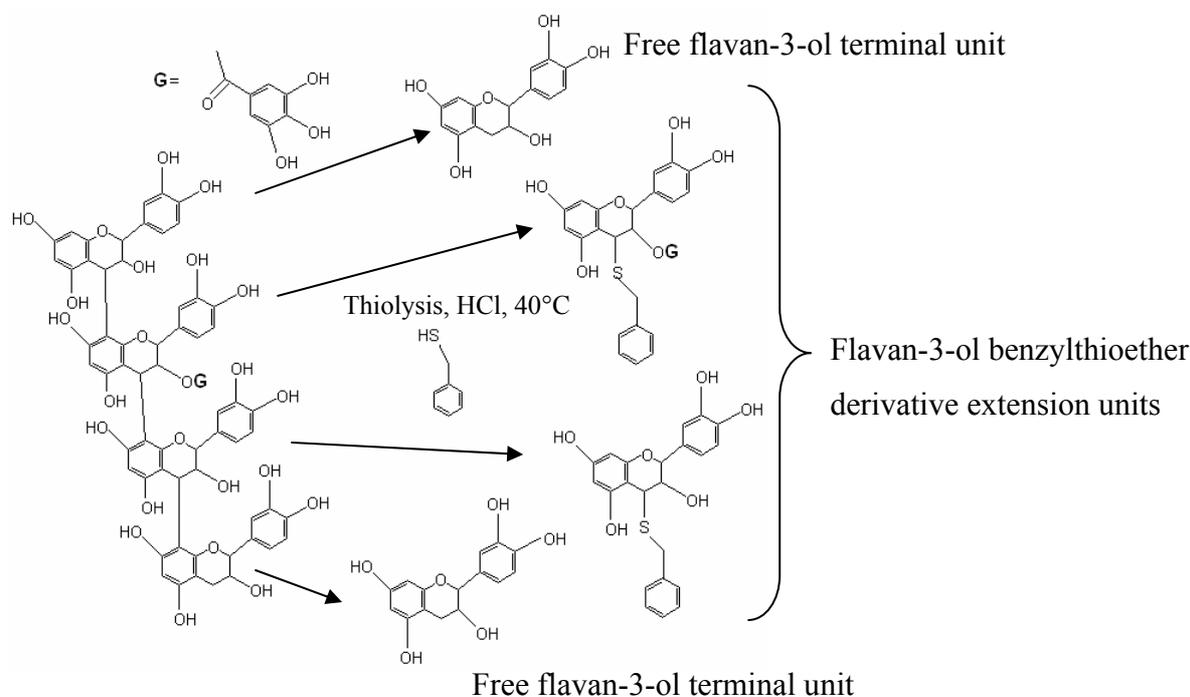


Figure 5.1.1. Thiolysis degradation of proanthocyanidins in the presence of toluene- α -thiol (Adapted from Gu *et al.* (2002))

Toluene- α -thiol is toxic, an irritant and has a strong persistent smell and for these reasons other nucleophiles such as phloroglucinol (Mathews *et al.*, 1997) and cysteamine (Torres & Lozano, 2001) have been used. Nevertheless toluene- α -thiol was used as it has been reported to give higher yields than phloroglucinol (Mathews *et al.*, 1997) and cysteamine was not used

because there is not enough literature on its use. To overcome the strong persistent smell, samples containing toluene- α -thiol were kept in sealed vials and the eluent from the HPLC was collected into a sealed container with a breather tube to take the smell outside the HPLC room.

Reversed-phase HPLC-MS analysis was applied to the thiolysis degradation products to identify and determine the constitutive unit composition and mean degree of polymerization of the proanthocyanidins in the fractions according to the method of Gu *et al.* (2003b). ESI-MS detection was used to identify the constitutive units especially the benzylthioether derivatives where authentic standards for these compounds were not commercially available. The relative constitutive unit composition and mean degree of polymerization were calculated from peak areas, ideally these should be calculated from molar concentration, but the non availability of authentic standards of benzylthioether derivatives was a limitation. However, the response factors of flavan-3-ol and their respective benzylthioether derivatives were reported to be the same at 280 nm therefore the peak areas could be used for the calculation (Gu *et al.*, 2002). The same procedure has been applied in the characterization of proanthocyanidins from mangosteen pericarps (Fu, Loo, Chia & Huang, 2007) and apples (Guyot, Marnet & Drilleau, 2001).

To provide a more reliable assessment of antioxidant activity it is useful to study the antioxidant effectiveness of an extract by more than one method (Xu *et al.*, 2007b; Macdonald *et al.*, 2006) as antioxidant activity assays proceed via different reaction mechanisms (Xu & Chang, 2008) as reviewed in Chapter 2, section 2.2.7. Therefore the antioxidant activities of the extracts were assessed with the ABTS and DPPH radical scavenging assays which are indirect methods and the ORAC assay which is a direct method (Roginsky & Lissi, 2005). The ORAC measures the ability of an antioxidant to scavenge radicals such as peroxy and hydroxyl radicals which are involved in oxidative damage in biological systems (Roginsky & Lissi, 2005). Biologically relevant assays that measure the effect of an antioxidant on biomarkers of oxidative stress were also used over and above the direct and indirect methods.

The ABTS assay was used because it is a relatively simple assay that measures the relative ability of an antioxidant to scavenge the ABTS \bullet^+ radical (Awika *et al.*, 2003) compared to

Trolox (a water soluble vitamin E analogue) and the data expressed as Trolox equivalent antioxidant capacity (TEAC) (Madhujith & Shahidi, 2005). It is a good method for evaluating both lipophilic and hydrophilic antioxidants (Rivero-Pérez *et al.*, 2007) because the radical is soluble in water and organic solvents (Alvarez-Suarez *et al.*, 2009). $ABTS^{\bullet+}$ is a stable radical that reacts energetically with H-atom donors such as phenolics (Roginsky & Lissi, 2005). The limitation is that the assay characterizes the capability of an antioxidant to react with the $ABTS^{\bullet+}$ rather than to inhibit an oxidative process and also the radical has poor selectivity because it reacts with any hydroxylated aromatic independent of its real antioxidant potential (Roginsky & Lissi, 2005). Therefore it may overestimate the antioxidant activity of an extract.

The DPPH method is a quick, simple and widely used assay for assessing the antioxidant activity of plant extracts with good repeatability (Awika *et al.*, 2003; Alvarez-Suarez *et al.*, 2009). The assay is based on the measurement of the reducing ability of an antioxidant towards the stable organic nitrogen DPPH $^{\bullet}$ radical (Madhujith & Shahidi, 2009). Reduction of DPPH by an antioxidant results in loss of absorbance at 515 nm and the degree of discolouration indicates the scavenging efficiency of the added substance (Cardador-Martinez *et al.*, 2002). The disadvantage of this assay is that the reaction of the DPPH $^{\bullet}$ molecule with antioxidants is very slow and non-linear to DPPH concentration (Brand-Williams *et al.*, 1995). The advantage of the DPPH assay is that the DPPH molecule is more selective than $ABTS^{\bullet+}$ in its reaction with H-donors (Roginsky & Lissi, 2005).

