Phenolic compounds in aqueous extracts of marama bean [*Tylosema esculentum* (Burchell) A. Schreiber] seed coat, sorghum (*Sorghum bicolor* L. Moench) bran and their bioactive properties

By

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DECLARATION

I Jeremiah S. Shelembe declare that the thesis, which I hereby submit for the degree PhD Food Science at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

SIGNATURE:.....

DATE: May 2012

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ABSTRACT

Phenolic compounds in aqueous extracts of marama bean [*Tylosema esculentum* (Burchell) A. Schreiber] seed coat, sorghum (*Sorghum bicolor* L. Moench) bran and their bioactive properties

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The marama bean is an underutilised legume growing wild in the arid and semi arid regions of Southern Africa. Sorghum is an important cereal crop in arid regions of the world. The seed coats of marama beans and bran of sorghum contain antioxidant phenolic compounds with potential health benefits. Aqueous extracts for possible use as antioxidant functional food ingredient were prepared from seed coats of marama beans and bran of condensed tannin sorghum by extracting with water (water extract) or with water acidified to pH 2 (acidified water extract). Aqueous extraction was used in order to obtain an extract free from organic solvents, safe for human consumption, and containing compounds representative of those that are available under aqueous conditions of the gastrointestinal tract. The extracts were analysed for total phenolic content, total flavonoid content, condensed tannin content and protein precipitation capacity using spectrophotometric methods and for individual phenolic compounds using HPLC-MS. Proanthocyanidins were characterised using thiolysis degradation in conjunction with HPLC-MS. The antioxidant activities of the extracts were measured using ABTS, DPPH and ORAC assays. Extracts were also evaluated for protective effect against free radical induced human erythrocytes haemolysis, oxidative DNA damage and human LDL oxidation.

Extracts from marama bean seed coats had significantly higher total phenolic content, total flavonoid content, condensed tannin content, protein precipitation capacity and phenolic compound concentration than equivalent extracts from condensed tannin sorghum bran. Three phenolic acids and three flavonols esterified to gallic acid were identified in the extracts from marama bean seed coats. Extracts from condensed tannin sorghum bran had six major phenolic acids, two phenolic aldehydes and three flavanones. Proanthocyanidins in extracts from marama bean seed coats were predominantly highly galloylated prodelphinidins while those in extracts from condensed tannin sorghum bran were procyanidins.

Extracts from marama bean seed coats had higher antioxidant activity and protective effects against free radical induced erythrocyte haemolysis and LDL oxidation compared to equivalent extracts from condensed sorghum bran. Extracts from condensed tannin sorghum bran showed some protective effect against oxidative DNA damage. However, extracts from marama bean seed coats gave inconclusive results probably due to prodelphinidins binding to DNA. Extraction of marama bean seed coats under acidic condition resulted in reduction in phenolic compound content, antioxidant activity and protective effect against erythrocyte haemolysis, possibly due to co-precipitation of phenolic compounds with interpolymer complex precipitate formed between highly galloylated condensed tannins and cell wall polysaccharides at pH 2. In contrast, extraction of condensed tannin sorghum bran under the acidic condition resulted in an extract with significantly higher phenolic content and protective effect against erythrocyte haemolysis than the water extract possibly due to enhanced extraction of free and esterified phenolics and release of bound phenolic compounds. Water extracts and acidified water extracts (from marama been seed coats or sorghum bran) did not show significant differences in their protective effect against oxidative DNA damage and LDL oxidation.

Extraction under acidic condition may be the preferred method for sorghum bran because it increases recovery of phenolic compounds, but not for marama bean seed coats because it causes reduction in phenolic compound content. The findings of this study show that the extracts have a potential to reduce oxidative stress which is implicated in many chronic diseases such as neurodegenerative diseases, cancer and cardiovascular disease. The extracts can be used in the development of functional foods with potential health benefits.

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CHAPTER 1

1. INTRODUCTION

The marama bean, [*Tylosema esculentum* (Burchell) Schreiber] is a tuberous legume plant native to the Kalahari region of Southern Africa (Mitchel, Keys, Madgwick, Parry & Lawlor, 2005). It is a long-lived perennial well adapted to the arid regions in Botswana, Namibia and South Africa (Hartley, Tshamekeng & Thomas, 2002) but also occurs in Angola, Zambia and Mozambique (Holse, Husted & Hansen, 2010). The bean has been an important food source for the people of the Kalahari Desert for centuries (Bower, Hertel, Oh & Storey, 1988) and a staple food for the Basarwa people of Kgalagadi, Botswana (Amarteifio & Moholo, 1998). Traditionally, marama beans are gathered by hand from the wild, roasted in hot sand and the cotyledons eaten as a snack (Amarteifio & Moholo, 1998; Holse *et al.*, 2010). The beans may also be ground and made into porridge after roasting or may be boiled and eaten as other beans before they are fully ripe (Holse *et al.*, 2010). Roasting or cooking render the bean cotyledon more palatable and significantly reduces trypsin inhibitor activity (Powel, 1987; Bower *et al.*, 1988; Maruatona, Duodu & Minnaar, 2010).

Generous consumption of whole grain on a regular basis has been linked to reduced risk of a number of chronic diseases (Liyana-Pathirana & Shahidi, 2005; Chen & Blumberg, 2008). Consumption of legumes, soybean in particular has been linked to reduced risk of coronary heart disease (Bazzano, He, Ogden, Loria, Vupputuri, Myers & Whelton, 2001). The physiological effects are attributed to the presence of abundant phytochemicals including phenolics (Cardador-Martinez, Loarca-Pina & Oomah, 2002). Phenolic compounds are non-nutritive micro-constituents having various biological functions, such as anti-oxidative, anti-inflammatory, anti-mutagenic (Suda, Ishikawa, Hatakeyama, Miyawaki, Kudo, Hirano, Ito, Yamakawa & Horiuchi, 2008; Cardador-Martinez *et al.*, 2002), anti-carcinogenic, anti-glycaemic, cholesterol lowering and antimicrobial activity (Im, Suh, Lee, Kozukue, Ohnis-Kameyama, Levin & Friedman, 2008).

It is considered important to increase antioxidant intake in the human diet, and one way of achieving this is by enriching food with phenolics extracted from natural sources (Ali-Farsi &

Lee, 2008). Seeds can be a valuable source of phenolics (Ali-Farsi & Lee, 2008) because of their phenolic content with potential health benefits (Soong & Barlow, 2005). Isolation and preparation of bioactive compounds from edible and non-edible plant tissue such as seed coats of moth beans (Siddhuraju, 2006) and sunflower kernels and shells (Weisz, Kammerer & Carle, 2009) to serve as potent natural antioxidants for industrial use has been suggested. Seed coats of legumes such as the common bean (Rannilla, Genovese & Lajolo, 2007), soybean (Xu & Chang, 2008), lentils and peas (Madhujith & Shahidi, 2005) have been shown to contain phenolic compounds. Marama bean seed coats have also been found to contain phenolic acid and flavonoid compounds (Chingwaru, Duodu, van Zyl, Schoeman, Majinda, Yeboah, Jackson, Kapewangolo, Kandawa-Schultz, Minnaar & Cencic, 2011). The seed coats are not consumed and make up 50% of the total weight of the seed (Holse *et al.*, 2010) compared to 9% in the common bean (Ranilla *et al.*, 2007) and 10% in soybean (Xu & Chang, 2008). Therefore, these seed coats are a potential waste material for the extraction of phenolic compounds that could be used as natural functional food ingredient with antioxidant activity for the functional food industry. This could diversify and increase the utilization of marama bean as it is an underutilised crop and increase the economic sustainability of communities in these arid regions.

It is important that natural antioxidants from their natural sources are extracted under conditions compatible with food requirements (Ali-Farsi & Lee, 2008). Organic solvents have limited use in the food industry and are undesirable due to their toxicity to human health (Tsuda, Mizuno, Ohshima, Kawakishi & Osawa, 1995). Also, phytochemicals extracted with organic solvents may not be representative of those that are actually bioacessible and bioavailable during human digestion under aqueous conditions (Chen & Blumberg, 2008). Aqueous extraction of bioactive compounds may represent a safer and preferred option for pharmaceutical and food grade commercial processes (Oomah, Corbé & Balasubramanian, 2010).

CHAPTER 2

2. LITERATURE REVIEW

2.1.1. The marama bean

The marama bean (Fig. 2.1.1A), also known as morama or gemsbok bean (Powel, 1987) belongs to the Fabaceae, subfamily Caesalpinioideae (Hartley *et al.*, 2002). It thrives under high temperature conditions and in poor quality sandy soils with low rain fall (Mitchel *et al.*, 2005). It has important traits for survival under extreme conditions which include conservation of water in the tuber, early shutting of stomata, closing of leaves and maintaining leaf functions in a few leaves under drought conditions (Mitchel *et al.*, 2005). For centuries the marama bean has been an important food source for the people of the Kalahari Desert (Bower *et al.*, 1988). The seeds (Fig. 2.1.1B) are high in protein (28.8-38.4%) and fat (32.0-41.9%) (Powel, 1987; Amarteifio & Moholo, 1998; Holse *et al.*, 2010). The proteins are comparable to soybean in essential amino acid content, with methionine as the limiting amino acid (Bower *et al.*, 1988). However marama bean protein has higher tyrosine and proline content than soybean (Amonsou, Taylor, Beukes & Minnaar, 2012).

A B

Figure 2.1.1. Marama bean plant (A) and marama bean pod with seeds (B) (Source: www.marama.life.uk)

The marama bean has been identified as a valuable crop with a potential for cultivation in the arid and semi-arid regions of the world and as a food source for both humans and animals (Powel, 1987; Bower *et al.*, 1988; Mitchel *et al.*, 2005). The agronomic potential of marama bean lies in the high nutrient value of the seed (Powel, 1987) and ability to produce harvestable materials in marginal soils under adverse conditions (Travlos & Karamanos, 2006). It has long been identified as a possible candidate for cultivation in arid and semiarid conditions of the world (Mitchel *et al.*, 2005) and it has been grown successfully in the arid region of Texas, USA (Powel, 1987).

2.1.2. **Chemistry of plant phenolic compounds and their content in marama bean seed coats and condensed tannin sorghum bran**

There are few reports on phenolic compounds in marama beans and therefore phenolic compounds in other legumes such as the common bean, lentils and soybean are reviewed. Phenolic compounds in the bran of condensed tannins sorghum cereal is also reviewed especially its condensed tannins which have been well characterized. Several studies have reported tannins in legumes with dark seed coats and therefore in this study bran from condensed tannin sorghum was included as a reference sample.

2.1.2.1. Phenolic compounds

Phenolic compounds are chemical substances that consist of an aromatic ring bearing one or more hydroxyl substitutes including functional derivatives such as esters, methyl ethers and glycosides (Harbone, 1989; Manach, Scalbert, Morand, Remesy & Jimenez, 2004; Roura, Andrés-Lacueva, Jàuregui, Badia, Estruch, Izqurdo-Pulido & Lameula-Raventós, 2005). These are secondary plant metabolites synthesized by the plant during normal growth and development and in response to stressful conditions (Naczk & Shahidi, 2004). Phenolic compounds provide defence against ultraviolet radiation, aggression by pathogens, pests and oxidative stress (Scalbert, Morand, Manach & Rémésy, 2002; Awika & Rooney, 2004; Manach *et al.*, 2004; Im *et al.*, 2008). Plant phenolics protect plant seeds against oxidative damage (Siddhuraju, 2006) and invading pathogens including yeast, fungi, virus and bacteria which might prevent the seeds from germinating (Soong & Barlow, 2005).

Plant phenolics are universally distributed through-out the plant but their concentration varies within the different tissues (Harbone, 1989). Phenolics exist in multiple forms such as free, esterified, glycosylated or polymerized and may also coexist as complexes with proteins,

carbohydrates, lipids and other plant components (Naczk & Shahidi, 2004; Manach *et al.*, 2004; Luthria & Pastor-Corrales, 2006). These compounds can be extracted from leaves, seeds, seed hulls, fruits, roots and stems of plants (Harbone, 1989).

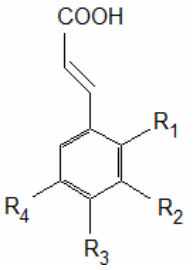
Dietary phenolic compounds occur as micro-constituents in many plant foods (Dabrowski & Sosulski, 1984; Hagerman, Riedl, Jones, Sovik, Ritchard, Hartfeld & Riecher, 1998) and they are major antioxidants in our diet (Ranilla *et al.*, 2007). The main sources are fruits, fruit juices, wine, tea, coffee, vegetables, cereals and legumes (Scalbert *et al.*, 2002). Phenolic compounds contribute to the bitterness, astringency, colour, flavour, odour and oxidative stability of food products (Careri, Mangia & Musci, 1998; Naczk & Shahidi, 2004). These molecules also participate in enzyme-catalyzed browning reactions that may adversely affect colour, flavour, and nutritional quality of food (Im *et al.*, 2008). Phenolic compounds through their antioxidant activity or as agents of other mechanisms are perceived to have beneficial effects on human health which includes anticarcinogenic and antimutagenic as well as reducing risk factors for cardiovascular disease such as low density lipoprotein (LDL) oxidation, platelet aggregation and inflammatory effects (Rice-Evans, Miller & Paganga, 1996; Vaher & Koel, 2003; Im *et al.*, 2008).

Phenolic compounds are classified into different classes or groups according to the number of phenol rings and the structural elements that bind the rings together (Manach *et al.*, 2004). These compounds can be broadly classified into simple phenols, phenolic acids (both benzoic and cinnamic derivatives), coumarins, stilbenes, flavonoids, hydrolysable and condensed tannins, lignans and lignin (Naczk & Shahidi, 2004; Manach *et al.*, 2004).

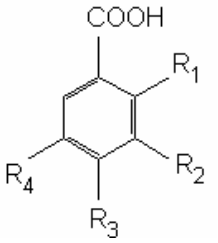
2.1.2.2. Phenolic acids

Phenolic acids are derivatives of benzoic and cinnamic acid (Fig. 2.1.2), that account for one third of phenolic compounds in food and they occur in free and bound forms (Manach *et al.*, 2004; Xu, Ye, Chen & Liu, 2007). These compounds differ from other phenolic compounds, due to the presence of a carboxylic group that confers acidic properties (Madhujith & Shahidi, 2005). Hydroxycinnamic acids, mainly *p*-coumaric, caffeic, ferulic and sinapic acids are amongst the most widely distributed phenylpropanoids in plant tissue (Manach *et al.*, 2004). Derivatives of hydroxybenzoic acid occur at low concentrations in a few edible

plants and are found in free form and/or as components of complex structures such as hydrolysable tannins (Manach *et al.*, 2004).



A. Cinnamic acid



B. Benzoic acid

Cinnamic acid derivatives	**R_1**	**R_2**	**R_3**	**R_4**
p-Coumaric acid	H	H	OH	H
o-Coumaric acid	OH	H	H	H
Caffeic acid	H	H	OH	OH
Ferulic acid	H	H	OH	OCH_3
Sinapic acid	H	OCH_3	OH	OCH_3

Benzoic acid derivatives	**R_1**	**R_2**	**R_3**	**R_4**
p-Hydroxybenzoic acid	H	H	OH	H
Vanillic acid	H	OCH_3	OH	H
Gallic acid	H	OH	OH	OH
Syringic Acid	H	OCH_3	OH	OCH_3
Protocatechuic acid	H	OH	OH	H
Gentisic acid	OH	H	H	OH
Salicylic acid	OH	H	H	H

Figure 2.1.2. Chemical structure and substitution pattern of representative phenolic acids (Adapted from Harbone (1989); Manach *et al.* (2004); Naczk & Shahidi (2004) and Awika & Rooney (2004)).

Having both carboxylic and hydroxyl groups, phenolic acids are rarely found in free form except in processed foods, and bound forms are glycosylated derivatives or esters of quinic, shikimic and tartaric acid (Manach *et al.*, 2004). These compounds may also form ester and

ether bonds with non-starch cell wall polysaccharides such as arabinoxylans (Smith & Hartley, 1983; Mueller-Harvey & Hartley, 1986; Bunzel, Ralph, Marita, Hatfield & Steinhart, 2001; Madhujith & Shahidi, 2005).

Phenolic acids in marama beans were found to be concentrated in the seed coat compared to the cotyledon (van Zyl, 2007; Schoeman, 2008). This was contrary to other legumes such as the common bean, lentils and soybean where phenolic acids are concentrated in the cotyledon (Dueñas, Hernández & Estrella, 2006; Ranilla *et al.*, 2007; Xu & Chang, 2008). Both cinnamic and benzoic acid derivative were reported in the seed coats of marama bean.

Cinnamic acid derivatives identified were caffeic, *p*-coumaric, sinapic, ferulic and cinnamic and benzoic acid derivatives were gallic, protocatechuic, 4-hydroxybenzoic, vanillic and syringic. Benzoic acid derivatives were the predominant phenolic acids in the seed coats. According to van Zyl (2007) protocatechuic acid was the major phenolic acid in the seed coat, while Schoeman (2008) reported that gallic acid was the major component. The difference could be due to differences in extraction methods, more gallic acid could have been released from bound forms during sequential hydrolysis in the study of Schoeman (2008).

Phenolic acids in cereal grains exist mostly in bound forms, esterified to arabinoxylans and also forming bridges between chains of hemicelluloses (Andreasen, Christensen, Meyer & Hansen, 2000; Manach *et al.*, 2004). The most abundant phenolic acid in cereal grains such as maize (*Zea mays* L.) (Li, Wei, White & Beta, 2007), sorghum (*Sorghum bicolor* (L.) Moench) (Hahn, Faubion & Rooney, 1983), wheat (*Triticum aestivum* L.) (Anson, Havenaar, Bast & Haenen, 2010) and rye (*Secale cereale* L.) (Andreasen *et al.*, 2000) is ferulic acid, followed by *p*-coumaric acid (Madhujith & Shahidi, 2005). Ferulic acid is mainly concentrated in the outer parts of the grain, chiefly in the aleurone layer and the pericarp (Manach *et al.*, 2004). Sorghum has been reported to contain both benzoic and cinnamic acid derivatives and these compounds exist in free and bound forms, but mainly in bound form (Hahn *et al.*, 1983). The phenolic acids are mainly concentrated in the bran, the outer layer of the kernel, which includes the pericarp, testa and aleurone layer (Dykes & Rooney, 2006). Benzoic acid derivatives identified in sorghum were gallic, protocatechuic, *p*-hydroxybenzoic and vanillic and cinnamic acid derivatives were ferulic, *p*-coumaric, cinnamic, caffeic and

sinapic (Hahn *et al.*, 1983; Awika & Rooney, 2004; Dykes & Rooney, 2006). Ferulic acid was the most predominant phenolic acid occurring mainly in bound form (Hahn *et al.*, 1983; Dykes & Rooney, 2006).

2.1.2.3. Flavonoids

Flavonoids are found in all parts of plants and are the largest group of secondary metabolites occurring widely in plants (Harbone, 1989). More than 9000 flavonoid compounds have been identified (Williams & Grayer, 2004).

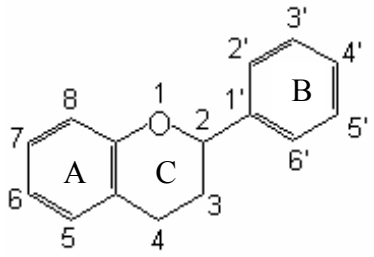
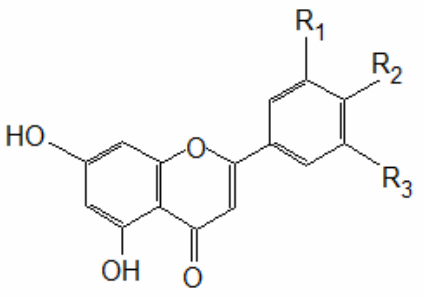
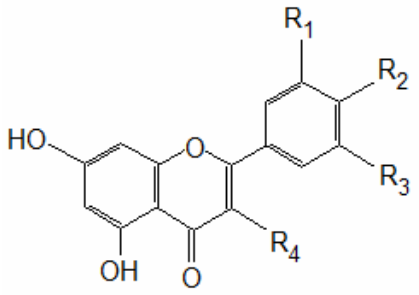


Figure 2.1.3. Generic structure of flavonoid compounds (Adapted from Wolfe & Liu (2008)).

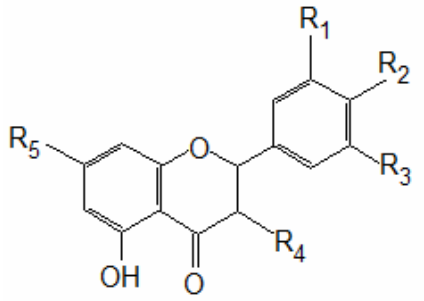
Flavonoids consist of a diphenylpropane (C_6-C_3-C_6) skeleton, which is basically two benzene rings (A and B rings) joined by a three carbon linear chain that form an oxygenated heterocycle (C ring) (Fig. 2.1.3) (Rice-Evans *et al.*, 1996; Manach *et al.*, 2004). While some flavonoids occur in free form, most exist in nature in glycoside forms and the most common sugar residue in the glycoside form is D-glucose, while the other sugars may be D-galactose, L-rhamnose, L-arabinose and D-xylose (Rice-Evans *et al.*, 1996; Careri *et al.*, 1998). Flavonoids are divided into six major subclasses (Fig. 2.1.4 and Table 2.1.1) depending on the type of heterocyle involved and the main subclasses are flavone, flavonol, flavanone, flavanol, anthocyanidin and isoflavone (Manach *et al.*, 2004).



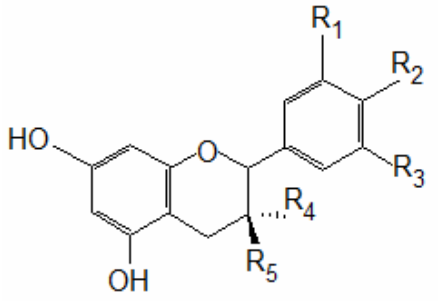
A. Flavone



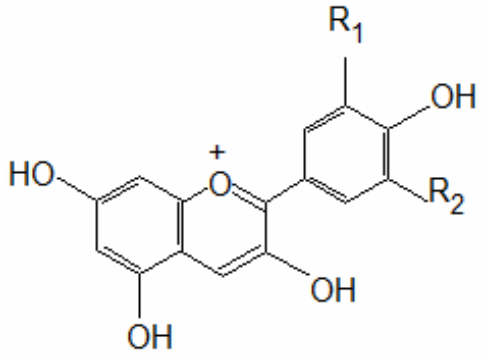
B. Flavonol



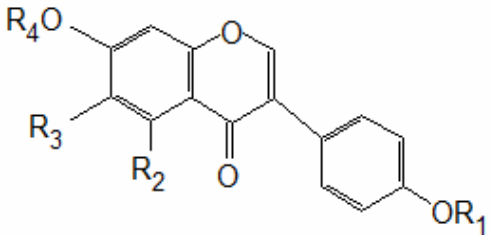
C. Flavanone



D. Flavanol



E. Anthocyanidin



F. Isoflavone

Figure 2.1.4. Basic chemical structures of flavonoid subclasses (Adapted from Naczk & Shahidi (2004)).

Table 2.1.1. Flavonoid subclasses and substitution pattern (structures shown in Fig. 2.1.3) of representative compounds (Adapted from Manach *et al.* (1989); Rice-Evans *et al.* (1996); Awika & Rooney (2004) and Naczk & Shahidi (2004))

Subclass	Compound	R_1	R_2	R_3	R_4	R_5
A. Flavone	Chrysin	H	H	H		
	Apigenin	H	OH	H		
	Luteolin	OH	OH	H		
B. Flavonol	Kaempferol	H	OH	H	OH	
	Quercetin	OH	OH	H	OH	
	Myricetin	OH	OH	OH	OH	
	Rutin	OH	OH	H	Rut.	
C. Flavanone	Naringin	H	OH	H	Rhaglc	OH
	Naringenin	H	OH	H	H	OH
	Taxifolin	OH	OH	H	OH	OH
	Eriodictyol	OH	OH	H	H	OH
	Hesperidin	OH	OCH_3	H	H	Rut
D. Flavanol	(+)-Catechin	OH	OH	H	OH	H
	(-) Epicatechin	OH	O H	H	H	OH
	(+) Gallocatechin	OH	OH	OH	H	OH
	(-) Epigallocatechin	OH	OH	OH	OH	H
	Afzelechin	H	OH	H	H	OH
	Epiafzelechin	H	OH	H	OH	H
E. Anthocyanin	Cyanidin	OH	H			
	Delphinidin	OH	OH			
	Pelargonidin	H	H			
	Malvidin	OCH_3	OCH_3			
	Petunidin	OCH_3	OH			
F. Isoflavone	Daidzin	H	H	H	H	
	Genistin	OH	OH	H	Glu	
	Daidzein	Glu	H	H	Glu	
	Genistein	Glu	OH	H	Glu	

Rhaglc- Rhamnose glucoside, Rut – Rutinose, Glu – Glucose

The main flavonoid compounds reported in the seed coats and cotyledon of marama bean were rutin, naringin, hersperidin, fisetin, myricetin, quercetin, and kaempferol, while catechin was only present in the cotyledon, and naringenin in the seed coat (Chingwaru *et al.*, 2011). Flavonoids were more concentrated in the seed coat (13036.5 mg/100 g) than the cotyledon (157.8 mg/100 g) (Chingwaru *et al.*, 2011).

Table 2.1.2. Major flavonoids reported sorghum grain (Adapted from Awika & Rooney (2004))

Flavanoid subclass	Compound
Flavones	Luteolin
	7-O-methyl luteolin
Flavanones	Naringenin
	Eriodictoyl
	Eriodictoyl 5-O-β-glucoside
Dihydroflavonol	Taxifolin
	Taxifolin 7-O-β-glucoside
Flavan-4-ols	Apiforol
	Luteoforol
Anthocyanins	Apigeninidin-5-glucoside
	Luteolinidin-5-glucoside
Anthocyanidins	Apigeninidin
	Luteolinidin
	7-O-methyl apigeninidin
	Fisetinidin

A number of flavonoid compounds have been identified in sorghum and the major compounds identified are listed in Table 2.1.2. Sorghum has unique anthocyanins, 3-deoxyanthocyanins that do not contain the hydroxyl group in position 3 on the C ring (Dykes & Rooney, 2006). Black sorghum has the highest levels of 3-deoxyanthocyanins in the pericarp, while flavan-4-ols are the main flavonoid compounds in the pericarp of sorghums with red pericarp (Dykes & Rooney, 2006).

2.1.2.4. Tannins

Tannins are high molecular weight phenolic plant metabolites and are widely distributed in plants, including many plants used for food. Tannins are characterized by their ability to complex strongly with carbohydrates and proteins (Hagerman & Butler, 1980; Hagerman *et al.*, 1998). Tannins play a role in the sensory properties and biological quality of foods (Dueñas, Sun, Hernández, Estrella & Spranger, 2003) such as grapes, wine (Prieur, Rigaud, Cheynier & Moutounet, 1994; Prior, Lazarus, Cao, Muccitelli & Hammerstone, 2001), cocoa (Prior *et al.*, 2001), legume seeds, cereals and cider (Mathews, Mila, Scalbert, Pollet, Lapierre, Hervé du Penhoat & Roland, 1997). Tannins are responsible for the astringency of many plant materials and when added or occurring naturally at high levels in foodstuffs decrease the nutritive value thereof (Strumeyer & Malin, 1975). Plant tannins are grouped into hydrolysable and condensed tannins (Strumeyer & Malin, 1975; Harbone, 1989; Hagerman *et al.*, 1998) and are differentiated by their structure and reactivity toward hydrolytic agents (Strumeyer & Malin, 1975). Gallotannins are the simplest hydrolysable tannins which are metabolites of polyol to which gallic acid units are ester linked to a sugar molecule (Fig. 2.1.5), which may also be joined to other galloyl units through C→C or C→O covalent bonds (Harbone, 1989). Gallotannins are readily cleaved by enzymes as well as dilute acids to liberate glucose and phenolcarboxylic acid such as gallic acid (Strumeyer & Malin, 1975).

Proanthocyanidins, also known as condensed tannins (Gu, Kelm, Hammerstone, Zhang, Beecher, Holden, Howtowitz & Prior, 2003a) are high molecular weight polyflavonoids (Hagerman & Butler, 1980; Putman & Butler, 1989). They are widely distributed in the plant kingdom (Dueñas *et al.*, 2003) and after lignin are the second most abundant natural phenolic compounds (Mathews *et al.*, 1997; Gu *et al.*, 2003a; Li & Deinzer, 2007). Proanthocyanidins are composed of dimers, oligomers and polymers of flavan-3-ol units (Mathews *et al.*, 1997; Gu *et al.*, 2003a). The flavan-3-ol units may be esterified to gallic acid to form 3-O-gallates (Ricardo da Silva, Rigaud, Cheynier, Cheminat & Moutounet, 1991; Li & Deinzer, 2007). Acid catalyzed depolymerisation of proanthocyanidins results in the formation of anthocyanidins (Porter, Hrstich & Chan, 1986; Li & Deinzer, 2007). The oligomer and polymer gross structure of proanthocyanidins is characterized by the nature of its constitutive extension and terminal flavan-3-ol units as well as the degree of polymerization (DP) which is the number of units in a polymer (Prieur *et al.*, 1994). Proanthocyanidins with a DP

between 2 and 10 are defined as oligomers and those greater than 10 are polymers (Gu, Kelm, Hammerstone, Beecher, Cunningham, Vannozzi & Prior, 2002).

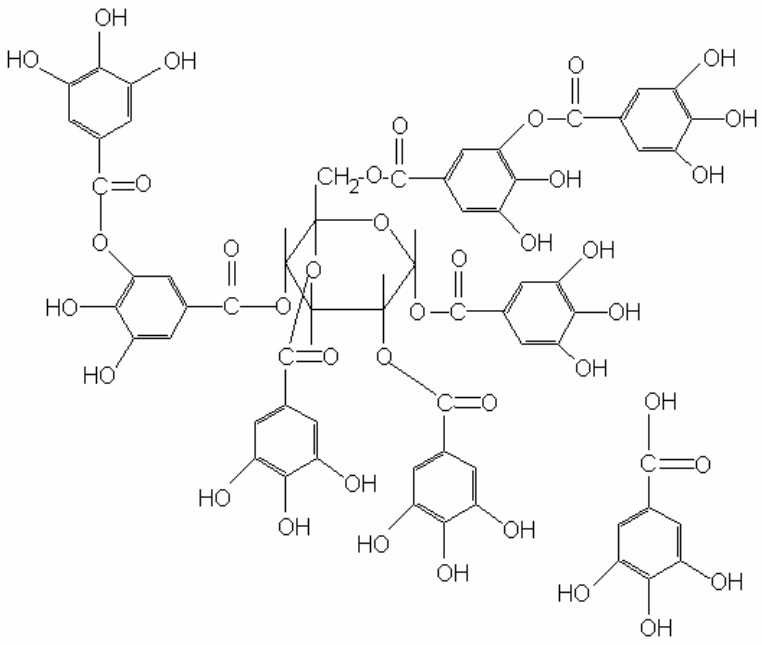
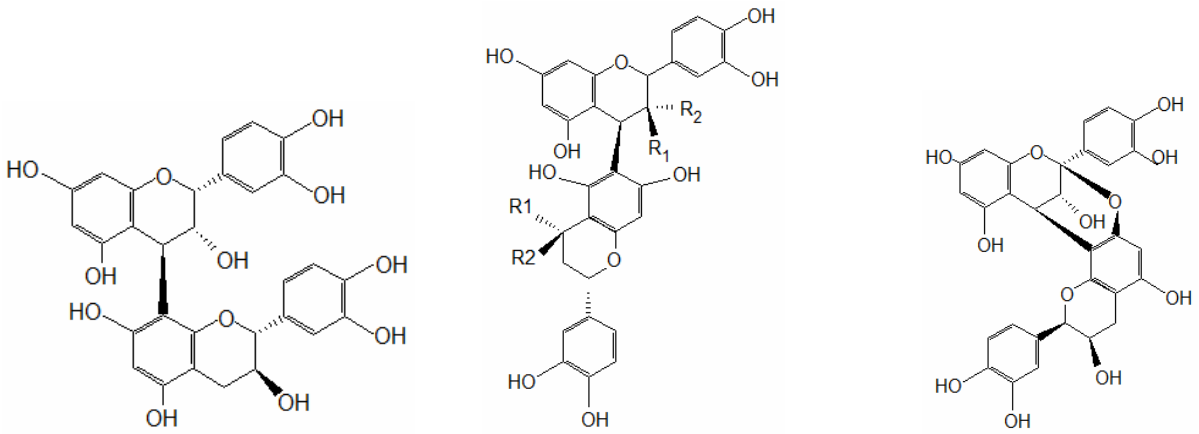


Figure 2.1.5. Hydrolysable tannin structure (Adapted from Manach *et al.* (1989) and Naczk & Shahidi (2004)).

The flavon-3-ol units are frequently linked through a single C4-C8 interflavan bond (Fig. 2.1.6 A), however C4-C6 interflavan bonds (Fig. 2.1.6 B) also exist and these single linked proanthocyanidins are B-type (Ricardo da Silva *et al.*, 1991; Gu *et al.*, 2003a; Dueñas *et al.*, 2006). The units can also be double linked through an additional ether bond between C2 and O7 (Fig. 2.1.6 C) and are known as A-type (Gu *et al.*, 2003a).

Proanthocyanidins in plants are divided into three main subclasses, namely procyanidins, propelargonidins and prodelphinidins. Procyanidins and prodelphinidins are the most predominant proanthocyanidins in food (Dueñas *et al.*, 2006).



A). Singly linked (4β→8) B-type dimers

B). Singly linked (4β→6) B-type dimer

C) Doubly linked (4β→8; 2 β→O→7) A-type dimer

Figure 2.1.6. Proanthocyanidin chemical structures showing the different linkages (Adapted from Prior *et al.* (2001)).

Proanthocyanidins consisting exclusively of flavan-3-ol units, (+)-catechin and/or (-)-epicatechin are designated procyanidins (Harbone, 1989; Gu *et al.*, 2003a; Xu & Chang, 2007). The simplest procyanidins are dimeric and the most common are the C4-C8 linked isomers accompanied by small amounts of C4-C6 linked isomers (Ricardo da Silva *et al.*, 1991). Propelargonidins contain subunits of afzelechin and/or epiafzelechin and prodelphinidins contain subunits of gallocatechin and/or epigallocatechin (Gu *et al.*, 2003a). Propelargonidins and prodelphinidins are not common in nature and usually coexist with procyanidins (Gu *et al.*, 2003a).

To date no tannins have been reported in marama beans. However, tannins have been found in other legumes such as the common bean (Gu *et al.*, 2003a; Ranilla *et al.*, 2007), lentils (Dueñas *et al.*, 2003), peas (Dueñas *et al.*, 2006) and soybean (Xu & Chang, 2008), especially those varieties with dark seed coats. Tannins in these legumes are essentially located in the seed coat (Dueñas *et al.*, 2006) and are predominantly procyanidins with catechin or epicatechin as subunits. Propelargonidin (less than 5%) with (epi)catechin and (epi)afzelechin subunits have also been reported in some common bean varieties (Beninger, Gu, Prior, Junk, Vanderberg & Bett, 2005). Prodelphinidins composed of epigallocatechin and traces of epigallocatechin-3-O-gallate have been reported in the seed coats of lentils (Dueñas *et al.*, 2003).

Condensed tannins are present in sorghums with pigmented testa. Grains from purple/red plants with thick pericarp have higher levels of condensed tannins than grains from tan plants with thin pericarp (Dykes, Rooney, Waniska & Rooney, 2005). Sorghums having condensed tannins are classified as type II and III (Awika & Rooney, 2004) and have a dominant gene B_1_B_2_which controls the presence or absence of the pigmented testa layer (Dykes *et al.*, 2005). Sorghum tannins are exclusively of the condensed type (Awika & Rooney, 2004) and are high molecular weight polymers composed of flavan-3-ols and/or flavan-3,4-diol linked mainly by C4 – C8 interflavan bonds and are B-type proanthocyanidins (Dykes & Rooney, 2006). There are also A-type proanthocyanidins which are composed of flavan-3-ol units linked by C4 – C8 interflavan bond and an additional ether bond between C2 and C7 (Dykes & Rooney, 2006). Gu *et al.* (2003a) reported that condensed tannins in sorghum (variety early Sumac) were procyanidins composed of catechin and epicatechin. The terminal units were predominantly catechin (9.3%) and epicatechin (2.5%). Extension units were almost exclusively epicatechin (88.2%) and the mean degree of polymerization was 8.4.

2.1.3. **Extraction of phenolic compounds**

Phenolic compounds (free, soluble esters and soluble glycosides) from plant material are commonly extracted with alcohols such as methanol and ethanol, acetone, water or their aqueous mixtures (Krygier, Sosulski & Hogge, 1982; Kamath, Chandrashekar & Rajin, 2004; Xu & Chang, 2007). Hexane and n-butanol (Chen & Blumberg, 2008) have also been used. The reasons for choosing any particular solvent system has not been justified (Xu & Chang, 2007). Subcritical water (Ibañez, Kubátová, Seňoráns, Cavero, Reglero & Hawthorne, 2003) and supercritical carbon dioxide (Tsuda *et al.*, 1995; Murga, Ruiz, Beltran & Cabezas, 2000) extraction have also been used to extract phenolic compounds from natural sources and are preferred over organic solvent extraction. Supercritical carbon dioxide, just like water is an ideal solvent for extracting compounds for use in food application because it is nontoxic, non-flammable, low cost and environmentally safe (Tsuda *et al.*, 1995) but it has the disadvantage of being non-polar requiring the use of co-solvents such as methanol or ethanol to extract polar compounds (Murga *et al.*, 2000).

