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).

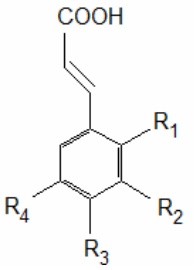
Dietary phenolic compounds occur as micro-constituents in many plant foods (Dabrowski & Sosulski, 1984; Hagerman, Riedl, Jones, Sovik, Ritchard, Hartfeld & Riecher, 1998) and they are major antioxidants in our diet (Ranilla *et al.*, 2007). The main sources are fruits, fruit juices, wine, tea, coffee, 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).

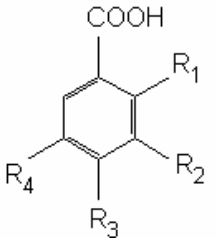
2.1.2.2. Phenolic acids

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).

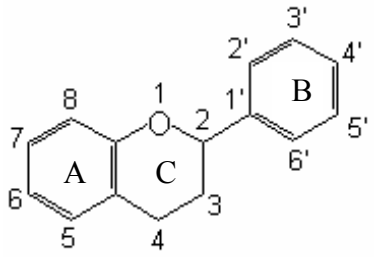
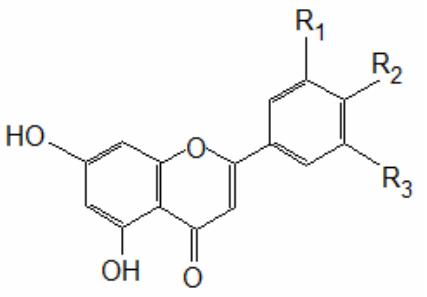
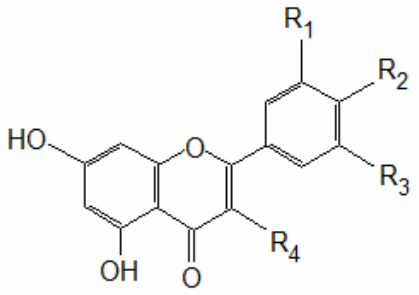


Figure 2.1.3. Generic structure of flavonoid compounds (Adapted from Wolfe & Liu (2008)).

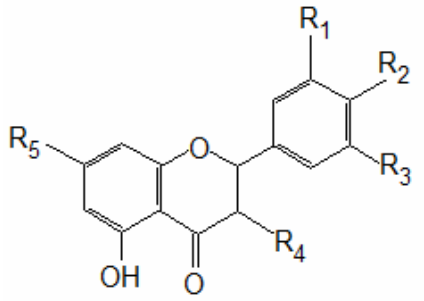
Flavonoids consist of a diphenylpropane (C_6-C_3-C_6) skeleton, which is basically two benzene rings (A and B rings) joined by a three carbon linear chain that form an oxygenated heterocycle (C ring) (Fig. 2.1.3) (Rice-Evans *et al.*, 1996; Manach *et al.*, 2004). While some flavonoids occur in free 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).



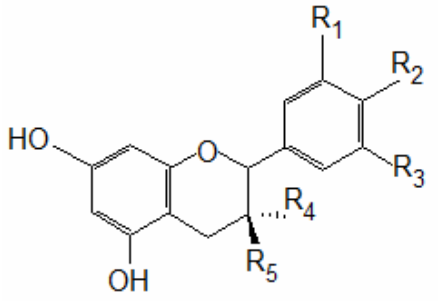
A. Flavone



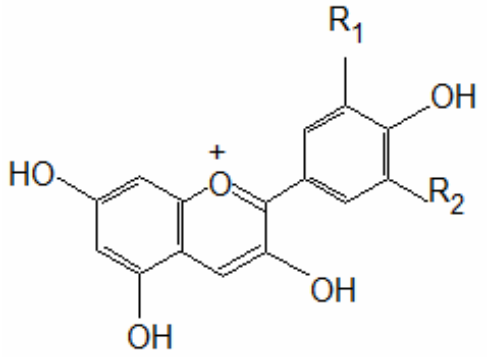
B. Flavonol



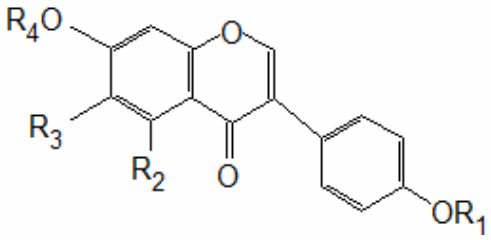
C. Flavanone



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).