The automated oxygen radical absorbance capacity (ORAC) assay was developed by Cao *et al.* (1997) using β -Phytoerthrin (β -PE) a protein isolated from *Porphyidium cruentum* as a probe. It was later modified by Ou *et al.* (2001) replacing β -PE with fluorescein (FL) (3',6'-dihydroxy-spiro[isobenzofuran-1[3H],9'[9H]-xanthene]-3-one). This method was used to measure the antioxidant radical scavenging activity of the extracts because unlike ABTS and DPPH assays it is a direct method that measures the antioxidant scavenging activity of an antioxidant against the biologically relevant peroxy radical induced by thermal decomposition of 2,2'-azobis (2-methyl-propionamide) dihydrochloride (AAPH) at 37 °C (Ou *et al.*, 2001; Madhujith & Shahidi, 2009). It is the only assay that combines both inhibition time and degree of inhibition into a single value (Madhujith & Shahidi, 2009), the ORAC value. The ORAC assay is a standardized method therefore results can be easily

compared across laboratories. However, the equipment needed is expensive (Awika *et al.*, 2003) which limits its availability in most laboratories (Macdonald *et al.*, 2006).

To investigate the potential health benefit due to antioxidant activity, it is necessary to further evaluate antioxidant activity in a relevant biological and/or cellular system or more complex animal models. The first level of testing is using simple biological systems that represent targets of oxidative damage. In this study the extracts were evaluated for the ability to protect erythrocyte membrane, and plasmid DNA against AAPH-induced oxidative damage and LDL against copper-induced oxidative damage. Erythrocytes were used as a model system for the evaluation of the protective effect of the aqueous extracts against free radical biomembrane oxidative damage because the membrane is rich in polyunsaturated fatty acids (Lanping, Zaiqun, Bo, Li & Zhongli, 2000) and therefore particularly susceptible to free radical-mediated lipid peroxidation (Manna *et al.*, 1999; Paiva-Martins, Fernandes, Santos, Silval, Borges, Rocha, Belo & Bogdanov, 2010). In this assay peroxy radicals generated by AAPH attack the RBC cell membrane by inducing lipid peroxidation and oxidation of membrane proteins (Takebayashi, Kaji, Ichiyama, Makino, Gohda, Yamamoto & Tai, 2007) and eventually causing haemolysis (Lanping *et al.*, 2000). The advantage of this method is its simplicity and the fact that from a small amount of blood sample many samples can be tested simultaneously. However, the challenge with this assay is acquiring fresh blood as blood older than three days was found to be no longer suitable for this assay. The disadvantage of this assay is poor repeatability when using different batches of blood because biologically originated substrates may contain different levels of endogenous chain breaking antioxidants such as vitamin E which may interfere with the assay (Roginsky & Lissi, 2005). To overcome this problem the same batch of blood was used for the two independent experiments.

AAPH-catalyzed single strand break of supercoiled circular plasmid vector pBR 322 DNA was used as an experimental model to evaluate the protective effect of the extracts against oxidative DNA damage. Peroxyl radicals generated by thermal decomposition of AAPH induce single and double strand breaks on supercoiled plasmid DNA resulting in relaxed circular DNA and linear DNA, respectively (Aronovitch *et al.*, 2007; Tang & Liu, 2008). Using agarose gel electrophoresis, the three forms of DNA are separated due to their differences in electrophoretic mobility (Aronovitch *et al.*, 2007). Some researchers have used DNA from calf thymus (Rivero-Pérez *et al.*, 2007) and others Caco-2 cells in the comet assay

(Bhat *et al.*, 2006) to evaluate the protective effect of antioxidants against free radical induced oxidative DNA damage. Using Caco-2 cells seems to be a more biologically relevant model system than using naked supercoiled plasmid DNA or DNA from calf thymus because *in vivo* antioxidants have to pass through the cell membrane, cytoplasm and then enter the nucleus and/or the mitochondria in order to protect DNA against oxidative damage. However, working with cell cultures is not as simple as with naked DNA. Besides using AAPH, oxidative DNA damage can be induced with hydroxyl radicals generated with hydrogen peroxide or copper. Generation of this radical using H₂O₂ and copper or iron is more biologically relevant because *in vivo* superoxide anion produced in the mitochondria is converted to hydrogen peroxide by superoxide dismutase which in turn is reduced to hydroxyl radical (OH[•]) which is catalyzed by transition metal (Turrens, 2003). The hydroxyl radical is the most highly reactive electrophile that can attack a variety of macromolecules including DNA causing oxidative damage (Sohal, 1997). Therefore the method can be improved by using H₂O₂ or Cu²⁺ or Fe²⁺ instead of AAPH which generate the peroxy radical which is involved in lipid peroxidation.

Copper-induced human LDL oxidation was used as an *in vitro* model to determine the protective effect of the aqueous extracts against LDL oxidation. The method is based on the induction of LDL oxidation with Cu²⁺ which leads to lipid peroxidation and decomposition of lipid peroxides to aldehydes and other compounds (Esterbauer *et al.*, 1992). The decomposition products or thiobarbituric reactive substances (TBARS) are then measured with the thiobarbituric assay (TBA assay). The TBA assay is the most frequently used method to assess the resistance of LDL to lipid oxidation (Schnitzer, Pinchuk, Bor, Fainaru, Samuni & Lichtenberg, 1998). It is also used to assess the degree of lipid peroxidation in LDL (Esterbauer *et al.*, 1992; Frankel, Bosanek, Meyer, Silliman & Kirk, 1998). However, the relevance of this *in vitro* LDL oxidizability test has been questioned because the test is done under strong oxidizing conditions and therefore it may have little relevance to initiation of atherogenesis which may occur at minimal oxidation conditions (Schnitzer *et al.*, 1998). A further criticism is that the assay does not account for the water soluble antioxidants such as vitamin C in serum or plasma. To overcome this problem the oxidation of LDL and formation of oxidation product has been monitored in plasma or serum after exposure to Cu (II) (Hodgson *et al.*, 1996; Schnitzer *et al.*, 1998). However, the use of plasma or serum may result in variable results depending on the nutritional status of the donor. This method requires isolation of LDL by preparative ultracentrifugation or use of expensive

commercially available isolated LDL which may be limiting factors in some laboratories. The method has also been criticized for low specificity and for the fact that heating of polyunsaturated fatty acids in hot acid, as applied in the TBA assay is very harsh and may result in autoxidation so TBARS may be formed during the assay itself. This was avoided by the addition of EDTA to chelate copper which catalyses this reaction (Esterbauer *et al.*, 1992).

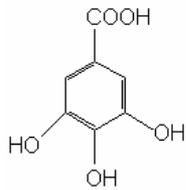
5.2. Discussion of study results

This study shows that the percentage yield of freeze-dried aqueous extracts from marama bean seed coats was significantly higher than that of equivalent extracts from condensed tannin sorghum bran. The total phenolic content, total flavonoid content and condensed tannin content of the extracts from marama bean seed coats were also significantly higher than that of equivalent extracts from condensed tannin sorghum bran. These findings suggest that marama bean seed coats may be a better raw material source for the extraction of phenolic compounds than condensed tannin sorghum (PAN 3860) bran. However, it is worth noting that during dehulling of the sorghum grain, components of the endosperm may contaminate the bran therefore affecting the phenolic content results. This may not be the case with marama as the seed coats are carefully and more easily separated by hand from the cotyledons.

