Chapter IV

General discussion

The general discussion chapter focuses on the methodologies used in this research project. The methods include the classification of sorghums into condensed tannin and condensed tannin-free varieties, determination and quantification of total phenols and condensed tannins from sorghum bran fractions and further evaluation of their antimicrobial activities. The effects of concentration of these phenolic fractions on antimicrobial activities of different bacterial species involved in this research project and the variations in the levels of inhibition exhibited by the Gram-positive and Gram-negative bacteria will also be further discussed. In this research project, the chlorox bleach test for classifying sorghum into condensed tannin and condensed tannin variety, vanillin-HCL and Folin-Ciocalteu methods for determining condensed tannins and total phenols respectively and, the paper disc diffusion method for determining antimicrobial activity have been used. However, scientific principles, advantages and limitations associated with the use of these methodologies need to be understood and are therefore further discussed.

4.1 Methodology evaluation

The Chlorox bleach test as described by Waniska *et al*. (1992) was used to classify the two sorghum varieties used in this research into condensed tannin or condensed tannin-free types. Boren and Waniska according to Waniska *et al*. (1992) mentioned that seed colour is not a good indicator of the presence of condensed tannin in sorghum and this necessitates a qualitative means to determine the presence of tannins in sorghum. The chlorox bleach test used in this project has been used in previous studies in determining the presence of tannins and determining whether a sorghum variety is a condensed tannin variety or not (Beta *et al*., 1999; Waniska *et al*., 1992).

The test supposes that if the kernels contain a pigmented testa layer then condensed tannins are present (Waniska *et al*., 1992). The pigmented testa is the innermost layer of the pericarp and is seen as the dark layer between the endosperm and the pericarp, when the kernel is scraped to remove the pericarp (Waniska, 2000). During the test, the components of the testa are oxidised to form black-coloured pigments. Sorghum grain is

immersed in a sodium hypochlorite solution (bleach) containing alkali. The solution dissolves the outer pericarp layer of sorghum grain, revealing the presence of a black-pigmented testa layer in the case of tannin sorghums, or its absence in the case of non-tannin sorghums (Price and Buttler, 1977; Waniska *et al*., 1992). Sodium hypochlorite in the bleach oxidises the components of the testa to form black-coloured pigments as indicated above.

Kernels that turn black are described as tannin-containing sorghums and may be classified under Type II or III sorghums whilst kernels that turn light yellow or white are described as tannin-free sorghums and are classified under Type I sorghums (Waniska *et al*., 1992). In this study, the red sorghum variety was found to be a condensed tannin sorghum whilst the white sorghum variety was found to be a condensed tannin-free sorghum variety.

The chlorox test is a simple test, does not require a lot of time (15-20 minutes) and is quite inexpensive (Waniska *et al*., 1992). However, this technique also has some limitations associated with it, which include among others lack of quantification of tannin content and an inability to differentiate between Type II and III sorghums. Proper quantitative tannin analysis should be done with a method such as the Vanillin-HCL method (Waniska *et al*., 1992).

A variety of methods can be used to determine the relative levels of phenolic compounds (Earp *et al*., 1981). The selection of the method depends largely on the type of phenolic compounds to be determined. Much confusion has prevailed because each method is specific for particular group(s) of phenols, and furthermore the extraction methods and treatment of samples affect the analytical values obtained within and among methods (Earp *et al*., 1981). This has made the quantitative comparison of phenol levels difficult, if not impossible (Earp *et al*., 1981). An acceptable method of analysis of phenolic compounds in sorghum should be relatively simple to perform, rapid, and give results that are reproducible from day to day and laboratory to laboratory (Maxson and Rooney, 1972).

The Folin-Ciocalteu method of Singleton and Rossi (1965) is used to quantify total phenols in the sorghum bran fractions. The method is based on the reducing power of phenolic hydroxyl groups. During the reaction, these phenolic hydroxyl groups react with the Folin-Ciocalteu phenol reagent to form a blue phosphotungstic-phosphomolybdic complex of undefined structure that can be detected spectrophotometrically (Waterman and Mole, 1994).