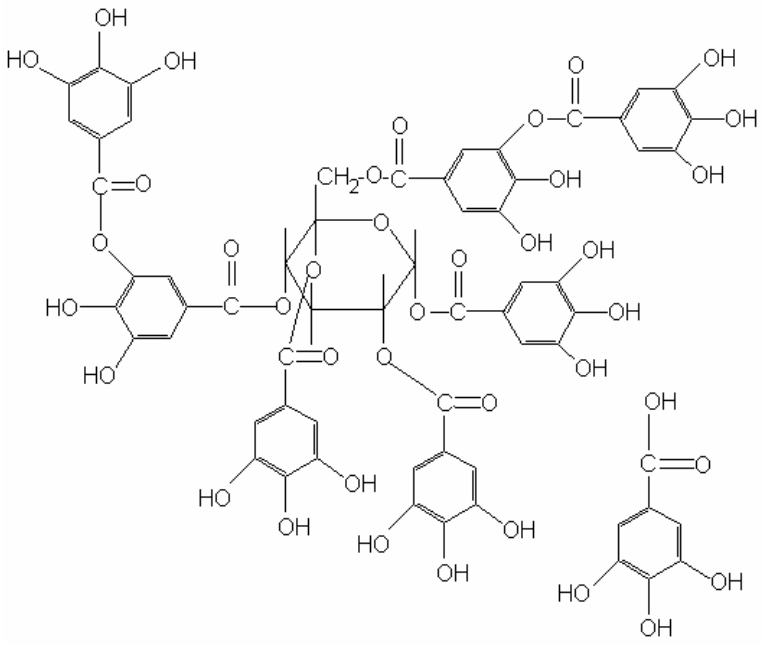
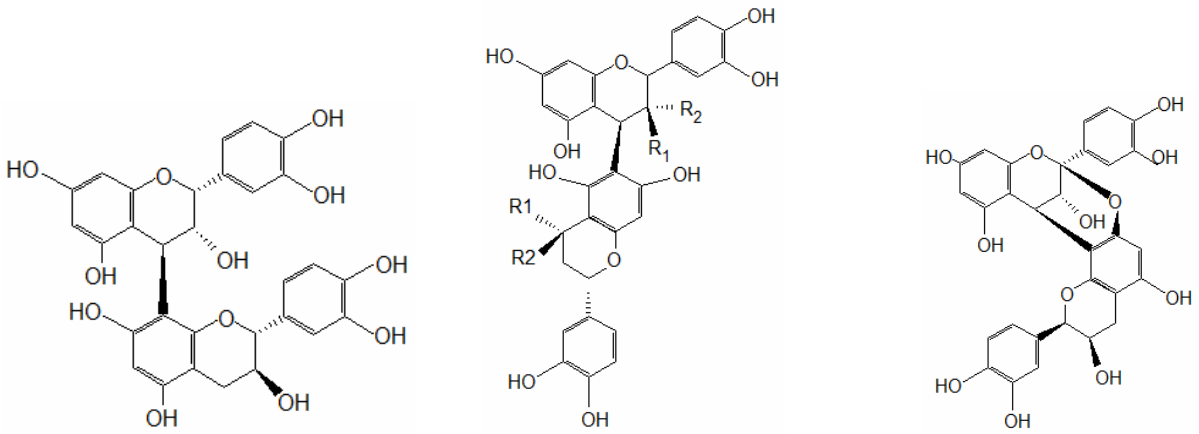


Figure 2.1.5. Hydrolysable tannin structure (Adapted from Manach *et al.* (1989) and Naczk & Shahidi (2004)).

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).

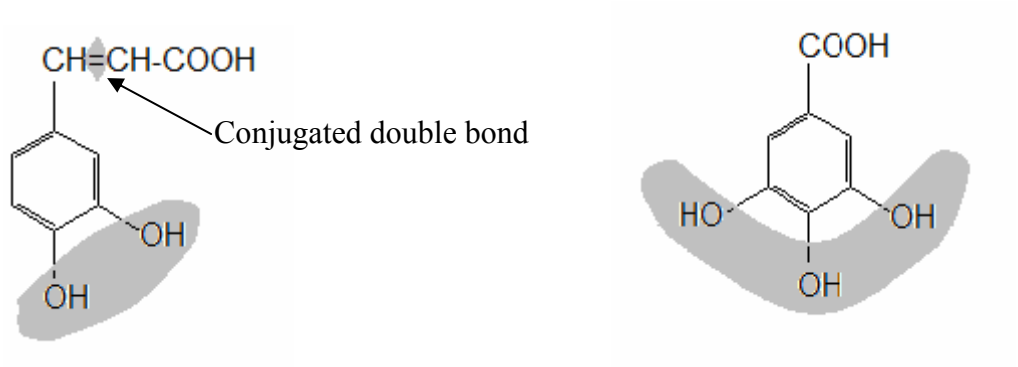
Extraction under conditions that mimic those prevailing in the gastrointestinal tract has been suggested to provide a more physiologically relevant approach as it may extract more water-soluble phenolic constituents and simulate the impact of digestive enzymes and conditions on polyphenols (Chen & Blumberg,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

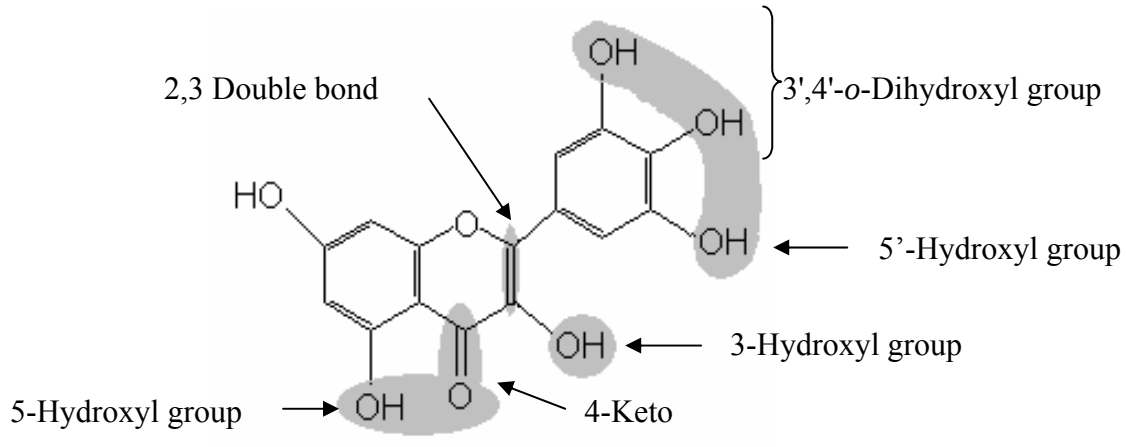


Figure 2.1.8. Flavonoid structure showing functional groups that affect antioxidant activity.

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.