Chapter 1

Introduction, statement of the problem and literature review

1.1 Introduction and statement of the problem

A wide range of preservation methods exist in food industries. Among the approaches used to achieve food preservation by preventing the growth of unwanted microorganisms is the use of chemical agents with antimicrobial activity (Sofos, Beuchat, Davidson and Johnson, 1998). These chemicals may include synthetic compounds, which are added intentionally to the foods or naturally occurring, biologically derived substances, the so-called naturally occurring antimicrobials (Sofos *et al*., 1998). Examples of synthetic additives include chemical antimicrobials such as formic and propionic acid (Duffy and Power, 2001).

Foodborne illnesses result in a major public health impact around the word (White, Zhao, Simjee, Wagner and McDermott, 2002) even in developed countries (Alzoreky and Nakahara, 2003). In the United States (US), according to the data published by the Centers for Disease Control and Prevention, foodborne diseases have been shown to account for approximately 76 million illnesses, 325, 000 hospitalisations, and 5000 deaths each year in the US alone (Mead, Slutsker, Dietz, McCaig, Bresee and Shapiro as cited by White *et al*., 2002). *Listeria* and *E. coli* O157:H7 among other bacteria were shown to account for 28 and 3 % of about 90 % of estimated food-related deaths respectively (White *et a*l., 2002). Increasing concern of outbreaks of food borne diseases due to pathogenic microorganisms and the suspected carcinogenic nature of synthetic additives, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) (Tauxe, Madavi and Salunkhe as cited by Jayaprakasha, Selvi and Sakariah, 2003) resulted in consumers increasing avoiding foods prepared with preservatives of chemical origin (Rauha, Remes, Heinonen, Hopia, Kähkönen, Kujala, Pihlaja, Viorela and Viorela, 2000). Natural alternatives are therefore needed to achieve sufficiently long shelf-life of foods and a high degree of safety with respect to foodborne pathogenic microorganisms (Rauha *et al*., 2000). Attention is therefore shifting towards the use of natural preservatives (Menon and Garg, 2001). Phenolic extracts from different plant sources such as green tea, cinnamon, curry, mustard, herbs, spices and grapes have been shown to

have antioxidant as well as antimicrobial activity (Sakanaka, Juneja and Taniguchi, 2000; Jayaprakasha *et al*., 2003; Baydar, Özkan, and Sağdiç, 2004).

Polyphenols are secondary plant metabolites, i.e. they are not directly involved in any metabolic process and are characterised by possession of a phenol group (Awika and Rooney, 2004). These compounds have been shown to have many favourable effects on human health such as lowering of human low-density lipoprotein (Frankel, Waterhouse and Teissedre, 1995) and tannins (a class of phenolic compounds) have also been reported to exert other physiological effects such as reducing blood pressure (Chung, Wong, Wei, Huang and Lin, 1998a). They also appear to be responsible for the astringency of many plant materials and can have an effect on the colour, appearance and nutritional quality when added to the diet or when found in high levels in certain foodstuffs (Strumeyer and Malin, 1975; Haslam, 1989; Hahn *et al*., 1984; Murty and Kumar, 1995; Bvochora, Danner, Miyafuji, Braun and Zvauya, 2004). Sorghum could be a source of phenolic compounds. A number of studies have been conducted on the phenolic composition of sorghum and it has been found to contain large amounts of phenolic compounds (Beta, Rooney, Marovatsanga and Taylor, 1999; Awika, Rooney, Wu, Prior and Cisneros-Zevallos, 2003). They are mostly concentrated in the outer layers of the sorghum kernel (Hahn, Rooney and Earp, 1984). There is however no information on the antimicrobial properties and inhibitory effects of sorghum phenolic extracts.

1.2 Literature review

1.2.1 Sorghum (*Sorghum bicolor* (L) Moench)

Doggett (1988) described sorghum (*Sorghum bicolor* (L) Moench) as an indigenous African cereal and traditional food crop. Like other cereals such as barley, maize, rice and wheat, sorghum belongs to the grass family, the *Gramineae* (Odibo, Nwanko and Agu, 2002). Sorghum kernel is considered a naked caryopsis, i.e. kernel losing its hulls or glumes after threshing (Serna-Saldivar and Rooney, 1995).

1.2.2 Sorghum production and utilisation

Sorghum is the fifth major cereal crop in the world after wheat, rice, corn and barley (Awika and Rooney, 2004). The world sorghum production was 59 million metric tones in 2003 (FAO, 2004) with the United States being the largest producer and exporter, accounting for 20 % of world production (Awika and Rooney, 2004). In the United States, grain sorghum is mainly utilised for animal feed (Lochte-Watson, Weller and Jackson, 2000). In contrast, sorghum is consumed as a staple by millions of people in Asia and Africa (Ratnavathi and Sashidhar, 1998) with more than 35 % of the crop grown directly for human consumption (Awika and Rooney, 2004).

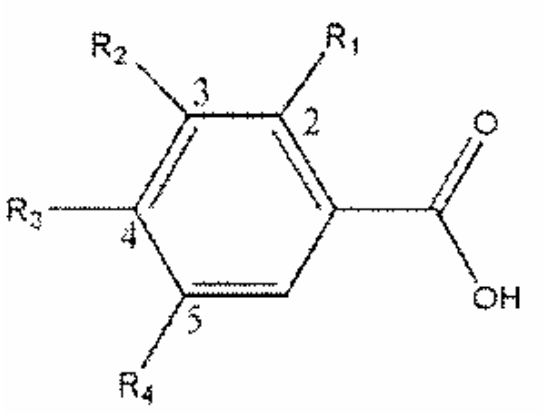
Sorghum processing entails partial or complete decortication of sorghum grains before further processing and consumption, though whole grains may also be directly dry-milled to give a range of products such as fine flour or meal, cracked grains and grits (Murty and Kumar, 1995). Traditionally, sorghum flour is used as food in the form of thin and thick porridges (Bvochora *et al*., 2004), snacks, cookies and other cultural foods (Awika and Rooney, 2004). The phenol-rich bran, which is a low-value, high-fiber by-product of the sorghum milling process, is often discarded or used as low-value animal feed (Murty and Kumar, 1995; Lochte-Watson *et al*., 2000). According to Hahn *et al*. (1984) all sorghums contain phenolic compounds, which can have an effect on the colour, appearance and nutritional quality of grain and sorghum products.

1.2.3 Phenolic compounds in sorghum

Phenolic compounds in sorghum may be divided into three major categories, namely, phenolic acids, flavonoids and the tannins (Hahn *et al*., 1984).

1.2.3.1 Phenolic acids

The phenolic acids of sorghum mainly exist as benzoic (**1-6**) or cinnamic acid (**7-11**) derivatives (Figure 1) and are mostly concentrated in the bran (outer covering of the grain) (Awika and Rooney, 2004). They exist mostly in bound forms (esterified to cell walls polymers), with ferulic acid (**8**) being the major bound phenolic acid in sorghum (Hahn *et al*., 1984). These compounds may occur as free acids or soluble and insoluble esters in sorghum (Hahn *et al*., 1984). The insoluble ester forms, located in the endosperm only, are thought to be linked with the endosperm cell walls (Hahn *et al*., 1984). A number of other phenolic acids have been identified in sorghum including *p*-coumaric (**10**) and sinapic (**11**) as the more abundant (Hahn and Waniska, Poe and Bandyopadhyay as cited by Awika and Rooney, 2004).



Benzoic acids (**1-6**)

Gallic acid (**1**): R_1=H,R_2=R_3=R_4=OH

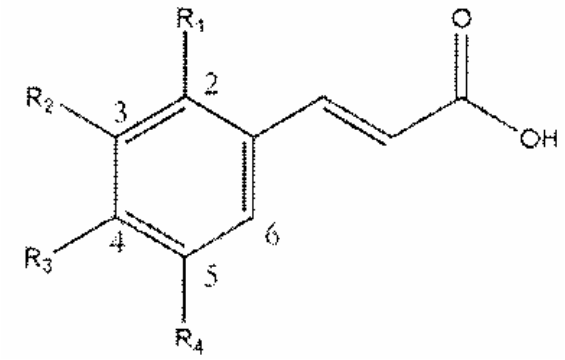
Gentisic acid (**2**): R_1=R_4=OH,R_2=R_3=H

Salicylic acid (**3**): R_1=OH,R_2=R_3=R_4=H

p-hydrobenzoic acid (**4**): R_1=R_2=R_4=H,R_3=OH

Syringic (**5**): R_1=H,R_2=R_4=OCH_3,R_3=OH

Protocatechuic (**6**): R_1=R_4=H,R_2=R_3=OH



Cinnamic acids (**7-11**)

Caffeic acid (**7**): R_1=R_4=H,R_2=R_3=OH

Ferulic acid (**8**): R_1=R_4=H,R_2=OCH_3,R_3=OH

o-coumaric acid (**9**): R_1=OH,R_2=R_3=R_4=H

p-coumaric acid (**10**):R_1=R_2=R_4=H,R_3=OH

Sinapic (**11**): R_1=H,R_2=R_4=OCH_3,R_3=OH

Figure 1. Some phenolic acid monomers identified in sorghum (adapted from Awika and Rooney, 2004).