The yield of an extract is dependant upon the type of solvent, its polarity, pH, extraction time, temperature and pressure as well as the chemical composition and chemical properties of the sample (Xu & Chang, 2007; Chen & Blumberg, 2008). Xu and Chang (2007) reported that

50% aqueous acetone extracts from eight different legumes gave higher total phenolic content compared to 80% acetone, acidic 70% acetone, 70% methanol, 70% ethanol and 100% ethanol. However, total flavonoid, condensed tannin content and DPPH radical scavenging activity were the highest in 80% acetone extracts. Water extracts from date seeds showed low total flavonoid content and total phenolic content compared to methanol and ethanol extracts (Ali-Farsi & Lee, 2008) due to low solubility of phenolic compounds in water. These authors also reported that extraction time beyond 30 minutes had no significant effect on total phenolic content and optimum extraction temperature was 50 °C but for 50% aqueous acetone the optimum temperature was 65 °C. Acetone and methanol are intermediate polar solvents and their mixtures were found to be more effective in extracting phenolic compounds from seed hulls of the common bean compared to ethanol, chloroform and ethyl acetate (Cardador-Martinez *et al.*, 2002).

During extraction, bound phenolic compounds may be released by alkaline or acid hydrolysis or both (Krygier *et al.*, 1982) or by enzymatic means using enzymes such as cellulase (Madhujith & Shahidi, 2005). Alkaline hydrolysis using sodium hydroxide solution at room temperature is the most commonly used method to release bound phenolic compounds (Dabrowski & Sosulski, 1984; Luthria & Pastor-Corrales, 2006; Ross, Beta & Arntfield, 2009; Madhujith & Shahidi, 2005). A major proportion of phenolic acid compounds in barley were found to exist in bound forms (Madhujith & Shahidi, 2005). The insoluble bound phenolic fraction from barley had higher total phenolic content and exhibited higher antioxidant activity compared to free and soluble conjugated phenolic fractions. In several sorghum varieties, phenolic acids were found to be more concentrated in the bound phenolic acid fraction compared to the free phenolic acid fraction (Hahn *et al.*, 1983). Alkaline hydrolysates from maize extracts had higher total phenolic content and antioxidant activity compared to methanol and HCl/methanol extracts because alkaline hydrolysis released soluble-conjugated and bound phenolic compounds in maize as free phenolic acids (Li *et al.*, 2007).

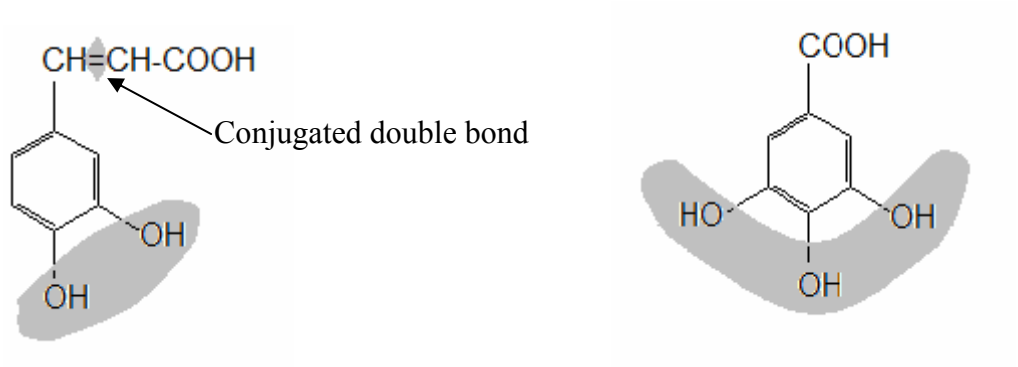
Extraction under conditions that mimic those prevailing in the gastrointestinal tract has been suggested to provide a more physiologically relevant approach as it may extract more water-soluble phenolic constituents and simulate the impact of digestive enzymes and conditions on polyphenols (Chen & Blumberg, 2008). Anson *et al.* (2010) used digestive juices and a

multi-compartmental *in vitro* model that simulated the upper gastrointestinal tract to obtained bioaccessible phenolic compounds from wheat fractions to investigate their antioxidant and antinflammatory potential. Chen and Blumberg (2008) reported that the total phenolic content of acidified methanolic extracts of almond skin were 4.8 fold more than aqueous gastrointestinal extracts and compounds such as catechin, epicatechin, kaempferol-3-O-glucoside, quercetin-3-O-glucoside, rutin, naringenin, kaempferol, quercetin and eriodictyol were absent in the gastrointestinal extracts. Oxygen radical absorbance capacity (ORAC) values of gastrointestinal extracts were lower than those of acidified methanolic extracts. The results demonstrated that compounds extracted with organic solvents may not be the same as those released in the gastrointestinal tract under aqueous conditions.

2.1.4. **Antioxidant activity and structure relationship**

The antioxidant activity of phenolic compounds is directly related to their structure (Dueñas *et al.*, 2006) and their scavenging potential is due to the ability of hydroxyl substituents to donate hydrogen (Siddhuraju, 2006). Phenolic acids with a catechol structure such as protocatechuic acid and caffeic acid (Fig. 2.1.7a) or with the pyrogallol structure such as gallic acid (Fig. 2.1.7b) were found to be potent antioxidants compared to compounds without the catechol or pyrogallol nucleus such as resorcyclic acid and 2,4,6 trihydroxybenzoic acid (Moran, Klucas, Grayer, Abian & Becana, 1997).

Antioxidant activity of phenolic compounds has been found to increase with increase in the number of free hydroxyl groups on the aromatic ring (Moran *et al.*, 1997). Fukumoto and Mazza (2000) reported that loss of one hydroxyl group from phenolic acids with three hydroxyl groups decreased the antioxidant activity slightly and loss of two hydroxyl groups significantly reduced activity. Methylation of gallic acid significantly reduced its effectiveness as an antioxidant in copper-mediated low density lipoprotein- (LDL) and serum oxidation experiments which indicated that hydroxyl groups were essential for antioxidant activity (Caccetta, Croft, Beilin & Puddey, 2000).



a) Caffeic acid, catechol structure b) Gallic acid, pyrogallol structure

Figure 2.1.7. Catechol and pyrogallol representative structures (Adapted from Moran *et al.* (1997)).

Antioxidant activity of phenolic acids was also found to increase with conjugation of the side chain on the aromatic ring (Fukumoto & Mazza, 2000). Caffeic acid was found to be the most potent inhibitor of copper-mediated LDL- and serum oxidation compared to gallic acid because of the presence of the conjugated double bond on the side chain of caffeic acid (Moran *et al.*, 1997). Cinnamic acid derivatives were found to have higher antioxidant activity than benzoic acid derivatives because of the presence of a conjugated double bond on the side chain (Fukumoto & Mazza, 2000). The presence of the catechol or pyrogallol groups facilitated donation of hydrogen atoms (Fukumoto & Mazza, 2000) and the presence of a conjugated double bond on the side chain of the phenyl ring increased resonance stabilization of the resulting phenoxy radical (Moran *et al.*, 1997).

The antioxidant activities of flavonoid compounds with the same basic chemical structure against lipophilic radicals were found to be proportional to the number of hydroxyl (OH) substitution (Cao, Sofic & Prior, 1997). Kaempferol, quercetin and myricetin which have four, five and six hydroxyl group substitution had ORAC absorbance activities of 2.7, 3.3 and 4.3 µM Trolox equivalent/ µM sample, respectively. Fukumoto and Mazza (2000) also reported that flavonoid compounds with three hydroxyl groups on the B ring (Fig. 2.1.7) had higher antioxidant activity, with the loss of one hydroxyl group activity decreased slightly, while the loss of two hydroxyl groups reduced activity significantly. Addition of a sugar moiety decreased the antioxidant activity of quercetin, cyanidin, pelargonidin and peonidin and the addition of a second sugar moiety to the aglycone further decreased the activity. However ,the presence of a third OH group in the B-ring in the 5′ position did not enhance

the antioxidant activity against aqueous phase radicals as in myricetin compared to quercetin (Rice-Evans *et al.*, 1996) and this was said to be due to pro-oxidant effects (Wolfe & Liu, 2008). Cao *et al.* (1997) noted that flavonoids with 3',4'-*o*-dihydroxy substitution, such as luteolin had much higher antioxidant activity compared to compounds without the 3',4'-*o*-dihydroxy configuration such as kaempferol. Terao, Piskula and Yao (1994) reported that compounds with 2,3 double bond and 4-keto group on C-ring (Fig. 2.1.8) had higher antioxidant activity compared to compounds with a single bond and without the 4-keto group. They reported that flavan-3-ols such as epicatechin and epicatechin gallate had a lower rate of peroxyl radical scavenging activity compared to other subclasses of flavonoid such as quercetin because of the absence of 2,3 double bond and 4-keto group on C-ring.

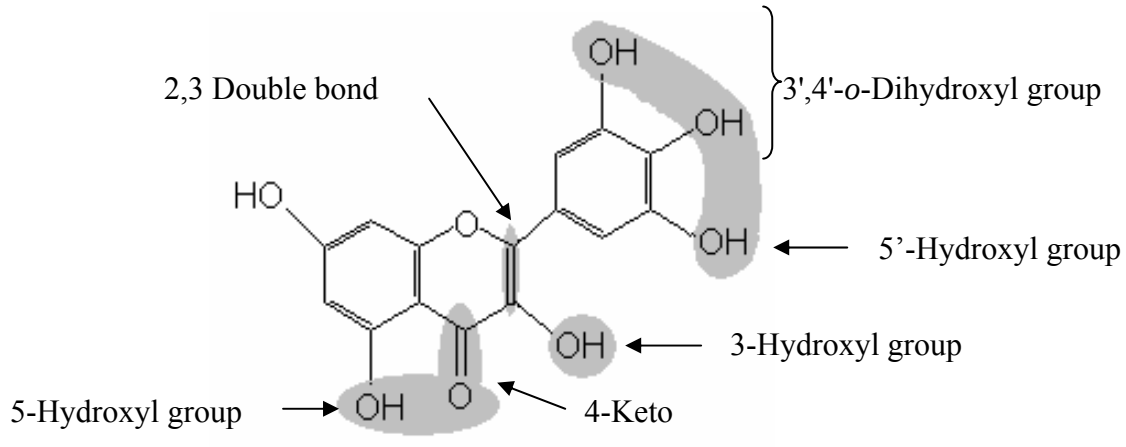


Figure 2.1.8. Flavonoid structure showing functional groups that affect antioxidant activity.

Fukumoto and Mazza (2000) also noted that the flavanols (+)-catechin and (-)-epicatechin had lower antioxidant activity than their flavonol and anthocyanindin counterparts because of the loss of the double bond in ring C resulting in less stability of unpaired electron delocalization. The 4-keto group and 5-hydroxyl group are important sites for chelation of transition metals which catalyse oxidation reactions (Wolfe & Liu, 2008). Flavonoids generally have higher antioxidant activity than phenolic acids and therefore are effective scavengers of free radicals (Fukumoto & Mazza, 2000).

Tannins are more potent antioxidants than monomeric compounds because of their high molecular weight and proximity of many aromatic rings and hydroxyl groups (Hagerman *et*

al., 1998). High molecular weight proanthocyanidins are unable to reach the inner tissue in their intact form (Rios, Gonthier, Rémésy, Mila, Lapierra, Lazarus, Williamson & Scalbert, 2003). However, a significant increase in concentration of low molecular weight procyanidin dimer B2 (epicatechin-(4β→8)-epicatechin), epicatechin and 3′-O-methyl-epicatechin was observed in plasma of rats, reaching peak concentration after 30 - 60 minutes of oral administration of purified procyanidin B2 dissolved in deionized water (Baba *et al*., 2007). This was an indication that procyanidin dimer B2 was rapidly absorbed in the upper digestive tract and that methylation and conjugation occurred in the intestinal mucosa (Baba *et al*., 2007). Procyanidin B2, epicatechin and catechin were found in the plasma of human subjects, reaching peak concentration after 2 hours of consumption of 0.375 g cocoa powder / kg of body weight in 300 ml water (Holt Lazarus, Sullards, Zhu, Schramm, Hammerstone, Fraga, Schmitz & Keen, 2002). These studies provided further evidence that procyanidin oligomer dimer B2, catechin, and epicatechin were absorbed into circulation and therefore their metabolites may confer health benefits.

Biological effects of high molecular weight proanthocyanidins may also be attributed to their metabolites produced by colonic bacteria. Consumption of chocolate by human subjects resulted in increased urinary excretion of 11 phenolic acids including ferulic, vanillic, m-hydroxybenzoic acid, p-hydroxybenzoic acid and huppuric acid as the major excretory metabolites (Rios *et al*., 2003). The increase was observed after 9 hours of consumption of chocolate, with the exception of vanillic acid indicating that the metabolites were likely to originate from microbial metabolism of proanthocyanidins in the colon which are then absorbed into circulation. This suggests that formation of the easily absorbed phenolic acids may contribute to the prevention of oxidative stress in inner tissues (Rios *et al*., 2003).

2.1.5. ***In vitro*** **antioxidant activity of legume extracts**

Antioxidants scavenge free radicals and reactive oxygen species and thus inhibit oxidative mechanisms that lead to degenerative diseases (Cardador-Martinez *et al.*, 2002). A number of *in vitro* studies have shown that extracts from legumes, especially the seed hulls or seed coats have antioxidant activity and may exert beneficial biological effects on biomolecules, cells and tissue. Methanolic extracts from seed coats of common beans (*Phaseolus vulgaris* L.) with red testa were shown to have a dose-dependent 2,2-diphenyl-1-picryhydrazyl (DPPH) radical quenching capacity which was significantly more effective than catechin and

butylated hydroxyanisole (BHT) at 1000 µm concentration (Cardador-Martinez *et al.*, 2002). Aqueous acetone extracts prepared from raw and heated moth beans [*Vigna aconitifolia* (Jacq.) Marechal] showed free radical scavenging activity against DPPH$^{\bullet}$ and 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid) radicals (ABTS$^{+\bullet}$) (Siddhuraju, 2006). The extracts also showed a dose-dependent superoxide anion radical ($O_2^{\cdot-}$) and hydroxyl radical ($OH^{\cdot}$) scavenging activity. Furthermore, these extracts exhibited a 54% lipid peroxidation inhibition activity in a linoleic acid emulsion system. Hot water extracts from mung beans (*Phaseolus radiates* L.), adzuki bean (*Phaseolus auresus* Roxb.), black bean (*Glycine max* (L.) Merr.) and rice beans (*Phaseolus calcaratus* Roxb.) showed a dose dependent scavenging effect on the superoxide anion (ranging from 35 – 90% inhibition), with black beans exhibiting the highest activity using the cytochrome C test (Lin, Wu, Wang, Yang & Chang, 2001). Acidified methanolic extracts from the seed coats of lentils (*Lens culinaris* L.) and dark peas (*Pisum sativum* L.) showed greater DPPH radical scavenging activity compared to their cotyledons (Dueñas *et al.*, 2006).

A significant amount of antioxidant activity in methanolic extracts prepared from seed coats of the common bean was found to be due to condensed tannins. Methanol extracts from genotypes with higher condensed tannin levels such as the red, light red and yellow brown seed coat genotypes had higher antioxidant activity compared to extracts from yellow seed coat genotypes without condensed tannins (Beninger & Hosfield, 2003). Extracts from black beans, lentils, small red beans and pinto beans with higher total phenolic content, total flavonoid content and condensed tannin content exhibited higher antioxidant capacity against copper-induced human LDL oxidation compared to yellow, green peas, chickpea and yellow soy beans with lower values (Xu, Yuan & Chang, 2007a). There was a high positive correlation between antioxidant activity and total phenolic content, flavonoid content and condensed tannin content. Flavonoids and condensed tannins played a significant role in the overall antioxidant activity of extracts from black beans, lentils, red kidney beans and pinto beans (Xu *et al.*, 2007a). Ranilla *et al.* (2007) reported that aqueous methanol extracts from seed coats of Brazilian and Peruvian bean cultivars had higher antioxidant activity against the DPPH radical compared to cotyledons and there was a high positive correlation between antioxidant activity and total phenolic content ($r = 0.88$) and condensed tannin content ($r = 0.86$).

2.1.6. **Oxidative stress and chronic diseases**

Free radicals play an important role in oxidative stress (Kamath *et al.*, 2004), which is thought to contribute to the development of various chronic diseases including atherosclerotic cardiovascular disease, several cancers (Hodgson, Croft, Puddy, Mori & Beilin, 1996; Xu & Chang, 2007) and neurodegenerative (Chen & Blumberg, 2008) and inflammatory conditions (Erlejman, Jaggers, Fraga & Oteiza, 2008). Reactive oxygen and nitrogen species (ROS/RNS) are continuously produced in the human body and are essential in energy metabolism, detoxification, chemical signalling and immune function and levels are controlled by endogenous enzymes such as superoxide dismutase, glutathione peroxidase and catalase (Jacob & Burri, 1996; Dimitrios, 2006). Impairment of the antioxidant defence systems that scavenge and minimize the formation and overproduction of free radicals leads to oxidative damage to cellular biomolecules such as DNA, lipids and proteins (Jacob & Burri, 1996; Baublis, Decker & Clydesdale, 2000; Kamath *et al.*, 2004).

Biomembranes are major sites of lipid peroxidation damage due to the presence of polyunsaturated fatty acids in the membrane phospholipids (Mak, Misra & Weglicki, 1983). Destruction of membrane lipids compromises the function of the membrane and affects the transmembrane passage of solutes and fluidity of the membrane (Reiter, 1998) and may affect the function of subcellular organelles such as mitochondria, microsomes and lysosomes (Cejas *et al.*, 2004). Lipid peroxides decompose to relatively stable toxic aldehyde products which may diffuse and attack biomolecules such as proteins, DNA and phospholipids forming intra and inter molecular covalent adducts (Cejas *et al.*, 2004) leading to decline in physiological functions and ultimately cell death. Erythrocytes as oxygen carriers are exposed to oxidative damage (Paiva-Martins, Fernandes, Rocha, Nascimento, Vitorino, Amado, Borges, Bello & Santos-Silva, 2009) and in some hereditary haemolytic anaemia, chronic oxidative stress is an important factor in the aetiology of the disease (Manna, Galletti, Cucciolla, Montedoro & Zappia, 1999). Chronic oxidative stress caused by impairment of endogenous antioxidant defence mechanisms or overproduction of reactive oxygen species (ROS) such as hydrogen peroxide (H_2O_2) and superoxide anion radical (O_2^-) outside or within red blood cells may lead to haemolysis (Paiva-Martins *et al.*, 2009).

Oxidative modification of LDLs by radicals is an early event in coronary heart disease (Heinonen, Meyer & Frankel, 1998). LDL is highly unstable because of its size, large surface area and high concentration of easily oxidized polyunsaturated fatty acids (Jacob &

Burri, 1996). LDL oxidation initiates a number of events which include platelet aggregation, injury of arterial endothelial cells and other events that facilitate the development of foam cells and fatty streaks, a key event in atherosclerosis (Anderson, Smith & Washnock, 1999; Baba, Osakabe, Natsume, Muto, Takizawa & Terao, 2007). Chronic inflammation of the arterial walls is also associated with the development of atherosclerosis and therefore inhibition of inflammatory cytokines and other mediators through drugs, diet and specific nutrients is considered to be of great benefit in the maintenance of vascular homeostasis and the prevention of atherosclerosis (Guo, Wise, Collins & Meydani, 2008).

Studies have shown that oxidative stress and reactive oxygen species might be involved in initiation events in cancer (Laparra, Vélez, Barberá, Farré & Montoro, 2008). The mitochondrial electron transport chain may leak electrons to O_2 leading to the formation of superoxide radical (O_2^-) which is dismuted by superoxide dismutase to hydrogen peroxide (Turrens, 2003), which diffuses freely to the nucleus (Lee, O'Connor & Pfeifer, 2002). Hydrogen peroxide in the presence of transition metal generates the most electrophilic and highly reactive hydroxyl radicals ($HO^{\bullet}$) in proximity to DNA causing "site specific" strand scission (Hagerman *et al.*, 1998; Bhat, Azmi, Hanif & Hadi, 2006) resulting in transition mutations which may lead to cancer (Lee *et al.*, 2002).

Reactive oxygen species are also implicated in the pathogenesis of several inflammatory conditions such as irritable bowel disease (IBD), a chronic inflammatory condition, which is a result of mucosal damage and alteration of intestinal barrier integrity by intracellular generation of reactive oxygen species (Erlejman *et al.*, 2008).

2.1.7. **Protective effect of polyphenolic antioxidants against chronic diseases**

Antioxidants play a significant role in the body's defence system against ROS (Liyana-Pathirana & Shahidi, 2005). Phenolic compounds show promise as health-promoting phytochemicals (Im *et al.*, 2008) because of their potential to modulate oxidative stress associated with chronic diseases (Paiva-Martins *et al.*, 2010). The antioxidant activity of phenolic compounds is attributed to the presence of phenolic hydroxyl groups that scavenge free radicals (Terao *et al.*, 1994), and bring about chelation of redox-active metals (Siddhuraju, 2006) and regeneration of α–tocopherols and other antioxidants by hydrogen donation (Abuja, Murkovic & Pfannhauser, 1998). Phenolic compounds may also provide a

protective effect through other mechanisms such as interacting with phospholipid membranes through hydrogen bonding to polar head groups and thus preventing access of lipid soluble oxidants to the hydrophobic region of the bilayer and by also modulating the response of cells to signalling molecules (Verstraeten, Keen, Schmitz, Fraga & Oteiza, 2003).

Aherne & O'Brien (2000) studied the protective effect of the flavonoids, quercetin and rutin against *tert*-butylhydroperoxide- and menadione-induced DNA single strand breaks in Caco-2 cells. Quercetin reduced the level of damage by 50% and rutin by 18% compared to controls without extracts. Both of these compounds scavenged ROS and chelated transition metals ions such as ferrous, ferric and cuprous ions thus providing DNA strand breakage protective effect. Transition metals have been proposed to be the catalysts for the initial formation of hydroxyl radicals via the Fenton reaction (Waris & Ahsan, 2006) while chelating agents may stabilize transition metals in the living systems thereby inhibiting hydroxyl radical generation and consequently reducing free radical damage (Siddhuraju, 2006).

A number of studies have shown that phenolic compounds can inhibit the oxidation of LDL. Meyer, Donovan, Pearson, Waterhouse & Frankel (1998) showed that hydroxycinnamic acids such as caffeic and ferulic acid were effective in inhibiting *in vitro* copper-catalyzed oxidation of human LDL. Protection was ascribed to antioxidant activity and also it was hypothesized that the antioxidants may block copper access to apolipoprotein B tryptophans via binding of the antioxidant to apolipoprotein B. The soybean isoflavones, genistein and daidzein and their metabolites equol and O-desmethylangolensin (O-DMA) were found to inhibit copper-catalyzed LDL oxidation in serum (Hodgson *et al.*, 1996). However the metabolites were more potent antioxidants than their parent compounds. Genistein and daidzein and their glycosides were also shown to inhibit copper-mediated LDL oxidation but the aglycones were more effective than the corresponding glycosides (Lee, Yang, Xu, Yeung, Huang & Chen, 2005). However, isoflavone aglycones and their glycosides were found to be weaker antioxidants compared to (-)-epicatechin from green tea. Consumption of a cocoa drink containing polyphenolic compounds by human subjects resulted in increased resistance of LDL to oxidation and there was a 23.4% increase in HDL-cholesterol plasma concentration (Baba *et al.*, 2007). The mechanism by which polyphenolic compounds elevated plasma HDL-cholesterol concentration was unclear but it was thought to be as a

result of increased expression and production of apoliprotein A1, a major protein component of HDL.

Epigallocatechin-3-O-gallate (EGCG) was found to inhibit growth of nasopharyngeal carcinoma cells in a dose-dependent manner (Yan, Yong-Guang, Fei-Jun, Fa-Qing, Min & Ya, 2004). The researchers reported that EGCG suppressed expression of epidermal growth factor receptor (EGFR) gene, which plays an important role in the production and development of cancers. EGCG inhibited the activation of transcription factor (NF-κB) and translocation of NF-κB (p65) protein from the cytoplasm to the nucleus to up-regulate the expression of the EGFR gene in nasopharyngeal carcinoma cell lines. This demonstrated an antitumor effect of phenolic extracts through inhibition of cell signal transduction pathway. In another experiment (+)-catechin was found to delay tumour onset in a linear dose dependent manner in a transgenic mouse model of neurofibromatosis, suggesting that catechin may play an important role in cancer prevention; however the mechanism could not be understood (Ebeler, Brenneman, Kim, Jewel, Webb, Chanon-Rodgriuez, Crammer, Levi, Ebeler, Islas-Trej, Kraus, Hinrichs & Clifford, 2002),

Experiments with intestinal epithelial Caco-2 cells showed that high molecular weight hexameric procyanidins, although not absorbed by cells inhibited expression of inducible nitric oxide synthase (iNOS) gene which plays a role in inflammatory processes such as in inflammatory bowel diseases (Erlejman *et al.*, 2008). The hexameric procyanidin interacted with cell plasma membrane leading to inhibition of transcription factor (NK-αB) activation and relocation of NF-κB (p50) protein from the cytoplasm into the nucleus and thus inhibiting expression of the iNOS gene which is responsible for the generation of the cell oxidant nitric oxide (NO) (Erlejman *et al.*, 2008). Verstraeten *et al.* (2003) showed that the phenolic compounds, flavan-3-ols and procyanidin can induce changes to membrane structure and these researchers speculated that these changes may modulate the response of cells to signalling molecules.

(+)-Catechin and quercetin were found to inhibit monocyte adhesion to human aortic endothelial cells through scavenging of reactive oxygen species, suggesting that these phenolic compounds may be responsible for the beneficial effects of flavonoid rich foods on cardiovascular disease risk (Koga & Meydani, 2001).

Polyphenols extracted from olive oil were shown to protect red blood cells against oxidative haemolysis initiated by peroxyl radicals ($ROO^{\bullet}$) generated by 2,2'-azobis [2-methyl-propionamidine] dihydrochloride (AAPH) (Paiva-Martins *et al.*, 2009). The peroxyl radical induced oxidation of lipids and proteins in the erythrocyte membrane and eventually caused haemolysis. Protection was through free radical scavenging activity and interaction with red blood cell membrane protein. Flavan-3-ols (+)-catechin and (-)-epicatechin protected liposomes against 2,2'-azinobis (2,4-dimethylvaleronitrile) (AMVN) induced lipid oxidation (Verstraeten *et al.*, 2003). According to the authors, the flavanols and procyanidins are thought to interact with membrane phospholipids through hydrogen bonding to polar groups of phospholipids and accumulate at the membrane surface maintaining membrane integrity by preventing access of deleterious molecules.

2.1.8. **Methods for the evaluation of antioxidant activity of extracts**

A number of methods have been developed to measure the antioxidant activity of foodstuffs and other substrates and these methods differ with regards to their reaction mechanisms. Some methods use the hydrogen atom transfer (HAT) mechanism which quantifies the capacity of an antioxidant to donate hydrogen atoms, and others use the single electron transfer mechanism (SET) which measures the reductive capacity of antioxidants (Huang, Ou & Prior, 2005; Prior, Wu, Schaich, 2005; Rivero-Pérez, Muñiz & González-Sanjosé, 2007). The methods may be divided into indirect and direct methods. Indirect methods are frequently used to study the ability of an antioxidant to scavenge some free radical that is not associated with real oxidative stress and direct methods measures the ability of an antioxidant to scavenge biologically relevant active free radicals that are involved in oxidative stress, such as peroxyl, superoxide anion and hydroxyl radicals (Roginsky & Lissi, 2005).

The ABTS radical cation scavenging assay is one of the most popular indirect methods (Roginsky & Lissi, 2005) used for determining the antioxidant capacity of plant extracts because of its simplicity. It uses the single electron transfer mechanism (Macdonald, Wood & Garg, 2006; Alvarez-Suarez, Tulipani, Romandini, Vidal & Battino, 2009). This assay measures the ability of an extract to scavenge $ABTS^{\bullet+}$ relative to a standard amount of Trolox (Liyana-Pathirana & Shahidi, 2005). The stable $ABTS^{\bullet+}$, a blue-green chromophore, is generated by potassium persulphate oxidation of $ABTS^{2-}$ at room temperature for 12–16 hours in the dark (Macdonald *et al.*, 2006; Siddhuraju, 2006). The extent of discolouration of

the chromophore by antioxidants or Trolox standard solution is measured spectrophotometrically at 734 nm because $ABTS^{\bullet+}$ has strong absorption in the range 600-750 nm and the results expressed as Trolox equivalents (Roginsky & Lissi, 2005).

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay is an easy and rapid indirect method that is commonly used to evaluate the antiradical activity of antioxidants (Brand-Williams, Cuvelier & Berset, 1995). $DPPH^{\bullet}$ produces a violet colour in methanol and on reduction by an antioxidant results in loss of absorbance (Xu & Chang, 2007) which is monitored spectrophotometrically at 515 nm (Macdonald *et al.*, 2006). The reaction proceeds via an electron transfer mechanism (Alvarez-Suarez *et al*., 2009) as opposed to a hydrogen transfer mechanism which was initially proposed by Brand-Williams *et al.* (1995). The disadvantage of this assay is the slow reaction with extracts which may take up-to 6 hours to reach steady state (Brand-Williams *et al.*, 1995).

The oxygen radical absorbance capacity (ORAC) assay is a robust, reliable and sensitive direct method that measures the ability of an antioxidant to scavenge the biologically relevant peroxyl radical generated by AAPH (Ou, Hampsch-woodill & Prior, 2001). The peroxyl radical attacks fluorescein (FL) (3',6'-dihydroxypiroisobenzofuran-13H,9',9H-xanthene-3-one) resulting in loss of fluorescence which is recorded at specific time intervals as the reaction goes to completion. The presence of an antioxidant compound inhibits free radical damage of fluorescein (Xu & Chang, 2007) by scavenging the radical and its protective effect is measured by assessing the area under the fluorescence decay curve (AUC) compared to that of a blank in which no antioxidant is present (Ou et at., 2001). The results are reported as Trolox equivalents using Trolox as a standard (Macdonald *et al.*, 2006). The area under curve (AUC) indicates the magnitude of inhibition and inhibition time by the antioxidant against oxidation of fluorescein by peroxyl radicals (Ou et at., 2001; Chen & Blumberg, 2008). The reaction proceeds via a hydrogen transfer mechanism (Alvarez-Suarez *et al.*, 2009).

Biologically relevant methods are those that evaluate the scavenging capacity of antioxidants towards reactive oxygen species and those that measure the effect of antioxidants on biomarkers of oxidative stress that are representative of damage to different cellular components, such as membrane, DNA and proteins (Rivero-Pérez *et al.*, 2007).

The inhibition of LDL oxidation assay is a direct and biologically relevant method that measures the end products of lipid peroxidation referred to as thiobarbituric acid reactive substances (TBARS) (Xu *et al.*, 2007a) which are biomarkers of oxidative stress. The LDL molecule has on average 2700 fatty acid molecules, of which half are polyunsaturated fatty acids, mainly linoleic acid and minor amounts of arachidonic and decosahexaenoic acid (Esterbauer, Gebicki, Puhl & Jürgens, 1992). LDL oxidation is a lipid peroxidation radical chain reaction that proceeds in three stages, initiation, propagation and decomposition. The first and second stages generate conjugated diene hydroperoxides as the main products and in the decomposition stage conjugated dienes react further to give the late stage products of lipid peroxidation which are measured by the thiobarbituric acid (TBA) assay (Abuja *et al.*, 1998). In this assay oxidation of isolated human LDL in phosphate buffer saline (PBS) pH 7.4 in the presence or absence of extract or Trolox standard is initiated with Cu^{2+} and the mixture incubated at 37 °C for a period of time (3-4 hours). The reaction is stopped by the addition of EDTA solution, proteins are precipitated with trichloroacetic acid and the late stage products of lipid peroxidation reacted with thiobarbituric acid to form pink chromophores which are measured spectrophotometrically at 532 nm (Xu *et al.*, 2007a).

The oxidative DNA damage assay is a biologically relevant assay that uses DNA damage as a biomarker of oxidative stress. Under oxidative stress super-coiled pBR322 plasmid vector DNA is converted into relaxed circular form due to single strand breaks and into linear form due to double strand breaks and these three forms are separated by agarose gel electrophoresis because of their differences in electrophoretic mobility (Aronovitch, Godinger, Israeli, Krishna, Samuni & Goldstein, 2007). Oxidative DNA breakage is initiated by the addition of Cu(II) (Rivero-Pérez *et al.*, 2007) or hydrogen peroxide to generate hydroxyl radicals (Aronovitch *et al.*, 2007) or AAPH solution to generate peroxyl radicals (Wei, Zhou, Cai, Yang & Liu, 2006) in the presence or absence of the test compound. The mixture is incubated at 37 °C for a period of time after which loading buffer is added and the different DNA forms separated by agarose gel electrophoresis. The ethidium stained bands are visualized and photographed under UV illumination at 300 nm and the intensity of the DNA bands on the photographs are measured using imaging software. The higher the intensity of the supercoiled band, the higher the antioxidant activity of the extract.

Biomembranes are highly susceptible to attack by oxygen radicals (Terao *et al.*, 1994). Red blood cell haemolysis has been used as a model system for the evaluation of the protective

effect of antioxidant compounds against biomembrane oxidative damage (Manna *et al.*, 1999; Somparn, Phisalaphong, Nakornchai, Unchern & Morales, 2007; Tang & Liu, 2008). In this assay isolated erythrocytes are suspended in PBS and oxidative stress of the biomembrane is induced by incubation with AAPH which generate the peroxyl radical (Tang & Liu, 2008) or with hydrogen peroxide which produces the hydroxyl radical (Paiva-Martins *et al.*, 2010) in the presence or absence of the phenolic compound or extract at 37 °C. Peroxidation of lipids and oxidation of proteins in the membrane leads to membrane rupture and haemolysis (Sato, Sato & Suzuki, 1999). The mixture is centrifuged and the degree of haemolysis is measured by taking absorbance readings of the supernatant at 405 nm.

2.1.9. **Gaps in knowledge**

Methanol and acidified methanol extracts from the seed coats of marama beans have been found to contain phenolic compounds (Chingwaru *et al.*, 2011) with possible application as a natural functional antioxidant food ingredient. However, phenolic compounds extracted with organic solvents may not be suitable for food application because these may be toxic to human health (Tsuda *et al.*, 1995). Also the extracted phenolic compounds may not be the same as those released under aqueous conditions that prevail in the gastrointestinal tract (Chen & Blumberg, 2008). Liyana-Pathirana and Shahidi (2005) showed that crude phenolic extracts can be prepared from wheat bran under aqueous conditions, and subjecting the extracts to gastric pH conditions (pH 2) resulted in higher total phenolic content and antioxidant activity due to release of bound phenolic compounds. Preparation of aqueous crude phenolic extracts from marama bean seed coats for application in food has not been investigated. However, the chemical composition of aqueous phenolic extracts are likely to differ from those of methanolic extracts therefore there is a need to determine the phenolic chemical composition and antioxidant activity of the aqueous extracts.

Condensed tannins (proanthocyanidins) have been shown to contribute significantly to the antioxidant activity of extracts from dark coloured seed coats of legumes such as the common bean (Cardador-Martinez *et al.*, 2002; Madhujith & Shahidi, 2005; Ranilla *et al.*, 2007), lentils (Dueñas *et al.*, 2006) and soybeans (Xu & Chang, 2008). A positive correlation between condensed tannin content and antioxidant activity has been reported (Beninger & Hosfield, 2003; Xu, Yuan & Chang, 2007b). Proanthocyanidins in legumes such as the common bean (Gu *et al.*, 2003a; Beninger *et al.*, 2005) and lentils (Dueñas *et al.*, 2003) have

been characterized and they have been found to be predominantly procyanidins which are composed of (+)-catechin and (-)-epicetechin subunits, with small amounts of prodelphinidins which are composed of gallocatechin and epigallocatechin subunits. No studies have been done to establish whether proanthocyanidins are present or not in the seed coats of marama bean, and if present there is a need to characterize their constitutive units in the polymers.

Isolated phenolic compounds and crude phenolic extracts from plant materials have been shown to have some protective effect on biological molecules such as low-density lipoprotein (LDL) (Abuja *et al.*, 1998; Meyer *et al.*, 1998; Rüfer & Kulling, 2006; Xu *et al.*, 2007a), DNA (Aherne & O'Brien, 2000; Rivero-Pérez *et al.*, 2007) and erythrocytes (Manna *et al.*, 1999; Tedesco, Russo, Nazzaro, Russo & Palumbo, 2001; Tang & Liu, 2008; Paiva-Martins *et al.*, 2009) against free radical oxidation. No studies have been done to determine if aqueous extracts from marama bean seed coats could protect biological molecules such as DNA, LDL and biomembrane against oxidative damage by radicals. These studies are important and would give an indication if the phenolic compounds in marama bean seed coats have a potential to protect the body against oxidative stress thereby contributing to the prevention of chronic diseases including cardio vascular disease, neurodegenerative disease and cancer.