A problem with the Folin-Ciocalteu method is that it is not very specific for particular groups of phenolic compounds but rather serves to quantify the total concentration of phenolic hydroxyl groups in the plant extract of interest (Waterman and Mole 1994; Schofield, Mbugua and Pell, 2001; Sun *et al*., 1998). In other words, this method does not differentiate between types of phenols but measures all phenols in the sorghum kernel.

The Folin-Ciocalteu method does however address some of the problems experienced with previously used methods for determining total phenolics in plant extracts. A problem such as occasional development of a white precipitate through the addition of lithium sulphate, that prevents a spectrophotometric measurement that has been associated with the Folin-Denis method of total phenols (Waterman and Mole, 1994).

The two sorghum varieties were evaluated for condensed tannins using the Vanillin-HCL method of Price *et al*., (1978). This method is based on the ability of condensed tannin units to react with vanillin reagent in the presence of mineral acids to produce a red colour, which is measured spectrophotometrically. The condensed tannin units that may react with the vanillin reagent are flavanols with a single bond between carbons 2 and 3 of the C ring (Earp *et al*., 1981), and also with the free meta-oriented OH groups on the B ring. Therefore, since condensed tannins are condensation products of flavan-3-ols and flavan-3,4-diols, they give a positive reaction with vanillin (Gupta and Haslam, 1980). The method is specific for the condensed tannin type. The hydrochloric acid (HCl) methanol extract seems to speed up the rate of extraction that in turn results in higher values of tannins (Earp *et al*., 1981). The reaction is critically dependent on timing and temperature and is thus technically more difficult (Waterman and Mole, 1994). The other

problem with this assay is that all the solutions must be in 100 % methanol as the assay is particularly sensitive to water (Waterman and Mole, 1994). The use of methanol as a solvent quenches the reaction of vanillin monomeric flavanols, such as catechin, so that the reaction is more specific for oligomers (tannins) (Beta *et al*., 1999).

There have been nearly as many methods used for determining antimicrobial activity as there are compounds (Parish and Davidson, 1993). The agar disc diffusion method was used to evaluate the antimicrobial activity of sorghum CPE against selected pathogenic bacteria. Diffusion methods are broadly used to investigate the antibacterial activity of natural substances and plant extracts (Rauha *et al*., 2000). Among others, the agar diffusion method is probably the most widely used method in determining the antimicrobial effects of an antimicrobial substance.

These assays are based however on the use of discs or holes as reservoirs containing solutions of substances to be examined (Davidson and Parish, 1989). The antimicrobial compound in a specific quantity is added to an agar plate on a paper disc, the compound diffuses through the agar resulting in a concentration gradient that is inversely proportional to the distance from the disc (Davidson and Parish, 1989; Parish and Davidson, 1993). The extent of inhibition is indicated by a zone of no growth around the disc that contains the substance that is being tested and is dependent upon the rate of diffusion of the compound and cell growth (Barry (1986) as cited by Davidson and Parish, 1989; Parish and Davidson, 1993). It is therefore required that the antimicrobial agent that is being evaluated need not be highly hydrophobic as the compound will not diffuse resulting in little or no inhibition (Davidson and Parish, 1989; Parish and Davidson, 1993). It is also required that the test microorganism selected grow quickly and uniformly as slow-growing strains were shown to produce large zones of inhibition and vice-versa (Parish and Davidson, 1993). In cases of solutions with low activity, however, a larger concentration or volume is needed (Rauha *et al*., 2000).

Even though the agar diffusion method is the most commonly used, the method has however, some limitations associated with it. It has been reported that the results of the agar diffusion method are normally qualitative and microorganisms are either susceptible, intermediate or resistant to the natural antimicrobial agent being tested depending on the size of inhibition zones expressed in millimeters (Davidson and Parish, 1989). Fang and Kung (1997) as cited by Gurira, (2004) have indicted that if a heavy inoculum size is used the growth of the bacteria might partially or totally mask the inhibition zone of the microorganism being tested. It was also mentioned that in instances where the inhibition zone extends over a large diameter, it is that the zone may cover the area of less inhibitory microorganism.