According to Awika and Rooney, (2004) most quantitative studies specific to phenolic acids are performed by chromatographic analysis. In sorghums, the levels of phenolic acids were shown not to correlate with the presence or levels of anthocyanins or tannins (Awika and Rooney, (2004). In a study done by Waniska, Poe and Bandyopadhyay, (1989) increased levels of free phenolic acids in certain sorghums with pigmented testa (containing tannins) compared to ones without pigmented testa were observed (Awika and Rooney, 2004). Levels of major phenolic acids in sorghum are given in Table 1.

Table 1. Contents of major phenolic acids in sorghum (adapted from Awika and Rooney, 2004)

Phenolic acid	µg/g (dry wt)[a]	Reference
Sorghum Grain		
Ferulic (**8**)	100-500	Hahn and Rooney (1986); Hahn (1984)
Sinapic (**11**)	50-140	Hahn et al. (1983)
p-Cuomaric (**10**)	70-230	Hahn et al. (1983)
Sorghum Bran		
Ferulic (**8**)	1400-2170	Hahn (1984)
Sinapic (**11**)	100-630	Hahn (1984)
p-Cuomaric (**10**)	0-970	Hahn (1984)

[a] Total (free and bound) measured by HPLC

1.2.3.2 Flavonoids

Flavonoids consist of two distinct units: a C6-C3 fragment from cinnamic acid forms the B-ring and a C6 fragment from malonyl-CoA forms the A-ring (Hahn *et al*., 1984; Waniska, 2000). Three major groups of flavonoids are the flavans with the basic structure as indicated in Figure 2, flavones (carbonyl at carbon 4, double bond between carbons 2 and 3) and flavonols (carbonyl at carbon 4, hydroxyl at carbon 3 and a double bond between carbons 2 and 3) (Hahn *et al*., 1984). The flavanones (carbonyl at carbon 4, no double bond between carbons 2 and 3 and no OH group at carbon 3) are also a group of flavonoids. The flavans are the major group of flavonoids in sorghum (Hahn *et al*., 1984)

with an OH group at carbon 3. The major flavans are leucoanthocyanidins (hydroxyls at carbons 3 and 4) and catechin (hydroxyl at carbon 3) (Hahn *et al*., 1984; Waniska, 2000).

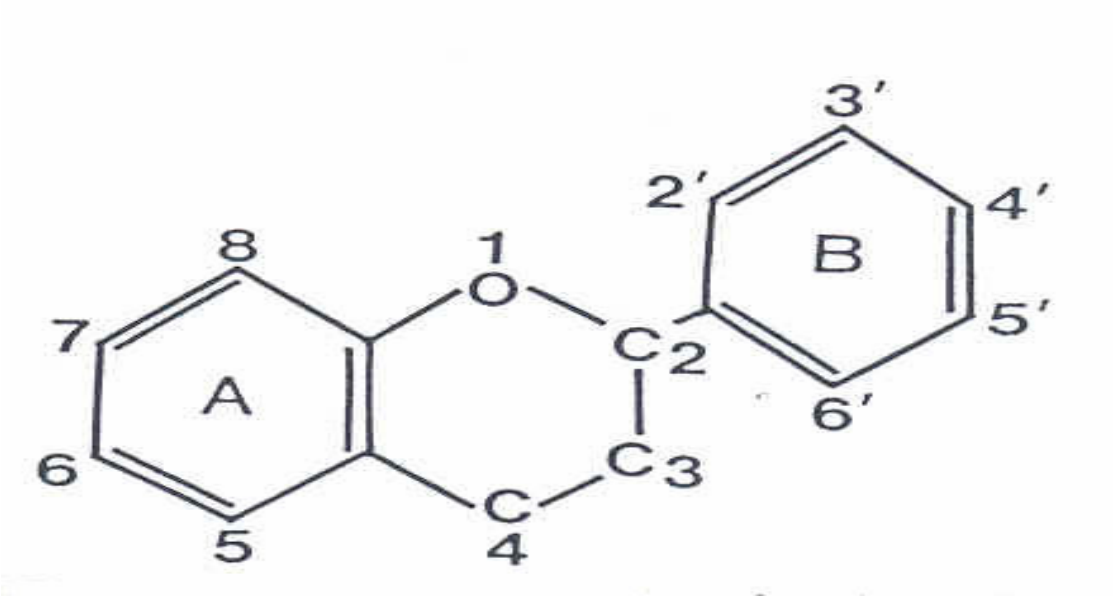


Figure 2. Basic flavonoid ring structure (Hahn *et al*., 1984).

The 3-deoxyanthocyanidins are the most common anthocyanins in sorghum, which include apigeninidin and luteolinidin, (Figure 3) (Sweeny and Iacobucci and Gous according to Awika and Rooney, 2004). However, these anthocyanins have a small distribution in nature and differ from the more widely distributed anthocyanidins in that they do not have a hydroxyl group at the C-3 position, (Figure 3) (Clifford as cited by Awika and Rooney, 2004).

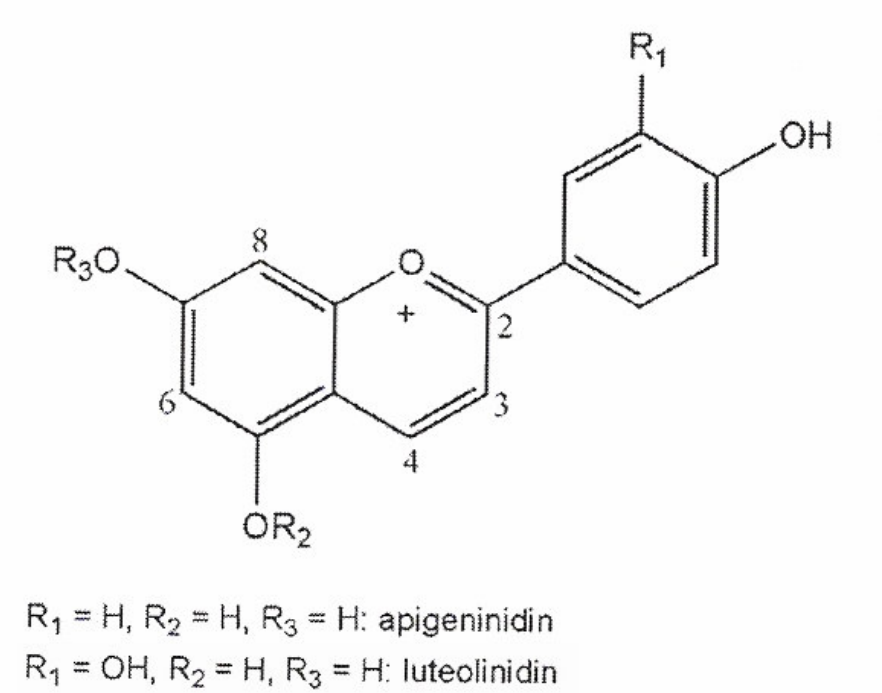


Figure 3. The 3-deoxyanthocyanidins in sorghum (adapted from Awika and Rooney, 2004).

Awika and Rooney, (2004) have indicated that effective quantification of anthocyanins is hampered by lack of appropriate standards, efficient extracting solvents and separation techniques. They have however in their previous work (Awika 2003) showed that acidified methanol extraction resulted in higher anthocyanins values than aqueous acetone extraction for sorghum anthocyanins. The anthocyanin levels in sorghum are given in (Table 2).