CHAPTER 3

3. HYPOTHESES AND OBJECTIVES

3.1. Hypotheses

3.1.1. Acidified water extracts prepared from marama bean seed coats and condensed tannin sorghum bran will have higher total phenolic content, flavonoid content, condensed tannin content, antioxidant activity, and a higher protective effect against erythrocyte haemolysis, LDL oxidation and oxidative DNA damage initiated by radicals compared to water extracts. This is because acidic condition during preparation of acidified water extracts will enhance extraction of free and esterified phenolic compounds and hydrolysis and release of bound phenolic compounds (Liyana-Pathirana & Shahidi, 2005; Li *et al.*, 2007). Phenolic compounds will exert antioxidant activity by scavenging free radicals and chelation of metal cations (Aherne & O'Brien, 2000).

3.1.2. Acidified water extracts prepared from marama bean seat coats and condensed tannin sorghum bran will have higher levels of free phenolic acids such as *p*-coumaric, ferulic and caffeic acids, and flavonoid aglycones compared to water extracts on analysis by HPLC-MS. The acids exist mostly in bound forms esterified to cell wall components (Manach *et al.*, 2004) and the flavonoids exist mostly as glycosides and upon pH treatment bound phenolic acids will be released as free phenolic acids (Liyana-Pathirana & Shahidi, 2005) and flavonoid glycosides hydrolysed to their aglycone forms (Careri *et al.*, 1998).

3.2. Objectives

3.2.1. To determine the effect of extraction under acidic condition on total phenolic, total flavonoid, condensed tannin contents and protein precipitation capacity of aqueous extracts prepared from marama bean seed coat and condensed tannin sorghum bran and to quantify and characterize their phenolic compounds using HPLC–MS.

3.2.2. To determine the effect of extraction under acidic condition on antioxidant activity of aqueous extracts prepared from marama bean seed coat and condensed tannin sorghum bran using the 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^{\bullet+}$), 2,2-diphenyl-1-

picrylhydrazyl (DPPH$^{\bullet}$) scavenging assays and oxygen radical absorbancy capacity (ORAC) assay.

3.2.3. To determine the effect of aqueous extracts prepared from marama bean seed coats and condensed tannin sorghum bran on AAPH-induced oxidative DNA damage and AAPH-induced human erythrocyte haemolysis and copper-catalysed human LDL oxidation.

CHAPTER 4

4. RESEARCH

4.1. Characterization of phenolic acid and flavonoid compounds in aqueous extracts prepared from marama bean seed coats and condensed tannin sorghum bran

Part of this chapter has been published in the International Journal of Food Science and Technology[1] and accepted for publication in the Journal of Food Biochemistry[2].

[1]Shelembe, J.S., Cromarty, D., Bester, M.J., Minnaar, A. and Duodu, K.G. (2012) Characterisation of phenolic acids, flavonoids, proanthocyanidins and antioxidant activity of water extracts from seed coats of marama bean (*Tylosema esculentum*) – an underutilised food legume. *International Journal of Food Science and Technology 47*, 648-655.

[2]Shelembe, J.S., Cromarty, D., Bester, M.J., Minnaar, A. and Duodu, K.G. (2012) Effect of acidic condition on phenolic composition and antioxidant potential of aqueous extracts from sorghum (*Sorghum bicolor*) bran. *Journal of Food Biochemistry* (in press)

4.1.1. Abstract

Marama bean is a legume plant growing wild in arid and semi arid regions of Southern Africa and sorghum is an important cereal crop for populations in arid and semi-arid regions of the world. Freeze-dried aqueous extracts with possible application as antioxidant functional food ingredients were prepared from marama bean seed coats and condensed tannin sorghum bran by extracting with water (water extract) or acidified water pH 2 (acidified water extract). The extracts were analyzed for total phenolic and flavonoid content using spectrophotometric methods and for phenolic acid and flavonoid compounds using HPLC-MS. Water and acidified water extracts from marama bean seed coats had 10 and 5 fold higher total phenolic and total flavonoid content, respectively than equivalent extracts from sorghum bran. Acidified water extract from marama been seed coats had 36, 33% and 38% lower total phenolic content, flavonoid content and overall phenolic compound concentration, respectively, compared to water extracts. The reduction in phenolic content was probably as a result of co-precipitation of phenolic compounds with other polymeric species. In contrast, acidified water extracts from sorghum bran had 15% and 19% higher total phenolic content and overall phenolic compounds concentration, respectively, compared to water extracts, but no significant difference in total flavonoid content was found. Acidic condition enhanced extraction of free, esterified phenolic compounds and released bound phenolic compounds resulting in increased phenolic compound content. Phenolic acids in extracts from marama bean seed coats were benzoic acid derivatives and flavonoids were flavanols esterified to gallic acid. In extracts from sorghum, phenolic compounds were benzoic and cinnamic acid derivatives, phenolic aldehydes and flavonoids from the flavanone subclass.

Kew words: Marama bean seed coat; Sorghum bran; Phenolic compounds; Total phenolic content; Total flavonoid content; HPLC-MS.

4.1.2. Introduction

Nowadays there is considerable interest in dietary phenolics because of their presumed role in the prevention of various degenerative diseases associated with oxidative stress (Scalbert, Morand, Manach & Rémésy, 2002). Oxidative stress is implicated in the development of various chronic diseases including atherosclerotic cardiovascular disease, several cancers (Hodgson, Croft, Puddy, Mori & Beilin, 1996; Xu & Chang, 2007), neurodegenerative conditions (Chen & Blumberg, 2008) and inflammatory conditions (Erlejman, Jaggers, Fraga & Oteiza, 2008). It is initiated by reactive oxygen and nitrogen (ROS/RNS) species. These free radicals are continuously produced in the body and are essential in energy metabolism, detoxification, chemical signalling and immune function and are controlled by endogenous enzymes such as superoxide dismutase, glutathione peroxidase and catalase (Jacob & Burri, 1996; Dimitrios, 2006). However, failure of the antioxidant defence systems to scavenge and minimize the formation of free radicals as well as the overproduction of free radicals leads to oxidative damage of biomolecules such as DNA, lipids and proteins in the human body (Jacob & Burri, 1996; Baublis, Decker & Clydesdale, 2000; Kamath, Chandrashekar & Rajin, 2004). Phenolic compounds are non-nutritive components found in many food and medicinal plants and have various biological functions such as antioxidative, anti-inflammatory and antimutagenic activities (Suda, Ishikawa, Hatakeyama, Miyawaki, Kudo, Hirano, Ito, Yamakawa & Horiuchi, 2008). The antioxidative functions are mediated by their ability to scavenge free radicals and to chelate of transition metals such as copper and iron (Aherne & O'Brien, 2000; Scalbert *et al.*, 2002) which catalyze oxidation reactions.

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The marama bean (*Tylosema* species) plant is drought-tolerant and has potential for cultivation as a food crop (Bower, Hertel, Oh & Storey, 1988). The bean is a potential source of value-added processed products similar to those from soy bean (Maruatona, Duodu & Minnaar, 2010). The seed coats of marama beans are not edible and are discarded as waste material during consumption/processing. A study has shown that organic extracts prepared from the seed coats of marama beans contain phenolic compounds with antioxidant activity. Total phenolic content of acidified methanol (1% HCl in methanol) extract prepared from marama bean seed coats was found to be 24.6 mg catechin equivalents/100 mg on a dry weight basis and phenolic compounds in the extract were phenolic acids and flavonoids (Chingwaru, Duodu, van Zyl, Schoeman, Majinda, Yeboah, Jackson, Kapewangolo, Kandawa-Shultz, Minnaar & Cencic, 2011).

Sorghum (*Sorghum bicolor* (L.) Moench) bran, a by-product of sorghum milling, is a potential source of natural antioxidant compounds with potential health benefits (Awika & Rooney, 2004). During milling, the grain is de-hulled to remove phenolic compounds which are concentrated in the pericarp or outer layers of the grain (bran) and this improves colour, reduces astringency and improves protein digestibility of products produced from the sorghum meal (Dlamini, Taylor & Rooney, 2007). Therefore the bran fraction is an ideal material source for the extraction of phenolic compounds. All sorghums contain phenolic acids and flavonoid compounds but only type II and III sorghums contain high molecular weight condensed tannins (Dykes & Rooney, 2006).

Phenolic compounds are commonly extracted with organic solvents. However, organic solvents may not be compatible with food because they have some degree of toxicity towards human health (Tsuda, Mizuno, Kawakishi & Osawa, 1995). Extraction with water might be a preferred option for pharmaceutical and food grade commercial processes (Oomah, Corbé & Balasubramanian, 2010). Water does not pollute the environment and does not require special management and it is cheaper compared to organic solvents (Llorach, Tomás-Barberán & Ferreres, 2004). Extraction under aqueous acidic condition (using acidified water) may help release bound phenolic compounds from complex carbohydrates and thus increasing yield of antioxidant phenolics (Liyana-Pathirana & Shahidi, 2005). Furthermore, acidification also simulates the pH condition of the stomach and can give an indication of the availability of phenolic compounds during the early stages of digestion in the stomach.

The objective of this study was to determine the effect of extraction under acidic condition on yield, total phenolic content, total flavonoid content and phenolic compound profile (phenolic acid and flavonoid compounds) of aqueous extracts from marama bean seed coats and condensed tannin sorghum bran.

4.1.3. Materials and methods

4.1.3.1. Materials

Marama beans from the 2008 season were obtained from Ghanzi province in Botswana. Condensed tannin sorghum (PAN 3860, a type III sorghum) was from the 2004 season and was cultivated in South Africa. Folin Ciocalteu reagent, sodium carbonate, 1 M hydrochloric acid solution, 1 M sodium hydroxide solution, 32% concentrated hydrochloric acid, HLPC

grade methanol, HPLC grade acetonitrile, ethyl acetate, aluminium chloride, sodium nitrate and sodium hydroxide were purchased from Merck Chemicals (Johannesburg, South Africa). Gallic acid, protocathechuic acid, (+)-catechin hydrate, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, *p*-coumaric acid, (-)-epicatechin-3-O-gallate, ferulic acid, taxifolin, naringen, hesperetin were purchased from Sigma-Aldrich (Johannesburg, South Africa).

4.1.3.2. Methods

4.1.3.2.1. Sample preparation

Marama beans 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 then ground into a powder using a Waring blender, sieved through a 500 µm aperture test sieve and packed under vacuum. The powdered samples were stored at 4 °C until extraction.

Five kilograms of condensed tannin sorghum grain was hand cleaned and sorted to remove diseased, broken grains and foreign materials. The grain was decorticated using a Prairie Research Laboratory (PRL) type sorghum dehuller (Rural Industries Innovation Center, Kanye, Botswana) for 5 min to obtain a bran yield of approximately 7%. Bran was separated from de-hulled grain by sieving through a 1000 µm aperture test sieve. The bran was ground into a powder using a Waring blender to pass through a 500 µm test sieve. Powdered bran sample was packed under vacuum and stored at 4 °C until extraction.

4.1.3.2.2. Preparation of extracts

Aqueous extracts from marama bean seed coats and condensed tannin sorghum bran were prepared with water adjusted to pH 2 (acidified water extracts) or water only (water extracts) according to the methods of Baublis *et al.* (2000) and Liyana-Pathirana and Shahidi (2005) with some modifications. Acidified water extracts were prepared by mixing 10 g sample with 150 ml distilled water and stirring for 30 min using a magnetic stirrer. The pH was adjusted to 2 using 1 M HCl and then incubated in a shaking water bath at 37 °C for 30 min. The pH was then adjusted to 6 using 1 M NaOH and incubation continued in a shaking water bath at 37 °C for 30 min. Water extracts were prepared by mixing 10 g sample with 150 ml distilled water containing 0.3 g NaCl (equivalent to the amount produced by the reaction

between 1 M HCl and 1 M NaOH added in acidified water extract) and stirring for 30 min using a magnetic stirrer. Extraction was further carried out in a shaking water bath at ambient temperature for 60 min. Both water and acidified water extracts were centrifuged at 7500 g, 25 °C for 15 min using a model Rotanta 460R centrifuge (Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany). The supernatant was decanted and freeze-dried. Extracts were weighed and stored at -20 °C in sealed zip-lock bags until analyzed.

4.1.3.2.3. Total phenolic content

Total phenolic contents of the extracts were determined using the Folin-Ciocalteu method (Singleton & Rossi, 1965). Freeze-dried marama bean seed coat (25 mg) and sorghum bran (75 mg) extracts were weighed into 25 ml volumetric flasks and made up to volume with distilled water. Catechin standard serial dilutions ranging from 0 to 1000 mg/L were prepared from a stock solution of catechin (1250 mg/L). Extract solution or catechin standard solution (0.5 ml) was added into a 50 ml volumetric flask containing 10 ml distilled water. Folin-Ciocalteu's reagent (2.5 ml) was added and the contents were mixed before 7.5 ml sodium carbonate solution (20 g/100 ml) was added within 1-8 minutes after the addition of Folin-Ciocalteu's reagent. The contents were mixed again and made-up to the mark with distilled water. Two hours after the addition of sodium carbonate solution the absorbance was measured at 760 nm using a T80+ UV-VIS spectrophometer (PG Instruments, Wibtoft, UK) and data acquired with UVWin software version 5.0.5 (PG Instruments, Wibtoft, UK). Total phenolic content were reported as mg CE/g (mg catechin equivalens per gram) on dry sample weight basis.

4.1.3.2.4. Total flavonoid content

Total flavonoid content was determined as described by Ali-Farsi and Lee (2008) with some modification. Five milligrams of freeze-dried marama bean seed coat and condensed tannin sorghum bran extracts were dissolved in 2 ml and 1 ml distilled water, respectively, to a final concentration of 2.5 mg/ml and 5 mg/ml, respectively. (+)-Catechin standard serial dilutions ranging from 0–0.8 mg/ml were prepared from a 1.2 mg/ml stock solution. Extract or catechin standard solution (30 µl) was added into the wells of a 96 well plate. Then 20 µl of 36 mM sodium nitrate, 20 µl 94 mM aluminium chloride and 100 µl of 0.5 M sodium hydroxide solutions were added to each well. Absorbance was read at 450 nm on a 96 well

plate reader (Bio-Tek EL X800, Bio-tek Instruments Inc, Winooski, USA). A calibration curve was prepared from catechin standards and flavonoid content reported as mg catechin equivalents per gram of sample (mg CE/g) on dry weight basis.

4.1.3.2.5. Characterization of phenolic acids and flavonoids in aqueous extracts by HPLC-MS

Extracts were subjected to acid catalyzed hydrolysis and extracted with ethyl acetate according to the methods described by Hahn, Faubion and Rooney (1983) and Svensson, Sekwati-Manang, Lutz, Schieber and Ganzle (2010) with some modification. Freeze-dried marama bean seed coat (6 mg) and condensed tannin sorghum bran (20 mg) extracts were dissolved in 5 ml of 2 M HCl. The mixture was heated in a shaking water bath at 98 °C for 1 hour. The solution was allowed to cool and phenolic acid and flavonoid compounds were extracted twice with 5 ml ethyl acetate using a separation funnel. After partitioning, the ethyl acetate fractions were combined and then evaporated to dryness in a rotary vacuum evaporator model Rotavapor RE 120 (Büchi Labortechnik AG, Flawil, Switzerland) at 30 °C. The residues (after evaporation of ethyl acetate) from marama bean seed coat and condensed tannin sorghum bran were re-dissolved in 2 ml and 1 ml methanol, respectively, filtered through a 0.2 µm PTFE filter and stored at 4°C until analysis.

*HPLC condition*s: The HPLC system consisted of a Waters 1525 binary pump, Waters 2487 dual wavelength absorbance detector (Waters Associates, Milford, USA) and a YMC-Pack ODS AM-303 (250 x 4.6 mm i.d., 5 µm particle size) reverse phase column (Waters Associates, Milford, USA). The HPLC conditions were according to Kim, Hyun, Kim, Park, Kim, Kim, Lee, Chun & Chung (2007). Mobile phase A was 0.1% acetic acid in water and mobile phase 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). Sample injection volume was 20 µl. Flow rate was maintained at 0.8 ml/min and total run time was 61 min. Phenolic compounds were detected at 280 nm and data acquired by Breeze system software (Waters Associates, Milford, USA). Calibration curves were prepared from phenolic standards serial dilutions and concentration of each compound in samples calculated and data was reported as µg/g of sample on dry weight basis.

HPLC/ESI-MS conditions: The system consisted of an Agilent 1100 series HPLC system with model G1312A binary pump, model G1322A degasser, model G1367A auto sampler, model G1316A column heater and model G1315B photo diode array detector (Agilent Technologies, Santa Clara, CA, USA). 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. The HPLC system was coupled to a triple quadrupole / linear ion trap mass spectrometer, model 4000 Q TRAP LC/MS/MS system (AB SCIEX, Concord, Canada). Injection sample volume was 20 µl and solvent gradient was the same as previously outlined under HPLC conditions except that column temperature was set at 30 °C and detector wavelength at 190-600 nm. The operating conditions for the mass spectrometer were set as follows: The source temperature was maintained at 400 °C, ion spray voltage at -4000 V (negative mode). 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 (EP) potential 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, Concord, Canada).

4.1.3.3. Statistical analysis

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

4.1.4. Results and discussion

4.1.4.1. Yield, total phenolic and total flavonoid content of aqueous extracts

Table 4.1.1 shows that water extracts from marama bean seed coats had the highest yield amongst all the extracts. Extraction of marama bean seed coats under acidic condition (acidified water extract) resulted in a 20% significant reduction in yield compared to extraction with water (water extract). In contrast, extraction of sorghum bran under acidic condition resulted in a 7% significant increase in yield compared to extraction with water.

The total phenolic and flavonoid content of water extract from marama bean seed coats were the highest amongst all the extracts following the same trend as the yield. Extraction of marama bean seed coats under acidic condition resulted in 36% and 33% reduction in total phenolic content and flavonoid content, respectively compared to water extracts.

Table 4.1.1. Effect of extraction under acidic condition on yield, total phenolic and total flavonoid content of aqueous extracts from marama bean seed coats and condensed tannin sorghum bran

Sample	% Yield[1]	Total phenolic content (mg CE[2] / g)	Total flavonoid content (mg CE/g)
Marama bean seed coat			
Water extract	13.4[3] d[4] ± 0.16	62.1 d ± 1.3	22.2 c ± 0.9
Acidified water extract	10.7 c ± 0.44	39.5 c ± 2.3	14.9 b ± 1.2
Sorghum bran			
Water extract	9.7 a ± 0.05	5.8 a ± 0.1	4.3 a ± 0.2
Acidified water extract	10.4 b ± 0.05	6.7 b ± 0.1	4.7 a ± 0.2

[1] Grams of freeze dried aqueous extract/100 g of dry sample weight

[2]CE, Catechin equivalent

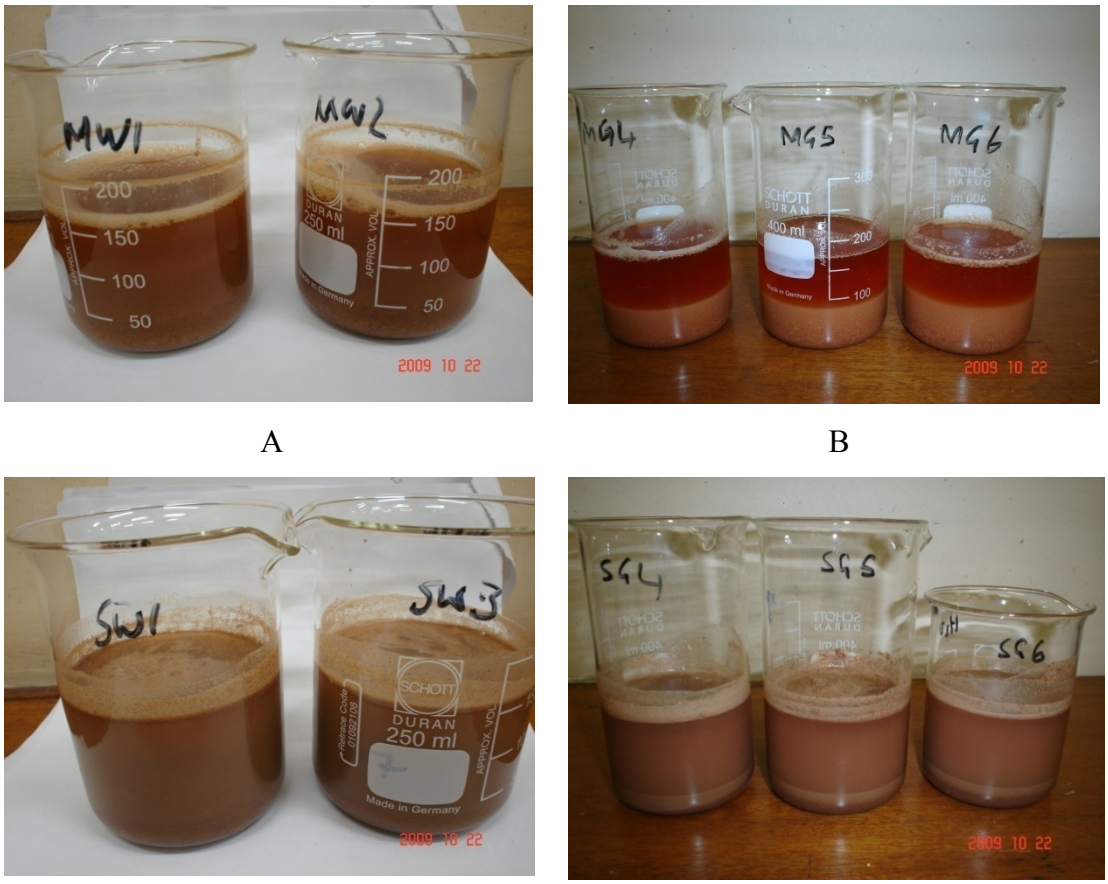
[3]Data are means ± standard deviations of four determinations, on dry weight basis

[4]Means followed by different letters in the same column are significantly different ($p \leq 0.05$)

In contrast, extraction of condensed tannin sorghum bran under acidic condition resulted in a 15% and 9% increase in total phenolic and flavonoid content, respectively. However the increase in flavonoid content was not significant ($p>0.05$) when compared to water extracts.

Water extract from marama bean seed coats appeared opaque without coagulation before removal of insoluble solids by centrifugation (Fig. 4.1.1A). However, in acidified water

extracts a precipitate was observed after acid treatment to pH 2 before removal of insoluble solids by centrifugation (Fig. 4.1.1B). The precipitate appeared to be as a result of a coagulation/flocculation process, leaving a transparent brownish supernatant. The precipitate was likely to be an interpolymer complex formed between non-ionic polymers such as cellulose or other hydroxyl containing polymers and ionic polymers (Khutoryanskiy, Dubolazov & Mun, 2009) such as tannins due to their ionic behaviour in solution (Roussy, Chastellan, van Vooren & Guibal, 2005).



A B

C D

Figure 4.1.1. Appearance of aqueous extracts from marama bean seed coats and condensed tannin sorghum bran after treatment; A) Marama water extract; B) Marama acidified water extract; C) Sorghum water extract; D) Sorghum acidified water extract.

These complexes are formed under acidic conditions (below pH 3) due to the formation of hydrogen bonds between ionic polymers and proton-accepting non-ionic polymers (Khutoryanskiy *et al.*, 2009). Low molecular weight compounds (such as phenolic acids and flavonoids) may be incorporated into the interpolymer complex through reversible non-

covalent binding (Molyneux & Vekavakayanondha, 1986). Therefore the decrease in yield, total phenolic and flavonoid content of acidified water extracts from marama bean seed coat could be as a result of co-precipitation of phenolic compounds with interpolymer complexes initiated by change in pH.

Acidified water extracts from condensed tannin sorghum bran (Fig. 4.1.1D) did not show as much coagulation and precipitation, and the medium was slightly cloudier and more opaque compared to the water extract (Fig. 4.1.1C). Liyana-Pathirana & Shahidi (2005) reported a two fold increase in yield and 2–5 fold increase in total phenolic content of extracts from soft and hard wheat bran, as a result of extraction under acidic condition. According to the authors the increase was attributed to the release of phenolic compounds esterified to non-starch polysaccharides such as arabinoxylans under simulated acidic conditions. Extraction of phenolic compounds following acid or base hydrolysis has been shown to results in extracts with higher levels of phenolic compounds (Hahn *et al.*, 1983; Madhujith & Shahidi, 2009), which is attributed to release of bound phenolic compounds. Therefore the increase in yield, total phenolic and flavonoid content in acidified water extracts from sorghum bran could be as a result of a similar process. However, in acidified water extracts from marama bean seed coats bound phenolic compounds released by acid hydrolysis were possibly co-precipitated with interpolymer complexes at low pH resulting in lower yield, total phenolic content and total flavonoid content.

The total phenolic content of water and acidified water extracts from marama bean seed coat were 75% and 84%, respectively lower than that of acidified methanolic extracts prepared from marama bean seed coats (246 mg catechin equivalent/g, dry weight basis) (van Zyl, 2007). This is because water is a poor extraction solvent for phenolic compounds and it can also extract soluble polysaccharides and proteins compared to methanol. The solubility of phenolic compounds in water is very low compared to organic solvents such as methanol and acetone (Ali-Farsi & Lee, 2008).

Kobue-Lekalake, Taylor and de Kock (2007) reported that the total phenolic content of infusion from bran of the same sorghum cultivar used in this study (PAN 3860) prepared by boiling in water for 20 min was 48.6 g tannic acid equivalents per kg (4.9% (w/w)). These results were comparatively higher than that of water extracts and acidified water extracts as shown in Table 4.1.1 (0.58% and 0.67% (w/w), respectively). This could be due to enhanced

extraction of phenolic compounds by boiling which breaks down cell walls and thus releasing compounds into extraction solution. Awika, Rooney, Wu, Prior and Cisneros-Zevallos (2003) reported that the total phenolic content of aqueous acetone extracts from bran of Hi tannin and Sumac (SU99) sorghum were 55 and 66 mg gallic acid equivalent/g on dry weight basis (5.5 % (w/w) and 6.6 % (w/w) respectively). Sikwese and Duodu (2007) reported that the total phenolic content of 100% methanol extracts from bran of a condensed tannin sorghum variety (*Phatafuli*) was 101 mg tannic acid equivalent/g on dry weight basis (10.1% (w/w)). The comparatively lower total phenolic content in the water and acidified water extracts from the condensed tannin sorghum bran may be mainly due to water being a poor solvent for the extraction of phenolic compounds compared to organic solvents (Ali-Farsi & Lee, 2008). Nevertheless, the amount of phenolic compounds in the aqueous extracts is an indication of the bio-available fraction i.e. phenolic compounds that may have health promoting effects when added to the diet. It must be mentioned that it is generally difficult to compare total phenolic contents in literature directly due to the fact that there is no uniformity in the standards used.

Total phenolic contents of water and acidified water extracts from marama bean seed coat were 10 and 5 fold respectively, higher than equivalent extracts from condensed tannin sorghum bran. Also, the flavonoid contents were 6 and 3 fold respectively, higher than equivalent extracts from condensed tannin sorghum bran.

4.1.4.2. Characterization of phenolic acid and flavonoid compounds by HPLC-MS

The retention times, mass spectra and λ_{max} of selected phenolic compound standards were determined under the same separation conditions as samples and the results are shown in Table 4.1.2.

4.1.4.2.1. Marama bean seed coats

The phenolic compound profiles of hydrolysed water and acidified water extracts from marama bean seed coats were similar. The HPLC and total ion current chromatograms are presented in Fig. 4.1.2 and the mass spectra data is presented in Fig. 4.1.3.

Table 4.1.2. Mass spectra and UV data for selected phenolic acid and flavonoid standard compounds

Peak No.	Compound	Molecular weight (g/mol)	t_R* (min)	*m/z* $[M-H]^-$ (% intensity)	Fragment ions *m/z* MS^n (% intensity)	HPLC-DAD (λ_{max} nm)
1	Gallic acid	170.12	7.1	169 (100)	125 (51)	216, 272
2	Protocatechuic acid	154.10	10.8	153 (100)	109 (55)	218, 260, 294
3	Catechin	290.29	14.1	289 (100)	-	208, 228, 278
4	*p*-Hydroxybenzoic acid	138.12	14.7	137 (100)	93 (53)	198, 208, 256
5	Vanillic acid	168.10	16.2	167 (100)	123 (27), 109 (13)	216, 260, 292
6	Caffeic acid	180.20	16.6	179 (100)	135 (61)	216, 324
7	*p*-Coumaric acid	164.20	21.4	163 (100)	119 (91)	212, 222, 310
8	(-)-Epicatechin 3-O-gallate	442.40	22.6	441 (100)	289 (15), 208 (36), 179 (25)	208, 324
9	Ferulic acid	194.19	23.0	193 (100)	178 (39), 149 (19), 134 (26)	216, 322
10	Taxifolin	304.25	24.1	303 (100)	285 (16)	202, 290
11	Naringenin	272.26	35.7	271 (100)	151 (7)	194, 288
12	Hesperetin	302.30	36.4	301 (100)	242 (8)	204, 288

t_R* - Retention time

- No framgment ion for parent compound

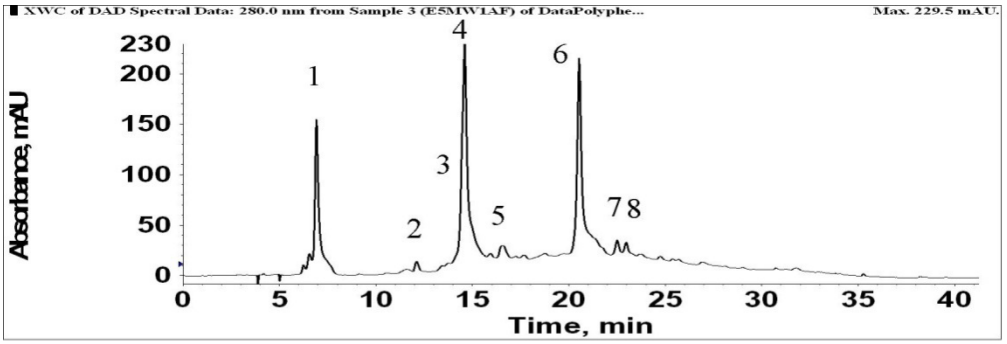
The identity of the major phenolic acids and flavonoids are summarized in Table 4.1.3. Peak 1 with the most abundant $[M-H]^-$ molecular ion at *m/z* 169.8 (100) (Fig. 4.1.3A) corresponded to gallic acid (MW=170.12). The major fragment ion at *m/z* 125.6 (63) corresponded to decarboxylated gallic acid ion. The retention time, fragmentation pattern and UV λ_{max} were similar to that of gallic acid standard (Table 4.1.2). Gallic acid has been reported in alkaline hydroylsates of seed coats from mung beans, lentils, faba beans, pigeon and cowpeas (Sosulski & Dabrowski, 1984). It has also been reported to occur as a component of condensed tannins in legumes in the form of (-)-epicatechin 3-O-gallate as extender and terminal units (Dueñas, Hernández & Estrella, 2006) and procyanidin gallate in red lentils (Amarowicz, Estrella, Herńandez, Dueñas, Agnieszka, Kosińiska & Pegg, 2009).

However, gallic acid was not found in aqueous acetone extracts from the seed coats of black soy beans (Xu & Chang, 2008) and aqueous methanol extracts from the seed coats of fifteen Brazilian and Peruvian bean cultivars (Rannilla, Genovese & Lajolo, 2007). From these reports it would appear that gallic acid does not occur in free form in the seed coats but as part of proanthocyanidin structure, which would explain its absence in aqueous methanol and acetone extracts and its presence in the alkaline hydrolysates.

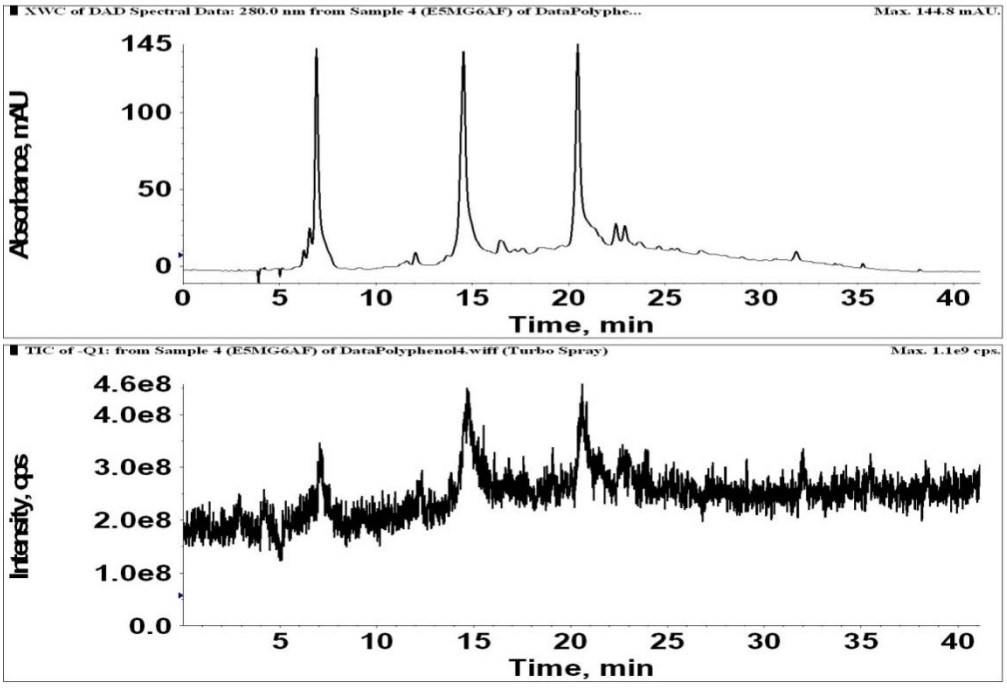
The compound eluting in peak 2 did not match any of the standards. The most abundant $[M-H]^-$ molecular ion at *m/z* 167.6 (100) (Fig. 4.1.3B) suggested that this compound could be homogentisic acid (MW = 168.14). The fragment ion at *m/z* 123.6 (100) corresponded to decarboxylated homogentisic acid $[M-H-44]^-$ molecular ion. Homogentisic acid has been reported in lentils (Zadernowski, Pierzynowska-Korniak, Ciepielewska & Forniak, 1992). The compound eluting in peak 3 with abundant $[M-H]^-$ molecular ion at *m/z* 137.6 (100) (Fig. 4.1.3C) corresponded to *p*-hydroxybenzoic acid (MW = 138.12). The retention time spectra matched that of *p*-hydroxybenzoic acid standard (Table 4.1.2). *p*-Hydroxybenzoic acid has been reported in the seed coats of black soybeans (Xu & Chang, 2008), lentils (Zadernowski *et al.*, 1992), mung beans, fababeans, pigeon pea, lima beans, chickpea and cowpeas (Sosulski & Dabrowski, 1984).

The compound eluting in peak 4 did not match any of the standard phenolic compounds. It had an abundant $[M-H]^-$ molecular ion at *m/z* 455.9 (100) (Fig. 4.1.3D) and fragment ion at

m/z 169.3 (10) and it was tentatively identified as methyl (epi)catechin-3-O-gallate (MW = 456).

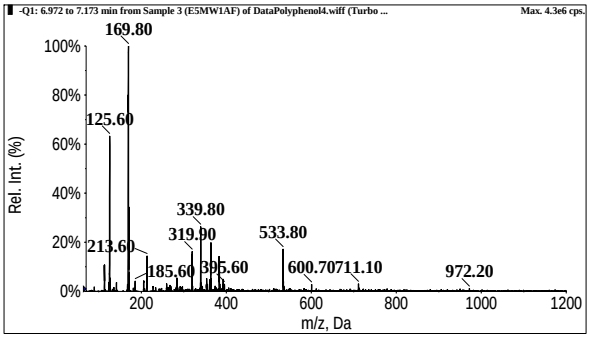


(A

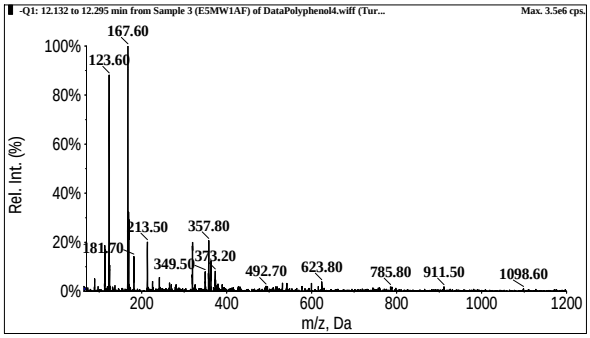


(B)

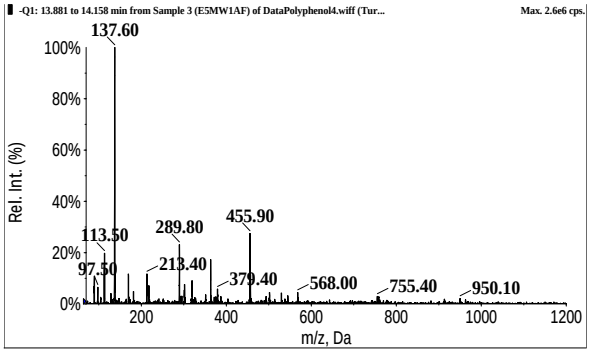
Figure 4.1.2. HPLC chromatogram recorded at 280 nm (top) and total ion current (TIC) chromatogram (bottom) of phenolic compounds in A) water and B) acidified water extracts from marama bean seed coats. Peak identities are shown in Table 4.1.3.