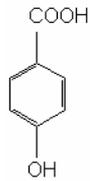
Phenolic compounds in aqueous extracts from marama bean seed coats were phenolic acids and flavonoids and the chemical structures are shown in Fig. 5.2.1. The phenolic acids identified in extracts from marama bean seed coat were benzoic acid derivatives and these have been reported in organic extracts from marama bean seed coats (Chingwaru *et al.*, 2011) with the exception of homogentisic acid. In this study gallic acid was the major phenolic acid and this finding was in agreement with Chingwaru *et al.* (2011).

Flavonoid compounds in aqueous extracts from marama bean seed coats found in this study were all flavonols esterified to gallic acid but these flavonoid compounds were not found in the study by Chingwaru *et al.* (2011). The differences could be due to differences in extraction solvents and methods used. In this study aqueous extraction was used and Chingwaru *et al.* (2011) used sequential hydrolysis in conjunction with organic solvent extraction.

Benzoic acid derivative

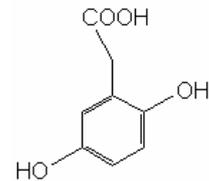


A) Gallic acid



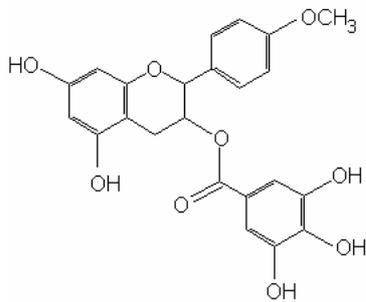
B) *p*-Hydroxybenzoic acid

Phenylacetic acid derivative

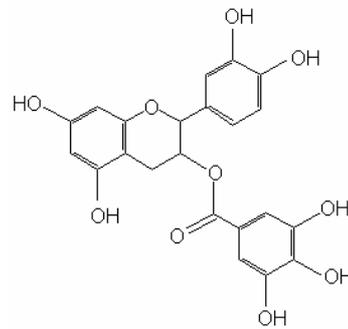


C) Homogentisic acid

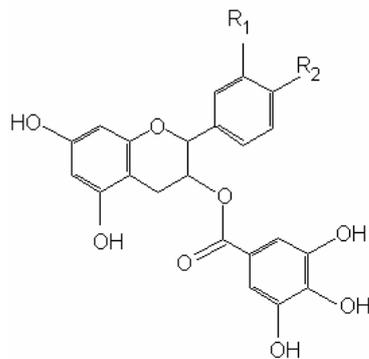
Flavanol



D) 4'-O-methyl (epi)afzelechin-3-O-gallate



E) (Epi)catechin-3-O-gallate



F)	R1	R2	Compound
	H	OCH ₃	4'-O- methyl (epi)catechin-3-O-gallate
	OCH ₃	H	3'-O- methyl (epi)catechin-3-O-gallate

Figure 5.2.1. Chemical structures of phenolic acid and flavonoid compounds identified in aqueous extracts from marama bean seed coats (Adapted from Harbone (1989), Wolfe and Liu (2008)).

However, these compounds may have been present in the organic extract prepared by Chingwaru *et al.* (2011) but due to lack of authentic standards and MS detection these compounds could not be identified.

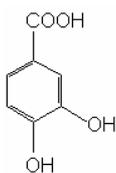
In this study it was not possible to distinguish between isomers using UV and MS detection without authentic phenolic compound standards. Methyl (epi)afzelechin-3-O-gallate (Fig. 5.2.1.D) had two isomers, with retention times of 16.7 and 20.5 min. These compounds could be methyl afzelechin-3-O-gallate and methyl epiafzelechin-3-O-gallate.

However, it was not possible to assign the compounds to their respective peaks. The chemical structure of methyl (epi)catechin-3-O-gallate (Fig. 5.2.1F) could not be deduced from the mass spectra data because it was not possible to locate the position of the methyl group. Therefore this compound could be one of four possible compounds from the fact that each of the possible compounds 3'-O-methyl (epi)catechin-3-O-gallate and 4'-O-methyl (epi)catechin-3-O-gallate may have two isomers each. (Epi)catechin-3-O-gallate had two isomers with retention times of 22.6 and 22.9 min. The first retention time matched that of (-)-epicatechin-3-O-gallate and therefore the compound in the second peak could be (+)-catechin-3-O-gallate.

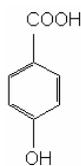
In the absence of authentic phenolic standards nuclear magnetic resonance (NMR) techniques could be used to differentiate between isomers especially when analysing an extract with unknown compounds.

Phenolic compounds found in aqueous extracts from sorghum bran were phenolic acids, phenolic aldehydes and flavonoids and the structures are shown in Fig. 5.2.2. In contrast to aqueous extracts from marama bean seed coats, phenolic acids in aqueous extracts from sorghum bran comprised both benzoic and cinnamic acid derivatives which have been previously identified in organic solvent extracts from sorghum grain (Hahn *et al.*, 1983; Awadelkareem, Muralikrishna, EL Tinay & Mustafa, 2009; Svensson *et al.*, 2010). Also the phenolic aldehyde compounds, protocatechualdehyde and *p*-hydroxybenzaldehyde as well as the benzoic acid derivative caffeoylglycerol identified in this study have all been previously identified in organic solvent extracts from sorghum grain (Svensson *et al.*, 2010). Flavonoid compounds were all from the flavanone subclass and have been previously identified in extracts from sorghum grain (Svensson *et al.*, 2010).

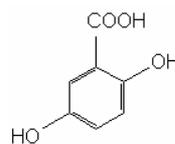
Benzoic acid derivative



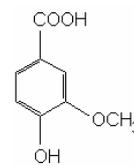
A) Protocatechuic acid



B) *p*-Hydroxybenzoic acid

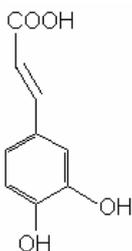


C) Gentisic acid

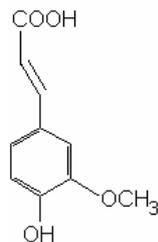


D) Vanillic acid
(Minor)

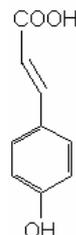
Cinnamic acid derivatives



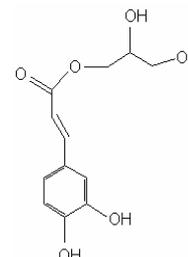
E) Caffeic acid



F) Ferulic acid

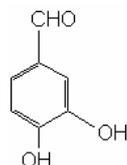


G) *p*-Coumaric acid (Minor)

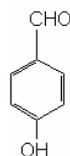


H) Caffeoylglycerol

Phenolic aldehydes

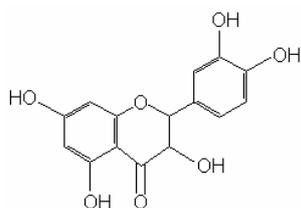


I) Protocatechualdehyde

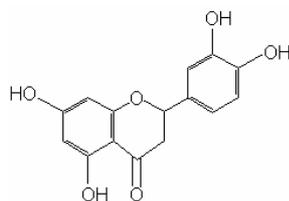


J) *p*-Hydroxybenzaldehyde

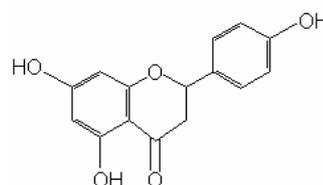
Flavanone



K) Taxifolin



L) Eriodictyol



M) Naringenin

Figure 5.2.2. Chemical structures of phenolic compounds identified in aqueous extracts prepared from condensed tannin sorghum bran (Adapted from Harbone (1989) and Manach *et al.*, 2004).