Table 2. Anthocyanin content of sorghum brans (adapted from Awika and Rooney, 2004)

Commodity	Content [a]	Major anthocyanidins	Source
Black sorghum bran	4.0-9.8	Apigeninidin and luteolinidin	b
Brown sorghum bran	1.6-3.9	Apigeninidin and luteolinidin	b
Red sorghum bran	3.3	Apigeninidin	b

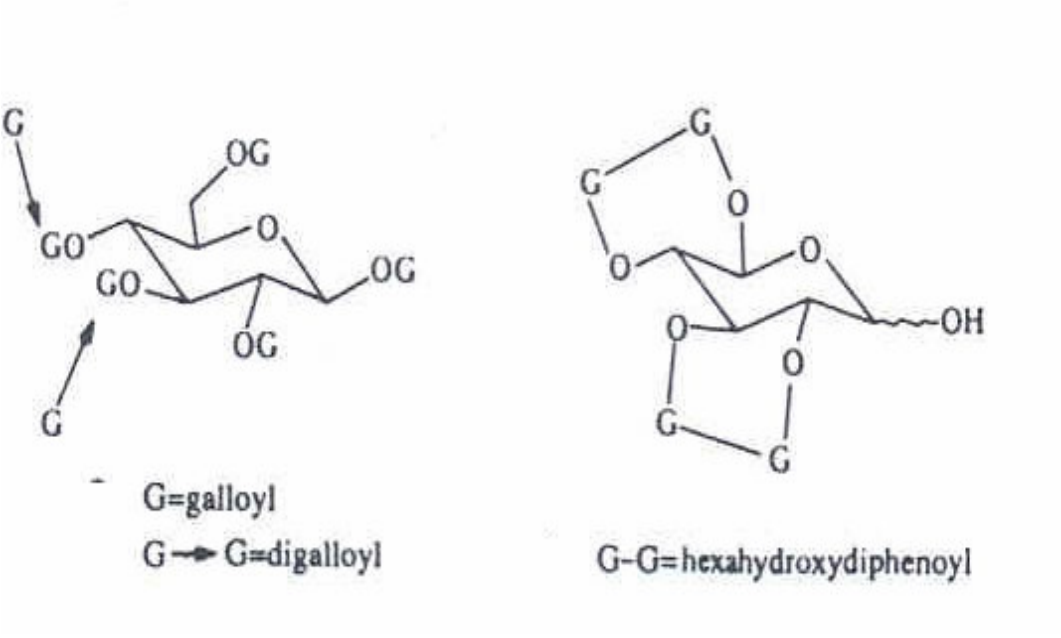
(b) Awika (2003)

[a]mg/g, fresh weight

1.2.3.3 Tannins

The tannins are high-molecular-weight phenolic compounds that have the ability to bind with proteins (Maxson and Rooney, 1972) and are commonly found in a large array of higher plant species (Scalbert, 1991). They are the most abundant phenolic compounds that can be extracted from seed of brown sorghums (Hahn *et al*., 1984).

The tannins are generally divided into hydrolysable tannins (galloyl and hexahydroxydiphenoyl esters and their derivatives) (Figure 4) and condensed tannins or proanthocyanidins (polymers of flavan-3-ols) (Figure 5) (Haslam, 1989; Awika and Rooney, 2004). Hydrolysable tannins are esters of phenolic acids and a polyol which is usually glucose (Scalbert, 1991). The phenolic acids are either gallic acid in gallotannins, for example Figure 4 (I) or other phenolic acids deriving from the oxidation of gallic acid in ellagitannins, for example Figure 4 (II) (Scalbert, 1991). Tannins of sorghum are almost exclusively of the "condensed" type (Awika and Rooney, 2004) and are present in sorghums with a pigmented testa (classified as type II and III sorghums) (Awika and Rooney, 2004). Apart from sorghums, the proanthocyanidins are also found in other food products such as tea and cocoa (Scalbert, 1991).



(I) (II)

Figure 4. Chemical structures of hydrolysable tannins, galloyl and hexahydroxydiphenoyl (Scalbert, 1991).

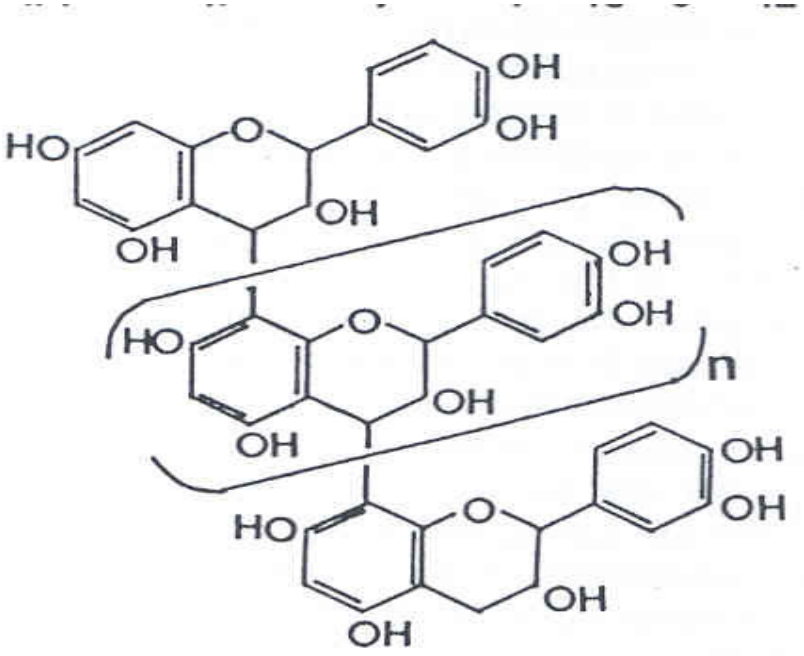


Figure 5. Structure of proanthocyanidin (tannin) polymer; (n=5-7) (Hahn *et al*., 1984).

An accurate characterisation of the sorghum tannins is essential to effectively predicts their potential effects on health and nutrition (Awika and Rooney, 2004). However, the complex structure of tannins often makes it difficult to isolate and characterise tannins effectively (Awika and Rooney, 2004). According to Awika and Rooney, (2004) lack of appropriate standards in the quantification of tannins, the choice of organic solvents and extraction procedures significantly affect results. Nevertheless, levels of 0.5-3.8 mg catechin equivalents/g for the tannin-free sorghum up to 68 mg catechin equivalents/g for the tannin sorghum as determined by the vanillin-HCl method on a dry basis of the sorghum grains have been reported (Jambubathan and Mertz; Hahn and Rooney; Agullo and Rodriguez; Awika as cited by Awika and Rooney, (2004).

1.2.4 Sorghum morphology with particular reference to phenolic compounds

The seed of sorghum is small and rounded (Kent, 1975) and consists of three distinguishing anatomical components: pericarp (outer layer), germ and endosperm (storage tissue), surrounded by a seed coat or testa (Serna-Saldivar and Rooney, 1995) and a relatively large scutellum, (Figure 6) adapted from Waniska, 2000.