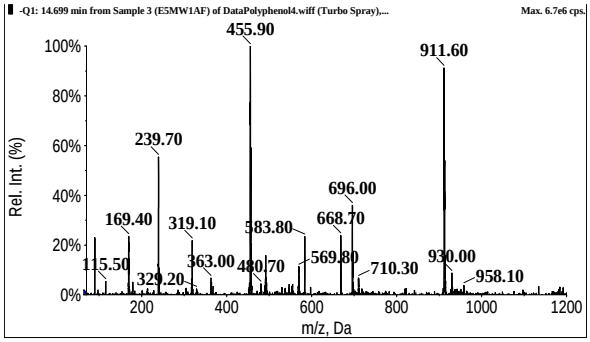
A) Gallic acid



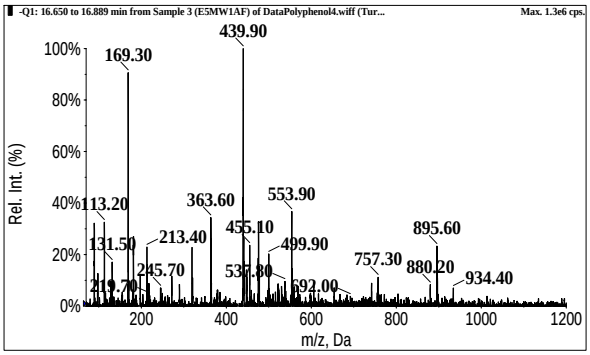
B) Homogentisic acid



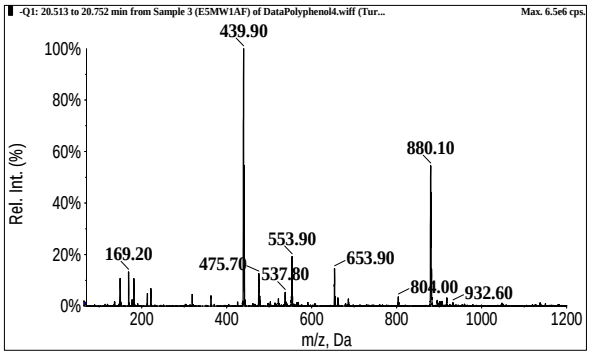
C) *p*-Hydroxybenzoic acid



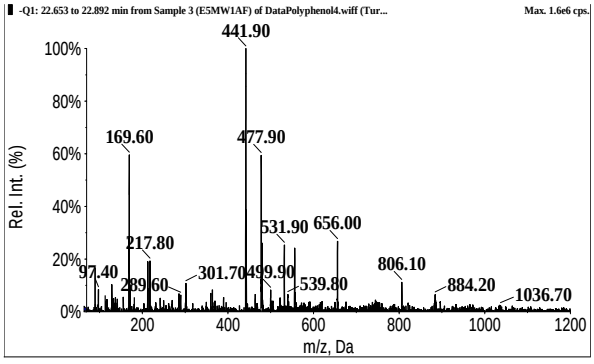
D) Methyl (epi)catechin-3-O-gallate



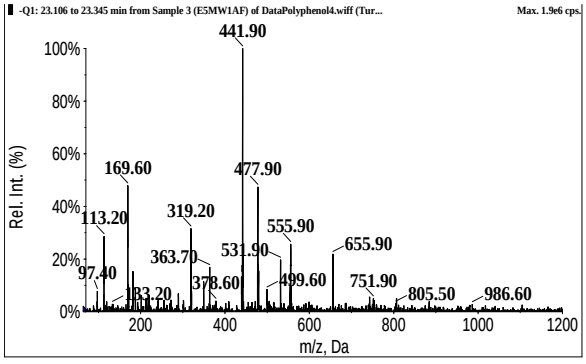
E) Methyl (epi)afzelechin-3-O-gallate



F) Methyl (epi)afzelechin-3-O-gallate



G) (-)-Epicatechin-3-O-gallate



H) (+)-catechin-3-O-gallate

Figure 4.1.3. Mass spectra of phenolic acid and flavonoid compounds in aqueous extracts from marama bean seed coats.

This compound has been previously identified in green tea (*Camelia sinensis*) by the same molecular ion *m/z* 455 (Bastos, Saldanha, Catharino, Sawaya, Cunha & Eberlin, 2007). However, the position of the methyl group could not be established by mass spectrometry so this compound could be 4'-O-methyl (epi)catechin-3-O-gallate or 3'-O-methyl (epi)catechin-3-O-gallate. Also the ion at *m/z* 239.7 (58) which appeared to be a major fragment ion could not be identified. It is likely that it was a molecular ion of another compound co-eluting with methyl (epi)catechin-3-O-gallate.

Peak 5 did not match any of the standard phenolic compounds. The mass spectra (Fig. 4.1.3E) showed an abundant $[M-H]^-$ molecular ion at *m/z* 439.9 (100) and fragment ion at *m/z* 169.3 (91). The molecular weight of this compounds was 16 Da (one oxygen) lower than that of methyl (epi)catechin-3-O-gallate and the compound was tentatively identified as methyl (epi)afzelechin-3-O-gallate (MW = 440).

In their work on identifying phenolic antioxidants Bastos *et al.* (2007) reported a similar molecular ion at *m/z* 439 for one of the phenolic compounds in the mass spectrum of ethanolic extracts from green tea. However, it could not be identified. Peak 6 which was the third largest peak had the same abundant $[M-H]^-$ molecular ion at *m/z* 439.9 (100) and fragment ion at *m/z* 169.2 (13) (Fig. 4.1.3F) as peak 5. This compound was tentatively identified as an isomer of methyl (epi)afzelechin-3-O-gallate.

The compound eluting in Peak 7 with abundant $[M-H]^-$ molecular ion at *m/z* 441.9 (100) (Fig. 4.1.3G) corresponded to (-)-epicatechin-3-O-gallate standard (MW=442) (Table 4.1.2). Fragmentation gave rise to *m/z* 289.6 (7) ion corresponding to epicatechin ion and *m/z* 169.6 (60) ion corresponding to gallic acid ion. Similar molecular and fragment ions have been observed for epicatechin-3-O-gallate from green tea (Miketova, Schram, Whitney, Kerns, Valcic, Timmermann & Volk, 1998) and oolong tea (Dou, Lee, Tzen & Lee, 2007). The compound eluting in peak 8 with abundant $[M-H]^-$ molecular ion at *m/z* 441.9 (100) (Fig.

4.1.3H) had the same molecular weight as (-)-epicatechin-3-O-gallate in peak 7, suggesting that it could be its isomer (+)-catechin-3-O-gallate. Fragmentation gave rise to *m/z* 289.6 (6) and *m/z* 169.6 (59) ions which corresponded to catechin and gallic acid ions, respectively.

Table 4.1.3. Phenolic acid and flavonoid compounds identified in aqueous extracts prepared from marama bean seed coats

Peak No	t_{R*} min	*m/z* $[M-H]^-$ (% Intensity)	Fragment ion m/z (% Intensity)	HPLC-DAD λ_{max} (nm)	Compound identity
1	7.2	169.8 (100)	125.6 (63)	214, 270	Gallic acid
2	11.8	167.6 (100)	123.6 (88)	208	Homogentisic acid
3	13.9	137.6 (100)	-	208, 278	*p*-Hydroxybenzoic acid
4	14.6	455.9 (100),	239.7 (58), 169.4 (10)	214, 280	Methyl (epi)catechin-3-O-gallate
5	16.7	439.9 (100)	169.3 (91)	210, 280	Methyl (epi)afzelechin-3-O-gallate
6	20.5	439.9 (100)	169.2 (13)	208, 280	Methyl (epi)afzelechin-3-O-gallate
7	22.6	441.9 (100)	289.6 (7), 169.6 (59)	208, 278	(-)-Epicatechin-3-O-gallate
8	22.9	441.9 (100)	169.6 (48)	208, 278	(+)-Catechin-3-O-gallate

*t_R-retention time

- No fragment ion for the parent compound was observed

The concentration of phenolic acid and flavonoid compounds in the aqueous extracts from the seed coats of marama bean is shown in Table 4.1.4. Methyl (epi)afzelechin-3-O-gallate isomers were the major compounds in the extracts accounting for at least 40% of total phenolic acid and flavanoid compound content. Methyl (epi)catechin-3-O-gallate was the second major compound accounting for at least 28% and gallic acid was third accounting for 10%. The concentration of all the phenolic compounds in acidified water extracts was significantly higher than that of water extracts. Extraction under acidic condition resulted in a significant reduction in concentration of all the compounds. The percentage reduction ranged from 24% for gallic acid to 54% for *p*-hydroxybenzoic acid. The overall reduction in phenolic acid and flavonoid compounds as a result of extraction under acidic condition was 38% and this was similar to the 36% reduction in total phenolic content as determined with the Folin Ciocalteu method. As explained earlier, the reduction could be as a result of phenolic compounds co-precipitating with interpolymer complex triggered by low pH.

Table 4.1.4. Effect of extraction under acidic condition (pH 2) on phenolic acid and flavonoid compounds in aqueous extracts from marama bean seed coat[1]

Peak No	Compound	Water extract µg/g	Acidified water extract µg/g
1	Gallic acid	1981.0 b[2] ± 178.0	1510.2 a ± 115.2
2	Homogentisic acid[3]	489.9 b ± 57.6	277.6 a ± 13.6
3	*p*-Hydroxybenzoic acid	1760.2 b ± 156.6	802.4 a ± 34.2
4	Methyl (epi)catechin-3-O-gallate[4]	5839.9 b ± 424.4	3265.5 a ± 250.6
5, 6	Methyl (epi)afzelechin-3-O-gallate[4]	8339.2 b ± 334.0	5464.7 a ± 502.6
7, 8	(Epi)catechin 3-O-gallate[4]	1744.5 b ± 159.7	1133.5 a ± 80.9
	Total	20154.7 b ± 1067.5	12453.9 a ± 673.9

[1] Results are means ± standard deviation of four determinations from two independent experiments on dry weight basis

[2] Means with different letters in the same row are significantly different ($p \leq 0.05$)

[3] Values expressed as protocatechuic acid equivalents

[4] Values expressed as (-)-epicatechin-3-O-gallate equivalents

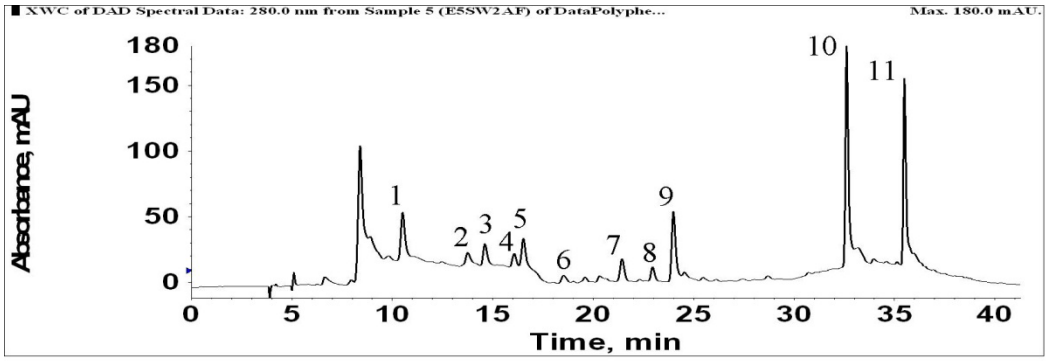
4.1.4.2.2. Condensed tannin sorghum bran

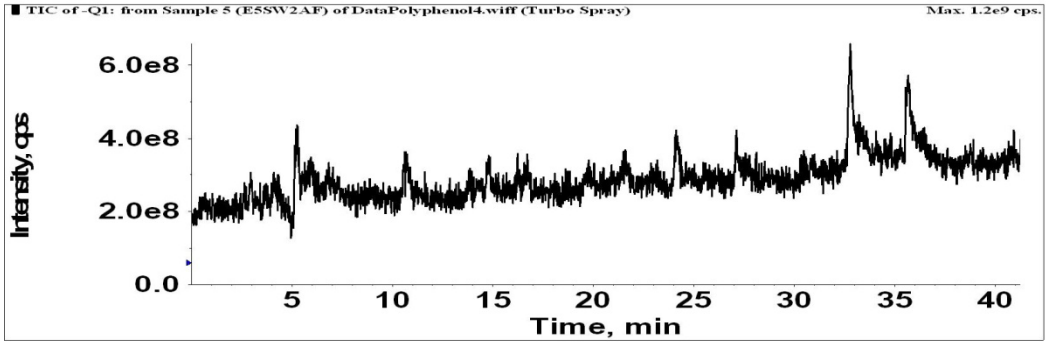
HPLC and total ion current chromatograms of phenolic compounds in water extracts and acidified water extracts from condensed tannin sorghums bran are shown in Fig. 4.1.4 and mass spectra data in Fig 4.1.5. The peak identities are shown in Tables 4.1.5. The profiles of the HPLC chromatograms and total ion current chromatograms for water and acidified water extracts were similar and eleven phenolic compounds were identified in the extracts.

Peak 1 with an abundant $[M-H]^-$ molecular ion at *m/z* 153.7 (100) (Fig. 4.1.5A, which corresponded to authentic protocatechuic acid standard (MW=154.10). On fragmentation it gave rise to $[M-H-44]^-$ ion at *m/z* 109.6 (31) as a result of decarboxylation. The retention time, mass spectra and λ_{max} were similar to that of protocatechuic acid authentic standard (Table 4.1.2).

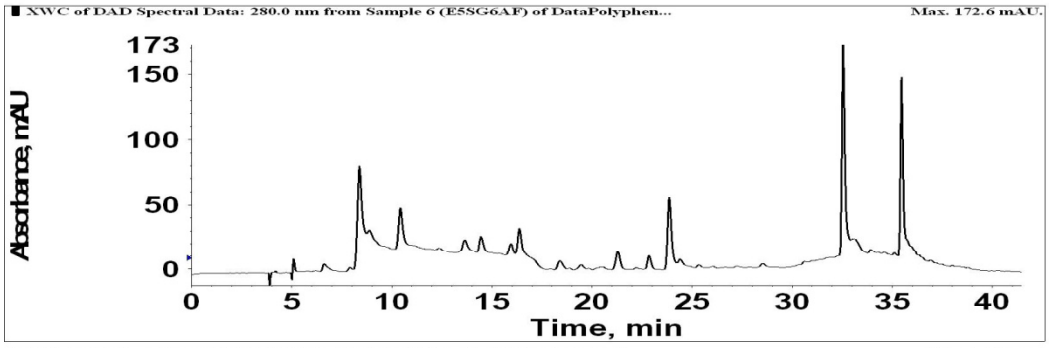
Peak 2 did not match any of the authentic phenolic standards. The most abundant $[M-H]^-$ molecular ion at *m/z* 137.6 (100) (Fig. 4.1.5B) suggested that it could be protocatechualdehyde (MW = 138.12). Protocatechualdehyde has been reported in aqueous methanol extracts from sorghum grain (Svensson *et al.*, 2010). The compound eluting in peak 3 with abundant $[M-H]^-$ molecular ion at *m/z* 137.6 (100) (Fig. 4.1.5C) corresponded to *p*-hydroxybenzoic acid (MW = 138.12) and on decarboxylation resulted in $[M-H-44]^-$ fragment ion at *m/z* 93.4 (61). The retention time, mass spectrum, and λ_{max} matched that of *p*-hydroxybenzoic acid authentic standard (Table 4.1.2).

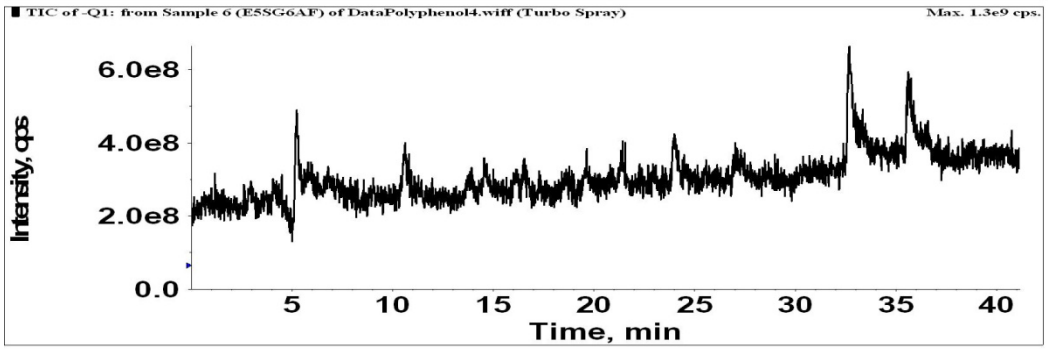
Compound in peak 4 did not match any of the phenolic standards. The abundant $[M-H]^-$ molecular ion at *m/z* 253.7 (100) (Fig. 4.1.5D) suggested that it could be caffeoylglycerol (MW = 254.08) which has been reported in aqueous methanol extract from sorghum grain (Svensson *et al.*, 2010). Traces of vanillic acid (MW=168.15) identified by $[M-H]^-$ molecular ion at *m/z* 167.6 appeared to co-elute with caffeoylglycerol at a retention time of approximately 15.9 min. Peak 5 with abundant $[M-H]^-$ molecular ion at *m/z* 179.6 (Fig. 4.1.5E) corresponded to caffeic acid standard (MW=180.2) (Table 4.1.2) and on decarboxylation gave rise to $[M-H-44]^-$ fragment ion at *m/z* 135.3 (60).





(A)





(B)

Figure 4.1.4. HPLC chromatogram recorded at 280 nm (top) and total ion current (TIC) chromatogram (bottom) of phenolic compound profile of A) water and B) acidified water extracts from condensed tannin sorghum bran.

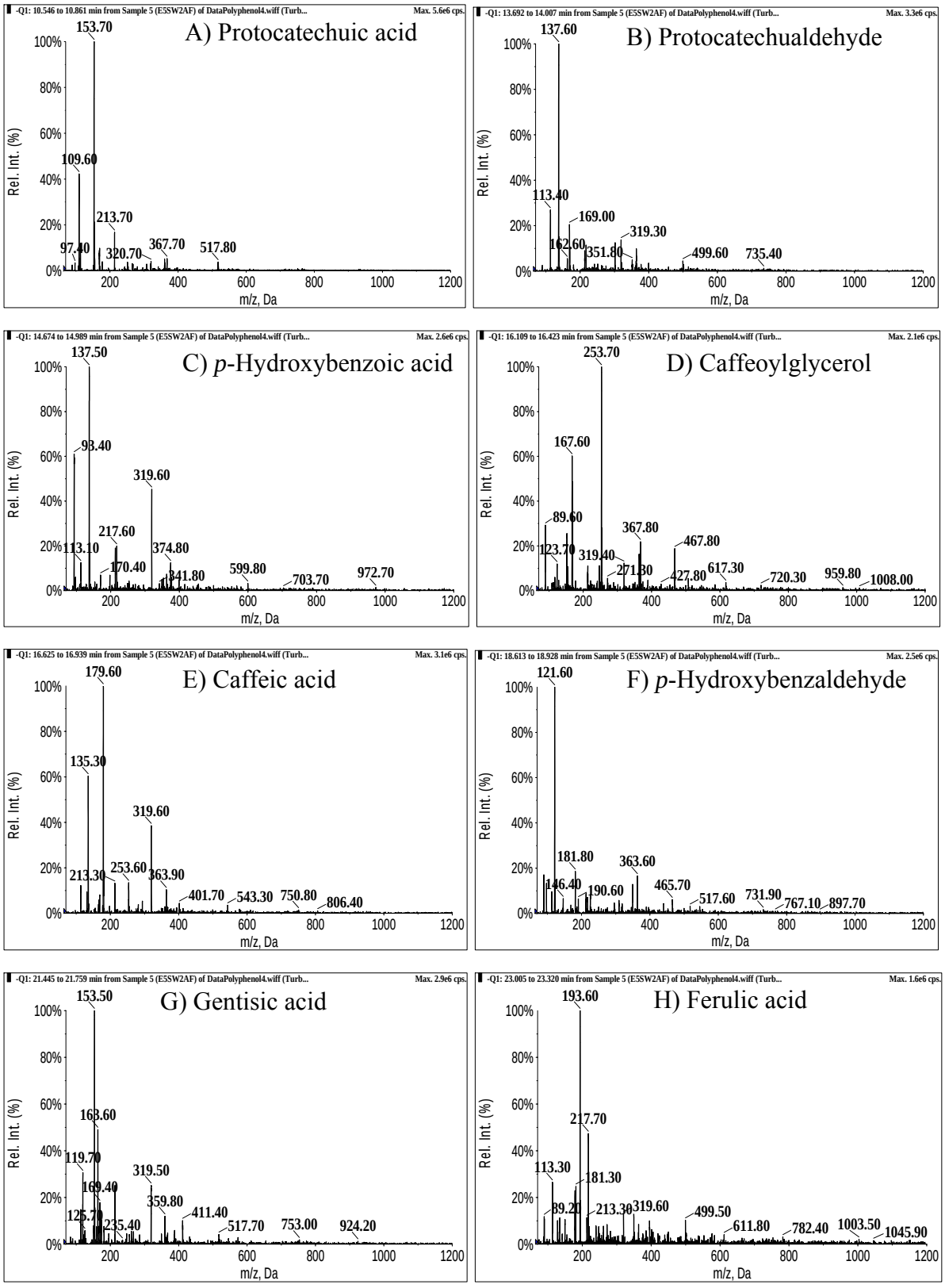


Figure 4.1.5. Mass spectra of phenolic acid and aldehyde compounds in water extracts prepared from condensed tannin sorghum bran.

Peak 6 did not match any of the phenolic standards. The abundant [M-H]$^-$ molecular ion at *m/z* 121.6 (100) (Fig. 4.1.5F) suggested that it could be *p*-hydroxybenzaldehyde (MW= 122.12) which has been reported in methanolic extracts from sorghum grain (Hahn *et al.*, 1983) and ethanol extracts from aerial parts of sorghum (Sène, Gallet & Doré, 2001). Peak 7 with abundant [M-H]$^-$ molecular ion at *m/z* 153.5 (100) (Fig. 4.1.5G) did not match any of the authentic phenolic standards and it was tentatively identified as gentisic acid (MW = 154.14). Gentisic acid has been reported in acidified acetonitrile extracts from Korean sorghum varieties (Chung, Kim, Yeo, Kim, Seo & Moon, 2011), aqueous methanol extracts from Sudanese sorghum cultivars (Awadelkareem, Muralikrishna, EL Tinay & Mustafa, 2009) and in extracts from sorghum aerial parts (Sène *et al.*, 2001). Co-eluting with this compound was the [M-H]$^-$ molecular ion at *m/z* 163.6 (49) which corresponded to *p*-coumaric acid standard (MW = 164.2) (Table 4.1.2) and [M-H-44]$^-$ fragment ion at *m/z* 119 (31) was as result of decarboxylation of the parent compound.

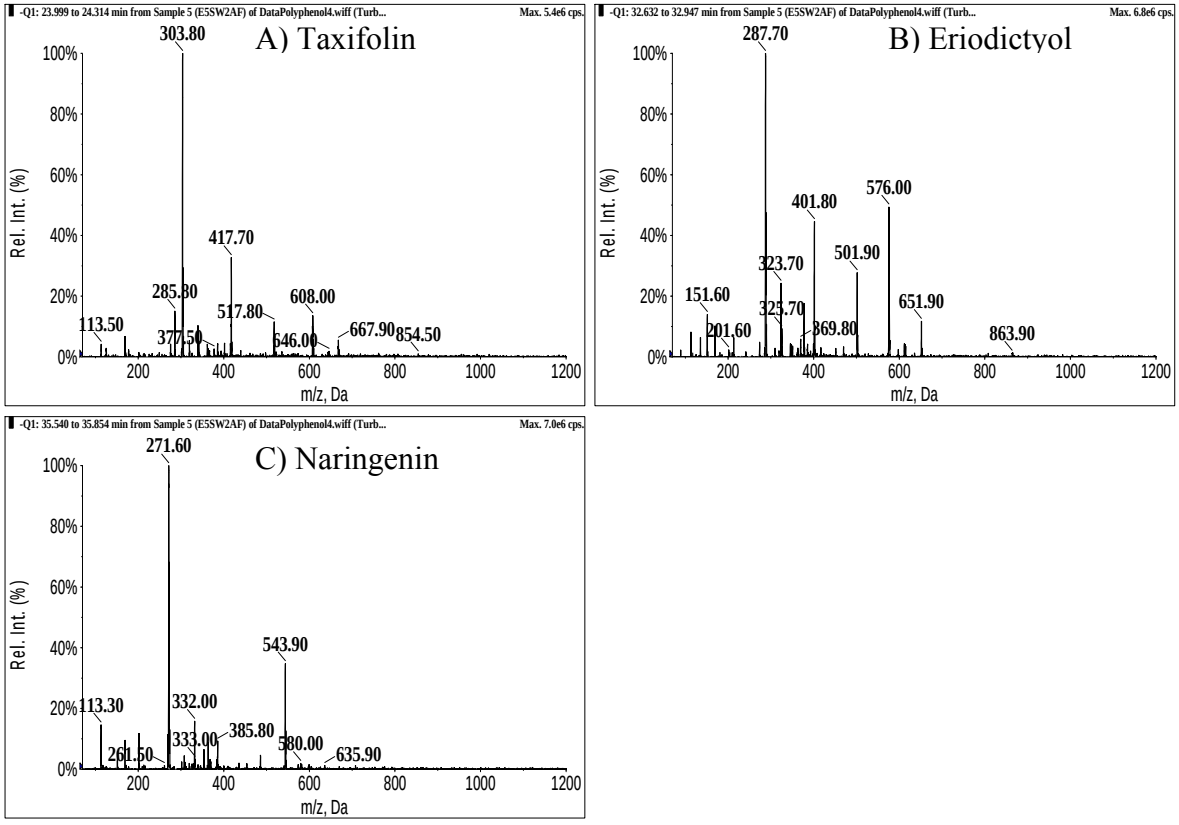


Figure 4.1.6. Mass spectra of flavonoid compounds in hydrolysed water extracts from condensed tannin sorghum bran.

Table 4.1.5. Mass spectra and UV data of phenolic compounds identified in aqueous extracts from condensed tannin sorghum bran

Peak No.	t_R* (min)	Compound Identity	*m/z* $[M-H]^-$ (% intensity)	Fragment ion *m/z* (% intensity)	HPLC-DAD λ_{max} (nm)
1	10.7	Protocatechuic acid	153.7 (100)	109 (31)	208, 260, 292
2	13.8	Protocatechualdehyde	137.6 (100)	-	208, 282
3	14.9	*p*-Hydroxybenzoic acid	137.5 (100)	93 (61)	208, 256
4	16.2	Caffeyolglycerol	253.7 (100)		208, 266,288
5	16.5	Caffeic acid	179 (100)	135 (60), 121 (15)	210, 288, 322
6	18.7	*p*-Hydroxybenzaldehyde	121.6 (100)	-	210, 280
7	21.4	Gentisic acid	153.6 (100)	-	210, 292
8	23.0	Ferulic acid	193.6 (100)	-	212, 322
9	24.0	Taxifolin	303.8 (100)	285 (15)	204, 288
10	32.6	Eriodictyol	287.7 (100)	151 (14), 135 (7)	202, 288
11	35.4	Naringenin	271.6 (100)	151(11)	121, 288

*t_R, Retention time; -, No corresponding fragment ion identified

The compound eluting in Peak 8 with abundant $[M-H]^-$ molecular ion at *m/z* 193.6 (100) (Fig. 4.1.5H) corresponded to ferulic acid standard (MW=194.18) (Table 4.1.2). The phenolic acids, protocatechuic acid, *p*-hydroxybenzoic acid, vanillic acid, caffeic acid, *p*-coumaric acid and ferulic acid have been previously identified in aqueous methanolic (Hahn *et al.*, 1983; Svensson *et al.*, 2010) and acidified acetonitrile (Chung *et al.*, 2011) extracts from sorghum grain as well as aerial parts of sorghum plant (Sène, Gallet & Doré, 2001).

The mass spectra of flavonoid compounds identified in sorghum bran aqueous extracts are shown in Fig. 4.1.6 and their identities and concentrations are shown in Tables 4.1.5 and

4.1.6, respectively. Peak 9 with abundant $[M-H]^-$ molecular ion at *m/z* 303.8 (100) and fragment ion at *m/z* 285 (15) (Fig. 4.1.6A) corresponded to taxifolin (MW = 304.25) standard (Table 4.1.2). The compound eluting in peak 10 did not match any of the authentic phenolic standards. Its abundant $[M-H]^-$ molecular ion at *m/z* 287.7 (100) (Fig. 4.1.6B) suggested that it could be eriodictyol (MW= 288.25). The compound eluting in peak 11 with abundant molecular ions at *m/z* 271.6 (100) and fragment ion at m/z 151.0 (11) (Fig. 4.1.6C) corresponded to naringenin authentic standard (MW=272.26) (Table 4.1.2). The three flavonoid compounds, taxifolin, eriodictyol and naringenin have been previously identified in organic extracts from sorghum grain (Gujer, Magnolato & Self, 1986; Svensson *et al.*, 2010).

Extraction of phenolic compounds from condensed tannin sorghum bran under acidic condition resulted in a significant increase in concentration of seven of the phenolic compounds (Table 4.1.6). The increase in concentration of phenolic acids ranged from 13% for caffeic acid to 36% for ferulic acid. Flavonoid content increased by 34, 41 and 48% for naringenin, erioditol and taxifolin, respectively.

The sum of all phenolic compounds identified in the acidified water extract was 19% higher than that of the water extract. This increase was similar to the 15% increase in total phenolic content as determined by the Folin Ciocalteu method. The increase could be as a result of enhanced extraction of free and esterified phenolic compounds and hydrolysis and release of bound phenolic compounds under acidic condition (Liyana-Pathirana & Shahidi, 2005).

The phenolic compound profiles of marama bean and condensed tannin sorghum bran were different. Three phenolic acids were identified in aqueous extracts from marama bean seed coats compared to six in sorghum bran aqueous extracts. The common phenolic acid in both marama bean seed coat and condensed tannin sorghum bran aqueous extracts was *p*-hydroxybenzoic acid. Sorghum bran aqueous extracts had two phenolic aldehydes and none were present in marama bean seed coat aqueous extracts.

Table 4.1.6. Effect of extraction under acidic condition on phenolic compounds in aqueous extracts from condensed tannin sorghum bran

			Compound concentration[1]	
Peak No.	t_R* (min)	Compound Identity	Water extract (µg/g)	Acidified water extract (µg/g)
1	10.7	Protocatechuic acid	281.5 a[2] ± 12.8	280.2 a ± 30.3
2	13.8	Protocatechualdehyde[3]	178.9 a ± 32.0	239.9 b ± 30.0
3	14.9	*p*-Hydroxybenzoic acid	368.7 a ± 40.9	384.2 a ± 51.1
4	16.2	Caffeyolglycerol[4] / vanillic acid	78.1 a ± 4.2	113.0 b ± 14.7
5	16.5	Caffeic acid	147.9 a ± 16.7	172.1 b ± 5.5
6	18.7	*p*-Hydroxybenzaldehyde[5]	49.2 a ± 7.5	95.4 b ± 6.5
7	21.4	Gentisic acid[4] / *p*-coumaric acid	6.4 a ± 1.9	5.6 a ± 0.6
8	23.0	Ferulic acid	10.9 a ± 1.6	17.2 b ± 1.8
9	24.0	Taxifolin	39.2 a ± 2.4	68.5 b ± 2.7
10	32.6	Eriodictyol[6]	78.0 a ± 9.5	134.2 b ± 11.6
11	35.4	Naringenin	99.7 a ± 9.4	152.5 b ± 14.9
		Total	1338.6 a ± 97.8	1663.4 b ± 97.2

t_R*- Retention time

[1]Results are means ± standard deviation of four determinations from two independent experiments on dry weight basis

[2]Means with different letters in the same row are significantly different ($p \leq 0.05$)

[3]Values expressed as protocatechuic acid equivalent

[4]Values expressed as caffeic acid equivalent

[5]Values expressed as *p*-hydroxybenzoic acid equivalent

[6]Values expressed as taxifolin equivalent

Flavonoid compounds were also different in marama been seed coat and condensed tannin sorghum bran aqueous extracts. In marama bean seed coat extracts flavonoid compounds

were flavanols esterified with gallic acid while in sorghum these were flavanones. Taxifolin sometimes may be classified as a flavanonol (also called dihydroflavonol or 3-hydroxyflavanone) because of the presence of a 3-hydroxyl group in its chemical structure (Harbone, 1989; Naczk and Shahidi, 2004). The concentration of phenolic compounds in water and acidified water extracts from marama bean seed coats were 15 and 7 fold higher, respectively than equivalent extracts from condensed tannin sorghum bran. This trend was similar to the total phenolic content as determined by the Folin Ciocalteu method where total phenolic content of water and acidified water extracts from marama bean seed coat were 10 and 5 fold higher than equivalent extracts from condensed tannin sorghum bran.

The decrease in levels of phenolic compounds in acidified water extracts from marama bean seed coats but corresponding increase for extracts from condensed tannin sorghum bran suggests that there may be differences in the polymer compound matrix in marama bean seed coats and condensed tannin sorghum bran. Marama bean seed coats possibly contain both ionic and non-ionic polymers at relatively high concentration which interact at low pH through hydrogen bonds resulting in interpolymer complex precipitate (Khutoryanskiy *et al.*, 2009). Phenolic compounds of which some may have been released from bound forms by acidic condition (Liyana-Pathirana & Shahidi, 2005) co-precipitated with the interpolymer complex (Molyneux & Vekavakayanondha, 1986).

4.1.5. Conclusion

Aqueous extracts from marama bean (*Tylosema esculentum*) seed coats have significantly higher levels of total phenolic content, total flavonoid content and phenolic compound concentration than aqueous extracts from the bran of condensed tannin sorghum, variety PAN 3860. Extraction of marama bean seed coats under acidic condition results in reduction in phenolic content possibly due to co-precipitation of phenolic compounds with polymeric species. However, extraction of condensed tannin sorghum bran under acidic conditions results in an increase in phenolic content. Acidic conditions effect release of bound phenolic compounds and depending on the type of high molecular weight polymeric species present in the sample matrix it may result in co-precipitation of phenolic compounds due to interpolymer complex precipitation triggered by low pH. Sorghum bran aqueous extracts contain phenolic acids, phenolic aldehydes and flavonoid compounds while extracts from marama bean seed coats contain phenolic acid and flavonoid compounds at relatively higher

concentrations. The flavonoid compounds in aqueous extracts from sorghum bran are flavanones while in aqueous extracts from marama bean seed coats, they are flavanols esterified to gallic acid.

Marama bean (*Tylosema esculentum*) seed coats may be a better material source for aqueous extraction of phenolic compounds than bran of condensed tannin sorghum, variety PAN 3860 because of the higher phenolic content of the resultant extracts. These extracts from marama bean seed coats are likely to have higher antioxidant activity and potential health benefits than extracts from the condensed tannin sorghum bran in view of the higher phenolic content. Extraction of sorghum bran under acid conditions may be the preferred method because it increases the recovery of phenolic compounds. In contrast it is not recommended for marama bean seed coats because it causes co-precipitation of phenolic compounds with other polymeric species.

4.2. Characterization of proanthocyanidins in aqueous extracts from marama bean seed coats and condensed tannin sorghum bran

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Shelembe, J.S., Cromarty, D., Bester, M.J., Minnaar, A. and Duodu, K.G. (2011) Characterisation of phenolic acids, flavonoids, proanthocyanidins and antioxidant activity of water extracts from seed coats of marama bean (*Tylosema esculentum*) – an underutilised food legume. *International Journal of Food Science and Technology 47*, 648-655.

4.2.1. Abstract

Legumes with dark seed coats have been shown to contain condensed tannins which are important dietary antioxidants. However, there are no reports on condensed tannins in marama bean seed coats therefore the need for this investigation. Condensed tannin content of seed coats from marama bean and bran from condensed tannin sorghum was determined in methanol and aqueous extracts using the vanillin-HCl and butanol-HCl assays. The capacity of the extracts to precipitate proteins was determined using the protein precipitation capacity assay. The constitutive unit composition of proanthocyanidins in aqueous acetone and aqueous extracts was determined using the thiolysis degradation method and HPLC-MS to identify the compounds. Methanol extracts from marama bean seed coats had 3.7 fold higher condensed tannin content and 7.8 fold higher protein precipitation capacities than methanol extracts from condensed tannin sorghum bran. Aqueous extracts from marama also had higher condensed tannin content and higher protein precipitation capacity than equivalent extracts from condensed tannin sorghum bran. Extraction under acidic conditions resulted in a significant reduction in condensed tannin content of extracts from marama bean seed coats while it had no significant effect on extracts from condensed tannin sorghum bran. The reduction may be as a result of precipitation of interpolymer complexes formed through hydrogen bonding between tannins and cell wall polysaccharide triggered by low pH. The constitutive units of proanthocyanidins in marama seed coats were epigallocatechin-3-O-gallate and epicatechin-3-O-gallate occurring as major extension and terminal units and epigallocatechin and epicatechin as minor extension unit components. Proanthocyanidins in aqueous extracts from condensed tannin sorghum bran were composed of catechin and epicatechin. In conclusion, marama bean proanthocyanidins are predominantly prodelphinidins due to the presence of (epi)gallocatechin units while sorghum bran proanthocyanidins, due to the presence of (epi)catechin units are procyanidins.