The findings of this study show that the method used to characterize phenolic compounds in the extracts was able to identify a wide range of compounds; phenolic acids (both benzoic acid and cinnamic acid derivatives), phenolic aldehydes and flavonoids. The use of MS detection made it possible to distinguish between compounds that had similar retention times. Caffeoylglycerol and vanillic acid had similar retention times. However, with MS detection it was possible to show that the main compound was caffeoylglycerol with the most abundant [M-H]⁻ molecular ion at m/z 253.7 (100) and vanillic acid was the minor compound with [M-H]⁻ molecular ion at m/z 167.6 (60). Gentisic acid and *p*-coumaric acid also had similar retention times and it was possible to show that gentisic acid was the main compound with the most abundant [M-H]⁻ molecular ion at m/z 153.5 (100) and that *p*-coumaric acid was the minor compound with [M-H]⁻ molecular ion at m/z 163.4 (50). These findings indicate that the concentration of both caffeoylglycerol and gentisic acid may have been overestimated by coelution with vanillic acid and *p*-coumaric acid, respectively.

Overall, extracts from marama bean seed coats had higher concentration of phenolic compounds than equivalent extracts from condensed tannin sorghum bran which again suggests that marama bean seed coats may be a better raw material source of phenolic compounds compared to condensed tannin sorghum bran.

Extraction under aqueous acidic condition of marama bean seed coats resulted in significantly lower percentage yield of freeze-dried acidified water extract compared to extraction with water only. It also resulted in significantly lower total phenolic content, flavonoid content and condensed tannin content of acidified water extracts compared to water extracts from marama bean seed coats as measured with spectrophotometric methods. HPLC-MS method also showed a significant reduction in overall concentration of individual phenolic compounds in acidified water extracts from marama bean seed coats compared to water extracts. In contrast, acidic condition resulted in significantly higher percentage yield of freeze-dried acidified water extracts compared to freeze-dried water extracts from condensed tannin sorghum bran. The total phenolic content of acidified water extracts from condensed tannin sorghum bran was significantly higher than that of water extracts but there were no significant differences in flavonoid and condensed tannin content as measured with spectrophotometric methods. The HPLC-MS results also show that generally the concentration of many of the phenolic compounds in acidified water extracts were higher than water extracts from condensed tannin sorghum bran.

The lower recovery of phenolic compounds in acidified water extract from marama bean seed coats was unexpected because under acidic conditions phenolic compound content would be expected to increase as acidic conditions enhance extractability of free and esterified phenolic compounds and also hydrolyse and release bound phenolics (Liyana-Pathirana & Shahidi, 2005) as stated in the hypotheses (Chapter 3, section 3.3.1). From this observation, it may be hypothesised that condensed tannins of marama bean seed coats may be chemically different from those of condensed tannin sorghum bran. Condensed tannins from marama bean seed coat may interact with cell wall polysaccharides such as arabinoxylans and pectins (Naczek & Shahidi, 2004) to form an insoluble interpolymer complex precipitate (Hanlin, Hrmova, Harbertson & Downey, 2010) and also co-precipitate other phenolic compounds at low pH resulting in an extract with lower phenolic content. However, extraction of sorghum bran under acidic conditions resulted in enhanced extractability of free and esterified phenolic compounds and release of phenolic compounds from bound forms (Liyana-Pathirana & Shahidi, 2005). These findings suggest that extraction of condensed tannin sorghum bran under acidic conditions may be the preferred method to increase phenolic content yield but not for marama bean seed coats.

An important finding in this study is that proanthocyanidins in extracts from marama bean seed coats were predominantly prodelphinidins, while those in extracts from condensed tannin sorghum bran were procyanidins as has previously been reported (Gu, Kelm, Hammerstone, Beecher, Holden, Haytowitz & Prior, 2003b). Proanthocyanidins in other legumes are mainly procyanidins (Dueñas *et al.*, 2006). Small amounts of properlagonidins in common beans (Beninger *et al.*, 2005) and prodelphinidins in lentils (Dueñas *et al.*, 2003) have also been reported.

Therefore this makes proanthocyanidins in marama bean seed coats different from those of other legumes. Based on the constitutive unit composition, the chemical structure of proanthocyanidins from marama bean seed coats appear to be a heteropolyflavan polymer structure (Fig. 5.2.3). This structure is highly galloylated consisting of 66–77% of (epi)gallocatechin-3-O-gallate and (epi)catechin-3-O-gallate constitutive units in the oligomer and polymer fractions.

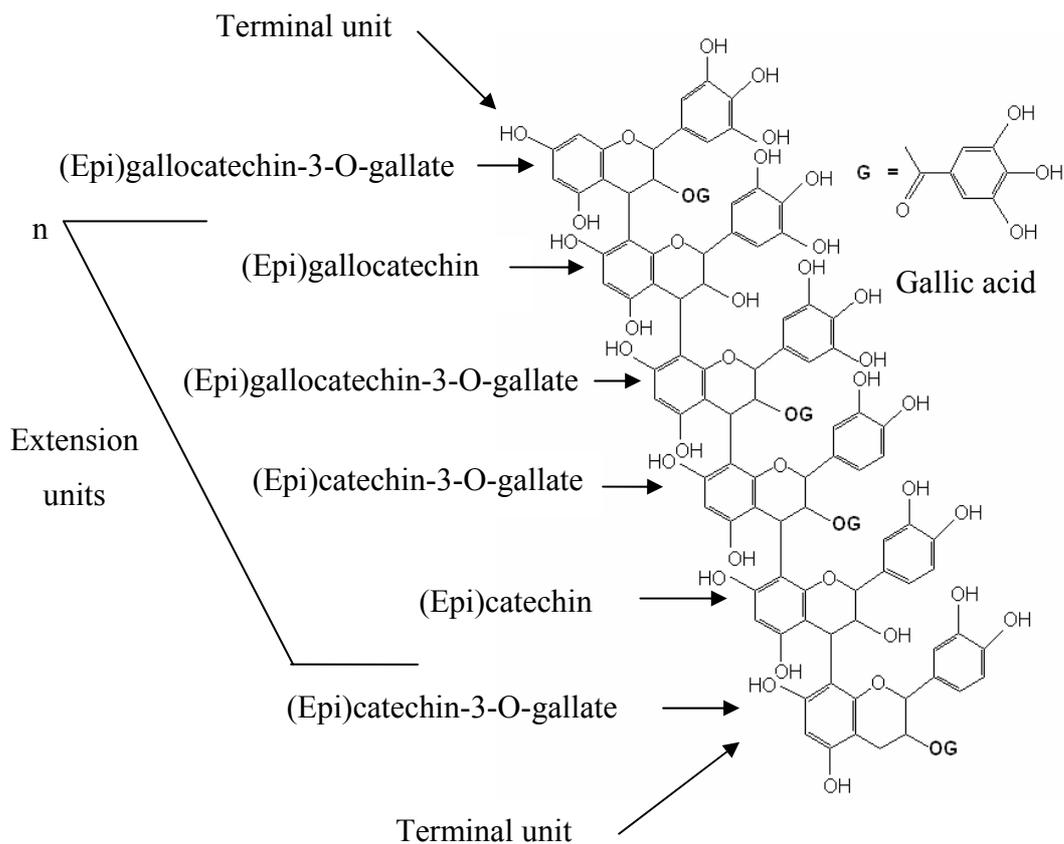


Figure 5.2.3. Proposed heteropolyflavan polymer structure of proanthocyanidins in extracts from marama bean seed coats, $1 < n < 8$ (Adapted from Yousef *et al.* (2006)).