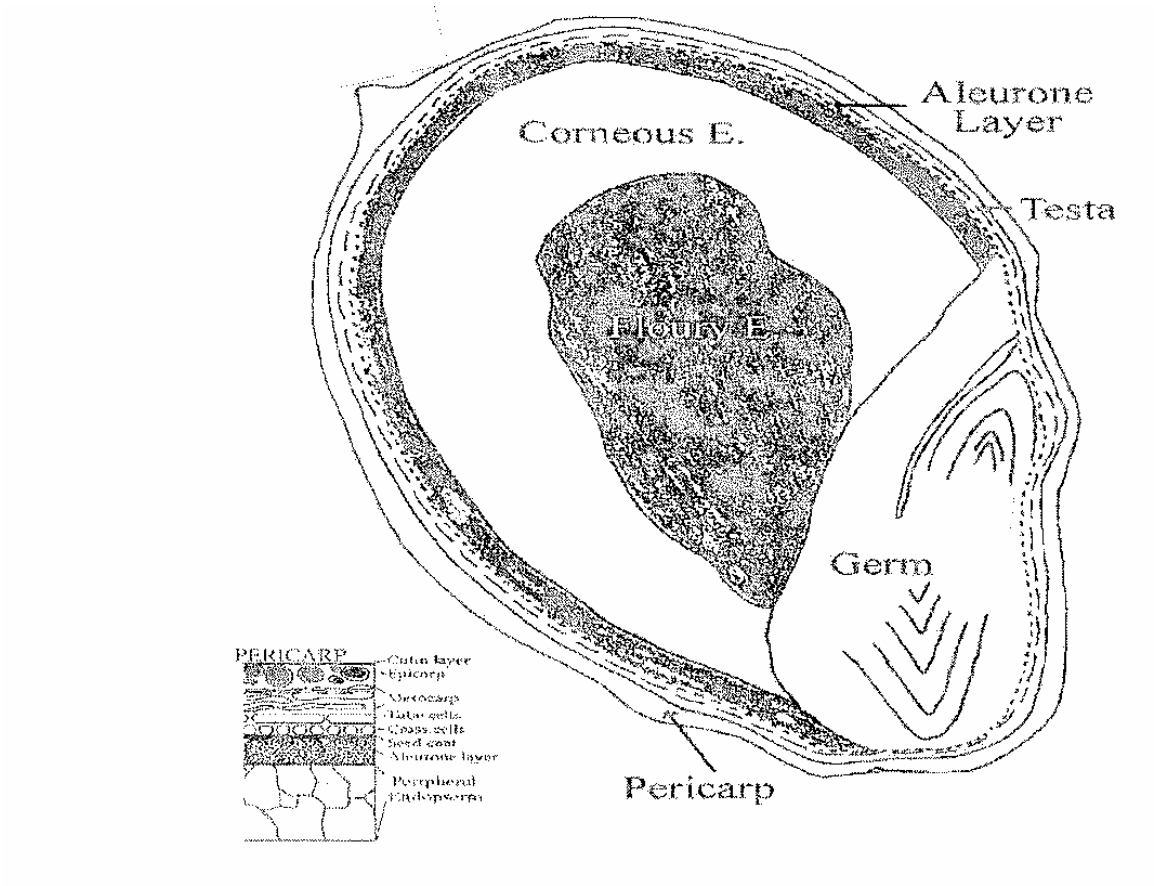


Figure 6. Diagram of sorghum caryopsis showing the pericarp [epicarp, mesocarp, testa] where phenolic compounds are found, endosperm (E) (aleurone layer, corneous, and floury) and the germ (adapted from Waniska, 2000).

1.2.4.1 Pericarp

The outermost layer of the sorghum caryopsis or pericarp consists of three layers: the epicarp, the mesocarp and the endocarp (Hoseney, 1994). The epicarp is the outermost layer and is generally covered with a thin layer of wax and often contains pigmented materials responsible for the colour of the pericarp (Serna-Saldivar and Rooney, 1995; Waniska, 2000).

The pericarp among other sorghum kernel anatomical parts forms part of the main components of the sorghum kernel with a prominent pigmented testa layer present underneath the pericarp in certain sorghums. The presence of a pigmented testa in

sorghum grain is associated with the presence of phenolic compounds, especially condensed tannins (Hahn *et al*., 1984; Waniska, Hugo and Rooney, 1992; Awika and Rooney, 2004). Ferulic acid is the major bound phenolic acid of sorghum thought to be associated with the cell walls of the grain (Hahn *et al*., 1984). According to Awika and Rooney, (2004) most of the phenolic acids in sorghum are esterified to the cell wall components and can only be extracted in meaningful quantities by alkaline hydrolysis and have been shown to account for over 85 % of total phenolic acids in sorghum (Hahn (1984) as cited by Awika and Rooney, 2004).

1.2.4.2 Endosperm

The endosperm is the largest portion of the sorghum grain (Hoseney, 1994). It consists of the aleurone layer as its outer layer, peripheral, corneous, and floury endosperm (Serna-Saldivar and Rooney, 1995). Composing the peripheral endosperm tissues are several layers of dense cells containing large quantities of proteins with small starch granules (Serna-Saldivar and Rooney, 1995). However, the corneous and floury endosperm cells are composed of starch granules, protein matrix, protein bodies and cell walls rich in β-glucans and hemicellulose (Serna-Saldivar and Rooney, 1995) with the starch granules and protein bodies embedded in the continuous protein matrix in the peripheral and corneous areas of the endosperm (Hoseney, Davis and Herbers as cited by Waniska, 2000). Besides phenolic acids (free acids, soluble and insoluble esters) that are concentrated in the outer layers of the kernel, there are insoluble tightly bound phenols, which are found in the endosperm cell walls of the sorghum kernel (Hahn *et al*., 1984).

1.2.4.3 Germ

The germ consists of the embryonic axis and the scutellum (Serna-Saldivar and Rooney, 1995; Waniska, 2000). The embryonic axis contains the new plant and is divided into a radicle and plumule whilst the scutellum is the single cotyledon of the sorghum caryopsis (Serna-Saldivar and Rooney, 1995; Waniska, 2000). The latter is the germ reserve tissue and contains large quantity of reserve nutrients, i.e. oil, protein, enzymes and minerals

and serves either as a bridge or connection between the endosperm and the germ (Serna-Saldivar and Rooney, 1995; Waniska, 2000). This component of the sorghum kernel does not contain any phenolic compounds.

1.2.5 Modes of classification of sorghums based on phenolic content

1.2.5.1 Classification based on extractable tannin content

Sorghums differ in their phenolic composition and content, with both genetics and environment affecting the kind and level of phenolic compounds (Awika and Rooney, 2004). Based on extractable tannin content, Awika and Rooney, (2004) classified sorghums as type I (no significant levels of tannins extracted by 1 % acidified methanol), type II (tannins extractable in 1 % acidified methanol, but not methanol 100 %) and type III (tannins extractable in both acidified methanol and methanol 100 %).

1.2.5.2 Classification based on presence or absence of condensed tannins

Sorghum may also be divided into condensed tannin- and condensed tannin-free depending on whether the sorghum grains contain condensed tannins or not (Waniska *et al*., 1992).

1.2.5.2.1 Condensed tannin sorghum

The chlorox bleach test is used to detect the presence of a pigmented testa layer in sorghum kernels. It is based on the assumption that if the kernels contain a pigmented testa layer then condensed tannins are present (Waniska *et al*., 1992). Kernels that have tannins exhibit the pigmented testa, which is brown to purple in colour, but becomes black after the chlorox bleach test (Waniska *et al*., 1992). This type of sorghum may be classified under type II sorghum variety where the tannins are present in pigmented testa or type III sorghum where the tannins are present in pigmented testa and pericarp (Waniska *et al*., 1992).

1.2.5.2.2 Condensed tannin-free sorghum

The condensed tannin-free sorghum is that which will test negative to the chlorox bleach test (Waniska *et al*., 1992). In this case, the kernels become light yellow or white (do not turn black) during the chlorox test due to lack of a pigmented testa, and hence no tannins (Waniska *et al*., 1992). Figure 7 shows a fluorescence photomicrograph of sorghum bran cross section, showing structural differences between non-tannin sorghum without a pigmented testa and tannin sorghum with a pigmented testa. Figure 8 summarises the relationship between the modes of classification of sorghums based on phenolic content.

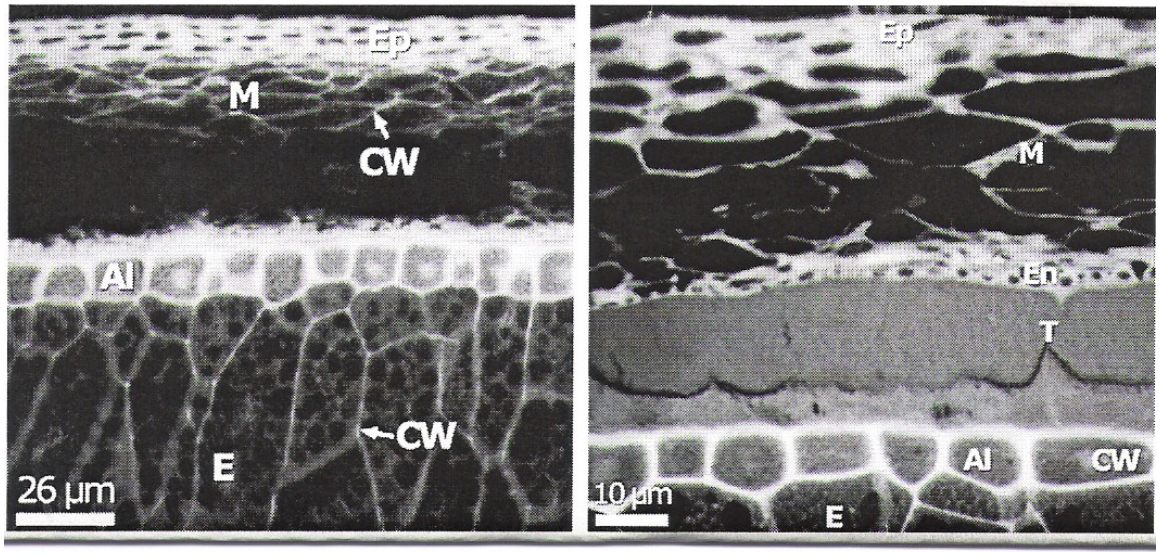


Figure 7. Fluorescence photomicrograph of sorghum bran cross-section, showing structural differences between a non-tannin sorghum without a testa (left) and a tannin sorghum with a pigmented testa (right). Al, aleurone layer; CW, cell wall; E, endosperm; En, endocarp; Ep, epicarp; M, mesocarp; T, pigmented testa (Awika and Rooney, 2004).