4.2 Relative levels of total phenols and condensed tannins in sorghum CPE

In this study, the red sorghum variety, confirmed to be a condensed tannin variety by the chlorox bleach test was found to contain higher levels of total phenols and condensed tannins than the white sorghum variety. This is because the red sorghum had higher amounts of phenolic hydroxyl groups due to the presence of condensed tannins. According to Gupta and Haslam, (1980), the condensed tannins are condensation products of flavan-3-ol and flavan-3,4-diol units. Typically, a condensed tannin molecule may contain from 7-10 flavan-3-ol or flavan-3,4-diol units. Such a molecule will therefore contain a large amount of phenolic hydroxyl groups that can react with the Folin-Ciocalteu phenol reagent. As indicated previously in Chapter 2 section 2.4.2, the greater the amount of phenolic hydroxyl groups (as found in condensed tannin type compounds), the greater the concentration of phenolic compounds to be detected by the Folin-Ciocalteu assay.

These flavan-3-ol and flavan-3, 4-diol units of condensed tannins also give a positive reaction with vanillin (Earp *et al*., 1981) and explains why the condensed tannin sorghum contained significant amounts of condensed tannins as compared to the condensed tannin-free sorghum. However, the presence of slight amounts of condensed tannins in the white condensed tannin-free sorghum variety could be attributed to the fact that a

number of compounds have been reported to give a positive vanillin reaction even though these compounds may not be condensed tannins. These compounds as indicated by the vanillin-HCl assay account for low levels (less than 1.0 catechin equivalent) of tannins that have been previously reported for sorghums without a pigmented testa (Maxon and Rooney, 1972; Buttler and Price, 1977; Earp *et al*., 1981). However, Earp *et al*. (1981) indicated that when blanks are subtracted, initial colour is removed, but this still does not eliminate the measurement of non-tannin, vanillin-positive compounds and therefore; determination of absolute levels of condensed tannins by these methods is impossible; rather, each of these tests is a relative measure of the tannins in sorghum. Previously, Blessin, VanEtten and Dimler as cited by Yasumatsu *et al*. (1965) also reported the pigment precursors in sorghum which become visible upon addition of acid or alkali. The pigments were shown to leach out during the steeping operation for wet milling and give a slightly pinkish off-colour to the product (Yasumatsu *et al*. 1965). However, the chemical structure of these materials were ill-defined but during their studies, Blessin *et al*. as cited by Yasumatsu *et al*., 1965 purified the main pigments using a procedure based on adsorption on a strongly basic ion-exchange resin and, a compound called fisetinidin was identified as one of the reaction products that resulted from the treatment of the anthocyanogens with 12N hydrochloric acid. These findings led to further investigation of the chemical structures of the pigments of sorghum (Yasumatsu *et al*., 1965). Sarkar and Howarth, (1976) reported a very positive reaction to vanillin test by the flavanols and dihydrochalcones. They indicated that a single bond between carbon 2 and 3 and free meta-oriented hydroxy groups on the B ring are an essential requirement of a positive reaction giving substantial amounts of colour development by the flavonols and dihydrochalcones (phloretin and phloridzin).

It was also observed from this study that total phenols and condensed tannins obtained in this research project were significantly higher than the values obtained in literature. This is probably because in this study, polyphenols were extracted from sorghum bran and not in whole sorghum grain. Phenolic compounds are mainly concentrated in the outer layers (pericarp and testa layers) of the sorghum kernel (Awika *et al*., 2003). The levels of phenols in the bran could be 4-5 times greater than in the whole grain (Awika *et al*., 2003). Using the Folin-Ciocalteu method of total polyphenols Awika *et al*. (2003) reported amounts of total polyphenols of 1 mg gallic acid equivalent/g and 5 mg gallic acid equivalent/g for the white grain and white bran respectively whilst amounts of 5 mg gallic acid equivalent/g and 20 mg gallic acid equivalent/g were reported for the red grain and red bran respectively.