The polymer structure of proanthocyanidins from sorghum bran was composed mainly of epicatechin extension units accounting for 67–84% and catechin terminal units accounting for 16–33% of total constitutive units in the oligomer and polymer fractions (Fig. 5.2.4). Gu *et al.* (2003b) reported that catechin terminal units accounted for 9% and epicatechin as extension and terminal units accounted for 91%. The higher percentage of terminal units versus extension units found in this study compared to the reported composition could be due to differences in extraction conditions. Extraction under aqueous condition resulted in extraction of shorter polymers with a lower mDP which ranged from 3.0 to 5.5 for oligomer and polymer fractions, respectively compared to 8.4 for proanthocyanidins extracted with aqueous acetone as reported by Gu *et al.* (2003b).

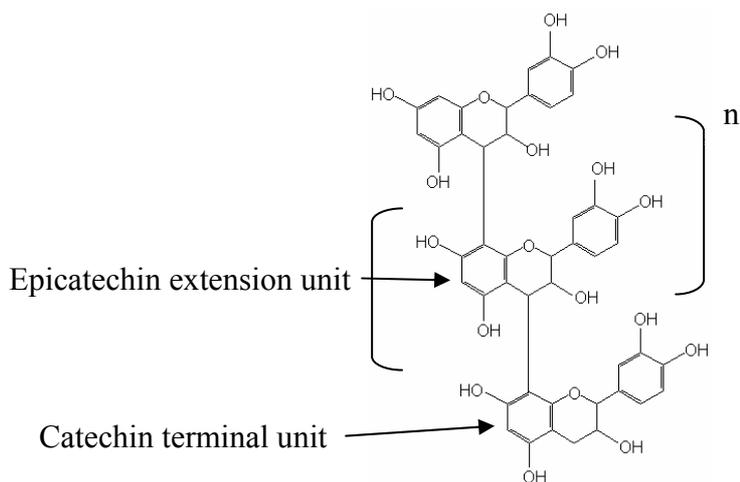


Figure 5.2.4. Chemical structure of proanthocyanidin polymer in extracts from condensed tannin sorghum bran, $1 < n < 5$ (Adapted from Hagerman *et al.* (1998) and Awika and Rooney (2004))

The observed differences in proanthocyanidin structure of marama bean seed coat and condensed tannin sorghum bran, allows for an explanation for the observed differences in yield, total phenolic content, phenolic compound concentration, condensed tannin content and protein precipitation capacity results. The presence of galloylated units in marama bean seed coat proanthocyanidins increases the number of hydroxyl groups in the chemical structure. Therefore the polymer may interact more with cell wall polysaccharides through hydrogen bonding of hydroxyl groups on A- or B-ring and on the gallic acid moieties with oxygen, hydroxyl and acetyl groups of polysaccharides and also possibly through hydrophobic interaction (Hanlin *et al.*, 2010) at low pH (Fig. 5.2.5). This could result in the formation of an insoluble interpolymer complex precipitate which could co-precipitate phenolic acid and flavonoid compounds. This could then lead to reduction in total phenolic content, total flavonoid content, phenolic compounds concentration, condensed tannin content and protein precipitation capacity of acidified water extracts from marama bean seed coats. Similar complexation of highly galloylated condensed tannins from persimmon fruit with pectin in solution has been reported (Taira, Ono & Matsumoto, 1997).

The proanthocyanidins from condensed tannin sorghum bran have comparatively less hydroxyl groups and therefore may interact less with other high polymer species at low pH.

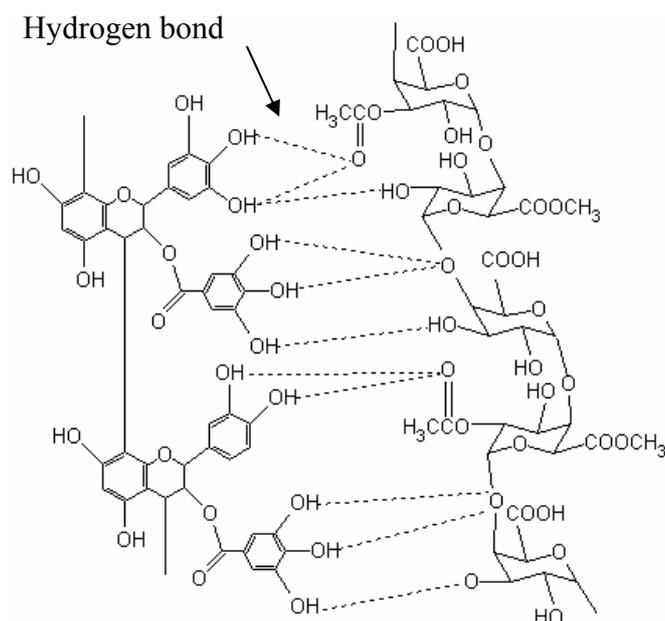


Figure 5.2.5. Hydrogen bonding between highly galloylated condensed tannins and cell wall polysaccharides to form interpolymer complex precipitate at low pH (Adapted from Hanlin *et al.* (2010))

Based on this, it may be hypothesized that the nature of proanthocyanidin polymeric species from condensed tannin sorghum bran did not have any major influence on the recovery of phenolic compounds and only the acidic conditions resulted in increased recovery of phenolic compounds of acidified water extracts from condensed tannin sorghum bran.

The phenomenon of co-precipitation of low molecular weight compounds with interpolymer complex precipitate has been demonstrated in water and waste water treatment experiments. Tannins were demonstrated to be good coagulant aid in water treatment experiment (Özacar & Şengil, 2002). In another experiment, tannins together with chitosan (a biopolymer) were shown to be good coagulants/flocculants for the removal of ink through co-precipitation with interpolymer precipitate in waste water treatment (Roussy, Chastellan, van Vooren & Guibal, 2005). Therefore proanthocyanidins from marama bean seed coats may find use in water and waste water treatment plants as natural coagulants/flocculants biopolymers.