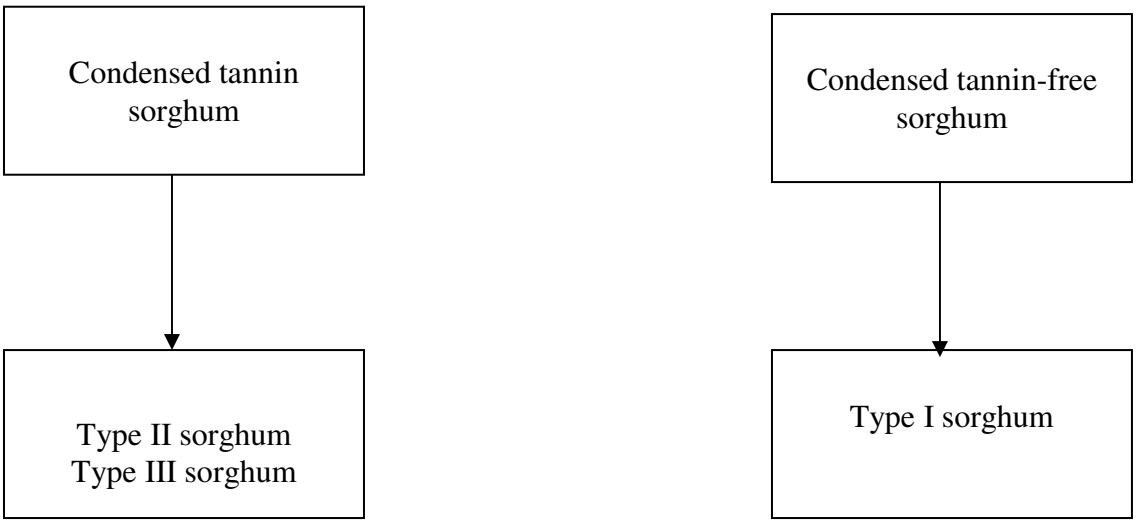


Figure 8. Relationship between the modes of classification of sorghums based on phenolic content.

1.2.6 Significance of phenolic compounds in sorghum

Phenolic acids may inhibit the growth of microorganisms and may also impart resistance to grain molds before and after grain maturity (Hahn *et al*., 1984). Awika and Rooney, (2004) also mentioned that phenolic acids have shown good antioxidant activity in vitro and are thought to contribute significantly to the health benefits associated with whole grain consumption. Brown cultivars of sorghum were shown to contain highest amounts of free phenolic acids and the compounds were more resistant to grain weathering (Waniska, 2000), therefore it is assumed that phenolic acids also offer a protective function to the grain against grain weathering. Pigmentation in the pericarp and testa is primarily due to the phenolic compounds, with the pericarp colour in sorghum due to the combination of anthocyanin and anthocyanidin pigments as well as other flavonoid compounds (Waniska, 2000). Tannins in sorghum grain were also shown to bind and precipitate proteins causing a reduction in nutritional value (Hahn *et al*., 1984). Grimmer, Parbhoo and McGrath (1992) reported the antimutagenicity of polyphenol-rich fractions from *Sorghum bicolor* grain, with the high molecular weight tannins showing highest anti-mutagenic activity than lower molecular weight tannins.

1.2.7 Natural antimicrobials in foods

An antimicrobial agent is a chemical or biological agent that either destroys or inhibits the growth of pathogenic and spoilage microorganisms (Adams and Moss, 1995). Natural antimicrobials may exhibit antimicrobial activity as additives in foods (Naidu, Bidlack and Crecelius, 2000) and include those that are present or derived from plant or animal tissues and those produced by microorganisms (http://www.cast-science.org/cast-science.lh/anti_sum.htm).

1.2.8 Determination of antimicrobial activity

There have been nearly as many methods used for determining antimicrobial efficacy, as there are compounds (Parish and Davidson, 1993). This makes it difficult to compare results from different laboratories and also even more complex to determine the potential success of an antimicrobial in food because of methods that are inappropriate or lack significance (Parish and Davidson, 1993). Despite the large number of methods available, the agar diffusion method has almost certainly been the most widely used method for determining the antimicrobial activity or for screening antimicrobial substances (Parish and Davidson, 1993; Tsai and Kondo, 2001) and has often been referred to as the disk assay (Davidson and Parish, 1989).

The agar diffusion method employs the application of an antimicrobial compound to an agar plate on a paper disk or in a well, followed by a diffusion of the compound through the agar, giving rise to a concentration gradient that is inversely proportional to the distance from the disk or well (Parish and Davidson, 1993). The activity of the antimicrobial compound against the indicator microorganism that has been inoculated into the agar will be indicated by a zone of no growth or inhibition zone (Davidson and Parish, 1989) with the size of the zone being dependent on the rate of diffusion and cell growth (Barry as cited by Davidson and Parish, 1989).

1.2.9 Antimicrobial activities and inhibitory effects of various phenolic compounds from different plant sources

The antimicrobial properties and inhibitory effects of different phenolic compounds from various plant sources on different microbial species have been studied (Scalbert, 1991; Jayaprakasha *et al*., 2003; Rauha *et al*., 2000; Sakanaka *et al*., 2000; Puupponen-Pimiä, Nohynek, Meier, Kähkönen, Heinonen, Hopia and Oksman-Caldentey, 2001; Baydar *et al*., 2004).

Baydar *et al*. (2004) reported the antimicrobial activity of phenolic compounds from grape seed extracts at a concentration of 20 % against *B. cereus* and *E. coli* bacteria. The inhibitory effect of tannic acid, ellagic acid and gallic acids on *L. monocytogenes* bacteria grown in cabbage juice at a concentration of 50 µg ml^{-1} and 250 µg ml^{-1} has been reported with total growth inhibition observed (Chung *et al*., 1993). In their study Baydar *et al*. (2004) also reported the antibacterial activities of grape seed extract at a concentration of 20 % on *L. monocytogenes* bacteria. There is however lack of information on the antimicrobial properties of phenolic compounds from sorghum on the growth of these microorganisms.

Sakanaka *et al*. (2000) found that green tea polyphenols could inhibit the development and growth of bacterial spores and the spoilage of canned drinks. The sample tested in this study was a mixture of green tea polyphenols, commercially available as "Sunphenon" prepared by extracting Japanese tea (*Camellia sinensis* var. *sinensis*) with hot water and then by partitioning with ethyl acetate (Sakanaka *et al*., 2000). The mixture was mainly composed of (+) –catechin (C, 3.5 %), (-)-epicatechin (EC, 7.0 %), (+)-gallocatechin (GC, 14.8 %), (-) epigallocatechin gallate (EGCg, 18.0 %), (-)-epicatechin gallate (ECg, 4.6 %), and (-)-gallocatechin gallate (GCg, 11.6 %) (Sakanaka *et al.,* 2000). Gallocatechin (GC) 18.8 %, epigallocatechin (EGCg) 18 %, gallocatechin gallate (GCg) 11.6 % were shown to inhibit the growth of *Bacillus* sp, while the growth of *Clostridium* sp was strongly inhibited by epicatechin galate (ECg) 4.6 %, gallocatechin galate (GCg) 11.6 % and epigallocatechin gallate (EGCg) 18 % (Sakanaka *et al.* 2000).

Epigallocatechin gallate, which is the main component of tea polyphenols, was shown to exhibit strong activity against both *B. stearothermophillus* and *C. thermoaceticum* (Sakanaka *et al*., 2000).

Baydar *et al*. (2004) also reported the inhibitory effects of phenolic compounds in grape seed extracts at 4 % and 20 % concentrations on *Aeromonas hydrophila*, *Bacillus brevis*, *Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Enterobacter aerogenes*, *Enterococcus faecalis*, *Escherichia coli*, *Klebsiella pneumoniae*, *Listeria monocytogenes*, *Mycobacterium smegmatis*, *Proteus vulgaris*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* bacteria. The grape seed extracts were found to contain 667.87 and 627.98 mg gallic acid equivalent/g total phenolics when extracted with a mixture of acetone: water: acetic acid (90:9.5:0.5) and ethyl acetate: methanol: water (60:30:10) respectively (Baydar *et al*., 2004). In a study done by Chung, Lu and Chou (1998b) tannic acid (a hydrolysable tannin) was found to be inhibitory to the growth of intestinal bacteria, *Bacteroides fragilis* ATCC 25285, *Clostridium crostridiiforme* ATCC 25537, *Clostridium perfringens* ATCC 13124, *Clostridium paraputrificum* ATCC 25870, *Escherichia coli* ATCC 25922, *Enterobacter cloacae* ATCC 13047 and *Salmonella* Typhimurium TA98 by acting like siderosphore (low molecular weight chelating agents that have the affinity for iron (III), bind and solubilise iron) to chelate iron from the medium and make iron unavailable for utilisation by the microorganisms.