Key words: Marama bean seed coat; Sorghum bran; Proanthocyanidin; Prodelphinidin; Procyanidin; Thiolysis

4.2.2. Introduction

Proanthocyanidins (condensed tannins) are widely distributed in the plant kingdom (Strumeyer & Malin, 1975) and they are widespread in many foods derived from plants (Hagerman, Riedl, Jones, Sovik, Ritchard, Hartfeld & Riechel, 1998; Serrano, Puupponen-Pimiä, Dauer, Aura & Saura-Calixto, 2009). Tannins are normally regarded as antinutrients because of their ability to precipitate proteins (Strumeyer & Malin, 1975), inhibit digestive enzymes and decrease absorption of minerals and vitamins (Kunyanga, Imungi, Okoth, Momanyi, Biesalski & Vadivel, 2011). However, proanthocyanidins have attracted increasing attention due to the rapidly increasing body of evidence associating this class of phenolic compounds with a wide range of health benefits (Lazarus, Adamson, Hammerstone & Schmitz, 1999). Proanthocyanidins are thought to be important dietary antioxidants (Hagerman *et al.*, 1998) and are the major phenolic constituents presumed to have a health-protective action (Guyot, Marnet & Drilleau, 2001).

Tannins are biologically active compounds that may have beneficial effects (Xu, Yuan & Chang, 2007). A diet supplemented with 1% (w/w) tannins from young persimmon fruit promoted faecal bile acid excretion in rats suggesting that tannins can be used clinically to reduce hypercholesterolemia (Matsumoto, Kadowaki, Ozaki, Takenaka, Ono, Yokoyama & Gato, 2011) often associated with cardiovascular disease. Condensed tannins were reported to inhibit α-amylase and α-glucosidase enzymatic activity thereby having the ability to delay intestinal glucose absorption and the onset of insulin-dependent diabetes mellitus (Kunyanga *et al.*, 2011). Procyanidins (dimer–hexamer) were shown to inhibit liposome oxidation in a polymer length dependent manner (Verstraeten, Keen, Schmitz, Fraga & Oteiza, 2003). Condensed tannins and flavonoid compounds from legumes (lentils, black beans, red kidney beans and pinto beans) were reported inhibit *in vitro* copper-catalysed LDL oxidation (Xu *et al.*, 2007) where LDL oxidation is a contributing factor in the development of cardiovascular disease.

Purification of condensed tannins is an important step in their characterization. Solid phase extraction is the most commonly used method for the purification of condensed tannins in plant extracts (Strumeyer & Malin, 1975; Hagerman & Butler, 1980; Prieur, Rigaud, Cheynier & Moutounet, 1994; Sun, Leandro, Ricardo da Silva & Spranger, 1998a; Prior, Lazarus, Cao, Muccitelli & Hammerstone, 2001; Dueñas, Sun, Hernández, Estrella & Spranger, 2003).

In the purified fractions constitutive unit composition and mean degree of polymerization of proanthocyanidin polymers have been determined by reverse-phase HPLC analysis of acid catalyzed degradation products. Acid catalyzed degradation of proanthocyanidins is carried out under mild conditions in the presence of a nucleophilic reagent such as toluene-α-thiol (benzyl mercaptan) (Prieur *et al.*, 1994; Guyot, Marnet, Laraba, Sanoner & Drilleau, 1998; Fu, Loo, Chia & Huang, 2007). This is an important analysis because it distinguishes between extension and terminal units in the polymer (Prieur *et al.*, 1994; Guyot *et al.*, 1998). Extension units are captured by benzyl mercaptan to form benzylthioether derivatives and terminal units are released as free flavan-3-ols (Prieur *et al.*, 1994) or flavan-3-O-gallates units (Yousef, Grace, Cheng, Belolipov, Raskin & Lila, 2006). HPLC coupled to mass spectrometry, with electrospray interface (ESI) has proven to be extremely useful for peak assignment and characterization of individual phenolic compounds (Weisz, Kammerer & Carle, 2009).

Marama beans are gathered by hand from the wild in arid regions of Southern Africa and the cotyledons are utilized as food (Amarteifio & Moholo, 1998) and the seed coats discarded. The seed coats contain phenolic compounds (Chingwaru, Duodu, van Zyl, Schoeman, Majinda, Yeboah, Jackson, Kapewangolo, Kandawa-Shultz, Minnaar & Cencic, 2011). However nothing is known about proanthocyanidins and their structural features in marama bean seed coats. Condensed tannins in other legumes are predominantly procyanidins consisting of catechin and epicatechin subunits, located in the seed coat (Dueñas, Hernández & Estrella, 2006). However, small amounts of propelargonidin in common bean varieties (Beninger, Gu, Prior, Junk, Vanderberg & Bett, 2005) and prodelphinidins in lentils (Dueñas *et al.*, 2003) have also been reported.

The aim of this study was to characterize and compare proanthocyanidins or condensed tannins in aqueous extracts prepared from seed coats of marama beans and bran of condensed tannin sorghum using high performance liquid chromatography and mass spectrometry.

4.2.3. Materials and methods

4.2.3.1. Materials

Chemicals. (+)-catechin, (-)-epicatechin, (-)-gallocatechin, (-)-epigallocatechin, (-)-epicatechin gallate, (-)-epigallocatechin gallate, bovine serum albumin, were purchased from

Sigma-Aldrich (Johannesburg, South Africa). Ethyl acetate, diethyl ether, benzyl mercaptan (toluene-α-thiol), HPLC grade methanol, HPLC grade acetonitrile, 32% hydrochloric acid, glacial acetic acid (analytical grade reagent), 1-butanol, ferric ammonium sulphate, vanillin, sodium chloride, sodium dodecyl sulphate, triethanolamine, ferric chloride, acetone, sodium hydroxide were purchased from Merck Chemicals (Johannesburg, South Africa).

4.2.3.2. Methods

4.2.3.2.1. Sample preparation

Marama bean seed coat and condensed tannin sorghum bran powders were prepared as described in section 4.1.3.2.1. Freeze-dried aqueous extracts (water extracts and acidified water extracts) from marama bean seed coats and condensed tannin sorghum bran powders were prepared as described in section 4.1.3.2.2. Aqueous acetone extracts were also prepared to compare with aqueous extracts because condensed tannins are usually extracted with aqueous acetone for characterization (Lazarus, Adamson, Hammerstone & Schmitz, 1999; Prior *et al.*, 2001; Gu, Kelm, Hammerstone, Beecher, Cunningham, Vannozzi, & Prior, 2002; Gu, Kelm, Hammerstone, Beecher, Holden, Haytowitz & Prior, 2003b).

4.2.3.2.2. Determination of condensed tannin content by vanillin-HCl method

The vanillin-HCl assay (Price, Van Scoyoc & Butler, 1978) was used for the determination of condensed tannin content of methanol extracts and freeze-dried aqueous extracts prepared from marama bean seed coat and condensed tannin sorghum bran. Methanol is the commonly used solvent in this assay (Price, Van Scoyoc & Butler, 1978). The 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 (Sarkar and Howarth, 1976; Dykes & Rooney, 2006). Methanol extracts were prepared by extracting 0.2 g marama bean seed coat or condensed tannin sorghum bran powder twice with 10 ml of 100% methanol. After the addition of each 10 ml portion of methanol, the mixture was shaken for 20 min using an orbital shaker model POS 300 (Grant Instruments Ltd, Shepreth, UK) and then centrifuged at 4500 rpm (5200 x g) for 10 min at 25 °C using a model Rotanta 460R centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) and the supernatants were decanted and combined. One millilitre of the methanol extract from marama bean seed coat and condensed tannin sorghum bran was diluted with 24 ml and 5 ml of 100% methanol,

respectively for analysis. Serial standard solutions of (+)-catechin were prepared in 100% methanol in a concentration range of 0 – 1000 mg/L. Vanillin reagent was prepared on the day required by mixing equal volumes of 1% vanillin in methanol and 8% concentrated HCl in methanol. Freeze-dried aqueous extracts from marama bean seed coat (35 mg) and condensed tannin sorghum bran (50 mg) were weighed into 100 ml and 25 ml volumetric flasks, respectively and made up to volume with 100% methanol to give a concentration of 0.35 and 2 mg/ml, respectively. Five millilitres of the vanillin-HCl reagent was added to 1 ml aliquot of sample or catechin standard solution. Sample blanks (without vanillin) were prepared by adding 5 ml of 4% HCl in methanol to 1 ml extract solutions. Absorbance was measured at 500 nm after 20 min from the time of addition of vanillin-HCl reagent using a T80+ UV-VIS spectrophometer and UVWin software version 5.0.5 (PG Instruments, Wibtoft, UK). Blank readings were subtracted from sample absorbance readings. Results were reported as mg catechin equivalent/g (mg CE/g) of dry sample weight.

4.2.3.2.3. Determination of condensed tannin content by Butanol-HCl assay

Condensed tannin content of methanol extracts and freeze-dried aqueous extracts was also determined with the butanol-HCl assay. The assay was performed as described by Porter, Liana, Hrstich and Chan (1986) with some modifications. In this assay proanthocyanidins are converted to anthocyanidins through an autoxidation reaction catalysed by Fe^{3+} following acid-catalysed cleavage of the interflavanoid bonds (Porter *et al.*, 1986). The anthocyanidins formed have a red colour and a maximum absorbance wavelength at around 550 nm (Sun *et al.*, 1998b). Methanol extracts were prepared by extracting 0.2 g marama bean seed coat or condensed tannin sorghum bran powder with 10 ml of 100% methanol. Mixture was shaken for 20 min on an orbital shaker model POS 300 and then centrifuged at 4500 rpm (5200 x g) for 10 min at 25 °C using a model Rotanta 460R centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany) and supernatant decanted. Five millilitre of supernatant was diluted with 20 ml methanol for analysis. Freeze-dried extracts (25 mg) were weighed into centrifuge tubes and 25 ml methanol was added to each tube. The tubes were shaken at 300 rpm for 20 min on an orbital shaker model POS 300. Samples were then centrifuged at 4500 rpm (5200 x g) for 10 min at 25 °C using model Rotanta 460 R centrifuge (Andreas Hettich GmbH & Co., Tuttlingen, Germany). One millilitre of sample supernatant, 0.2 ml ferric reagent (2% (w/v) $NH_4Fe(SO_4)_2$ $12H_2O$ in 2 M HCl) and 6 ml of 5% (v/v) 32% concentrated HCl in 1-butanol were added into glass test tube. The test tube was covered

with a glass marble, heated in a boiling water bath for 60 min, cooled and absorbance measured at 550 nm using a T80+ UV-VIS spectrophometer and UVWin software version 5.0.5 (PG Instruments, Wibtoft, UK). Results were reported as absorbance units per gram (Abs_{550}/g) of sample on dry weight basis.

4.2.3.2.4. Determination of protein precipitation capacity

The protein precipitation capacity assay (Hagerman & Butler, 1978) was used to evaluate the ability of tannins in methanol extracts and aqueous extracts from marama bean seed coats and condensed tannin sorghum bran to precipitate proteins. This assay is based on the capacity of tannins to interact with proteins (added in excess) to form a tannin-protein complex precipitate (Hagerman & Butler, 1978). The tannin-protein precipitate is then dissolved in sodium dodecyl sulphate-triethanolamine solution and ferric ions (added as ferric chloride) react with tannins in the complex in the alkaline solution to form a violet complex with maximum absorbance at 510 nm. Methanol extracts were prepared by extracting 0.2 g marama bean seed coat or condensed tannin sorghum bran powder twice with 10 ml methanol. After the addition of each 10 ml portion of methanol, the mixture was shaken for 20 min on an orbital shaker model POS 300 and then centrifuged at 4500 rpm (5200 x g) for 10 min at 25 °C using a model Rotanta 460R centrifuge and the supernatant was then decanted. Supernatants were combined. One millilitre of the methanol extract from marama bean seed coat was diluted with 4 ml of methanol and extract from sorghum was analyzed without dilution. Freeze-dried aqueous extract (25 mg) from marama bean seed coat and condensed tannin sorghum bran were dissolved in 20 ml and 2 ml distilled water to give a concentration of 0.25 and 2.5 mg/ml, respectively. Two millilitres of bovine serum albumin (1 mg/ml) in 0.2 M acetate buffer solution, pH 5 containing 0.17 M sodium chloride and 1 ml of methanol or aqueous extract solution were added into the centrifuge tube, mixed and allowed to stand for 15 min. The tubes were centrifuged at 5000 g for 15 min at 25 °C and the pellet carefully washed with 2 ml of 0.2 M acetate buffer solution pH 5. The pellet was then dissolved in 4 ml of 1% (w/v) sodium dodecyl sulphate-5% (w/v) triethanolamine solution. One millilitre of 0.01 M ferric chloride reagent in 0.01 M HCl solution was added and absorbance measured at 510 nm after 20 min using T80+ UV-VIS spectrophometer and UVWin software version 5.0.5. Results were reported as absorbance units at 510 nm per gram (Abs_{510}/g) on sample dry weight basis.

4.2.3.2.5. Identification of terminal and extension units in proanthocyanidins by HPLC-MS

Proanthocyanidins in aqueous acetone extracts and aqueous extracts were subjected to acid catalysed thiolysis degradation in the presence of benzyl mercaptan nucleophile under mild conditions to preserve flavan-3-O-gallate units and the products analysed by HPLC-MS. (Gu, Hammerstone, Zhang, Beecher, Holden, Howtowitz & Prior, 2003a).

4.2.3.2.5.1. Fractionation of proanthocyanidins using Sep-Pak C_{18} cartridges

Proanthocyanidins in aqueous acetone extracts and aqueous extracts from marama bean seed coat and condensed tannin sorghum bran were fractionated into monomer (FI), oligomer (FII) and polymer (FIII) fractions using Sep Pak C_{18} cartridges according to the method of Sun *et al.* (1998a). Aqueous acetone extracts were prepared as described by Gu *et al.* (2002) with some modification. One gram of marama bean seed coat or condensed tannin sorghum bran powder was extracted with 10 ml acetone/water/acetic acid (70:29.5:0.5, v/v/v) solution by vortexing for 30 sec and shaking for 50 min on an orbital shaker model POS 300. The mixture was centrifuged at 3500 rpm (3150 x g) for 15 min at 25 °C using a model Rotanta 460R centrifuge (Andreas Hettich GmbH & Co. KG, Tuttlingen, Germany). Acetone in six millilitres of supernatant was evaporated off at 25 °C under vacuum (200 tor) using a vacuum rotary evaporator model Rotavapor RE 120. Residue was made up to 6 ml with distilled water adjusted to pH 7 with 1 M NaOH. Fifty milligrams of freeze-dried extracts was dissolved in 6 ml distilled water adjusted to pH 7.0 and then sonicated for 5 sec. Samples were subjected to solid phase extraction by passing through two preconditioned neutral Sep Pak C_{18} (plus short, 0.7 ml, 360 mg) and SepPak tC_{18} (plus short, 0.8 ml, 400 mg) cartridges (Waters Associates, Milford, USA) connected in series under vacuum. The inferior C_{18} was at the top and the superior tC_{18} at the bottom. Cartridges were activated with 6 ml methanol (Tian, Nakamura, Cui & Kayahara, 2005; Walsh, Haak, Bohn, Tian, Schwartz & Failla, 2007) and reconditioned with 12 ml distilled water adjusted to pH 7 with 0.1 M NaOH solution. Phenolic acids and sugars (Fraction 0) were eluted with 10 ml distilled water adjusted to pH 7. Mixture of monomer and oligomer proanthocyanidins (Fraction I + II) was eluted with 15 ml ethyl acetate. Polymeric proanthocyanidins (Fraction III) were eluted with 5 ml methanol. The ethyl acetate solution was evaporated to dryness under vacuum (200 tor) at 25 °C using a rotary vacuum evaporator model Rotavapor RE 120 (Büchi Labortechnik AG, Flawil, Switzerland) and residue re-dissolved in 2 ml distilled water and re-deposited on

to the same cartridges after preconditioning with 12 ml distilled water adjusted to pH 7. Monomers (Fraction I) were eluted with 15 ml diethyl ether. Oligomeric proanthocyanidins (Fraction FII) were eluted with 5 ml methanol.

4.2.3.2.5.2. Thiolysis degradation of proanthocyanidins

Thiolysis degradation of oligomeric (FII) and polymeric (FIII) fractions was carried out according to the method of Prieur *et al.* (1994) modified by Gu *et al.* (2003a). A 50 µl aliquot of oligomeric or polymeric fraction, 50 µl of methanol acidified with concentrated HCl (3.3% v/v) and 100 µl of toluene-α-thiol (5% v/v in methanol) were added into safety lock eppendorf tube. The tube was 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. Reaction mixtures were kept at -20 °C until analysis.

4.2.3.2.5.3. Determination of mean degree of polymerization

Separation of oligomeric (FII) and polymeric (FIII) thiolysis degradation products in the reaction mixtures was carried out according to Gu *et al.* (2002) with some modification. Briefly, 20 µl reaction mixture was injected into an HPLC system (Water Associates, Milford, USA) consisting 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 (Water Associates, Milford, USA). Solvent system consisted of mobile phase A (2% acetic acid in water, v/v) and B (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 ml/min. The detector was set at 280 nm and the chromatograms acquired and monitored by Waters Breeze system software, version 3.30 SPA (Water Associates, Milford, USA). The mean degree of polymerization was calculated from peak area using the following equation adapted from Gu et al.,(2002).

mDP = { total area of benzylthioether derivatives/ total area of flavan-3-ol units} + 1

4.2.3.2.5.4. Identification of thiolysis degradation products by HPLC-MS

Thiolysis degradation products were analyzed by HPLC-MS to confirm their identity according to the method described by Gu *et al.* (2002) with some modification. The chromatographic system used consisted of an Agilent 1100 series HPLC system (Agilent Technologies, Santa Clara, CA) with model G1312A binary pump, model G1322A degasser, model G1367A auto sampler, model G1316A column heater and model G1315B photo diode array detector, reverse phase column (YMC-Pack ODS AM-303, 250 x 4.6 mm i.d., 5 µm particle size), (Water Associates, Milford, USA) and a triple quadruple / linear ion trap mass spectrometer, model 4000 Q TRAP LC/MS/MS (AB SCIEX, Concord, Canada). Solvent system consisted of mobile phase A (2% acetic acid in water) and mobile phase B (methanol) delivered at 0.5 ml/min in a linear gradient of B from 15 – 80% in 45 min, 80% – 15% in 10 min and 15% for 10 min. Injection volume was 20 µl, column temperature was 40 °C and detector wavelength was set at 275-285 nm. The operating conditions for the mass spectrometer were as follows: Source temperature was maintained at 400 °C, spray voltage at -4000 V (negative mode). Nitrogen at a pressure of 35 psi was used as a nebulizer gas (GS1) and at 40 psi as heating gas (GS2) and at 23 psi as curtain gas (CUR). Declustering (DP) and entrance potential (EP) were set at -60 V and -10 V, respectively. The mass of compounds was scanned from *m/z* 70 to *m/z* 1200 and chromatograms acquired and monitored by Analyst software, version 1.5 (Applied Biosystems and MDS Analytical Technologies, Concord, Canada)

4.2.3.3. Statistical analysis

Results from two independent experiments performed in duplicate were expressed as means ± 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, USA) was used for statistical data analysis and significance was accepted at $p \leq 0.05$.

4.2.4. Results and discussion

4.2.4.1. Condensed tannin content and protein precipitation capacity of methanolic extracts

The condensed tannin content and protein precipitation capacity of methanolic extract from marama bean seed coat were higher than that of methanolic extract from condensed tannin sorghum bran (Table 4.2.1). Condensed tannin content of marama bean seed coat determined with the vanillin-HCl method was 3.7 fold higher than that of condensed tannin sorghum bran. However the protein precipitation capacity of methanolic extract from marama bean seed coats extract was 7.8 fold higher than that of methanolic extract from condensed tannin sorghum bran which may be an indication that condensed tannins in marama bean seed coats have a higher affinity for proteins compared to condensed tannins from sorghum bran.

Table 4.2.1. Condensed tannin content and protein precipitation capacity of methanol extracts from marama bean seed coat and condensed tannin sorghum bran

Extract	Vanillin-HCl assay (mg CE[1]/g)	Butanol-HCl assay (Abs_{550} units/g)	Protein precipitation capacity (Abs_{510} units/g)
Marama seed coat	1226.4[2]b[3]± 16.3	198.6b ± 15.8	226.6 b ± 13.9
Sorghum bran	331.1a ± 9.6	119.3a ± 5.0	29.1a ± 1.1

[1]CE, Catechin equivalents

[2]Data are means ± standard deviations of six determinations, on dry weight basis

[3]Means followed by different letters in the same column are significantly different ($p \leq 0.05$)

Compared to other legumes the condensed tannin content of the methanol extracts from marama bean seed coats were higher than levels reported in methanol extracts from seed coats of dark coloured common beans which ranged from 216 to 449 mg CE/g on wet weight basis depending on cultivar (Ranilla, Genovesse & Lajolo, 2007) and seed coats of black soybean which was approximately 45 mg CE/g of seed coat (Xu & Chang, 2008).

Condensed tannin content of sorghum bran was comparable to that reported in methanol extracts from *Phatafuli* (a condensed tannin sorghum cultivar from Malawi) sorghum bran which was 451 mg CE/g, dry weight (Sikwese & Duodu, 2007). However, it was higher than

that reported for 70% acetone extract from sorghum variety Sumac bran which was approximately 175 mg CE/g (Awika, McDonough & Rooney, 2005). The differences could be due to genetics and environmental factors which have been suggested to influence condensed tannin content in sorghum (Awika, Rooney, Wu, Prior & Cisneros-Zevallos, 2003). The protein precipitation capacity of sorghum bran was comparable to that reported for whole grain methanol extracts from NK300, a type III condensed tannin sorghum, which was 2.84 absorbance units per gram of whole grain (Hagerman & Butler, 1978) which translated to 28.4 absorbance units per gram of bran, assuming that bran account for 10% of total grain weight (Sikwese & Duodu, 2007).

4.2.4.2. Condensed tannin content and protein precipitation capacity of aqueous extracts

Water extracts from marama bean seed coats had the highest condensed tannin content measured with both the vanillin-HCl and the Butanol-HCl methods and protein precipitation capacity amongst all the extracts (Table 4.2.2). The condensed tannin contents of acidified water extracts from marama bean seed coats were 2 and 2.3 fold lower than water extracts as measured with the vanillin-HCl and butanol-HCl assays, respectively. The reduction in condensed tannins could be as a result of their interaction with cell wall polysaccharides (Hanlin, Hrmova, Harbertson & Downey, 2010) through the formation of strong hydrogen bonds at low pH resulting in the formation of an insoluble interpolymer complex precipitate (Khutoryanskiy, Dubolazov & Mun, 2009) as mentioned earlier (section 4.1.4.1). However, there were no significant differences in condensed tannin content between water extracts and acidified water extracts from condensed tannin sorghum bran as condensed tannins from sorghum bran probably interacted with cell wall polysaccharides to a lesser extent compared to condensed tannins from marama bean seed coats under acidic condition.

Condensed tannin content of water extract and acidified water extract from marama bean seed coats were 19 and 9 fold respectively higher, as measured with the vanillin-HCl method and 7 and 4 fold, respectively higher as measured with the butanol-HCl assay than equivalent extracts from condensed tannin sorghum bran. The vanillin-HCl assay gave a measure of the amount of monomeric, oligomeric and polymeric species in the sample, while the butanol-HCl assay gave a measure of the amount of anthocynins formed from the hydrolysis and autoxidation of tannins.

The protein precipitation capacity of acidified water extracts from marama bean seed coats were 2 fold lower than that of water extracts. The significant reduction in protein precipitation capacity was proportional to the reduction in condensed tannin content, which was also by 2 fold as a result of extraction under acidic conditions. However, there were no significant differences in protein precipitation capacities between water extracts and acidified water extracts from condensed tannin sorghum bran and this may be due to the lack of significant differences in tannin contents between the extracts. Water extracts and acidified water extracts from marama bean seed coats showed a 47 and 37 fold higher protein precipitation capacities respectively compared to equivalent extracts from condensed tannin sorghum bran.

Table 4.2.2. Effect of extraction under acidic condition on condensed tannin content and protein precipitation capacity of aqueous extracts from marama bean seed coats and condensed tannin sorghum bran

Extract	Vanillin-HCl assay (mg CE[1]/g)	Butanol-HCl assay (Abs_{550} units/g)	Protein precipitation capacity (Abs_{510} units/g)
Marama bean seed coat			
Water	199.9[2] c[3] ± 1.1	122.0 c ± 3.0	83.9 c ± 2.7
Acidified water	99.2 b ± 3.7	52.7 b ± 4.7	48.8 b ± 1.0
Sorghum bran			
Water	10.5 a ± 1.9	13.3 a ± 1.1	1.8 a ± 0.1
Acidified water	13.9 a ± 1.9	14.6 a ± 2.0	1.3 a ± 0.2

[1]CE, Catechin equivalents

[2]Data are means ± standard deviations of six determinations, on dry weight basis

[3]Means followed by different letters in the same column are significantly different ($p \leq 0.05$)

The higher protein precipitation capacity may not be accounted for by the condensed tannin content only. It appears that marama bean seed coat proanthocyanidins may have a higher affinity for proteins to form complexes compared to condensed tannin sorghum bran proanthocyanidins.

4.2.4.3. Characterization of condensed tannins in extracts

4.2.4.3.1. Marama bean seed coat proanthocyanidins

Total ion current chromatograms and HPLC chromatograms of thiolysis degradation products of proanthocyanidins in the polymer fraction (FIII) from aqueous acetone, water and acidified water extracts from marama bean seed coats had similar phenolic compound profiles (Fig. 4.2.1) and the identities of the component peaks are shown in Table 4.2.3.

The chromatograms were similar to those of thiolysis degradation products of proanthocyanidins from the oligomeric fraction (FII) (data not shown). The chromatograms showed six distinct peaks labelled 1 to 6 and the mass spectrum of each compound is shown in Fig. 4.2.2.

The compound eluting in Peak 1 with an abundant $[M-H]^-$ molecular ion at *m/z* 457.7 (100) (Fig. 4.2.2A) was identified as (epi)catechin-3-O-gallate (MW = 458.37). The peak also showed a fragment ion at *m/z* 305.9 (14) which was identified as the deprotonated epigallocatechin ion and at *m/z* 169.5 (21) corresponding to the gallic acid ion. This compound has been identified in aqueous methanol extracts from adzuki beans also by the molecular ion $[M-H]^-$ ion at *m/z* 457 (Amarowicz, Estrella, Hernández & Troszyńska, 2008). Similar fragmentation pattern for epigallocatechin-3-O-gallate was reported in green tea (Miketova, Schram, Whitney, Kerns, Valcic, Timmermann, Volk, 1998) and oolong tea (Dou, Lee, Tzen & Lee, 2007) extracts.

Compounds eluting in Peak 2a and 2b with abundant molecular ion at *m/z* 441.8 (100) (Fig. 4.2.2B and 4.2.2C) were identified as isomers of (epi)catechin-3-O-gallate (MW=442.37). Fragmentation resulted in ions at *m/z* 289.5 (10) and at *m/z* 169.3 (40) which were identified as (epi)catechin and gallic acid ions. The retention time and mass spectrum of (-)-epicatechin-3-O-gallate standard was the same as that of peak 2b (Data not shown) therefore 2a could be (+)-catechin-3-O-gallate. Miketova *et al.* (1998) reported similar mass spectra data for (epi)catechin-3-O-gallate with *m/z* values at 441(100), 289 (16) and 169 (18).

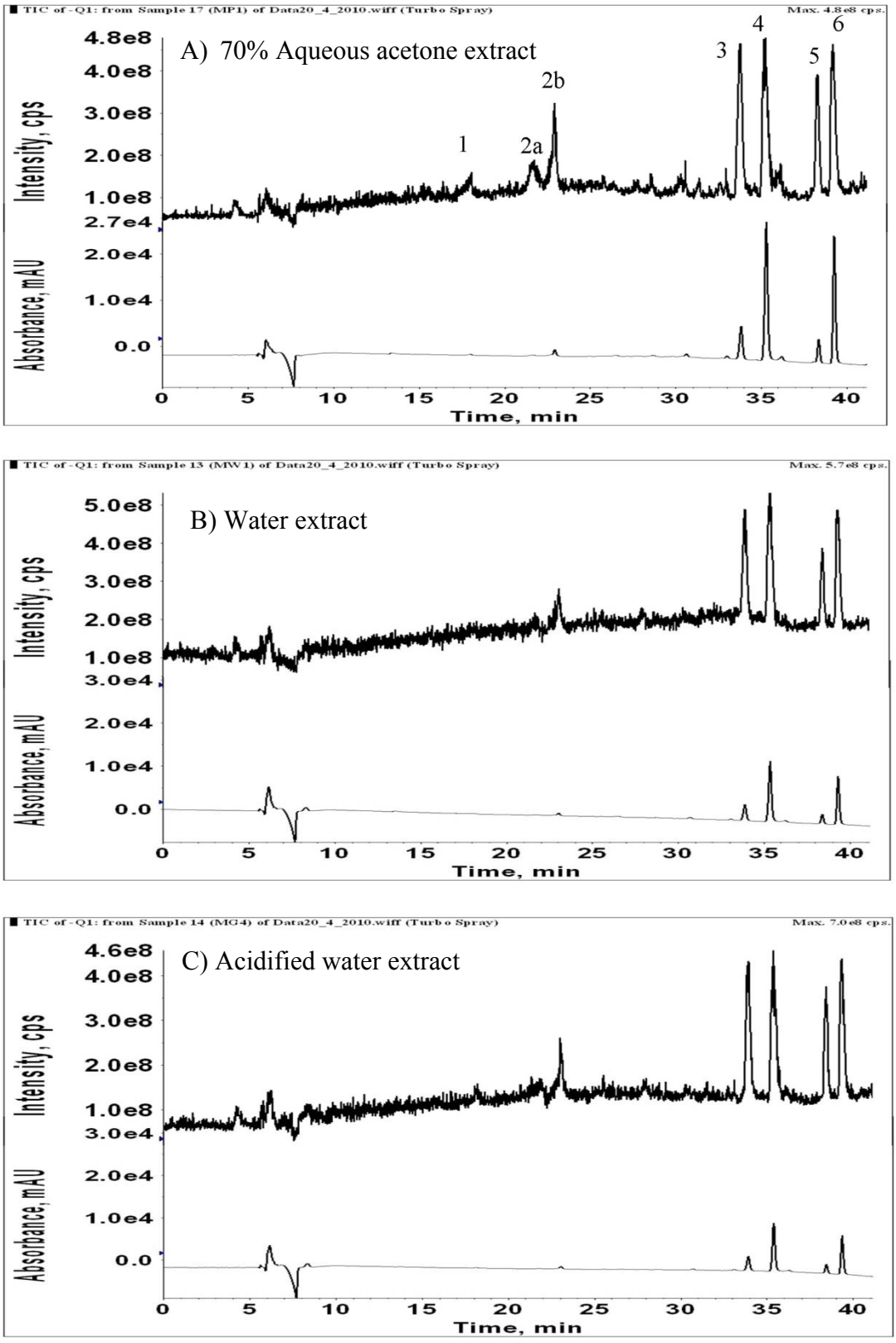
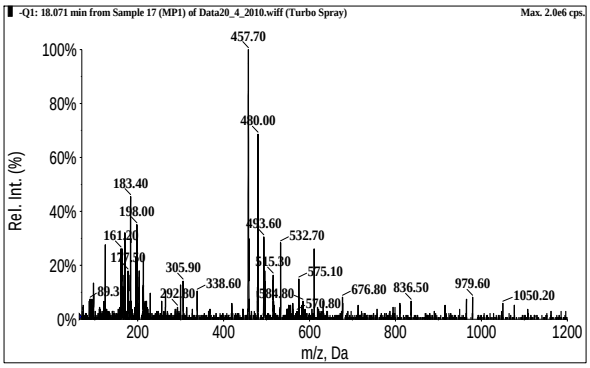
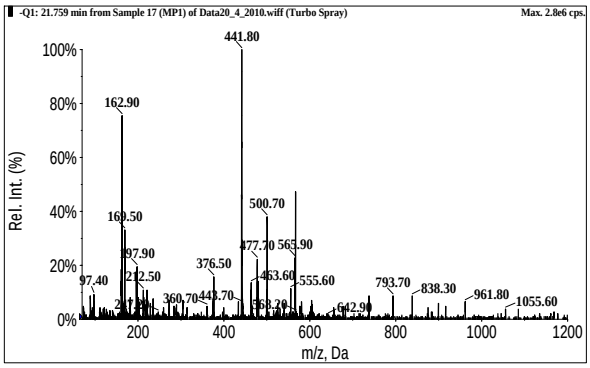


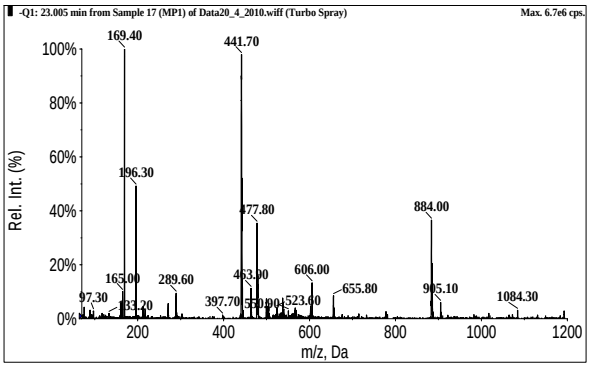
Figure 4.2.1. Total ion current chromatogram (top) and HPLC chromatogram (bottom) recorded at 280 nm of thiolysis degradation products of proanthocyanidins in polymer fraction (FIII) from extracts prepared from marama beans seed coats.



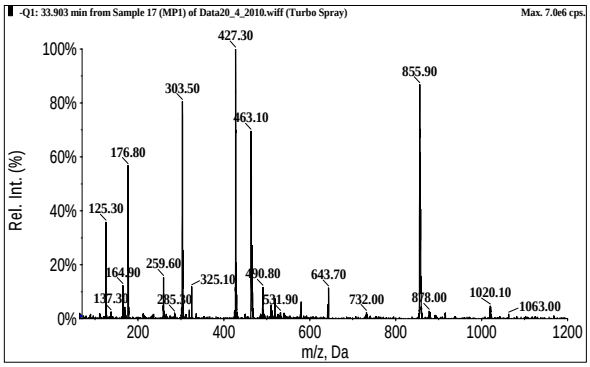
A) (Epi)gallocatechin-3-O-gallate



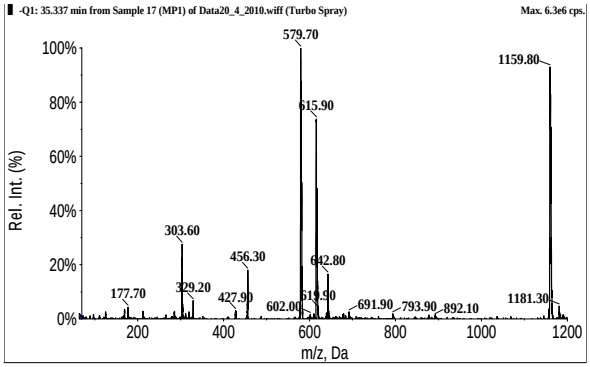
B) (Epi)catechin-3-O-gallate



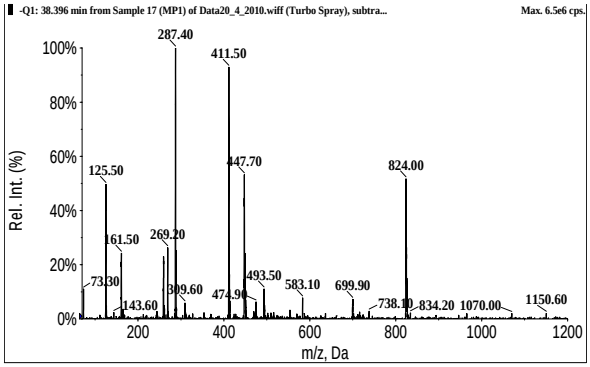
C) (Epi)catechin-3-O-gallate



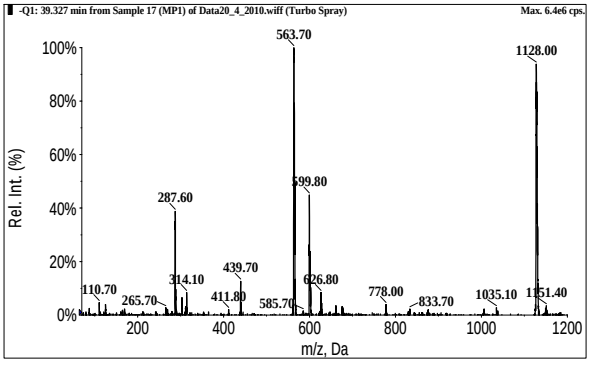
D) (Epi)gallocatechin benzylthioether



E) (Epi)gallocatechin-3-O-gallate benzylthioether



F) (Epi)catechin benzylthioether



G) (Epi)catechin-3-O-gallate benzylthioether

Figure 4.2.2. Mass spectra of thiolysis degradation products of proanthocyanidins in acetone extracts from marama bean seed coats.