Antioxidant activities of aqueous extracts from marama bean seed coats as measured by the ABTS, DPPH and ORAC assays were higher than those of equivalent extracts from condensed tannin sorghum bran. This suggests that aqueous extracts from marama bean seed coats may provide better protective effect against free radical oxidative damage. The higher antioxidant activity of extracts from marama bean seed coats could be attributed to the higher concentration of galloylated phenolic compounds compared to equivalent extracts from condensed tannin sorghum bran. Amongst the phenolic acids, gallic acid (Fig. 5.2.1A) appeared to have contributed significantly to the antioxidant activity of the extracts because of its higher concentration and pyrogallol structure which make it a more potent antioxidant than *p*-hydroxybenzoic acid and homogentisic acid which lack the pyrogallol or catechol structure (Moran *et al.*, 1997; Fukumoto & Mazza, 2000). The flavonoid compounds (Fig. 5.2.1) were also potent antioxidants due to esterification to gallic acid which adds more hydroxyl groups which results in increased antioxidant activity (Rice-Evans *et al.*, 1996). Methyl (epi)afzelechin-3-O-gallate and methyl (epi)catechin gallate were the main flavonoid phenolic compounds found in extracts from marama bean seed coats. However, they are weaker antioxidants than (epi)catechin-3-O-gallate due to the absence of the 3',4'-*o*-dihydroxyl groups (Wolfe & Liu, 2008).

Extracts from condensed tannin sorghum bran had a wider range of phenolic acids (Fig. 5.2.2) compared to extracts from marama bean seed coats. Some of the phenolic acids such as the cinnamic acid derivatives are potent antioxidant due to the presence of a conjugated double bond next to the phenyl ring (Rice-Evans *et al.*, 1996). The phenolic compounds, protocatechuic acid, caffeic acid and caffeoylglycerol have the catechol structure therefore may have contributed significantly to the antioxidant activity of the extracts from condensed tannin sorghum bran. However, these compounds occurred at low concentrations. The flavonoid compounds (flavanones) in the extracts from condensed tannin sorghum bran are potent antioxidants mainly because all have the 4-keto group and 5-hydroxyl group, taxifolin and eriodictyol also have the 3',4'-*o*-dihydroxyl groups and taxifolin also has the 3-hydroxyl group which are important groups for antioxidant activity (Rice-Evans *et al.*, 1996). The flavanones with the 4-keto group and either a 5-hydroxyl or 3-hydroxyl group are effective chelators of transition metals which catalyses oxidative chain reactions (Wolfe & Liu, 2008).

The highly galloylated condensed tannins in extracts from marama beans seed coats (Fig. 5.2.3) are more effective antioxidants than condensed tannins in extracts from condensed

tannin sorghum bran (Fig. 5.2.4) because of the additional hydroxyl groups (Hagerman *et al.*, 1998). According to Rice-Evans *et al.* (1996) the antioxidant activity of flavanols is more dependant on the number of hydroxyl groups on the structure as there is no electron delocalization between A and B rings due to the saturation of the heterocyclic ring.

The findings of this study demonstrated that aqueous extracts from marama bean seed coats and condensed tannin sorghum bran could potentially protect biomolecules against oxidative stress. Oxidative stress is caused by various reactive oxygen species such as superoxide anion, singlet oxygen, hydrogen peroxide, peroxynitrite anion, peroxy radical and the highly reactive hydroxyl radical (Waris & Ahsan, 2006). The pathway for the formation of these radicals and how these could initiate events leading to the development of chronic diseases is shown in Fig. 5.2.6. The superoxide anion radical is generated primarily during oxidative phosphorylation in the mitochondria (Sohal, 1997) and also via a number of enzymatic processes within the cell, such as xanthine oxidase and NADH oxidases (Turrens, 2003). This radical is quickly dismutated to hydrogen peroxide (H_2O_2) by a family of metalloenzymes called superoxide dismutases or it may react with nitric oxide ($NO\bullet$) produced by the breakdown of arginine to citrulline by nitric oxide synthesis to generate the peroxynitrite radical ($ONOO\cdot$) (Turrens, 2003). H_2O_2 is normally eliminated by conversion to water by the enzymes catalase and glutathione peroxidase (Waris & Ahsan, 2006). If H_2O_2 is not eliminated it is converted to the highly reactive electrophile, the hydroxyl free radical ($HO\cdot$) through the Fenton reaction catalysed by transition metals such as copper and iron (Sohal, 1997). The $HO\cdot$ radical reacts rapidly with any molecule within its vicinity such as DNA, membrane lipids, and carbohydrates (Reiter, 1998). The reaction of this radical with polyunsaturated fatty acids initiates lipid peroxidation, a self propagating chain reaction. The presence of transition metals such as iron catalyses the decomposition of lipid peroxides to peroxy and alkyl radicals which may abstract H^+ from lipids leading to further lipid breakdown (Reiter, 1998).