Such compounds as methyl gallate and propyl gallate, which are ester forms of gallic acid, were also found to be inhibitory to the growth of the intestinal bacteria mentioned above (Mila, Scalbert and Expert, 1996). Nohynek, Alakomi, Puupponen-Pimiä, Saarela and Oksman-Caldentey, (s.a) also reported the antimicrobial effects of phenolic berry extracts on Gram-negative bacteria, *Salmonella enterica* sv. TyphimuriumVTT E-981151 and *S. enterica* sv. Infantis VTT E-97738. There is however lack of information on the antimicrobial activity of phenolic compounds from sorghum.

1.2.10 Mechanisms of antimicrobial activity of phenolic compounds

Various mechanisms have been proposed to explain the antimicrobial activity of phenolic compounds (Scalbert, 1991). These include inhibition of extracellular microbial enzymes and substrate deprivation, alterations in cell wall permeability, an increase in the hydrogen ion activity of the microbial environment and perhaps most importantly, chelation of essential minerals, particulary iron with a concomitant destruction of the microbial oxidative metabolic system (Marouchoc, Singleton and Chung, Wei and Johnson as cited by O'Connell and Fox, 2001).

1.2.10.1 Astringency: Inhibition of enzymes and substrate deprivation

The antimicrobial properties of the tannins could be due to their complexation with enzymes (Scalbert, 1991). The astringent character of tannins has been indicated to can induce complexation with enzymes. Many microbial enzymes were found to be inhibited when raw culture filtrates or purified enzymes were mixed with tannins, for instance, cellulases (Lyr, Benoit and Starkey and Mole and Waterman as cited by Scalbert, 1991) and an antimicrobial effect thus effected.

1.2.10.2 Chelation of essential minerals

Microorganisms growing under aerobic conditions have been reported to require iron for performing a variety of functions including reduction of ribonucleotide precursor of DNA and formation of heme (Chung *et al*., 1998b). However, iron uptake is made difficult by the great insolubility of ferric iron and its derivatives, which leaves little free iron available for transport and for utilisation by microorganisms as it readily forms insoluble hydroxides (Cloete, 1999).

Plants, animals or microorganisms have developed elaborate mechanisms to compete for this resource (Cloete, 1999). Many microorganisms produce siderophores, which are low molecular weight molecules that are able to complex ferric iron (Fe^{3+}) and supply it to

the cell (Cloete, 1999). These iron-transport molecules are normally either hydroxamates or phenolate–catecholates. For instance, ferrichrome is the catecholate produced by many fungi; and enterobactin is the catecholate formed by *E. coli* (Cloete, 1999).

Microorganisms produce siderophores when little iron is available in the medium (Cloete, 1999). Once the iron-siderophore complex has reached the cell surface, it binds to a siderophore-receptor protein, then the iron is either released to enter the cell directly or the whole iron-siderophore complex is transported inside (Cloete, 1999). For *E. coli*, the siderophore receptor is in the outer membrane of the cell envelope (Cloete, 1999). When the iron reaches the periplasmic space, it moves through the plasma membrane with the aid of other proteins not specified (Cloete, 1999). After the iron has entered the cell, it is reduced to ferrous form (Fe^{2+}) (Cloete, 1999).

The tannins have also been reported to act like the siderophores to chelate iron from the medium and therefore make the iron unavailable for the growth of microorganisms (Chung, Wei and Johnson, 1998c) and hence prevent the microorganisms from growing. Most tannins contain more than two *o*-diphenol groups in their molecules and are therefore capable of chelating various metal ions such as ferric or cupric ion (Chung *et al*., 1998c). Tannins will therefore decrease the availability of essential metal ions for microorganisms just as tannins reduce the absorption of nutrients in rats given tannin-rich beverages like tea or cocoa (Chung *et al*., 1998c). Mila *et al*. (1996) also reported that a polyphenol molecule with its several *o*-dihydroxyphenyl chelating groups can bind several iron (III) ions with each iron (III) forming a lattice as shown in Figures 9 and 10 (Scalbert, 1991; Mila *et al*., 1996). This eventually results in the co-precipitation of iron and polyphenols and in the removal of the iron (III) from the solution with the lattice forming (Scalbert, 1991; Mila *et al*., 1996). In brief, phenolic compounds or polyphenols, using the tannins as an example, have been reported to be superior chelators of iron with which they form precipitates (Mila *et al*., 1996) and thus exert an antimicrobial effect through iron deprivation.

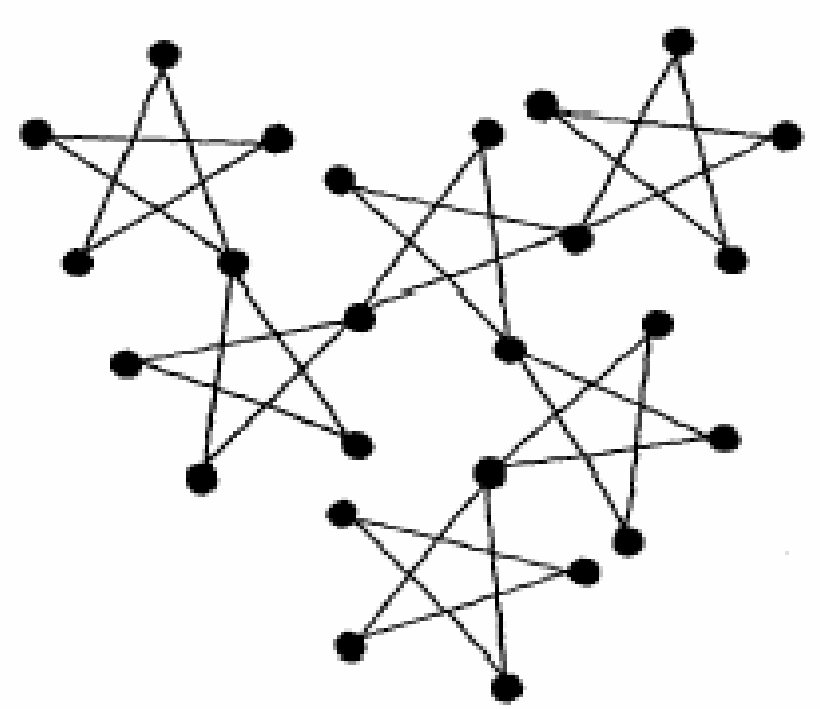


Figure 9. Polyphenol/iron (III) lattice formed upon complexation of iron (III) (dark spot) by polyphenol (star) containing five *o*-dihydroxyphenyl functional groups (triangles) (Mila *et al*., 1996).