Table 4.2.3. Mass spectra data of thiolysis degradation products of proanthocyanidins in extracts from marama bean seed coats

Peak No.	t_R* (min)	Identification	Molecular Weight	*m/z* $[M-H]^-$ (% intensity)	*m/z* MS^n (% intensity)
Terminal units release as free flavon-3-ol					
1	18.1	(Epi)gallocatechin-3-O-gallate	458	457.7 (100)	305.9 (14), 169.6 (32)
2	23.0	(Epi)catechin-3-O-gallate	442	441.8 (100)	289.5 (10), 169.3 (40)
Extension units released as benzylthioether derivatives					
3	33.9	(Epi)gallocatechin benzylthioether	428	427.7 (100)	303.5 (80)
4	35.3	(Epi)gallocatechin-3-O-gallate benzylthioether	580	579.8 (100)	457.0 (14), 303.6 (20), 169.6 (11)
5	38.4	(Epi)catechin benzylthioether	412	411.7 (90)	287.7 (100)
6	39.3	(Epi)catechin-3-O-gallate benzylthioether	564	563.5 (100)	439.8 (17), 287.7 (53), 169.1 (38)

*t_R – retention time

Extension units released as benzylthioether derivatives from marama bean seed coat proanthocyanidins are shown as peaks 3 to 6 (Fig. 4.2.1). Peak 3 with abundant $[M-H]^-$ molecular ion at *m/z* 427.3 (100) (Fig. 4.2.2D) and fragment ion at m/z 303.5 (80) was identified as (epi)gallocatechin benzylthioether (MW=428).

Compound in Peak 4 with abundant $[M-H]^-$ molecular ion at *m/z* 579.8 (100) (Fig. 4.2.2E) was identified as (epi)gallocatechin-3-O-gallate benzylthioether (MW=580). Fragmentation gave rise to *m/z* 303.6 (20) and *m/z* 169.3 (11) which corresponded to (epi)gallocatechin ion and gallic acid fragment ions, respectively. Similar fragmentation patterns for (epi)gallocatechin benzylthioether and (epi)gallocatechin-3-O-gallate benzylthioether have been reported in thiolysis degradation products of a highly galloylated proanthocyanidin polymer from persimmon fruit (Li, Laveverence, Trombley, Xu,Yang, Tian, Reed & Hagerman, 2010).

The compound eluting in peak 5 with $[M-H]^-$ molecular ion at *m/z* 411.7 (90) and major fragment ion at *m/z* 287.7 (100) (Fig. 4.2.2F) was identified as (epi)catechin benzylthiother (MW=412). Peak 6 with abundant $[M-H]^-$ molecular ion at *m/z* 563.8 (100) was identified as (epi)catechin-3-O-gallate benzylthioether (MW=564) (Figure 4.2.2.G). Fragment ion at *m/z* 439.8 (17) corresponded to (epi)catechin-3-O-gallate ion after cleavage of thiol group, at *m/z* 287.7 (44) corresponded to (epi)catechin ion and at *m/z* 169.1 (35) corresponded to galloyl group. Gu *et al.* (2003b) also identified epicatechin benzylthioether by molecular ion at *m/z* 411 and fragment ion at *m/z* 287 and epicatechin-3-O-gallate benzylthioether by the molecular ion at *m/z* 563 and fragment ion at *m/z* 439 in grape proanthocyanidin thiolysis degradation products.

4.2.4.3.2. Condensed tannin sorghum bran proanthocyanidins

Total ion current (TIC) and HPLC chromatograms of thiolytic degradation products of proanthocyanidins in polymer fraction (FIII) of aqueous acetone, water and acidified water extracts from condensed tannin sorghum bran are shown in Fig. 4.2.3 and the identities of the peaks are shown in Table 4.2.4. The compound profiles of the three extracts were similar. The total ion current and HPLC chromatograms of thiolytic degradation products of proanthocyanidins from the oligomer fraction (FII) (data not shown) were similar to those of the polymer fraction (FIII).

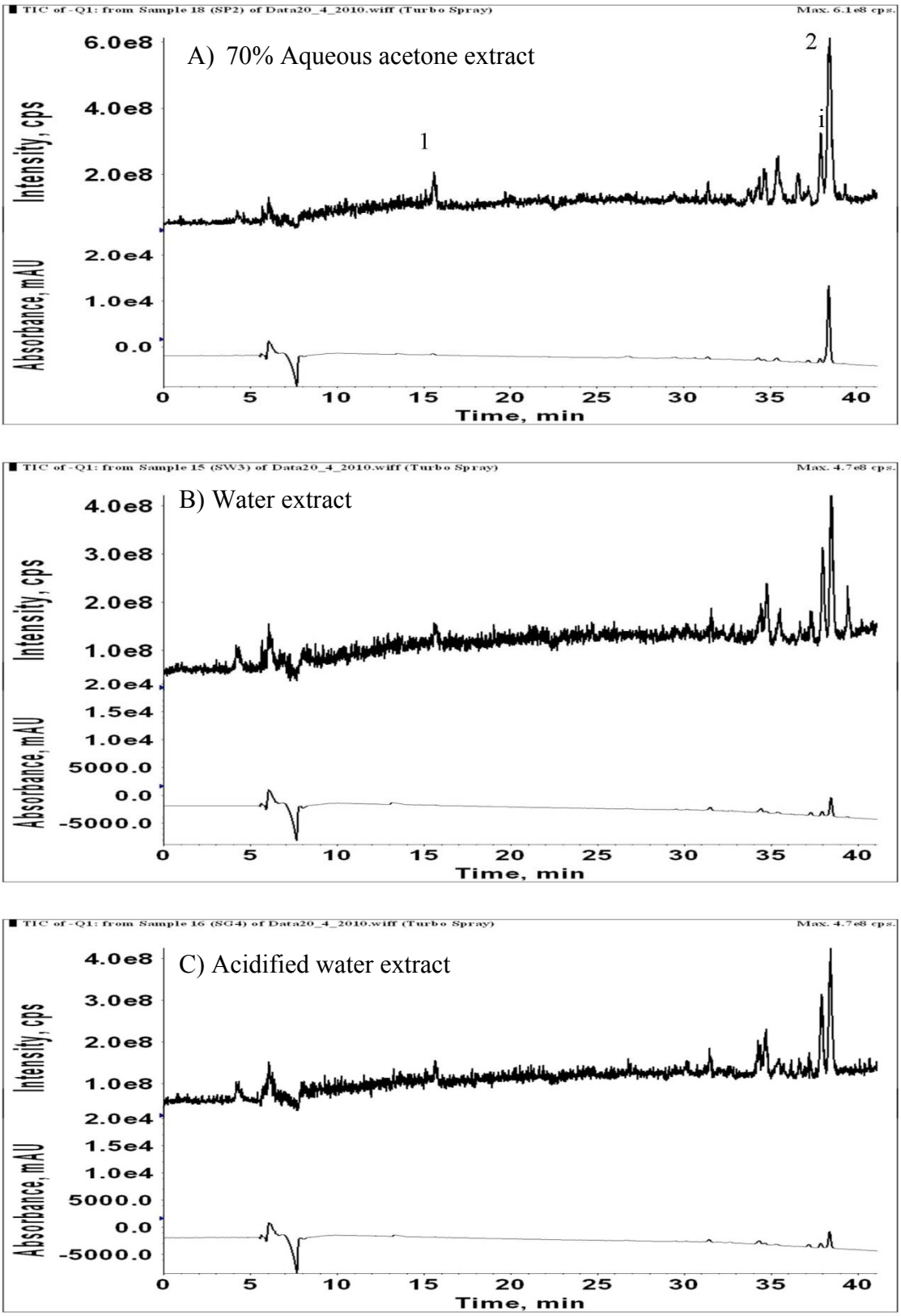
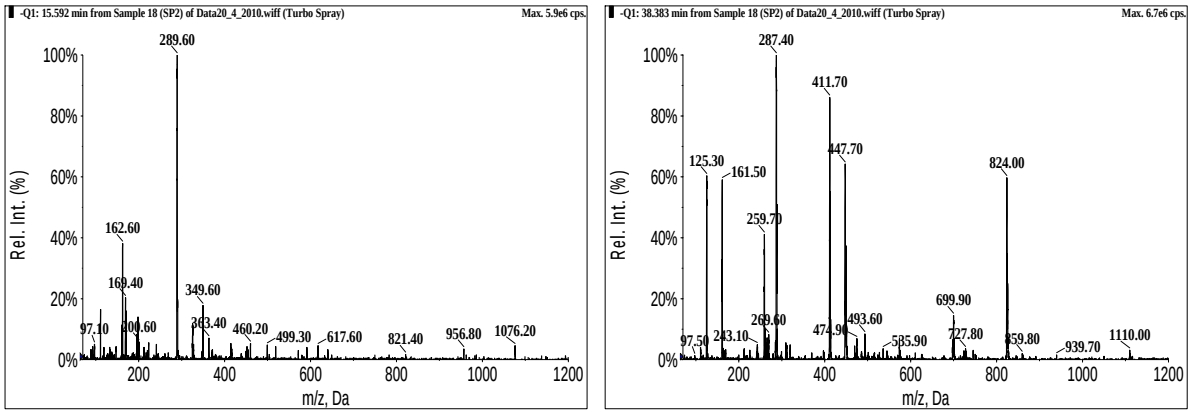


Figure 4.2.3. Total current ion (top) and HPLC (bottom) chromatograms of thiolysis degradation products of proanthocyanidins in polymer fraction from extracts prepared from condensed tannin sorghum bran.



A) (+)-Catechin B) (-)-Epicatechin benzylthioether

Figure 4.2.4. Mass spectra of thiolysis degradation products of proanthocyanidins in acetone extracts from condensed tannin sorghum bran.

Table 4.2.4. Mass spectra data of thiolysis degradation products of proanthocyanidins from condensed tannin sorghum bran

Peak No.	t_R* (min)	*m/z* $[M-H]^-$ (intensity,%)	*m/z* MS^n (intensity, %)	Identification
1	15.6	289.6 (100)	-	(+)-catechin
2	38.4	441.8 (100)	287.2 (100)	(Epi)catechin benzylthioether

*t_R – retention time

- No fragment ion of parent compound was observed

Peak 1 with abundant molecular ion at *m/z* 289.6 (100) (Fig. 4.2.4A) corresponded to (+)-catechin (MW=290.27) occurring as a terminal units. (+)-Catechin standard showed abundant molecular ion at *m/z* 290.1 and its retention time was the same as that for peak 1 therefore it was concluded that peak 1 was (+)-catechin. Duo *et al.* (2007) also identified (+)-catechin by the molecular ion at *m/z* 289 in oolong tea extracts. Terminal units in proanthocyanidins of condensed tannin sorghum bran were reported to be composed of (+)-catechin accounting for 89% and (-)-epicatechin accounting for 11% (Gu *et al.*, 2002). In this work (-)-epicatechin was not detected, probably because it occurred in low amounts.

The extension unit benzylthioether derivative from condensed tannin sorghum bran proanthocyanidins is shown as peak 2 (Fig. 4.2.4B). The compound in peak 2 with molecular

ion at *m/z* 411.8 (90) and major fragment ion at m/z 287.4 (100) was identified as (epi)catechin benzylthioether (MW=412). (-)-Epicatechin extension units released as (epi)catechin benzylthioether were identified by the parent ion at *m/z* 411 and fragment ion at *m/z* 287, in cocoa, sorghum bran, lowbush blueberry and cranberry proanthocyanidin thiolytic degradation products (Gu *et al.*, 2002). The peak marked (i) (Fig. 4.2.3A) had an abundant molecular ion at *m/z* 577(100) and was identified as A-type procyanidin dimer (MW=578) which has been previously reported in sorghum (Awika & Rooney, 2004).

4.2.4.3.3. Flavan-3-ol composition and mean degree of polymerization of proanthocyanidins in extracts

Flavan-3-ol composition and mean degree of polymerization (mDP) of proanthocyanidins in marama bean seed coat and condensed tannin sorghum bran extracts were calculated from HPLC chromatogram peak areas and are presented in Table 4.2.5. The structural composition of proanthocyanidins from marama bean seed coat was different from that of condensed tannin sorghum bran. The constitutive units in marama bean seat coat proanthocyanidins were (epi)gallocatechin-3-O-gallate, (epi)catechin-3-O-gallate, (epi)gallocatechin and epicatechin while in sorghum these were (+)-catechin and (-)-epicatechin. Marama bean seed coat proanthocyanidins differed from those of other legumes such as the common bean and lentils because of the higher percentage of galloylated subunits. Terminal units of marama bean seed coat proanthocyanidins were epigallocatechin-3-O-gallate and (epi)catechin-3-O-gallate, while in pinto beans, small red beans and red kidney beans were reported to be catechin (7.3–14.5%) and epicatechin (0.4–1.1%) (Gu *et al.*, 2003b) and in lentils were reported to be catechin (8.6–11.9%), epicatechin (1.3-1.7%) and traces of epicatechin-3-O-gallate (Dueñas *et al.*, 2003).

Extension units in marama bean seed coats proanthocyanidins were predominantly epigallocatechin-3-O-gallate and (Epi)catechin-3-O-gallate, while (Epi)gallocatechin and (epi)catechin (EC) were present as minor extension unit components, suggesting that the proanthocyanidins in the seed coats are predominantly prodelphinidins.

Table 4.2.5. Constitutive unit composition (relative percentage by peak area) of proanthocyanidins in polymer (FIII) and oligomer (FII) fractions from aqueous extracts from marama bean seed coat and condensed tannin sorghum bran[1]

		% Terminal units released as free flavan-3-ol			% Extension units released as benzylthioether derivatives				
	Extract	C[2]	EGCG	ECG	EGC	EGCG	EC	ECG	mDP
Marama bean seed coat									
FIII	Water	-	2.7 a[3] ± 0.2	8.2 a ± 0.7	6.6 a ± 1.2	43.3 d ± 0.7	5.7 a ± 0.4	33.5 b ± 1.5	9.2 e ± 0.6
	Acidified water	-	3.9 a ± 0.5	10.3 b ± 0.8	7.0 a ± 1.4	40.0 c ± 2.2	6.2 a ± 0.1	32.6 b ± 0.2	7.1 d ± 0.5
FII	Water	-	5.9 b ± 0.8	13.6 c ± 0.8	8.2 a ± 2.1	35.1 b± 0.8	7.0 a ± 0.5	30.1 a ± 2.1	5.1 b ± 0.3
	Acidified water	-	7.5 b ± 1.5	18.7 d ± 2.2	7.1 a ± 1.2	29.3 a ± 2.7	8.0 a ± 1.5	29.4 a ± 1.9	3.8 a ± 0.2
Sorghum bran									
FIII	Water	18.4 a ± 2.5	-	-	-	-	81.6 d ± 2.5	-	5.5 bc ± 0.7
	Acidified water	15.8 a ± 1.8	-	-	-	-	84.2 d ± 1.8	-	6.4 cd ± 0.7
FII	Water	26.8 b ± 2.1	-	-	-	-	73.2 c ± 2.1	-	3.7 a ± 0.3
	Acidified water	33.4 c ± 1.6	-	-	-	-	66.6 b ± 1.6	-	3.0 a ± 0.1

[1] Data are means and standard deviations of four determinations from two independent experiments.

[2] Abbreviations: C, catechin; EGCG, (epi)gallocatechin-3-O-gallate; ECG, (epi)catechin-3-O-gallate; EGC, (epi)gallocatechin; EC, (epi)catechin; mDP, mean degree of polymerization

[3] Means with different letters in the same column are significantly different ($p \leq 0.05$).

- Not detected

In comparison, extension units of proanthocyanidin from pinto beans, small red beans and red kidney beans were found to be composed of epicatechin (43.1–73.9%), catechin (5.4-33.2%) and (epi)afzelechin (8.7–14.6%) (Gu *et al.*, 2003b) and those in polymer fraction from the seed coats of lentils were composed of 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 constitutive unit composition of sorghum bran proanthocyanidins were similar to those reported by Gu *et al.* (2003b) except that in this study (-)-epicatechin occurring as terminal unit was not detected. These authors reported that terminal units in sorghum bran proanthocyanidins were predominantly (+)-catechin (89%) and (-)-epicatechin (11%) and extension units were exclusively (-)-epicatechin.

Aqueous extracts from marama bean seed coats had a higher mDP compared to equivalent extracts from condensed tannin sorghum bran. Extraction under acidic conditions of marama bean aqueous extracts resulted in a significant reduction in the mDP of proanthocyanidins in both oligomer (FII) and polymer (FIII) fractions. The reduction in mDP may be as a result of interaction of high molecular weight proanthocyanidins with cell wall polysaccharides (Hanlin *et al.*, 2010) at low pH resulting in the formation of an insoluble interpolymer complex precipitate (Khutoryanskiy *et al.*, 2009) as mentioned earlier (section 4.1.4.1) resulting in extraction of lower molecular weight polymers. The higher molecular weight proanthocyanidin polymers may be more involved in the interaction because of their higher number of hydroxyl groups that may form hydrogen bonds with other polymers causing precipitation. The mDP of marama bean seed coat proanthocyanidins was similar to that of other legumes such as the common bean and lentils. The mDP of proanthocyanidins in aqueous acetone extracts from pinto beans, small red beans and red kidney beans ranged from 6.3–12.0, depending on variety (Gu *et al.*, 2003b) and in polymer and oligomer fractions from lentils it ranged from 6.9–9.4 and 3.8–4.7, respectively (Dueñas *et al.*, 2003). Extraction under acidic conditions did not have a significant effect on the mDP of proanthocyanidins in acidified water extracts from condensed tannin sorghum bran. The change in pH had little or no effect on the formation of hydrogen bonds and eventual precipitate formation when compared to proanthocyanidins from marama bean seed coats. This may be due to the lower number of hydroxyl groups on the procyanidin polymer as well as the lower condensed tannin content.

The mDP of proanthocyanidins from condensed tannin sorghum bran were lower than that of aqueous acetone extracts from Sumac sorghum, a condensed tannin sorghum cultivar, which was reported to be 8.4 (Gu *et al.*, 2002). This indicated that lower molecular weight polymers were extracted under aqueous condition compared to extraction with organic solvent. The mDP obtained for oligomeric (FII) and polymeric (FIII) fractions from both marama bean seed coats and sorghum bran were lower than 10 which suggested that the proanthocyanidins in both fractions obtained from the Sep Pak catridges were all oligomers.

4.2.5. Conclusions

Aqueous extracts from marama bean (*Tylosema esculentum*) seed coats have higher condensed tannin content and protein precipitation capacity than equivalent extracts from bran of condensed tannin sorghum variety PAN 3860. Proanthocyanidins in marama bean seed coats are predominantly prodelphinidins and those from condensed tannin sorghum bran are procyanidins. Prodelphinidins have more hydroxyl groups within their polymer structure than procyanidins and this may render the polymer more water soluble than procyanidins resulting an extract with higher condensed tannin content. The higher number of hydroxyl groups in the polymer structure of prodelphinidins also results in higher interaction with proteins probably through hydrogen bonding resulting in higher protein precipitation capacity compared to procyanidins from condensed tannin sorghum bran.

Extraction under acidic condition results in a decrease in condensed tannin content of aqueous extracts from seed coats of marama beans. However, it does not have any significant effect on condensed tannin content of aqueous extracts from condensed tannin sorghum bran. The decrease in condensed tannin content of acidified water extracts from marama bean seed coats may be due to the large number of hydroxyl groups on the prodelphinidin structure which allows the polymer to interact with other high molecular weight cell wall polysaccharides through hydrogen bonding resulting in interpolymer complexation and precipitation at low pH.

4.3. *In vitro* antioxidant activity and protective effect of aqueous extracts prepared from marama bean seed coat and condensed tannin sorghum bran against oxidative damage of biological molecules

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[1]Shelembe, J.S., Cromarty, D., Bester, M.J., Minnaar, A. and Duodu, K.G. (2011) Characterisation of phenolic acids, flavonoids, proanthocyanidins and antioxidant activity of water extracts from seed coats of marama bean (*Tylosema esculentum*) – an underutilised food legume. *International Journal of Food Science and Technology 47*, 648-655.

[2]Shelembe, J.S., Cromarty, D., Bester, M.J., Minnaar, A. and Duodu, K.G. (2011) Effect of acidic condition on phenolic composition and antioxidant potential of aqueous extracts from sorghum (*Sorghum bicolor*) bran. *Journal of Food Biochemistry* (in press)

4.3.1. Abstract

Water and acidified water extracts prepared from marama bean seed coats and condensed tannin sorghum bran for possible use as natural dietary antioxidant were evaluated for free radical scavenging activities using the 2,2'-azinobis- (3-ethyl-benzothiazoline-6-sulphonic acid (ABTS), 2,2-diphenyl-1-picryhydrazyl (DPPH) and oxygen radical absorbance capacity (ORAC) assays. The extracts were also evaluated for their protective effect against free radical oxidative damage of biomolecules using physiological relevant assays. These were AAPH-induced human red blood cell haemolysis, AAPH-induced supercoiled plasmid pBR 322 DNA oxidative damage, and copper-catalyzed LDL oxidation assays. Aqueous extracts from marama bean seed coats had higher free radical scavenging activities than equivalent extracts from condensed tannin sorghum bran. Extraction of marama bean seed coats under acidic conditions resulted in an extract with lower free radical scavenging activity, while it had no significant effect on extracts from condensed tannin sorghum bran. The free radical scavenging activity of the extracts correlated with total phenolic, flavonoid and condensed tannin contents. Extracts from marama bean seed coats protected against AAPH-induced red blood cell haemolysis and copper-catalyzed LDL oxidation at lower concentration than equivalent extracts from condensed tannin sorghum bran. Extracts from condensed tannin sorghum bran showed a clear protective effect against AAPH-induced oxidative DNA damage. However, extracts from marama bean seed coats gave inconclusive results probably due to prodelphinidins binding to DNA causing poor mobility of the DNA within the agarose gel. In conclusion, the extracts from marama bean seed coats and condensed tannin sorghum bran have a potential to reduce oxidative stress implicated in the development of chronic diseases.

Keyword: Marama bean seec coat; Sorghum bran; Antioxidant activity; Erythrocyte haemolysis, Oxidative DNA damage; LDL oxidation

4.3.2. Introduction

Numerous studies have shown that a number of non-nutrient dietary compounds, known as antioxidants might have a beneficial role in protecting against the development of chronic degenerative diseases (Paiva-Martins, Fernandes, Rocha, Nascimento, Vitrino, Amado, Borges, Belo & Santos-Silva, 2009) associated with oxidative stress (Gheldof & Engeseth, 2002; Somparn, Phisalaphong, Nakornchai, Unchern & Morales, 2007) including cardiovascular disease (CVD) (Hodgson, Croft, Puddy, Mori & Beilin, 1996), neurodegenerative diseases and cancer (Somparn *et al.*, 2007). Oxidative stress is caused by reactive oxygen species (ROS) which are harmful by-products produced during normal cell metabolism (Liyana-Pathirana & Shahidi, 2005; Dimitrios, 2006). Excessive generation of ROS can modify or cause oxidative damage to DNA, proteins and polyunsaturated fatty acids (Heo & Lee, 2005) in the human body and these changes have been implicated in the pathogenesis of human chronic diseases (Jacob & Burri, 1996).

Lipid peroxidation is a major cause of many pathological effects such as CVD, cancer, brain dysfunction and the ageing process (Lim, Cheung, Ooi & Ang, 2002). The major sites of lipid peroxidation damage are biomembranes due to the presence of polyunsaturated fatty acids in the membrane (Mak, Misra & Weglicki, 1983). Destruction of membrane lipids compromises the function of membrane localized receptors and channels and it also affects the transmembrane passage of solutes and fluidity of the membrane (Reiter, 1998) and thereby affecting the function of the cell and subcellular organelles such as mitochondria, microsomes and lysosomes (Cejas, Casado, Belda-Iniesta, Catro, Espinosa, Redondo, Sereno, García-Cabezas, Vara, Domínguez-Cáceresi, Perona & González-Barón, 2004). Lipid peroxides decomposes to relatively stable toxic aldehyde products which can diffuse and attack biomolecules such as proteins, DNA and phospholipids forming intra and inter molecular covalent adducts (Cejas *et al.*, 2004) leading to decline in physiological functions of the cell and ultimately cell death.

In European countries CVD is ranked as the number one cause of death, accounting for 48% of all deaths in 2008 (European Heart Network, 2008) and worldwide it is also a rapidly growing problem (de Lima Portella, Barcelos, de Bern, Carratu, Bresolin, da Rocha & Soares, 2008). It is reported that in South Africa, between 1997 and 2004, 195 people died per day because of some form of heart and blood vessel disease (CVD) (Steyn, 2007). LDL oxidation is implicated in the development of coronary heart disease (Regnström, Ström,

Moldeus & Nilsson, 1993). Oxidized LDL is recognizable by the non down-regulating macrophage scavenger receptor (Mosinger, 1995) resulting in the uptake of the oxidized LDL by the macrophage (foam cells) (Jacob & Burri, 1996) and smooth muscle cells leading to the formation of fatty streaks (Baba, Osakabe, Kato, Natsume, Yasuda, Kido, Kukuda, Muto & Kondo, 2007). In recent years there has been great interest in antioxidants that retard LDL oxidation (Paiva-Martins *et al.*, 2009). In human subjects, phenolic compounds in cocoa powder increased resistance of LDL to oxidation (Baba *et al.*, 2007) and those found in virgin olive oil resulted in a decrease in LDL oxidation markers (de la Torre-Carbor, Chávez-Servin, Jaúregui, Castellote, Mamuela-Raventós, Nurmi, Paulsen, Gaddi, Kaikkonen, Zunt, Fitó,Covas & López-Sabater, 2010) demonstrating their potential to prevent atherosclerotic processes which lead to CVD.

Worldwide cancer is the leading cause of deaths and in 2008 it accounted for 13% of all deaths (WHO, 2011). Most cancers begin with somatic mutation of normal cells (Jacob & Burri, 1996) which may be initiated by oxidative DNA damage (Laparra, Vélez, Barberá, Farré & Montoro, 2008) caused by ROS (Ames, 1983). Phenolic compounds of plant origin have been shown to have anticancer and apoptosis inducing properties (Zhang, Spitz, Tomlinson, Schabath, Minna & Wu, 2002). Green tea extracts were found to have chemo-protective effect against DNA damage (Bhat, Azmi, Hanif & Hadi, 2006) and epigallocatechin-3-O-gallate from tea was found to inhibit the growth of nasopharyngeal carcinoma cells in a dose-dependent manner (Yan, Yong-Guang, Fei-Jun, Fa-Qing, Min & Ya, 2004).

The aims of this study were (1) to determine the effect of extraction under acidic condition on free radical scavenging activity of aqueous extracts from marama bean seed coats and condensed tannin sorghum bran, (2) to determine the protective effect of the extracts against erythrocyte membrane, LDL and plasmid DNA oxidative damage.

4.3.3. Materials and methods

4.3.3.1. Materials

Marama beans were obtained from Botswana, Ghanzi province from the 2008 season and condensed tannin sorghum was from the 2004 season grown in South Africa (Section 4.1.3.1). Supercoiled plasmid vector pBR322 DNA, agarose (D1 LE) and bromophenol blue

were purchased from Whitehead Scientific (Pty) Ltd (Johannesburg, South Africa). Potassium persulphate, ethylenediaminetetraacetate (EDTA), Tris(hydroxymethyl) aminomethane, glacial acetic acid, saccharose, sodium chloride, potassium chloride, sodium hydrogen phosphate 1-hydrate ($NaH_2PO_4.H_2O$), di-sodium hydrogen phosphate (Na_2HPO_4), methanol, copper sulphate, thiobarbituric acid, sodium hydroxide, trochloroacetic acid were purchased from Merck (Pty) Ltd South Africa (Johannesburg, South Africa). 2,2'-azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid (ABTS), ethidium bromide, 2,2'-Azobis (2-methyl-propionamidine) dihydrochloride (AAPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboylic acid (Trolox), fluorescein (FL), 2,2-diphenyl-1-picryhydrazyl (DPPH), human low density lipoprotein (LDL) were supplied by Sigma-Aldrich (Pty) Ltd (Johannesburg, South Africa).

4.3.3.2. Methods

4.3.3.2.1. Sample preparation and extraction

Marama bean seed coats and condensed tannin sorghum bran powder were prepared as described in section 4.1.3.2.1 and their freeze-dried aqueous extracts were prepared as described in section 4.1.3.2.2.

4.3.3.2.2. ABTS free radical scavenging assay

Total antioxidant activities of the extracts were determined using the $ABTS^{\bullet+}$ radical scavenging assay (Awika, Rooney, Wu, Prior & Cisneros-Zevallos, 2003; Siddhuraju, 2006; Thaipong, Boonprakob, Crosby, Cisneros-Zevallos & Byrne, 2006; Xu, Ye, Chen & Liu, 2007). In this assay, phenolic antioxidants react directly with preformed stable ABTS radical cations causing decrease in absorbance or colour of the radical (Hagerman, Riedl, Jones, Sovik, Ritchard, Hartfeld & Richel, 1998). The stable radical is generated by potassium persulphate oxidation of $ABTS^{2-}$ to form a blue-green $ABTS^{\bullet+}$ chromophore (Macdonald, Wood & Garg, 2006). The $ABTS^{\bullet+}$ radical cation stock solution was produced by mixing 5 ml of 7.4 mM ABTS solution and 5 ml of 2.45 mM potassium persulphate solution and allowed to react in the dark at room temperature for 16 hours. $ABTS^{\bullet+}$ radical cation working solution was prepared by diluting 5 ml of the $ABTS^{\bullet+}$ radical cation stock solution with 145 ml Phosphate buffer saline (PBS) pH 7.4 (0.2 M Na_2HPO_4, 0.2 M NaH_2PO_4 and 150 mM NaCl).

Freeze-dried aqueous extracts from marama bean seed coat (25 mg) and condensed tannin sorghum bran (50 mg) were weighed into a 25 ml volumetric flask and made up to volume with PBS to give a concentration of 1 mg/ml and 2 mg/ml, respectively and then further diluted to 0.1 mg/ml and 0.4 mg/ml respectively. Trolox standard serial dilutions (0–1000 µm Trolox) were prepared in PBS. To 100 µl of diluted sample extract or Trolox standard solution 2850 µl of $ABTS^{\bullet+}$ radical working solution was added and allowed to react for 30 min and the absorbance measured at 734 nm using a T80+ UV-VIS spectrophometer (PG Instruments, Wibtoft, UK). Results were reported as micromoles of Trolox equivalents/g (µmol TE/g), on dry sample weight basis.

4.3.3.2.3. DPPH free radical scavenging assay

The antioxidant activity of the extracts was also determined with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay (Brand-Williams, Cuvelier & Berset, 1995) which measures the ability of an antioxidants to quench the $DPPH^{\bullet}$ radical (Cardador-Martinez, Loarca-Pina & Oomah, 2002; Nuengchamnong, Krittasilp & Ingkaninan, 2009). DPPH is a free radical that is stable at room temperature which upon reduction by an antioxidant results in loss of absorbance at 515–517 nm (Brand-Williams *et al.*, 1995). The degree of discolouration indicates the scavenging efficiency of the added antioxidant (Xu & Chang, 2007). The $DPPH^{\bullet}$ stock solution was prepared by weighing 12 mg of DPPH into a 50 ml volumetric flask and made to volume with methanol. Ten millilitres of this stock solution was further diluted with 50 ml methanol to obtain an absorbance value of approximately 1.1 at 515 nm. Trolox standard serial dilutions ranging from 0 to 1000 µM were prepared in methanol. A 1 mg/ml solution in methanol of each extract was prepared. These were further diluted with methanol to final working concentrations of 0.5 and 0.1 mg/ml for extracts from marama bean seed coats and condensed tannin sorghum bran, respectively. To 150 µl of the diluted sample extract solution or Trolox solution, 2850 µl of DPPH working solution was added and the tubes were sealed and covered with foil. Samples were shaken for 60 min and Trolox standards for 15 min and absorbance was measured at 515 nm using a T80+ UV-VIS spectrophometer. Results were reported as micromoles of Trolox equivalents/g (µmol TE/g), on dry sample weight basis.

4.3.3.2.4. Oxygen radical absorbance capacity assay (ORAC)

The method of Ou, Hampsch-Woodill and Prior (2001) was used to measure the oxygen radical absorbance capacity of the extracts with some modifications. In this method fluorescein (FL) is used as a probe which is attacked by peroxyl radicals generated by 2,2'-Azobis (2-methyl-propionamidine) dihydrochloride (AAPH) resulting in loss of fluorescence which is recorded at specific time intervals as the reaction goes to completion. The presence of an antioxidant compound inhibits free radical damage of fluorescein (Xu & Chang, 2007) and its protective effect is measured by assessing the area under the fluorescence decay curve (AUC) (Ou *et al.*, 2001).

Freeze-dried marama bean seed coat and condensed tannin sorghum bran aqueous extracts were dissolved in distilled water at a concentration of 0.12 mg/ml and 0.15 mg/ml, respectively. Trolox standards ranging from 0 to 1 mM were prepared in distilled water. FL stock solution was prepared by dissolving 3.76 mg FL in 50 ml PBS pH 7.4 (prepared as described in section 4.3.3.2.2). FL working solution was made by mixing 120 µl FL stock solution, 5 ml PBS and 45 ml distilled water. AAPH solution (74 mM) was prepared by dissolving 80 mg AAPH in 4 ml distilled water. Trolox standard or sample extract (10 µl) was added into 96 well plate in triplicates. A FL-AAPH solution was prepared by mixing the 4 ml of AAPH solution with 16 ml of FL working solution and immediately after mixing, 200 µl was added to each well containing Trolox, or extract. For blank and negative control 200 µl FL working solution and 200 µl FL-AAPH solution respectively was added to wells containing 10 µl distilled water. The well plates were placed in a FLUOstar OPTIMA multifunctional plate reader (BMG LABTECH GmbH, Ortenberg, Germany) set at 37 °C. Fluorescence at 485 nm excitation and 520 nm emission (Macdonald *et al.*, 2006; Thaipong *et al.*, 2006) was recorded at every 5 min interval for 245 min. Area under curve (AUC) was calculated for samples and standards using Microcal Origin software, version 6 (Microcal Software Inc, Northampton, USA). AUC for samples and standards were subtracted from AUC for blank. Antioxidant activity for samples was calculated from Trolox calibration curves and results reported as micromoles Trolox equivalents/g (µmol TE/g) on dry weight basis.

4.3.3.2.5. AAPH-mediated red blood cell haemolysis assay

Aqueous extracts were evaluated for their capacity to protect biomembrane against oxidative damage by free radicals using human red blood cell as a model system. In this assay haemolysis of red blood cell membrane is induced with AAPH (Paiva-Martins *et al.*, 2009). AAPH decomposes at physiological temperature and generates peroxyl radicals (Somparn *et al.*, 2007) which cause peroxidation of lipids and oxidation of proteins in the membrane leading to membrane rupture or formation of haemolytic holes (Sato, Sato & Suzuki, 1999) and resultant leakage of cell contents. Antioxidants that scavenge peroxyl radicals would reduce the rate of red blood cell haemolysis.

The red blood cell haemolysis assay was performed according to Tang and Liu (2008). Human red blood cells were obtained from the Department of Pharmacology of the University of Pretoria and were suspended in isotonic phosphate buffer saline PBS, pH 7.4 (8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 3 mM KCl and 0.137 M NaCl) in a ratio of 1:1, stored at 4 °C and used within 3 days. Prior to use, the red blood cell suspension was centrifuged at 2750 rpm (1184 x g) for 3 min using model Z300 centrifuge (Hermle Labortechnik GmbH, Wehingen, Germany) and the supernatant removed and discarded. Red blood cells were washed 3 times by adding isotonic PBS in a ratio of 1:1 and centrifuging at 2750 rpm (1184 x g) for 3 min and with each step the supernatant was discarded. Freeze-dried aqueous extracts from marama bean seed coat and condensed tannin sorghum bran were dissolved in distilled water at a concentration of 1.2 and 12 mg/ml, respectively. Extract solutions from marama bean seed coats were further diluted to three different concentrations 10, 40 and 80 µg/ml concentrations and extracts solution from sorghum bran were further diluted to 100, 200 and 400 µg/ml concentrations. For treatments 100 µl isotonic PBS, 10 µl red blood cells, 40 µl extract solution and 40 µl of 110 mM AAPH solution (prepared in isotonic PBS) were added into 1.5 ml safe lock eppendorf tubes. For sample blanks 140 µl isotonic PBS, 10 µl red blood cells and 40 µl extract were added into each tube. Positive controls were prepared by adding 140 µl isotonic PBS, 10 µl red blood cells and 40 µl of 110 mM AAPH solution into tubes and negative controls were prepared by adding 180 µl isotonic PBS and 10 µl red blood cells. Tubes were sealed, mixed by vortexing and incubated at 37 °C for 16 h. After incubation the tubes were mixed by vortexing and centrifuged at 2750 rpm (1184 x g) for 3 min. Supernatant (50 µl) was transferred into a 96 well plate and absorbance measured at 405 nm on a Bio Tek ELx 800 plate reader (Biotek Instruments Inc, Winooski, USA).