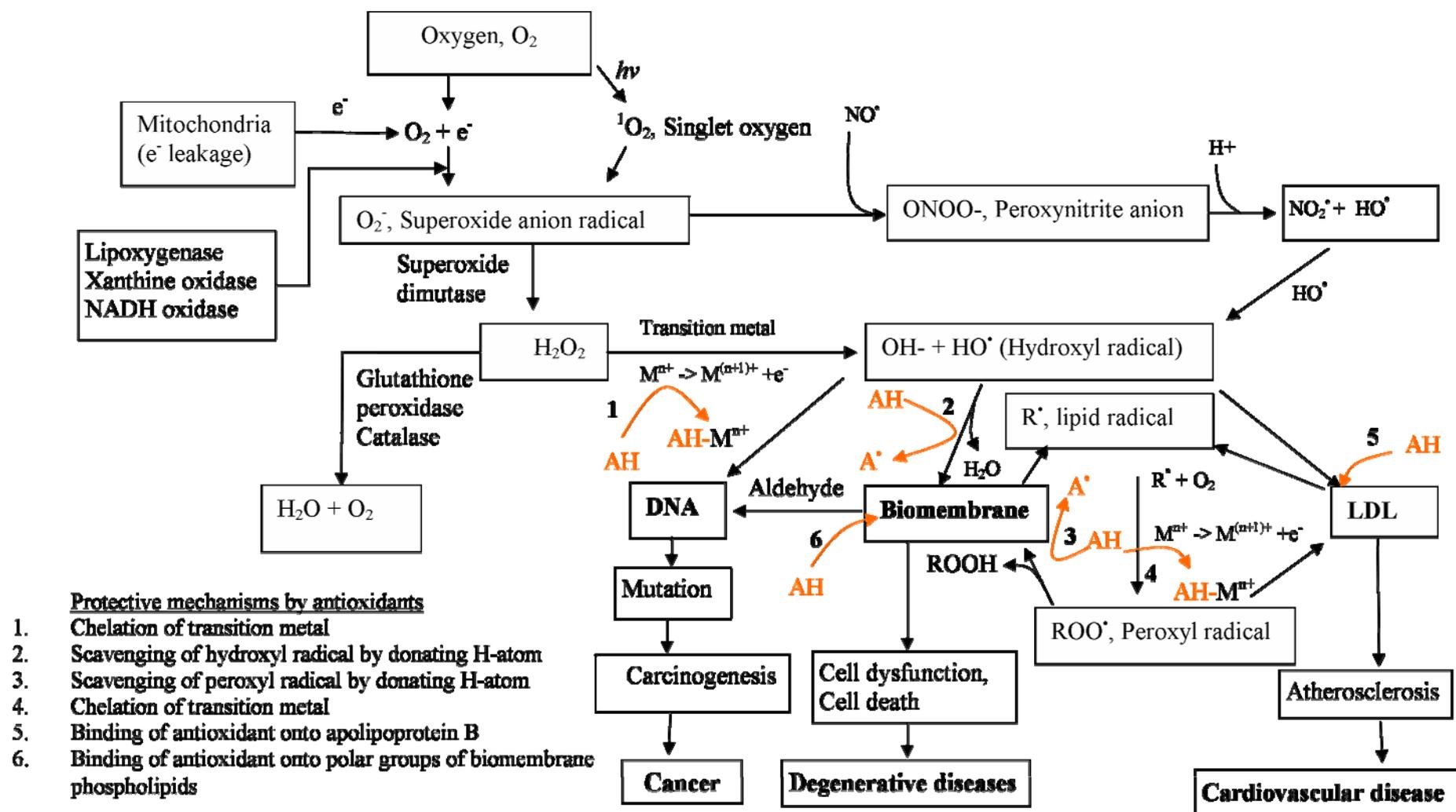


Figure 5.2.6. Mitigation of oxidative stress caused by reactive oxygen species by antioxidants shown as 1 to 6. AH, antioxidant and A[•], oxidized antioxidant. (Adapted from Esterbauer *et al.* (1992), Reiter (1998) and Waris & Ahsan (2006))

The effects of the extracts on induced oxidative damage of erythrocytes, DNA and LDL as used as biomarkers of oxidative stress in this study is summarized in Fig. 5.2.7. Aqueous extracts from marama bean seed coats and condensed tannin sorghum bran protected against AAPH-induced erythrocyte haemolysis. This model system demonstrated that these extracts could potentially protect against biomembrane oxidative damage. Extracts from marama bean seed coats were effective at lower concentrations than equivalent extracts from condensed tannin sorghum bran because of their higher phenolic content, highly galloylated phenolic compounds and antioxidant activity. Phenolic compounds in the extracts may protect against biomembrane oxidative damage by scavenging chain-propagating lipid peroxy radicals (Fig. 5.2.6, (3)) (Terao *et al.*, 1994) and/or prevent access of oxidants to the bilayer thereby limiting the propagation of lipid oxidation in the hydrophobic region of the membrane (Fig. 5.2.6, (6)) (Verstraeten *et al.*, 2003). Lipid peroxidation initiated by reactive oxygen species is implicated in neurological disorders, degenerative (Adibhatla & Hatcher, 2010) and cardiovascular disease and the aging process (Lim, Cheung, Ooi & Ang, 2002). Besides membrane damage lipid peroxidation may lead to production of aldehyde products which may covalently bind to proteins through reaction with thiol groups and therefore altering their function (Adibhatla & Hatcher, 2010). Aldehyde compounds may also diffuse to the cell nucleus and form adducts with DNA therefore activating mutagenic events associated with carcinogenesis (Cejas, Casado, Belda-Iniesta, Castro, Espinosa, Redondo, Sereno, García-Cabezas, Vara, Domínguez-Cáceresi, Perona & González-Barón, 2004).

In this study extracts from condensed tannin sorghum bran showed some protective effect against AAPH-induced supercoiled plasmid (pBR 322) DNA damage, (Fig. 5.2.7). This demonstrated the potential of the extracts to protect against free radical oxidative DNA damage which may lead to mutations and altered gene expression that may lead to the development of cancer (Lappara *et al.*, 2008). Other studies have shown similar protective effect of extracts and isolated phenolic compounds against oxidative DNA damage. Red wines showed protective effect against copper-catalysed oxidative calf thymus DNA damage (Rivero-Pérez *et al.*, 2007). Similarly free, soluble conjugated and insoluble-bound phenolic extract fractions from barley were reported to inhibit DNA strand scission (Madhujith & Shahidi, 2009). Chlorogenic acid protected DNA against AAPH induced oxidative damage (Tang & Liu, 2008). The protective effect may be through free radical scavenging activity (Fig. 5.2.6 (2)) and chelation of transition metals (Fig. 5.2.6 (1)) (Aherne & O'Brien, 2000).

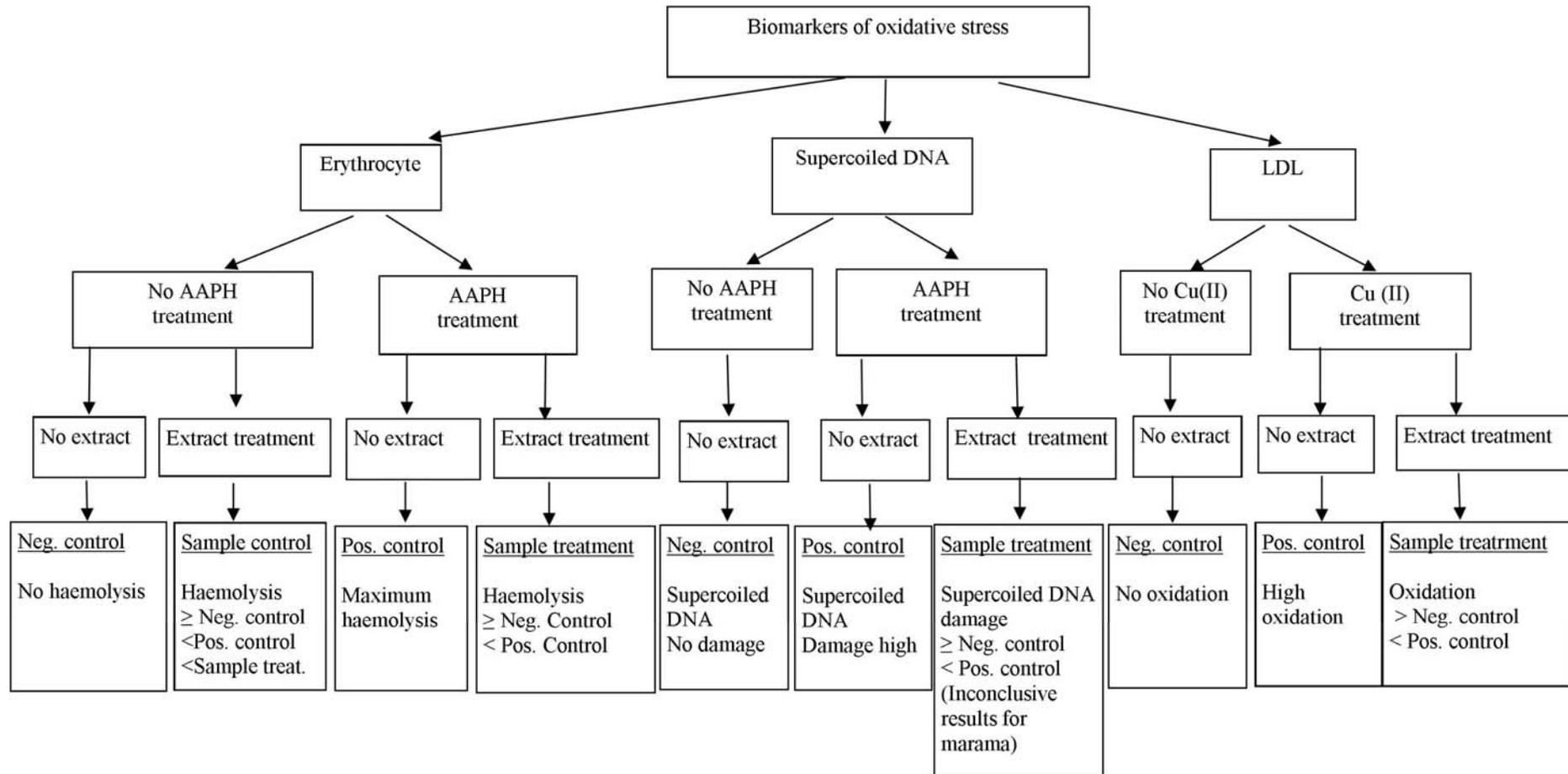


Figure 5.2.7. Summary of the effects of aqueous extracts from marama bean seed coats and condensed tannin sorghum bran against induced oxidative damage of biomembrane, DNA and LDL as biomarkers of oxidative stress