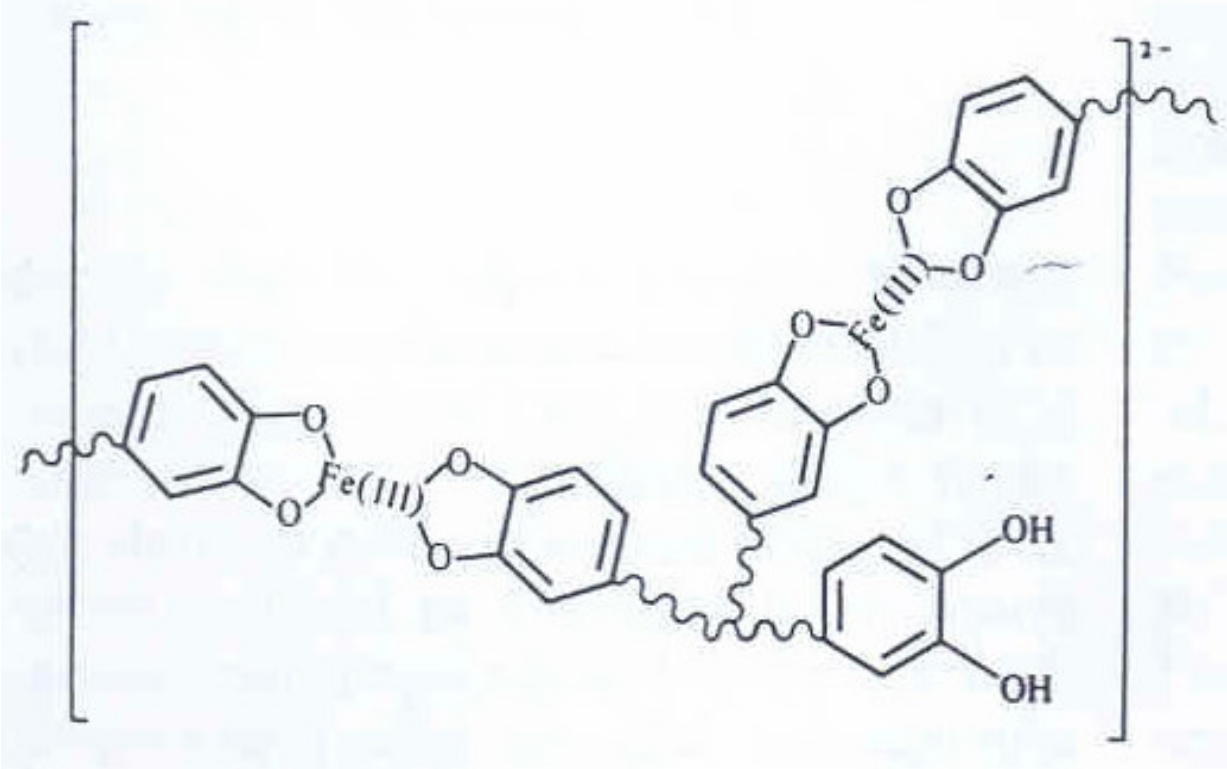


Figure 10. Insoluble tannin-ferric ion complexes (Scalbert, 1991).

1.2.11 Foodborne pathogens

Even though many foodborne infections have been controlled, the burden of foodborne disease remains substantial (Tauxe, 2002). A wide variety of microbial pathogens continue to contaminate human foods thereby causing illnesses after the consumption of such foods or consumption of the toxins (Tauxe, 2002), which the particular microbial pathogen involved, is capable of producing. Foodborne pathogens include among others enteric bacteria, aerobes and anaerobes as well as viral pathogens (Tauxe, 2002), some of which are transmitted through foods while others are capable of being spread through a number of ways on addition to foods (Tauxe, 2002). Foodborne pathogens occur widely in nature, which makes it difficult to prevent these pathogens from entering raw foods (Chung, Stevens, Lin and Wei, 1993). However, one way to prevent the growth of pathogens in foods is to use antimicrobial agents (Chung *et al*., 1993). Examples of

foodborne pathogens include among others *B. cereus*, *E. coli* and *L. monocytogenes,* which are microorganisms of concern in this project.

1.2.11.1 *Bacillus cereus* and its characteristics

B. cereus is a Gram-positive, aerobic to facultative and spore-forming, rod-shaped bacterium commonly found in soil, air, dust, water and vegetation and therefore, in many raw and processed food materials (Adams and Moss, 1995; Andersson, Rönner and Granum, 1995; Dufrenne, Bijwaard, Te Giffel, Beumer and Notermans, 1995; Granum and Lund, 1997; Jay, 2000).

For more than 40 years, the bacterium has been recognised as a causative agent of food poisoning and has frequently been linked with foodborne illness as a result of the production of toxins, with dairy, meat and vegetable products being the most common vectors (Ghelardi, Celandroni, Salvetti, Barsotti, Baggiani and Senesi, 2002). Its ubiquitous occurrence in the natural environment makes it possible for this organism to produce endospores (Ghelardi *et al*., 2002) and therefore difficult to eliminate it from foods.

1.2.11.1.1 Growth requirements of *B. cereus* with reference to temperature, pH and water activity

B. cereus is widespread in nature and is easily spread to many types of foods especially those of plant origin (Valero, Fernández and Salmerón, 2003). The organism grows over a temperature range of 8 to 50 °C, optimally around 28 to 35 °C (Adams and Moss, 1995) and is capable of growing well after cooking and cooling of foods (<48 °C) (Granum, 1997; Granum and Lund, 1997). The ability of some psychrotrophic strains of *B. cereus* to grow at temperatures as low as 4 to 6 °C has been shown to be a problem in chilled products such as milk and other dairy products and in “long-life” ready-to-eat or fresh chilled foods (Røssland, Borge, Langsrud and Sørhaug, 2003). The organism does not

have marked tolerance for low pH with a minimum of 5.0 to 6.0 depending on the acidulant, or water activity, minimum of approximately 0.95 (Adams and Moss, 1995).

1.2.11.1.2 Pathogenicity and foodborne illnesses associated with *B. cereus*

B. cereus pathogenicity is linked to the production of toxins that are responsible for causing two distinct types of food-borne illness, classified as: a diarrhoeal syndrome seen occasionally following consumption of milk and other dairy products and emetic syndrome (vomit-inducing) (Kramer and Gilbert, 1989; Granum, 1997; Kotiranta, Lounatmaa and Haapasalo, 2000). For both types of food poisoning the food involved has frequently been heated, and food poisoning manifests as a result of spore germination during heat treatment (Granum and Lund, 1997). This in turn leads to a substantial growth of *B. cereus* in the absence of competing flora (Granum and Lund, 1997). Desserts, meat dishes and dairy products are the foods most frequently associated with diarrheal illness, whereas rice is the most common vehicle of emetic illness (Granum, 1997).

1.2.11.1.2.1 Diarrhoeal syndrome

This syndrome is relatively mild and occurs as a result of production of emetic toxins by growing cells in the food with a minimal infective dose ranging generally between 10^5 and 10^7 cells (total dose) (Granum and Lund, 1997). It is characterised by distinctive symptoms, which include among others, infrequent nausea with vomiting being rare, cramplike abdominal pains, as well as watery diarrhoea (Kramer and Gilbert, 1989; Jay, 1992). The symptoms can only develop within an incubation period of 8-16 h after ingestion of contaminated food (Kramer and Gilbert, 1989; Agata, Ohta and Yokoyama, 2002), and can only last for 12-24 h (Kramer and Gilbert, 1989). Transmission of this type of food poisoning has been associated with foods such as vegetables, meat and milk products (Granum and Lund, 1997; Kotiranta *et al*., 2000).

1.2.11.1.2.2 Emetic syndrome

Emetic syndrome is more severe compared to diarrhoeal syndrome and results from consumption of an emetic heat-stable toxin (Jay, 1992; Jay, 2000). The syndrome transpires as a result of production of complex enterotoxins during vegetative growth of *B. cereus* in the small intestines (Granum and Lund, 1997). It is characterised by nausea and vomiting after 1-5 h of ingestion of the toxin followed by infrequent diarrhea of 6-24 h duration (Kramer and Gilbert, 1989). The symptoms can only last for less than 24 h (Kramer and Gilbert, 1989). The total infective dose appears to vary between 10^5 and 10^8 viable cells or spores per gram (Granum and Lund, 1997). Consequently, any food having more than 10^3 *B. cereus* per gram cannot be regarded as entirely safe for consumption (Granum and Lund, 1997). This kind of food poisoning has been shown to manifest as a result of insufficient heating of rice-containing dishes (Andersson *et al*., 1995) and has been largely associated with the consumption of foods such as rice and pasta (Granum and Lund, 1997; Kotiranta *et al*., 2000).

1.2.11.1.3 Prevalence in foods and epidemiological issues

Foods most frequently contaminated with *B. cereus* include among others milk products, in addition to rice and spices (Granum, 1997). According to Røssland *et al*. (2003), Wong, Chen and Chen reported the occurrence of *B. cereus* in ice cream (52 %), milk powders (29 %), fermented milks (17 %) and pasteurised milks and fruit flavoured milks (2 %) in 1988.

A number of foodborne diseases in industrial countries are as a result of *B. cereus* even though the exact number of food poisoning cases is not known since it is not a reportable illness and not constantly diagnosed (Kotiranta *et al*., 2000). Kotiranta *et al*. (2000) associated the pathogenicity of *B. cereus* in gastrointestinal and nongastrointestinal infections with the ability of this microorganism to produce toxins. The percentages of foodborne illnesses caused by *B. cereus* differed from country to country with the United States accounting for 1.3 % of the bacterial food poisoning cases between the years 1972-

1982 (Kotiranta *et al*., 2000). Kotiranta *et al*. (2000) and Kramer and Gilbert, (1989) also reported that *B. cereus* has caused 17.8 % of the total bacterial food poisonings in Finland, 11.5 % in the Netherlands, 0.8 % in Scotland, 0.7 % in Japan and 15.0 % between 1960-1968 in Hungary. The problems caused by *B. cereus* arise as a result of the fact that *B. cereus* is an ubiquitous microorganism in the environment and can easily contaminate any of the production or processing systems (Kotiranta *et al*., 2000).