4.3.3.2.6. AAPH-mediated oxidative DNA damage

The protective effects of marama bean seed coat and condensed tannin sorghum bran aqueous extracts on oxidative DNA damage induced by AAPH were assessed by the method as described by Wei, Zhou, Cai, Yang & Liu (2006) with some modification. Under oxidative stress supercoiled plasmid pBR322 DNA is converted into relaxed circular form due to single strand breaks and into linear form due to double strand breaks and these three forms are separated by agarose gel electrophoresis because of their differences in electrophoretic mobility (Aronovitch, Godinger, Israeli, Krishna, Samuni & Goldstein, 2007).

DNA solution was prepared by diluting 10 µg supercoiled plasmid pBR322 DNA with 100 µl Tris-acetate buffer pH 8 working solution and vortexed. Treatment samples were prepared by adding 2.5 µl DNA solution, 5 µl sample extract or Trolox solution and 5 µl of 11 µm AAPH solution into eppendorf tubes. Positive controls were prepared by adding 2.5 µl DNA solution, 5 µl Tris-acetate buffer, pH 8 and 5 µl of 11 µm AAPH into eppendorf tubes and negative controls by adding 2.5 µl DNA solution and 10 µl Tris-acetate buffer solution. Tubes were vortexed and then incubated for 1.5 h at 37 °C. Twelve microliters of loading buffer (40% sucrose and 0.025% bromophenol) solution was added into each tube and vortexed. Twenty microlitres of sample was loaded into the well in the gel in electrophoresis apparatus (Owl Scientific Inc, Worburn, USA) containing 80 mM Tris-acetate buffer pH 8. The gel was run at 60 V, 30 mA for 2 h using electrophoresis power supply unit model EPS 301 (Amersham Pharmacia Biotech, Uppsala, Sweden). DNA bands were visualized and photographed on a UV transilluminator (Vilber Laurmat, Marne la Vallee, France). Band intensities were measured using Image Tool for Windows software version 3.00 (The University of Texas Health Center, San Antonio, USA) and % intensity was calculated relative to the negative control (without AAPH).

4.3.3.2.7. Copper-catalyzed human low density lipoprotein oxidation

The effect of extracts against copper-catalyzed human low density lipoprotein (LDL) oxidation was evaluated using the thiobarbituric assay (TBA assay) (Rüfer & Kulling, 2006; Xu, Yuan, & Chang, 2007a). Freeze-dried marama bean seed coat and condensed tannin sorghum bran aqueous extracts were dissolved in distilled water at a concentration of 1.2 mg/ml and 6 mg/ml, respectively. Extract solutions were further diluted into three different concentrations, 10, 40 and 80 µg/ml for extract solution from marama bean seed coats and

100, 250 and 500 µg/ml for extract solution from condensed tannin sorghum bran. For treatments, 2 µl of LDL solution (5.5 mg/ml), 168 µl 0.02 M PBS solution and 10 µl sample extract or Trolox solution were added into eppendorf safety lock tubes and oxidation was initiated by adding 20 µl of 100 mM copper sulphate solution. Positive controls were prepared by adding 2 µl LDL, 178 µl PBS and 20 µl copper sulphate solutions. Negative controls were prepared by adding 2 µl LDL and 198 µl PBS solutions. Tubes were sealed and incubated in a water bath at 37 °C for 3 h. After incubation, 200 µl of 10 mM EDTA, 200 µl of 20 % (w/v) trichloroacetic acid solution and 200 µl of 0.67 % (w/v) thiobarbituric acid solution in 0.2 M NaOH were added and sealed. Tubes were then heated at 80 °C (Lim *et al.*, 2002) for 30 min in a water bath and after cooling were centrifuged at 1500 g for 15 min and the supernatant was then transferred into a 1 ml cuvette. Absorbance was measured at 532 nm using a T80+ UV-VIS spectrophometer.

4.3.3.3. Statistical analysis

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

4.3.4. Results and discussion

4.3.4.1. Antioxidant capacity

Water extracts from marama bean seed coats exhibited the highest antioxidant activity amongst all the extracts as determined with the $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ radical scavenging and ORAC assays (Table 4.3.1).

Extracts from marama bean seed coats had higher antioxidant activity than equivalent extracts from condensed tannin sorghum bran. Water extracts from marama bean seed coats had 35, 9 and 4 fold higher antioxidant activities than water extracts from condensed tannin sorghum bran as measured with the DPPH, ABTS and ORAC assays, respectively. Acidified water extract from marama bean seed coats had 19, 5, and 2 fold higher antioxidant activities than acidified water extract from condensed tannin sorghum bran as measured by DPPH, ABTS and ORAC assays, respectively. The DPPH antioxidant activities of marama bean

aqueous extracts were lower but comparable to those reported for methanol extracts from the seed coats of common beans with dark seed coats which had the highest antioxidant activity averaging 518 µmol TE/g, on wet weight basis amongst 28 cultivars (Rannilla, Genovese & Lajolo, 2007).

Table 4.3.1. Effect of extraction under acidic condition on antioxidant activity of aqueous extracts from marama bean seed coats and condensed tannin sorghum bran

Extract	ABTS (µmol TE/g)	DPPH (µmol TE/g)	ORAC (µmol TE/g)
Marama bean seed coat			
Water extract	707.1[1] c[2] ± 10.6	492.4 c ± 17.9	439.3 c ± 34.9
Acidified water extract	343.2 b ± 21.3	325.4 b ± 13.8	273.1 b ± 42.3
Condensed tannin sorghum bran			
Water extract	75.2 a ± 7.6	14.0 a ± 0.8	111.1 a ± 20.1
Acidified water extract	70.7 a ± 2.3	17.0 a ± 1.0	134.9 a ± 13.5

[1]Data are means ± standard deviations on dry weight basis of four determinations from two independents experiments.

[2]Means with different letters in the same column are significantly different ($p \leq 0.05$).

However, they were higher than that of acidified acetone extracts from seed coats of black soybean which was approximately 165 µmol TE/g on dry weight basis (Xu & Chang, 2008). The ORAC values for both marama bean aqueous extracts were lower than that reported for acidified acetone extract from seed coats of black soybeans, which was approximately 450 µmol TE/g on dry weight basis (Xu & Chang, 2008).

The $DPPH^{\bullet}$, $ABTS^{\bullet+}$ scavenging activities and ORAC values of aqueous extracts from sorghum bran were lower than values reported for aqueous acetone extracts from sorghum variety Sumac (SU99) bran which were 716 µmol TE/g, 768 µmol TE/g and 3124 µmol TE/g (Awika *et al.*, 2003) on dry weight basis, respectively. The differences could be due to differences in cultivars, extraction methods and solvents. Water is a poor solvent for extracting phenolic compounds compared to organic solvents such as acetone and methanol and their aqueous mixtures (Yu, Ahmedna & Goktepe, 2005).

Acidified water extract from marama bean seed coats had 2, 1.6 and 1.5 fold lower antioxidant activities as determined with the $ABTS^{\bullet+}$, $DPPH^{\bullet}$ scavenging assays and ORAC assay, respectively compared to water extracts. The lower antioxidant activity was as a result of lower phenolic content which may have been caused by interpolymer precipitation and co-precipitation of phenolic compounds triggered by low pH as discussed in section 4.1.4.1. Extraction of condensed tannin sorghum bran under acidic condition caused a slight increase in $DPPH^{\bullet}$ scavenging activity and ORAC value, however, this increase was not statistically significant and there were also no significant differences in $ABTS^{\bullet+}$ scavenging activities.

There was a strong correlation between phenolic content as reported in sections 4.1.4.1 and 4.1.4.2 and antioxidant activity of aqueous extracts from marama beans seed coats and condensed tannin sorghum bran (Table 4.3.2). Similar Pearson's correlation coefficients between phenolic content and ABTS ($r = 0.99$) and DPPH ($r = 0.98$) have been reported in acidified methanol and aqueous 70% acetone extracts, respectively from sorghum (Dykes, Seitz, Rooney & Rooney, 2009). The results show that ORAC values were more correlated to condensed tannin content. A high correlation ($r^2 = 0.994$) between ORAC values and procyanidins levels were reported in cocoa extracts (Adamson, Lazarus, Mitchell, Prior, Cao, Jacobs, Kremers, Hammerstone, Rucker, Ritter & Schmitz, 1999).

DPPH free radical scavenging activity was strongly correlated to total phenolic content, total flavonoid content and the sum of phenolic compound concentration. Xu and Chang (2008) also found similar results where DPPH was strongly correlated to total phenolic content ($r = 0.99$) and total flavonoid content ($r = 0.98$) and ORAC values showed lower correlations to total phenolic content ($r = 0.81$) and total flavonoid ($r = 0.77$), and poor correlation to phenolic acids ($r = 0.49$) and the flavanol catechin ($r = 0.06$). The difference were explained by the authors to be due to differences in reaction mechanisms, ORAC is based on the hydrogen transfer mechanism whereas DPPH is based on the electron transfer mechanism.

$ABTS^{\bullet+}$ scavenging activity was strongly correlated to condensed tannin content as measured with the vanillin-HCL method. Hagerman *et al.* (1998) reported that procyanidins (tannins) were more effective quenchers of the $ABTS^{\bullet+}$ because of the high molecular weight and proximity of many aromatic rings and hydroxyl groups.

Table 4.3.2. Pearson's correlation coefficients between antioxidant activities and phenolic contents determined with different methods

	ORAC	DPPH	ABTS
Total phenolic content (Folin Ciocalteu method)	0.965(**)	0.995(**)	0.984(**)
Condensed tannin content (Vanillin-HCl method)	0.980(**)	0.981(**)	0.997(**)
Total flavonoid content (Colorimetric method)	0.957(**)	0.991(**)	0.985(**)
Sum of phenolic compounds (HPLC method)	0.967(**)	0.994(**)	0.984(**)

** Correlation is significant at the 0.01 level (2-tailed), N=16

4.3.4.2. Effect of extracts on AAPH-mediated red blood cell haemolysis

Aqueous extracts from marama bean seed coats inhibited AAPH-induced red blood cell haemolysis in a dose dependent manner as shown in Fig. 4.3.1. Water and acidified water extracts from marama bean seed coats at 10 µg/ml concentrations resulted in a 16 and 20% reduction in haemolysis, respectively and at 80 µg/ml concentrations reduction in haemolysis was as much as 64 and 57%, respectively compared to positive control. Acidified water extracts from marama bean seed coats had significantly lower protective effect at 40 and 80 µg/ml concentrations than water extracts which is similar to the relative phenolic contents and antioxidant activities of these samples.

The results also show significant differences between the negative control and sample controls especially the water extracts indicating that the extracts on their own were also causing red blood cell haemolysis. This may be ascribed to the presence of homogentisic acid in the extracts (section 4.1.4.2.1) which has been reported to cause haemolysis when incubated with blood (Heoedus & Nayak, 1994). Homogentisic acid is metabolized by homogentisic acid oxidase during normal catabolism of phenylalanine and tyrosine in the liver (Gutzmer, Herbst, Kiehl, Kapp & Weiss, 1997) therefore it may not be expected to cause any harm when ingested. Homogentisic acid is also present in honey used for food depending on the source of nectar (Cabras, Angioni, Tuberoso, Floris, Reniero, Guilou & Ghelli, 1999).

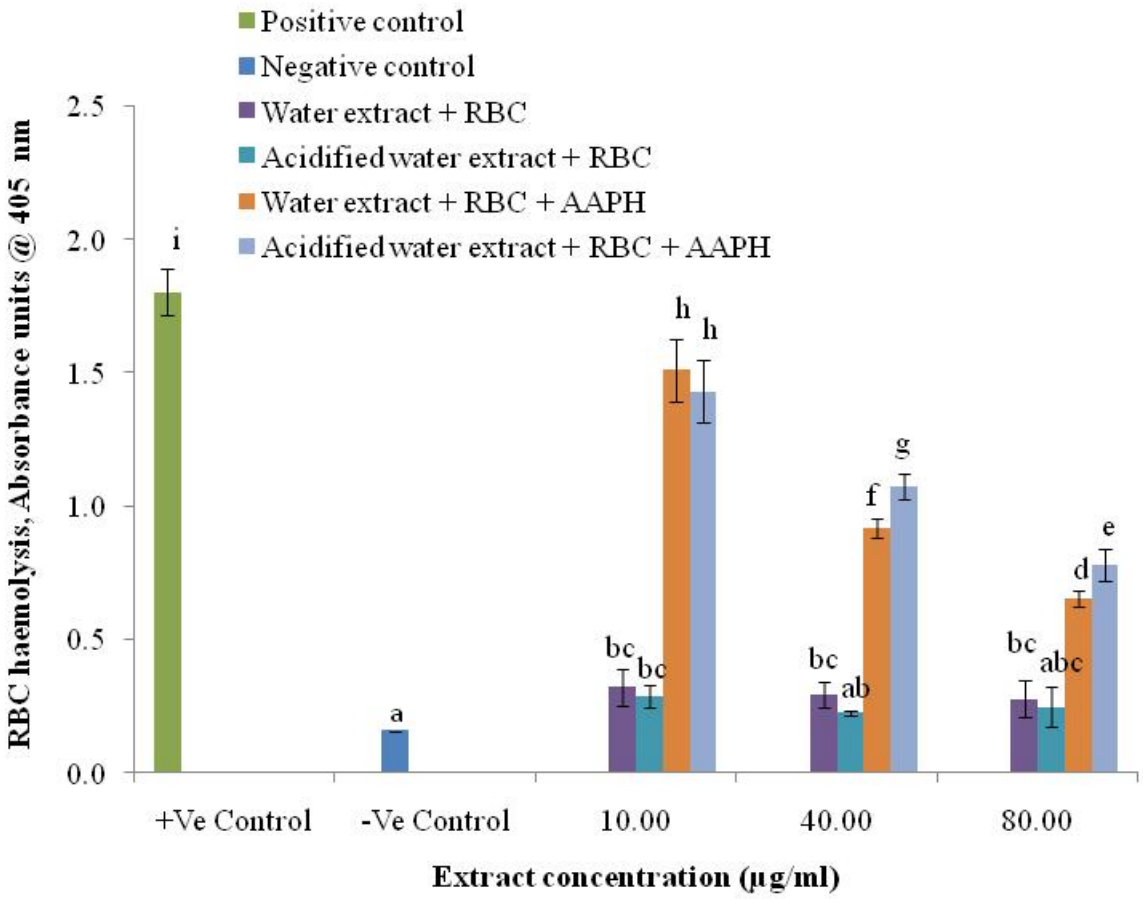

Figure 4.3.1. Effect of water and acidified water extracts prepared from marama bean seed coats on AAPH-mediated human red blood cell haemolysis. Each bar on the graph is an average of four determinations from two independent experiments. Error bars represent standard deviation of the mean. Bars with different letters are significantly different ($p \leq 0.05$).

Aqueous extracts from condensed tannin sorghum bran also inhibited AAPH-induced red blood cell haemolysis in a dose dependent manner as shown in Fig. 4.3.2. Treatment with water and acidified water extracts at 100 µg/ml concentrations resulted in 42 and 52% reduction in haemolysis, respectively and at 400 µg/ml concentrations by as much as 72 and 77%, respectively compared to positive control. Acidified water extracts showed a significantly higher protective effect at all concentration levels compared to water extracts

(Fig. 4.3.2). This may be due to the significantly higher total phenolic content and phenolic compound concentrations as measured with the Folin Ciocalteu method (section 4.1.4.1) and HPLC methods (section 4.1.4.2), respectively compared to water extracts. There was no significant difference between the negative control and sample controls indicating that the extracts from condensed tannin sorghum bran did not cause any haemolysis.

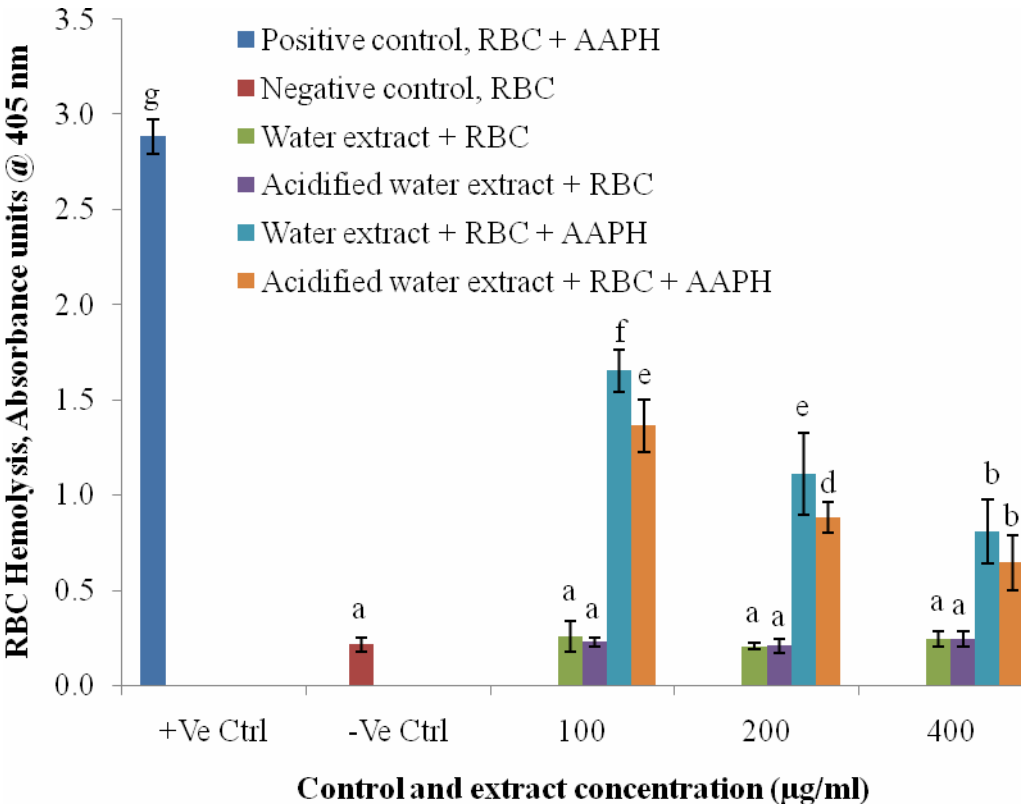

Figure 4.3.2. Effect of water and acidified water extracts from condensed tannin sorghum bran on AAPH-mediated human red blood cell haemolysis. Each bar on the graph is an average of four determinations from two independent experiments. Error bars represent standard deviation of the mean. Graph bars with different letters are significantly different ($p \leq 0.05$).

Extracts from marama bean seed coats were effective at lower concentrations 10–80 µg/ml (Figure 4.3.1) compared to extracts from condensed tannin sorghum bran which were effective at 100–400 µg/ml (Fig. 4.3.2). This is because extracts from marama bean seed

coats had higher phenolic content and antioxidant activity compared to equivalent extracts from condensed tannin sorghum bran.

Phenolic compounds from olive oil (Paiva-Martins, Fernandes, Santos, Silval, Borges, Rocha, Belo, & Bogdanov, 2010) and green tea (Lanping, Zaiqun, Bo, Li & Zhongli, 2000) showed a similar dose response protective effect against H_2O_2 and AAPH-induced red blood cell haemolysis, respectively. The protective effect of phenolic compounds in the extracts against red blood cell biomembrane oxidation is through antioxidant activity (Paiva-Martins *et al.*, 2009) by reducing lipid peroxides (Lin, Wu, Wang, Yang & Chang, 2001). Condensed tannins and flavonoid compounds in the extracts may also provide protection by accumulating at the membrane surface through hydrogen bonding to polar groups of membrane phospholipids thus preventing access of the deleterious peroxyl radicals to the hydrophobic region of the bilayer (Verstraeten, Keen, Schmitz, Fraga & Oteiza, 2003). The results suggest that phenolic compounds in the extracts have a potential to reduce oxidative stress on cell biomembranes which are the main sites of lipid peroxidation (Mak *et al*., 1983). Low molecular weight proanthocyanidins in the extracts, such as dimers, may be absorded in the upper gastrointestinal tract in their intact form (Holt, Lazarus, Sullards, Zhu, Schramm, Hammerstone, Fraga, Schmitz & Keen, 2002). However, high molecular weight condensed tannins (oligomers and polymers) may not be absorbed into circulation in their intact form (Rios, Gonthier, Rémésy, Mila, Lapierra, Lazarus, Williamson & Scalbert, 2003). They may therefore only directly provide protection to cells lining the gastrointestinal tract against oxidative damage and may also play a unique role in sparing other antioxidants and thus indirectly increasing antioxidant levels in other tissues (Hagerman *et al.*, 1998). However, the high molecular weight proanthocyanidins may be metabolised into phenolic acid metabolites by colonic bacteria and these metabolites may be absorbed into circulation and therefore may confer health beneficial effects (Rios et al., 2003; Saura-Calixto, Pérez-Jiménez, Touriño, Serrano, Fuguet, Torres & Goñi, 2010).

4.3.4.3. Effect of aqueous extracts on AAPH-mediated oxidative DNA damage

The effects of water and acidified water extracts from marama bean seed coats and condensed tannin sorghum bran on AAPH-induced oxidative supercoiled plasmid pBR 322 DNA damage are shown in Fig. 4.3.3.

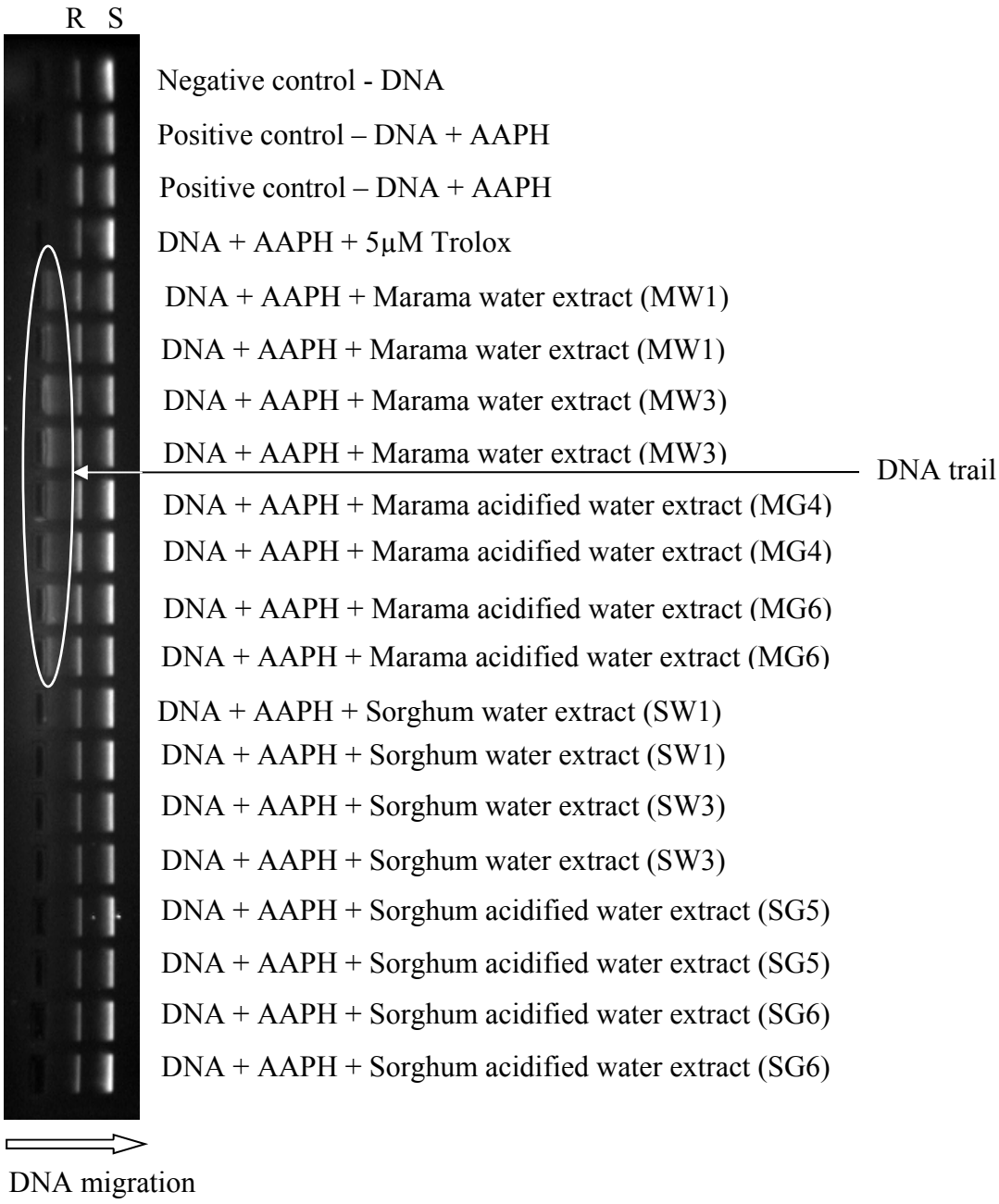

Figure 4.3.3. Effect of water and acidified water extracts from marama bean seed coats and condensed tannin sorghum bran on AAPH-induced oxidative supercoiled plasmid pBR 322 DNA damage. The S band represents supercoiled DNA and R band represents relaxed circular DNA.

Supercoiled DNA shown as S band had higher mobility than relaxed circular DNA shown as R band. In the negative control sample (without AAPH) the intensity of the super-coiled DNA band (S) was set to 100% and the intensities of the other bands were calculated relative to this band. Addition of 11 µmol AAPH solution to super-coiled plasmid DNA (positive control) caused single strand breaks resulting in decrease in intensity of the supercoiled DNA

band (S) to a mean value of 86 % of negative control and there was a slight increase in intensity of the relaxed circular DNA band (R) (Fig. 4.3.3).

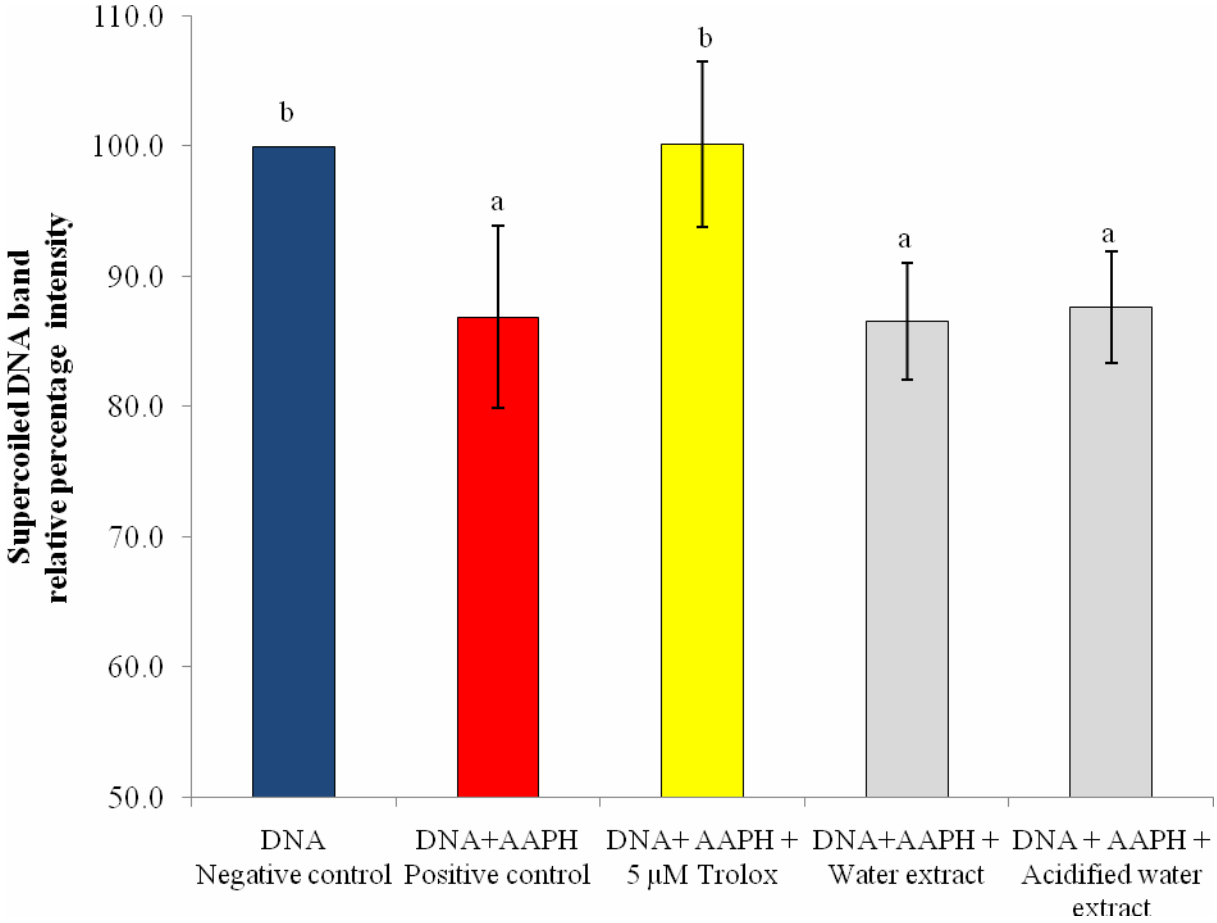

Figure 4.3.4. Effect of water and acidified water extracts from marama bean seed coats on AAPH-induced oxidative supercoiled plasmid pBR 322 DNA damage. Each bar on the graph is an average of four determinations from two independent experiments. Error bars represent standard deviation of the mean. Graph bars with different letters are significantly different ($p \leq 0.05$).

Addition of 5 µmol Trolox solution (5 µm Trolox + AAPH) resulted in protection against APPH-mediated single strand breaks and the intensity of the supercoiled DNA band was not significantly different from the negative control. Treatment with both water and acidified extracts from marama bean seed coats affected the mobility of DNA. All the lanes treated with extracts from marama bean seed coats (8 µg/ml) left behind a trail of DNA from the

wells resulting in lower intensities of the R and S bands especially the water extracts with higher levels of condensed tannin content (Fig. 4.3.3). However, all lanes treated with aqueous extracts from condensed tannin sorghum (200 µg/ml) did not show this trail of DNA.

Water and acidified water extracts from marama bean seed coats did not appear to provide protection against AAPH-mediated oxidative supercoiled DNA damage because there was no significant difference between the positive control and samples treated with the aqueous extracts (Fig. 4.3.4). However, it would appear that these results were inconclusive because there was poor migration of the DNA bands through the gel leaving behind a trail. The interference in the lanes treated with extracts from marama bean seed coats could be as a result of an interaction between DNA and the galloylated condensed tannins (section 4.2.5.3.1) in the extracts possibly through hydrogen bonding to form a complex with a higher molecular mass and low mobility. Condensed tannins in plant tissue have been reported to bind to DNA upon cell lyses during isolation of DNA for research work (Maliyakal, 1992). Also hydrolysable tannins have been reported to bind to DNA from calf thymus and the binding was attributed to the large number of hydroxyl groups and the high molecular weight (Labieniec & Gabryelak, 2006).

Treatment with water and acidified water extract solutions (200 µg/ml) from condensed tannin sorghum bran resulted in super-coiled DNA band (S) intensities of 96 and 97 %, respectively (Fig. 4.3.5). There was no significant difference between these bands and the negative control and the 5 µm Trolox treated sample indicating that both extracts from condensed tannin sorghum bran provided some protection against AAPH-mediated single strand breaks.

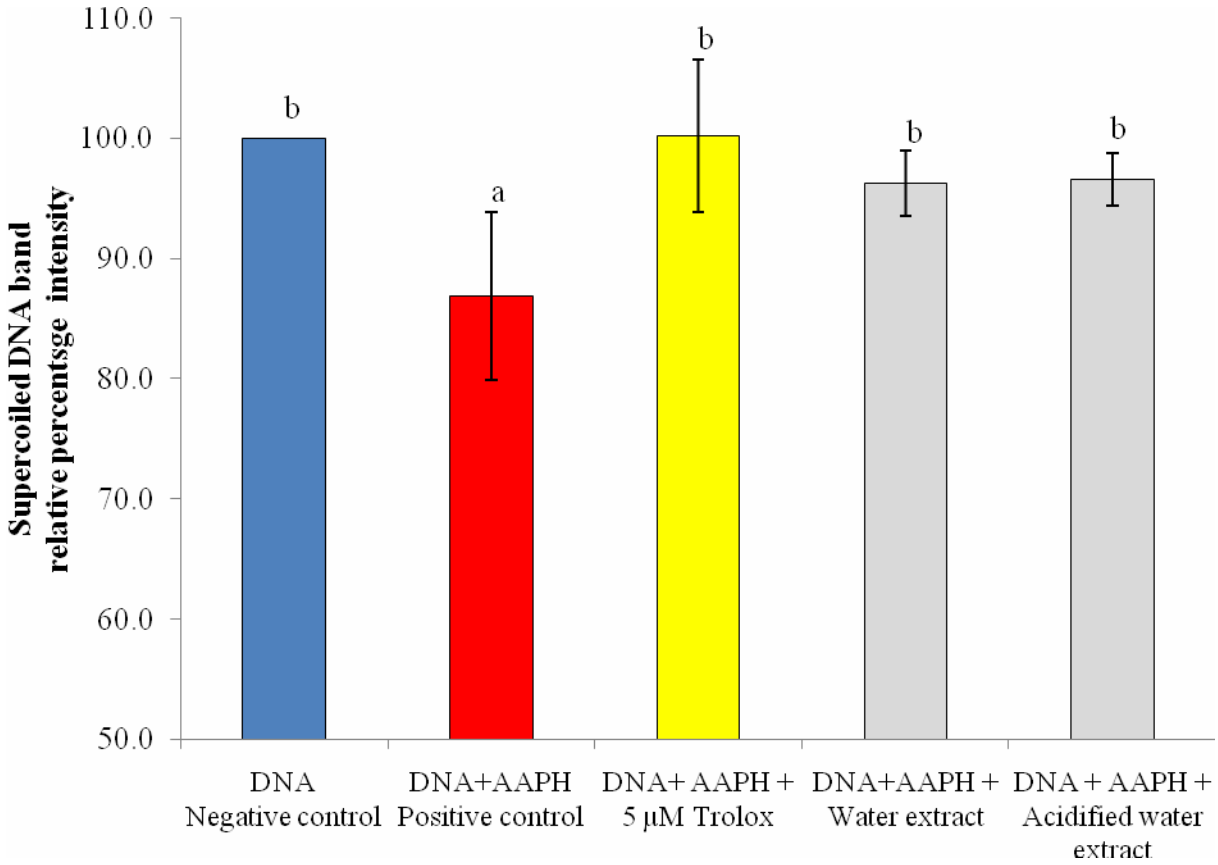

Figure 4.3.5. Effect of water and acidified water extracts (200 µg/ml) from condensed tannin sorghum bran on AAPH-induced oxidative supercoiled plasmid pBR 322 DNA damage. Each bar on the graph is an average of four determinations from two independent experiments. Error bars represent standard deviation of the mean. Graph bars with different letters are significantly different ($p \leq 0.05$).

The phenolic compounds epigallocatechin-3-O-gallate, epigallocatechin and epicatechin isolated from green tea were reported to have a protective effect against AAPH-induced supercoiled plasmid vector DNA (pBR 322) oxidative damage (Wei *et al.*, 2006). In another study the phenolic compounds caffeic acid, hydroxytyrosol and tyrosol from olive oil were also reported to have a protective effect against H_2O_2 induced oxidative DNA damage in cell lines from human prostate epithelium in a dose dependent manner (Quiles, Farquharson, Simpson, Grant & Wahle, 2002). The protective effect of phenolic compounds against oxidative DNA damage may be through free radical scavenging activity and transition metal ion chelation (Aherne & O'Brien, 2000). The results of this study suggest that the extracts by

virtue of their phenolic compound content may play a role in the prevention of cancer which may be initiated by free radical oxidative DNA damage.

4.3.4.4. Effect of extracts on copper-catalyzed human LDL oxidation

In this assay LDL oxidation was initiated with Cu (II) and the breakdown products of lipid peroxides, thiobarbituric reactive substances (TBARS) which are the late stage products of LDL oxidation (Xu *et al.*, 2007) were measured at the end of the incubation period using the TBA assay. Incubation of human LDL with copper sulphate solution (positive control) caused oxidation of LDL and produced maximum TBARS (Fig. 4.3.6 and Fig. 4.3.8).