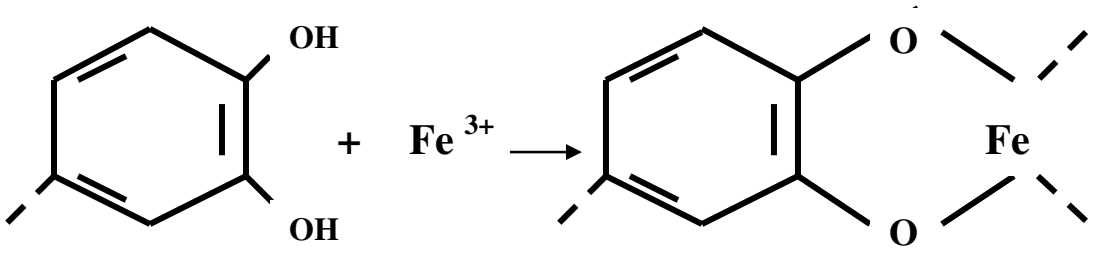
4.3 Antimicrobial activities of sorghum CPE and the effects of phenolic compound concentration and bacterial species on inhibition

In this study the effect of sorghum CPE from bran fractions of condensed tannins and condensed tannin-free sorghum bran fractions on *B. cereus* ATCC 1178, *E coli* ATCC 25922 and *L. monocytogenes* ATCC 7644 were examined using the paper disk diffusion method. The CPE from the condensed tannin sorghum which was found to contain higher levels of total phenols and condensed tannins exhibited greater antimicrobial activity against the tested bacteria, *B. cereus* ATCC 1178 and *L. monocytogenes* ATCC 7644 as compared to the CPE from the condensed tannin-free sorghum. The highest inhibitory effect of CPE from condensed tannin sorghum was observed at a concentration of 20 % whilst the CPE from the condensed tannin-free sorghum only showed a slight inhibition at a concentration of 20 %. The higher antimicrobial activity exerted by the condensed tannin sorghum CPE could be attributed to its higher concentration of phenolic compounds (Section 2.4.2, Table 3). Similarly, Baydar *et al*. (2004) attributed the inhibitory effects of grape seed extracts and bagasse extracts to their phenolic compounds content.

The CPE from condensed tannin sorghum showed strong antimicrobial activity against Gram-positive bacteria, *B. cereus* ATCC 1178 and *L. monocytogenes* ATCC 7644 but were ineffective against Gram-negative *E. coli* ATCC 25922 bacteria. On the other hand the CPE from the condensed tannin-free sorghum only showed slight inhibitory effects against Gram-positive, *B. cereus* ATCC 1178 and *L. monocytogenes* ATCC 7644 at a concentration of 20 % but was also ineffective against Gram-negative *E. coli* ATCC 25922 bacteria. The inhibitory effects of sorghum CPE against Gram-positive bacteria *B. cereus* ATCC 1178 and *L. monocytogenes* ATCC 7644 could be attributed to the absence of the outer membrane in Gram-positive bacteria that could have presented a permeability barrier to the entrance of foreign materials (i.e. phenolic compounds) into the cell of the bacteria and hence result in the bacteria being resistance to growth inhibition by phenolic compounds.

The inhibitory effects of CPE from sorghum bran fractions may be further attributed to the ability of phenolic compounds to bind with proteins and act like siderophores, thus chelating iron and making it unavailable for the microorganism and thereby preventing the growth of the microorganism.

According to McDonald, Mila and Scalbert (1996) phenolic compounds such as proanthocyanidins (condensed tannins) can bind directly with metal ions due to the presence of an orthodihydroxyphenyl group, (Figure 16). Therefore, any phenolic compounds (either flavonoid or condensed tannin type) with an orthodihydroxyphenyl group can bind a metal. Condensed tannins would contain several orthodihydroxyphenyl groups and so would bind more iron compared to monomers. This may explain why the CPE from condensed tannin sorghum had a higher inhibition of microbial growth than that from the condensed tannin-free sorghum.



Orthodihydroxyphenyl group Complex

Figure 16. Possible reaction between orthodihydroxyphenyl group of phenolic compound and Fe^{3+} ions (adapted from McDonald *et al*., 1996).

Nevertheless, additional studies need to be done to investigate the mechanisms involved in the inhibitory effect of sorghum CPE against bacteria and how the sorghum CPE may exhibit their antimicrobial activity in practical food systems.