1.2.11.2 *Escherichia coli* and its characteristics

E. coli is a Gram-negative and non-sporing rod (Adams and Moss, 1995), which accounts for the majority of the normal flora of the human gut (Kuntz and Kuntz, 1999). The organism was first described by Dr Theodor Escherich in 1885 as a bacterium that is part of the normal flora of the intestinal tract of humans and other warm blooded animals (Padhye and Doyle, 1992). Generally *E. coli* strains are harmless commensals; nevertheless, some strains are pathogenic and are capable of causing diarrhoeal diseases (Doyle, Zhao, Meng and Zhao, 1997).

1.2.11.2.1 Growth requirements of *E. coli* with reference to temperature, pH and water activity

E. coli is a typical mesophile growing from 7-10 °C up to 50 °C with an optimum around 37 °C, although there have been reports of some enterotoxigenic *Escherichia coli* (ETEC) strains growing at temperatures as low as 4 °C (Adams and Moss, 1995). The organism shows no marked heat resistance and has a near-neutral optimal pH for growth, but growth is possible down to pH 4.4 (Adams and Moss, 1995). The minimum water activity (a_w) for growth is 0.95 (Adams and Moss, 1995).

1.2.11.2.2 Pathogenicity and foodborne illnesses associated with *E. coli*

E. coli O157:H7 is a major foodborne pathogen, which is capable of causing well-known illnesses such as hemorrhagic colitis, hemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic pupura (TTP) (Doyle and Padhye, 1989; Doyle 1991).

Hemorrhagic colitis is characterised by a sudden onset of severe crampy abdominal pain followed within 24 h by watery diarrhoea, which later becomes grossly bloody with little or no fever (Doyle, 1991). On the other hand, HUS, which is a leading cause of acute renal failure in children, is characterised by production of bloody diarrhoea and patients with HUS often develop central nervous system diseases characterised by coma (Doyle, 1991). TTP is also a severe disorder with clinical and pathologic features similar to HUS but central nervous system involvement is usually a major feature in TTP (Doyle and Padhye, 1989; Padhye and Doyle, 1992). An enterohemmoragic strain of *E. coli* has also been shown to cause serious cases of food poisoning (Nørrung, 2000).

1.2.11.2.3 Prevalence in foods and epidemiological issues

The incidence and prevalence of enterohemorrhagic *E. coli* strains in meat, milk, poultry and seafood products are highly variable but more foodborne outbreaks are linked to beef than to any other single food source (Jay, 2000).

The occurrence of *E. coli* O157:H7 was found in 3.7 % of 164 beef, 1.5 % of 264 pork, 1.5 % of 263 poultry, and 2.0 % of 205 lamb samples (Doyle and Schoeni according to Jay, 2000). Doyle and Schoeni also conducted a survey on retail meats from grocery stores in Madison in the United States for *E. coli* O157:H7 after the suspicion of hamburger as the vehicle in most outbreaks of hemorrhagic colitis, according to Padhye and Doyle, (1992). The organism was isolated from roughly 2 % of beef, poultry, pork and lamb samples whilst meats from stores in Calgary, Canada, yielded considerably higher levels of contamination (Padhye and Doyle, 1992).

1.2.11.3 *Listeria monocytogenes* and its characteristics

L. monocytogenes is a Gram-positive, facultatively anaerobic, non-sporulating and rod-shaped bacterium (Pelczar, Reid and Chan, 1977; Adams and Moss 1995). *L. monocytogenes* differs from most known foodborne pathogens because of its ubiquitous nature and resistance to diverse environmental conditions including low pH, high salt concentrations (Rocourt, BenEmbarek, Toyofuku and Schlundt, 2003) and ability to grow in the cold (Kathariou, 2002). It is because of this characteristic, that this pathogen is of major and particular concern for the safety of refrigerated and ready-to-eat foods consumed without reheating, cooking or both (Kathariou, 2002).

L. monocytogenes has been recognised as the cause of human illness for more than 60 years (Rocourt and Bille as cited by Meng and Doyle, 1998) with consumption of contaminated foods implicated as a primary vehicle for transmission of listeriosis (Rocourt and Cossart, 1997). Since 1981, food has been identified as the vehicle of a number of important (>30 cases) outbreaks of listeriosis investigated (Rocourt and Cossart, 1997). Among the world's population the elderly, pregnant women and human immunodeficiency virus-infected individuals are the most vulnerable to the opportunistic infection by *L. monocytogenes* (Kathariou, 2002).

1.2.11.3.1 Growth requirements of *L. monocytogenes* with reference to temperature, pH and water activity

L. monocytogenes can grow over a wide temperature range from 0-42 °C with an optimum between 30 and 35 °C (Adams and Moss, 1995). *L. monocytogenes* can grow in the presence or absence of air and in foodstuffs within a pH range of 4.5 to 9.2, at water activities above 0.92 (Nørrung, 2000). The bacterium grows well at refrigeration temperature and in minimal nutrients, and is able to survive and multiply on plants, and in soil and water (Meng and Doyle, 1998). It is as a result of this wide distribution and survival under refrigeration temperatures and acidic conditions that *L. monocytogenes* has

the ability to contaminate food along the different stages of production, processing and manufacture, and distribution (Meng and Doyle, 1998).

1.2.11.3.2 Pathogenicity and foodborne illnesses associated with *L. monocytogenes*

L. monocytogenes is a bacterial pathogen, which can cause serious illness such as septicaemia and meningitis (Pelczar *et al*., 1977; Nørrung, 2000). Although listeriosis occurs infrequently, at an incidence rate below 10 cases per million, its fatality rate is high, up to 70 % in highly susceptible individuals, such as immuno-compromised individuals suffering from cancer and acquired immuno deficiency syndrome (Nørrung, 2000).

1.2.11.3.3 Prevalence in foods and epidemiological issues

The microorganism is prevalent in nature and can be found as a temporary inhabitant of the intestinal tract of humans and animals (White *et al*., 2002). Its widespread nature also permits a rather simple access to food products during different phases of production, processing, manufacturing and distribution (White *et al*., 2002). Contaminated foods are suspected to be the primary source of *L. monocytogenes* following the association of this microbe with a number of large foodborne outbreaks (Farber and Peterkin, 1991). According to Kozak, Balmer, Byrne and Fisher, (1996) epidemiological evidence and outbreaks have pointed to dairy products as suspected vehicles for cases of listeriosis. An outbreak associated with raw milk soft cheese in France in 1995 has been reported (Rocourt and Bille as cited by Meng and Doyle, 1998).

However, listeriosis has now been associated with consumption of a wide range of contaminated foods including fruits and vegetable products, meat (fresh and frozen), poultry and seafood products (Farber and Peterkin, 1991; Jay, 2000). This is probably because these foods are processed and stored at temperatures that would rather support the growth of *L. monocytogenes* and also because some of these foods are consumed without further cooking. The microorganism has also been isolated from an array of food

products, including fresh vegetables (11 %), raw meats (13 %), raw milk (3-4 %), dairy products (3 %), eggs and sea food products (Farber and Peterkin, 1991). Nonetheless, some foods appear to be of greater risk than others, mainly those that support the growth of the microorganism and are ready-to-eat and stored at refrigeration temperature for a long period (Meng and Doyle, 1998). Foods that are known to be supportive of the pathogen growth have always been implicated in human outbreaks associated with *L. monocytogenes* (Nørrung, 2000) and epidemic illnesses have largely been linked to refrigerated processed (ready-to-eat) foods consumed without prior cooking or reheating (White, Zhao, Simjee, Wagner and McDermott, 2002).

1.2.12 Hypothesis and objectives

1.2.12.1 Hypothesis

Phenolic extracts from sorghum bran fractions from condensed tannin and condensed tannin-free sorghum may exhibit antimicrobial properties because they bind with proteins, i.e. in this case extracellular microbial enzymes.

1.2.12.2 Objectives

1. To determine the levels of phenolic compounds from bran fractions of condensed tannin sorghum and condensed tannin-free sorghum types.
2. To determine the effect of sorghum crude phenolic extracts on the growth of *B. cereus*, *E. coli* and *L. monocytogenes*.