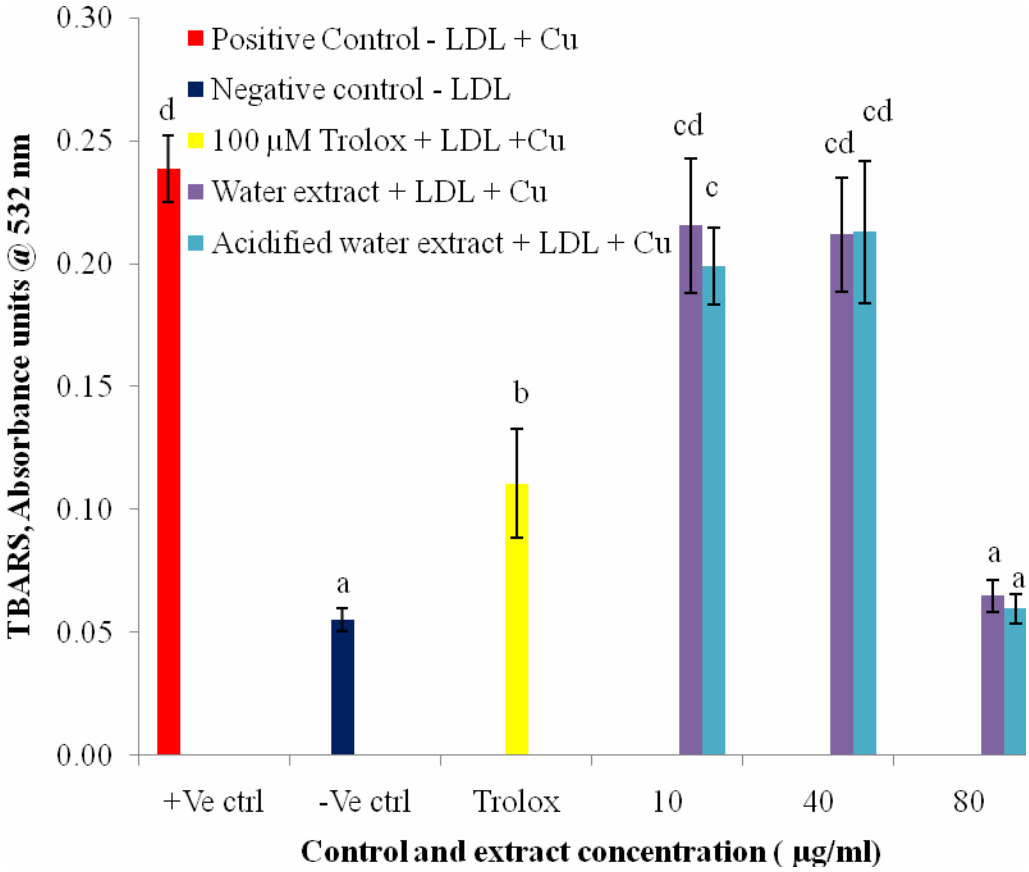

Figure 4.3.6. Effect of water and acidified water extracts prepared from marama bean seed coats on copper-catalyzed LDL oxidation determined with the thiobarbituric (TBA) assay. Bars are means of four determinations from two independent experiments. Error bars represent standard deviation of the mean. Bars with different letters are significantly different ($p \leq 0.05$).

Addition of 100 µm Trolox reduced LDL oxidation by 54% of positive control. Treatment with water and acidified water extracts from marama bean seed coats at 10 and 40 µg/ml concentrations resulted in 11–17% reduction in TBARS levels, however the TBARS levels were not significantly different from the positive control (Fig. 4.3.6). Treatment with water and acidified water extracts at 80 µg/ml concentrations resulted in 73 and 75% significant reduction in TBARS levels, respectively. The reduction was higher than that of 100 µm Trolox and the levels of TBARS were not significantly different from that of the negative control (without AAPH) which suggests that there was almost complete inhibition of LDL oxidation. There were no significant differences in the level of inhibition between water and acidified water extracts at all concentrations even though there were significant differences in phenolic compound content and antioxidant activity between the extracts. In Fig. 4.3.7 a possible explanation is provided as to why extracts with different levels of phenolic contents could exhibit similar TBARS levels. LDL oxidation is a lipid peroxidation reaction which is characterized by three phases; initiation (lag phase), propagation and decomposition. Antioxidants such as phenolic compounds lower the rate of initiation by scavenging lipid peroxyl radicals (Abuja, Murkovic & Pfannhauser, 1998) thereby prolonging the lag phase during incubation (Esterbauer, Gebicki, Puhl, & Jürgens, 1992). The extracts at 10 and 40 µg/ml concentrations had short lag phases and allowed the reaction to go through all the three lipid peroxidation phases resulting in similar TBARS levels. Both extracts at 80 µg/ml concentrations suppressed the initiation phase through-out the incubation period resulting in similar low TBARS levels.

Similar results were reported in an experiment investigating the protective effect of an antioxidant compound, oxime against copper-catalyzed LDL oxidation (de Lima Portella *et al.*, 2008). The authors found that at low concentrations levels (0.5, 1, 2 and 3 µM) the TBARS levels were not significantly different amongst treatments and the positive control even though the lag phases as measured by conjugated diene formation were significantly different amongst treatments and the positive control at all concentrations except at 0.5 µM. However at 5 µM there was a significant reduction in TBARS compared to the other treatments and the positive control.

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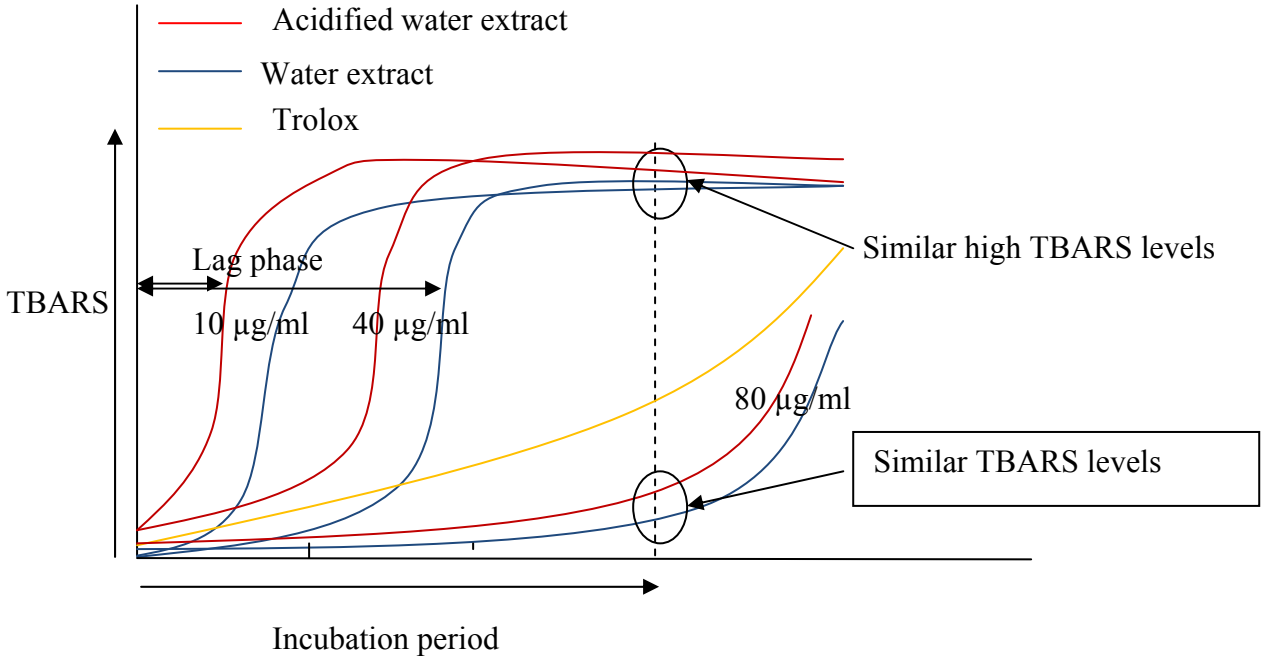

Figure 4.3.7. LDL oxidation kinetic curves showing how two extracts with significantly different antioxidant concentration could exhibit similar TBARS levels (Adapted from de Lima Portella *et al*. (2008)).

Addition of water and acidified water extracts from condensed tannin sorghum bran also showed a similar trend as extracts from marama bean seed coats (Fig. 4.3.8). Treatment with 100 and 250 µg/ml extract concentrations resulted in a 12–20% reduction in LDL oxidation and the TBARS levels at these concentrations were significantly different from that of the positive control. Treatment with water and acidified water extracts at 500 µg/ml concentration resulted in 72 and 71% significant reduction in TBARS levels, respectively and the levels were not significantly difference from that of the negative control (without AAPH) indicating an almost complete inhibition. The inhibition at this level was higher than that of 100 µm Trolox treated samples.

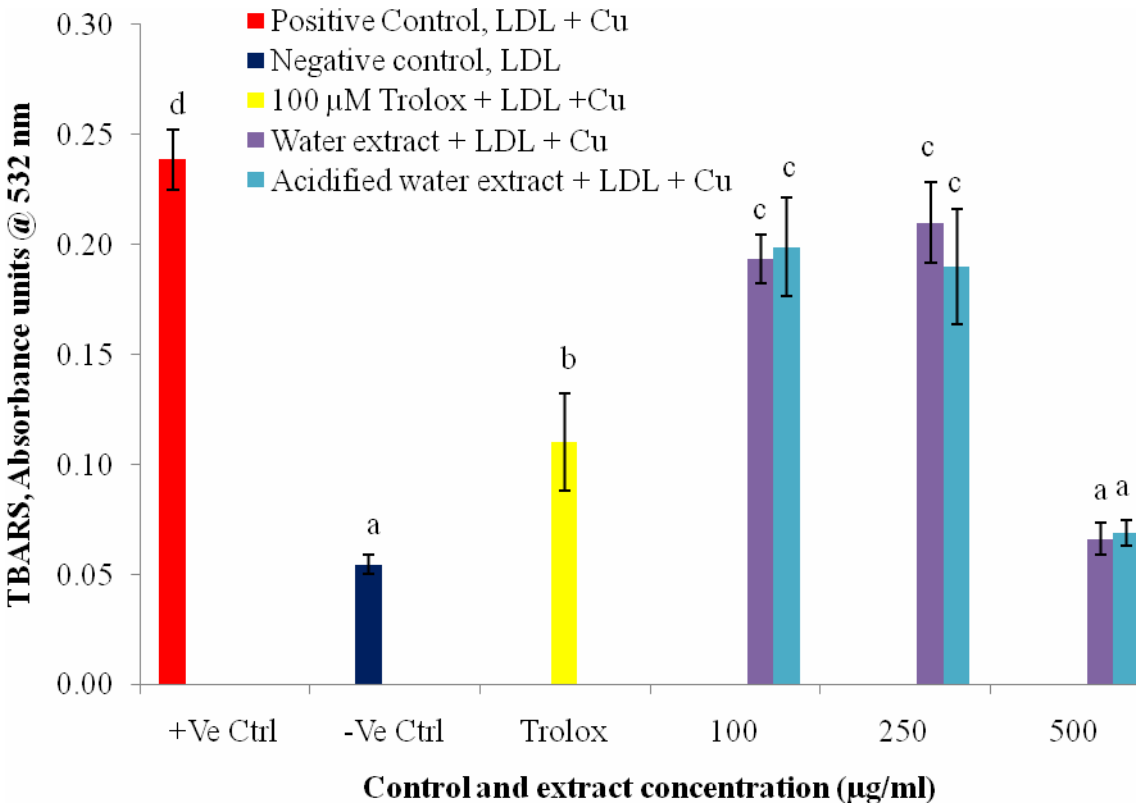

Figure 4.3.8. Effect of aqueous extracts prepared from condensed tannin sorghum bran on copper-catalyzed LDL oxidation using thiobarbituric assay (TBA assay). Bars are means of four determinations from two independent experiments. Error bars represent standard deviations. Bars with the same letter are not significantly different ($p \leq 0.05$).

Phenolic compounds protect against LDL oxidation by lowering the rate of initiation by scavenging lipid peroxyl radicals (Abuja *et al.*, 1998) interrupting propagation of free radicals and thus acting a chain breaking antioxidants (Reiter, 1998). These compounds may also provide protection through chelation of copper ions and by stabilizing LDL structure through interaction with apolipoprotein B thereby preventing binding of copper ions to the particles (Rüfer & Kulling, 2006).

The results show that water and acidified water extracts from marama bean seed coats were more effective than equivalent extracts from condensed tannin sorghum bran. This effect was observed at a concentration of 10–80 µg/ml for extracts from marama bean seed coats compared to 100-500 µg/ml for extracts from condensed tannin sorghum bran. This is due to

the higher phenolic content and antioxidant activity of extracts from marama bean seed coats compared to equivalent extracts from condensed tannin sorghum bran.

The results did not show a dose-response effect. Also there were no significant differences between water and acidified water extract treated samples, especially samples treated with extracts from marama bean seed coat which had significantly different phenolic content and antioxidant activity. The reason is that antioxidants only prolong the lag phase of lipid peroxidation during the incubation period (Abuja *et al.*, 1998; Esterbauer, Gebicki, Puhl & Jürgens, 1992) and if the reaction goes through all the phases of lipid peroxidation the amount of TBARS produced becomes dependent on the initial amount of LDL present and not on the extract concentration.

Other plant extracts from elderberry (Abuja *et al.*, 1998), grape juice (Frankel, Bosanek, Meyer, Silliman & Kirk, 1998) have been shown to inhibit LDL oxidation *in vitro*. Purified phenolic compounds extracted from plant material such as hydroxycinnamic acids from grape (Meyer, Donovan, Pearson, Waterhouse & Frankel, 1998), isoflavonoids and their metabolites from soybean (Hodgson *et al.*, 1996; Rüfer & Kulling, 2006) have also been shown to have a protective effect against LDL oxidation. Overall, the results provide some evidence that extracts from marama bean seed coats and condensed tannin sorghum bran, by virtue of their content of phenolic compounds could provide protection against LDL oxidation, which is a risk factor in cardiovascular disease.

4.3.5. Conclusions

Aqueous extracts from marama bean seed coats have higher free radical scavenging activity than equivalent extracts from condensed tannin sorghum bran because of their higher phenolic compound content. Extraction of marama bean seed coats under acidic condition results in an extract with lower free radical scavenging activity because of lower phenolic content as a result of co-precipitation of phenolic compounds with interpolymer complex precipitate which may be as a result of interaction between prodelphinidins (condensed tannins) and cell wall polysaccharides. In contrast, extraction of condensed tannin sorghum bran under acidic condition does not have a significant effect on free radical scavenging activity, probably because of the similar levels of condensed tannins in water and acidified water extracts.

Extracts from marama bean seed coats are effective at lower concentration against biomembrane oxidative damage than equivalent extracts from condensed tannin sorghum bran due to their higher phenolic compound content and antioxidant activity. Extracts from condensed tannin sorghum bran show a clear protective effect against oxidative DNA damage. However, this assay is not suitable for the evaluation of extracts from marama bean seed coats as the prodelphinidins in the extracts bind to DNA causing poor mobility of the bands in the agarose gel resulting in inconclusive results. The extracts have a potential to lower the risk of cancer by inhibiting free radical oxidative DNA damage in cells. Further more, these extracts can potentially prevent atherosclerosis associated with cardiovascular disease by inhibiting LDL oxidation. Aqueous extracts from marama bean may be more effective at lower concentration than those from condensed tannin sorghum bran because of their higher concentration of phenolic compounds. In conclusion, both marama bean seed coat and condensed tannin sorghum bran extracts have the potential to protect biomolecules against oxidative damage.

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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 (Naczk & 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 propeties. However, proteins are low in seed coats of legumes (Rodriquez & Mendoza, 1990). The protein content of marama 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 marama bean cotyledons have been shown contain high levels of tryrosine (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 marama 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 marama 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 $NH_4Fe(SO_4)_2$ 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.

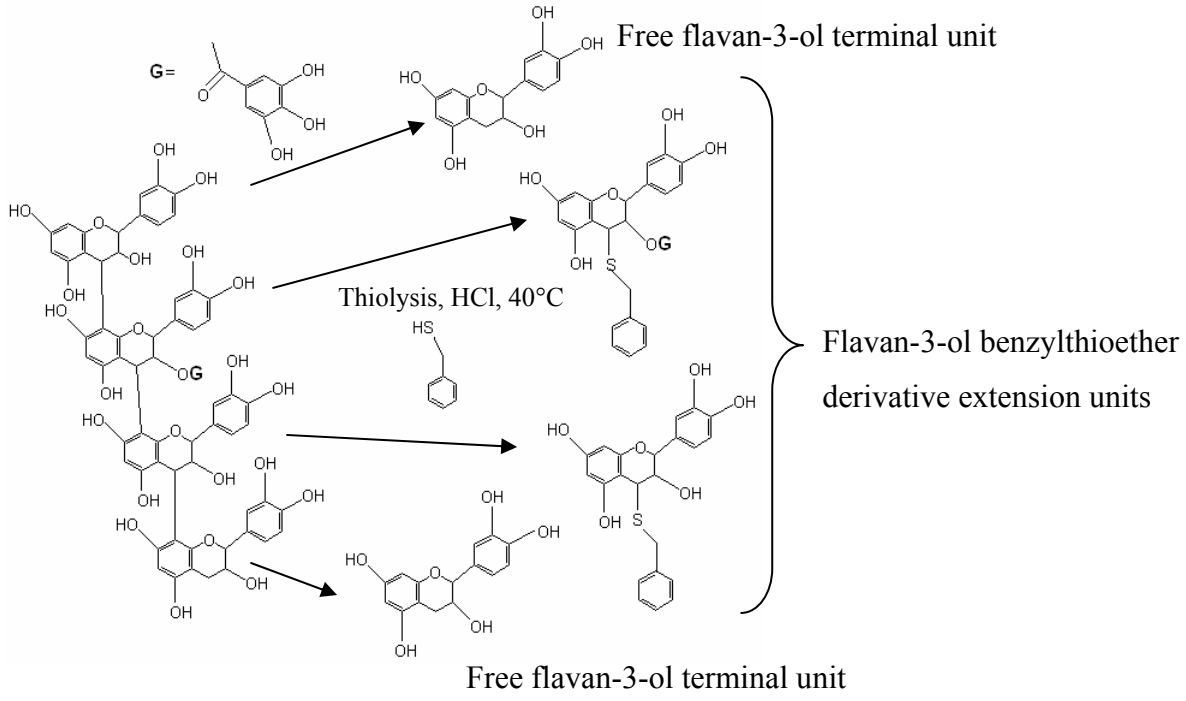


Figure 5.1.1. Thiolysis degradation of proanthocyanidins in the presence of toluene-α-thiol (Adapted from Gu *et al.* (2002))

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 peroxyl 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-Phycoerthrin (β-PE) a protein isolated from *Porphydium 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 peroxyl radical induced by thermal decomposition of 2,2'-azobis (2-methyl-propionamidine) dihydrochloride (AAPH) at 37 °C (Ou et at., 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 peroxyl 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 H_2O_2 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 ($OH^{\bullet}$) 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 H_2O_2 or Cu^{2+} or Fe^{2+} instead of AAPH which generate the peroxyl 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 Cu^{2+} 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).

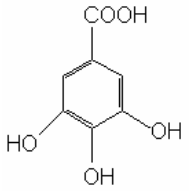
5.2. Discussion of study results

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.

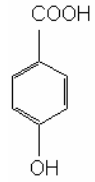
Phenolic compounds in aqueous extracts from marama bean seed coats were phenolic acids and flavonoids and the chemical structures are shown in Fig. 5.2.1. The phenolic acids identified in extracts from marama bean seed coat were benzoic acid derivatives and these have been reported in organic extracts 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

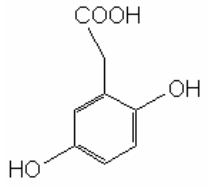


A) Gallic acid



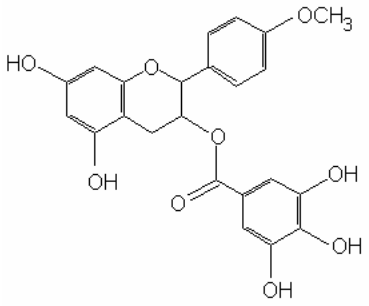
B) *p*-Hydroxybenzoic acid

Phenylacetic acid derivative

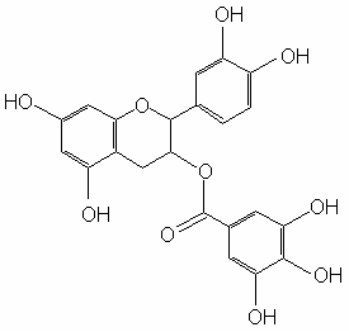


C) Homogentisic acid

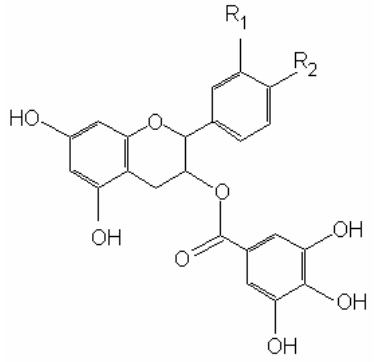
Flavanol



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.

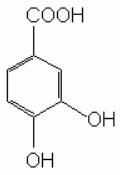
In this study it was not possible to distinguish between isomers using UV and MS detection without authentic phenolic compound standards. Methyl (epi)afzelechin-3-O-gallate (Fig. 5.2.1.D) had two isomers, with retention times of 16.7 and 20.5 min. These compounds could be methyl afzelechin-3-O-gallate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

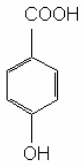
In the absence of authentic phenolic standards nuclear magnetic resonance (NMR) techniques could be used to differentiate between isomers especially when analysing an extract with unknown compounds.

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).

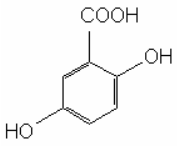
Benzoic acid derivative



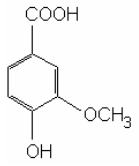
A) Protocatechuic acid



B) *p*-Hydroxybenzoic acid

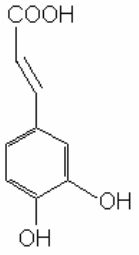


C) Gentisic acid

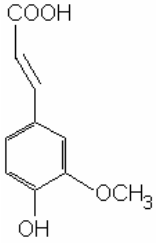


D) Vanillic acid (Minor)

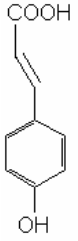
Cinnamic acid derivatives



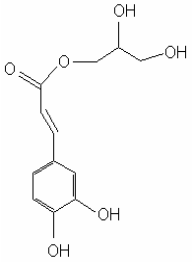
E) Caffeic acid



F) Ferulic acid

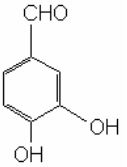


G) *p*-Coumaric acid (Minor)

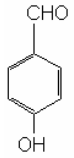


H) Caffeoylglycerol

Phenolic aldehydes

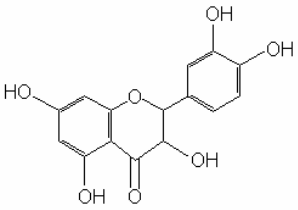


I) Protocatechualdehyde

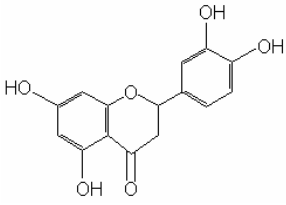


J) *p*-Hydroxybenzaldehyde

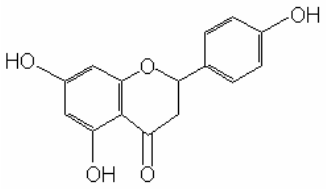
Flavanone



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 (Naczk & 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.

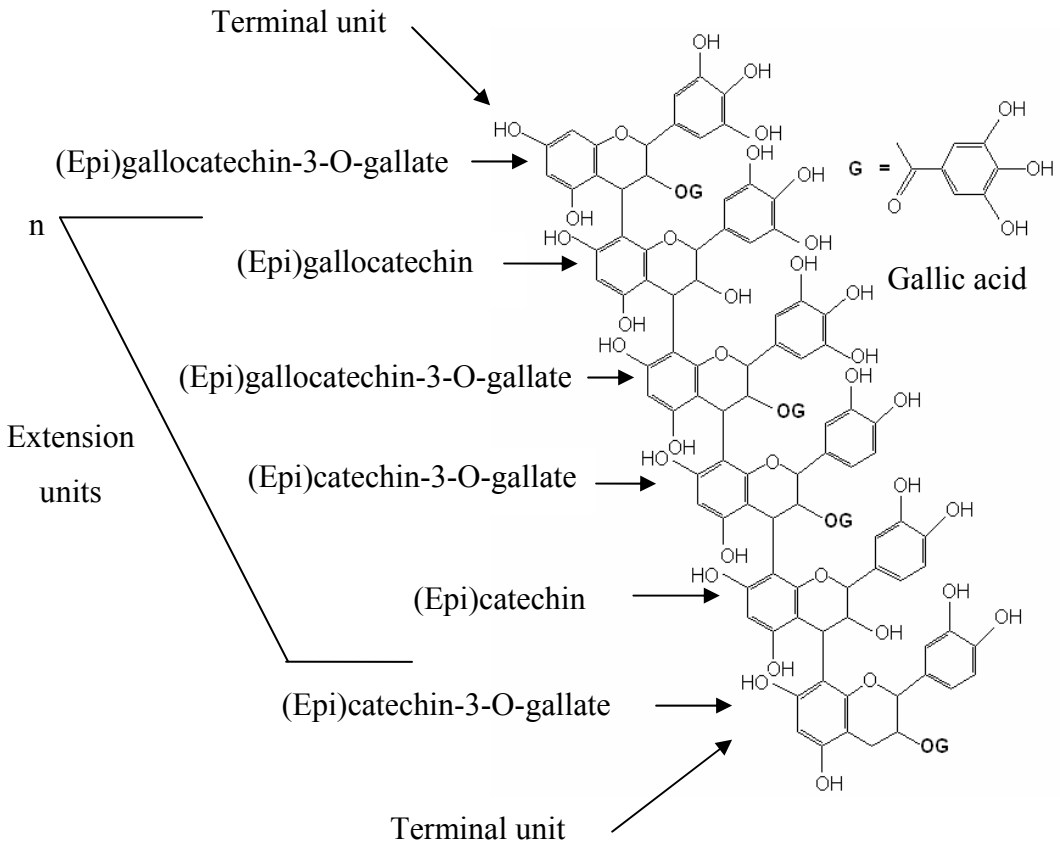


Figure 5.2.3. Proposed heteropolyflavan polymer structure of proanthocyanidins in extracts from marama bean seed coats, $1 < n < 8$ (Adapted from Yousef *et al.* (2006)).

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).

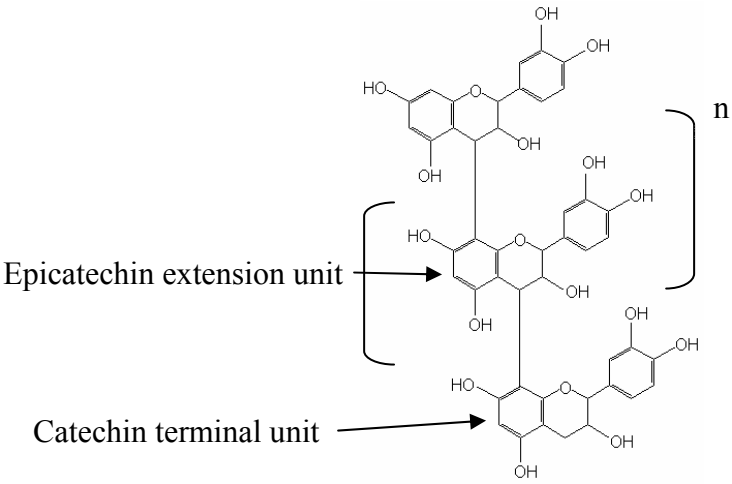


Figure 5.2.4. Chemical structure of proanthocyanidin polymer in extracts from condensed tannin sorghum bran, 1 < n < 5 (Adapted from Hagerman *et al.* (1998) and Awika and Rooney (2004))

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.

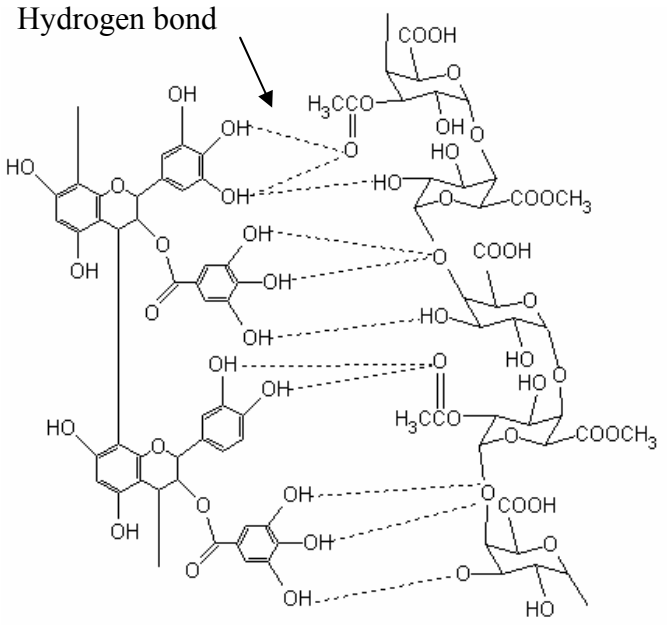


Figure 5.2.5. Hydrogen bonding between highly galloylated condensed tannins and cell wall polysaccharides to form interpolymer complex precipitate at low pH (Adapted from Hanlin *et al.* (2010))

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 precipite 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, peroxyl 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•) produced by the breakdown of arginine to citrulline by nitric oxide synthesis to generate the peroxynitrite radical ($ONOO^-$) (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 ($HO^•$) through the Fenton reaction catalysed by transition metals such as copper and iron (Sohal, 1997). The $HO^•$ 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 peroxyl and alkyl radicals which may abstract H^+ from lipids leading to further lipid breakdown (Reiter, 1998).

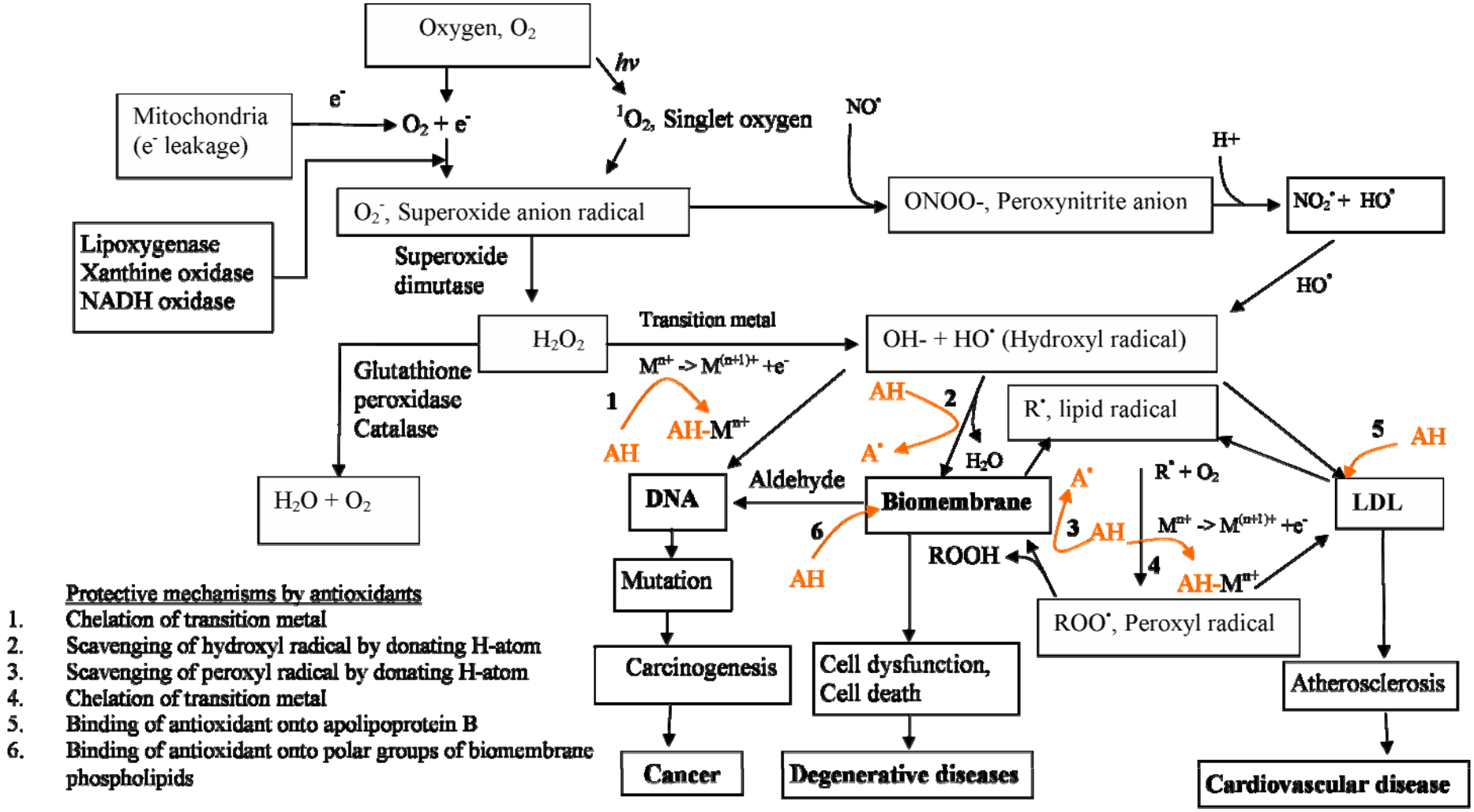


Figure 5.2.6. Mitigation of oxidative stress caused by reactive oxygen species by antioxidants shown as 1 to 6. AH, antioxidant and $A^•$, oxidized antioxidant. (Adapted from Esterbauer *et al.* (1992), Reiter (1998) and Waris & Ahsan (2006))

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 peroxyl 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).

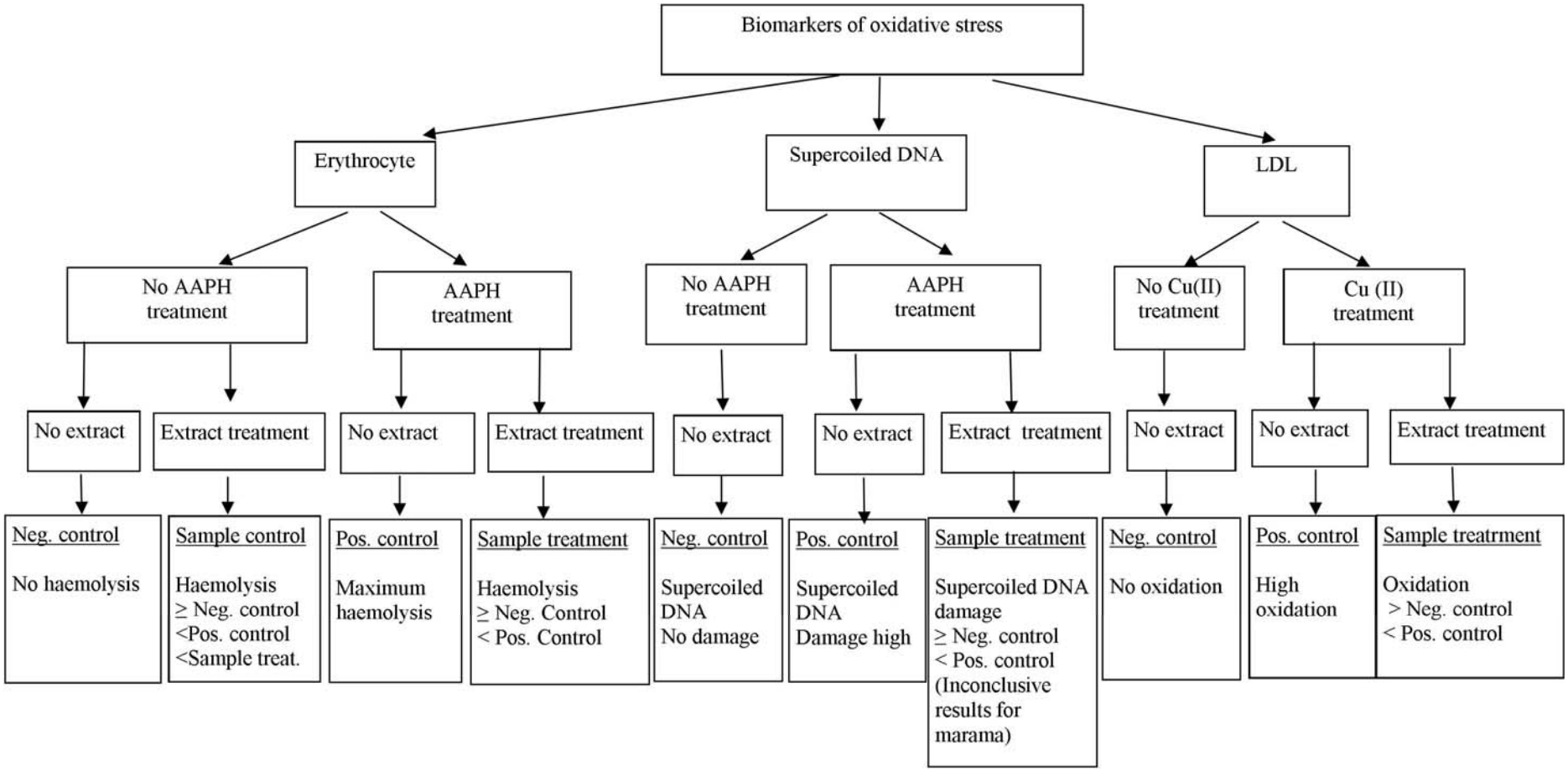


Figure 5.2.7. Summary of the effects of aqueous extracts from marama bean seed coats and condensed tannin sorghum bran against induced oxidative damage of biomembrane, DNA and LDL as biomarkers of oxidative stress

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, Lapierre, 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), peroxyl 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.

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8. PUBLICATIONS

Shelembe, J. S. Cromarty, D., Bester M. J., Minnaar, A. & Duodu, K. G. (2012). Characterization of phenolic acids, flavonoids, proanthocyanidins and antioxidant activity of water extracts from seed coats of marama bean [*Tylosema esculentum*] – an underutilized food legume. *International Journal of Food Science and Technology*, *47*, 648-655.

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