Extracts from marama bean seed coats did not show protective effect against free radical oxidative DNA damage, however these results were inconclusive. It appears that the high levels of galloylated condensed tannins present in the extracts interacted with DNA to form complexes. This resulted in DNA precipitation in the wells, poor DNA migration through the agarose gel and lower intensities of relaxed circular and supercoiled DNA bands thereby affecting the results of the experiment. Tannins have been reported to interfere with the isolation of DNA and RNA from cells of plants with high levels of condensed tannins. Maliyakal (1992) reported that it was notoriously difficult to isolate DNA and RNA from plant cells because of tannins binding to RNA and DNA upon cell lyses contaminating the DNA or RNA, therefore making it useless for research work. Tannins have been shown to bind to calf thymus DNA and this binding has been reported to be facilitated by the high number of hydroxyl groups on the tannin structure and high molecular size of tannins giving rise to greater hydrophobic interaction (Labieniec & Gabryelak, 2006). Similarly, the galloylated condensed tannins in marama bean seed coats may bind DNA and thereby interfere with the results of the experiment. This interference can be avoided by removing the tannins in the extracts by solid phase extraction. Only phenolic acid and flavonoid compounds would remain in the extracts. This may be more representative of *in vivo* effects as condensed tannins (except low molecular weight proanthocyanidins such as dimers) in their intact form are not absorbed into circulation (Rios, Gonthier, Rémésy, Mila, Lapiere, Lazarus, Williamson & Scalbert, 2003). Only phenolic acid and flavonoid compounds and their metabolites would reach inner tissues and cells to exert beneficial effects.

The findings of this study indicate that the presence of extracts from marama bean seed coats and condensed tannin sorghum bran inhibited copper-catalyzed human LDL oxidation (Fig. 5.2.7). Extracts from marama bean seed coats were more effective at lower concentration compared to extracts from condensed tannin sorghum bran because of the higher levels of phenolic content and antioxidant activity. The protective effect may be through scavenging of hydroxyl radicals (Fig. 5.2.6 (1)), peroxy radicals (Fig. 5.2.6 (3)) (Abuja *et al.*, 1998), chelation of transition metals (Fig. 5.2.6 (1 and 4)), and also by interaction of phenolic compounds with apolipoprotein B particle (Fig. 5.2.6 (5)) preventing binding of copper ion onto the particle (Rüfer & Kulling, 2006).

This study has shown that the aqueous extracts from both marama bean seed coats and condensed tannin sorghum bran contain phenolic compounds that have the potential to

protect biological molecules against oxidative damage. This may be through free radical scavenging activity, metal chelation and binding of antioxidant compound onto the surface of biological molecules such as biomembrane and LDL and thereby preventing access of deleterious free radical species as depicted in Fig. 5.2.6. The results of this study therefore suggest that aqueous extracts from marama bean seed coats and condensed tannin sorghum bran have a potential to inhibit oxidative stress, which is implicated in the development of various chronic diseases.

CHAPTER 6

6. CONCLUSIONS AND RECOMMENDATIONS

Aqueous extracts from marama bean seed coats have higher phenolic compound content therefore may be a better material source for the extraction of antioxidant phenolic compounds than bran of condensed tannin sorghum variety PAN 3860. Phenolic acid and flavonoid compound profiles of the two sources are different. Phenolic acids in extracts from marama bean seed coats are benzoic acid and phenylacetic acid derivatives, and flavonoid compounds are flavanols esterified to gallic acid. Gallic acid and methyl (epi)afzelechin-3-O-gallate are the major phenolic acid and flavonoid compounds, respectively. In extracts from condensed tannin sorghum bran phenolic acids are both benzoic and cinnamic acid derivatives, and flavonoid compounds are flavanones. *p*-Hydroxybenzoic acid and naringenin are the major phenolic acid and flavonoid compounds, respectively.

Proanthocyanidins in extracts from marama bean seed coats are predominantly prodelphinidins and those in extracts from condensed tannin sorghum bran are procyanidins. The prodelphinidins in marama bean seed coats are highly galloylated which increases the number of hydroxyl groups in the polymer structure and therefore interact to a greater degree with other high molecular weight polymers through hydrogen bonding and hydrophobic interactions to form interpolymer complex and tannin-protein complex precipitates.

Extraction of marama bean seed coats under acidic condition results in an extract with lower phenolic compound content, antioxidant activity and lower protective effect against biomembrane oxidative damage. In contrast, extraction of condensed tannin sorghum bran under acid condition results in an extract with higher phenolic content and protective effect against biomembrane oxidative damage. Extracts from condensed tannin sorghum bran show some protective effect against oxidative DNA damage, however, extracts from marama bean seed coats give inconclusive results because of the high levels of galloylated condensed tannins which bind to DNA thus affecting the results. It is recommended that solid phase extraction should be used to remove the condensed tannins from the extracts prior to use in this assay.

Extracts from marama bean seed coats are effective at lower concentrations in protecting against free radical LDL oxidation than equivalent extracts from bran of condensed tannin sorghum variety PAN 3860 because of their higher phenolic content. The TBA assay does not show a dose-response effect because TBARS are measured at the end of the incubation period. It is recommended that samples be taken at specific time intervals during the incubation period to measure TBARS. Extraction under acid condition is recommended for condensed tannin sorghum bran but not for marama bean seed coats. These findings indicate that the first and second hypotheses (Chapter 3, section 3.1) seem to hold true for condensed tannin sorghum bran but not for marama bean seed coat.

To further investigate the antioxidant effects of these extracts the use of cell lines such as the Caco-2 cell line can be considered. The Caco-2 cell line is considered as a physiologically relevant cell line as it represents cells of the colon. This cell line can be used to determine the cellular uptake and protective effects of the extracts against oxidative damage. Animal studies using rats or mice should also be done to investigate the absorption, distribution, metabolism and excretion and possible toxicity of the extracts.

The extracts may be added to food as functional antioxidant food ingredients in order to increase the potential health benefits. However, it is recommended that the effect of the extracts on protein digestibility and sensory quality of food products be studied in view of the condensed tannin content and protein precipitation capacity of the extracts, especially extracts from marama bean seed coats.

In conclusion, the aqueous extracts have a potential to reduce oxidative stress implicated in the development of chronic diseases such as neurodegenerative diseases, cancer and cardiovascular diseases.