Chapter II

Extraction of phenolic compounds and quantification of the total phenol and condensed tannin content of bran fraction of condensed tannin and condensed tannin-free sorghum varieties

2.1 Abstract

Defatted bran fractions prepared from a condensed tannin sorghum variety (red) and a condensed tannin-free sorghum variety (white) were analysed for their content of total phenols and condensed tannins. Total phenols were determined using the Folin-Ciocalteu method and condensed tannins with the vanillin-HCL method. Total phenols and condensed tannins of the bran fractions were extracted with aqueous acetone (75 % v/v) and acidified methanol (1 % HCL v/v in methanol) respectively, using a bran-to-solvent ratio of 1:4 (w/v). Red sorghum bran contained a higher amount of total phenols and condensed tannins (33.18 mg tannic acid equivalent/g and 117.98 mg catechin equivalent/g of the bran fractions) respectively than white sorghum bran (6.81 mg tannic acid equivalent/g and 8.52 mg catechin equivalent/g of the bran fractions) respectively.

Keywords: Sorghum; Bran fractions; Total phenols; Condensed tannins

2.2 Introduction

Sorghum (*Sorghum bicolor* (L) Moench) is an indigenous African cereal and traditional food crop (Dogget, 1988). Like other cereals such as barley, maize, rice and wheat, sorghum belongs to the grass family, the *Gramineae* (Odibo *et al.*, 2002). Sorghum is the fifth major cereal crop in the world after wheat, rice, corn and barley in terms of production and utilisation and the third leading cereal crop in the United States (Hahn *et al*., 1984). 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 *et al*., 2000). In contrast, sorghum is consumed as a staple by millions of people in Asia and Africa with more than 35 % of the crop grown directly for human consumption (Awika and Rooney, 2004; Ratnavathi and Sashidhar, 1998).

Sorghums contain phenolic compounds, which are secondary plant metabolites i.e. they are not directly involved in any metabolic process (FAO, 1995) and are characterised by possession of a phenol group. These compounds 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 naturally in high levels in certain foodstuffs (Strumeyer and Malin, 1975; Haslam, 1989; Hahn *et al*., 1984; Murty and Kumar, 1995; Bvochora *et al*., 2004). Phenolic compounds in sorghum may be categorised into three broad groups; the phenolic acids, the flavonoids and the tannins, and are located primarily in the outer layers of the sorghum kernel (pericarp, testa and aleurone) (Hahn *et al*., 1984). Therefore, their amounts may be lowered during practices such as sorghum decortication.

Phenolic compounds, especially, the tannins have been reported to inhibit digestive enzymes, affect the utilisation of vitamins and minerals and are capable of binding and precipitating proteins causing a reduction in nutritional value (Hahn *et al*., 1984; FAO, 1995; Chung et al., 1998c; Chavan, Shahidi and Naczk, 2001). They have therefore been

regarded as antinutrients and considered nutritionally undesirable (Hahn *et al*., 1984; FAO, 1995; Chung *et al*., 1998c; Chavan *et al*., 2001). However, these compounds are also believed to have some favourable effects on human health, such effects as the lowering of human low-density lipoprotein, reduction of heart diseases and cancer (Baydar *et al*., 2004).

Generally, 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, a product of the sorghum milling process, is often discarded or used as animal feed (Murty and Kumar, 1995; Lochte-Watson *et al*., 2000).

Microbial activity is the principal mode of spoilage of many foods and it is often responsible for the loss of quality and safety due to the actions of microorganisms (Jayaprakasha *et al*., 2003; Baydar *et al*., 2004). In trying to circumvent this problem, the use of synthetic additives is one of the methods that has been adopted (Jayaprakasha *et al*., 2003; Baydar *et al*., 2004). However recently, interest has been focused on the use of plant extracts rather than synthetic additives to prevent spoilage of foods (Baydar *et al*., 2004) as they sometimes show antioxidant as well as antimicrobial activity (Smid and Goris as cited by Jayaprakasha *et al*., 2003). Extracts of herbs and spices are mostly used for this purpose (Smid and Goris as cited by Jayaprakasha *et al*., 2003).

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 *et al*., 2000; Jayaprakasha *et al*., 2003; Baydar *et al*., 2004). Their potential for use as preservatives has been documented and interest has been focused on the use of these extracts in the preservation of food (Jayaprakasha *et al*., 2003; Baydar *et al*., 2004). This presents an opportunity for the use of sorghum bran as a source

of phenolic compounds with antimicrobial activity. There is however no information on the antimicrobial properties and inhibitory effects of sorghum phenolic extracts. The objectives of this study were therefore to determine the levels of phenolic compounds in extracts prepared from bran fractions of condensed tannin and condensed tannin-free sorghum varieties in preparation for further evaluation of their potential antimicrobial activity.

2.3 Materials and methods

2.3.1 Sorghum grain samples

A white sorghum (Ws) variety obtained from AGRICOL, Pretoria, South Africa and a Red sorghum (Rs) variety (Red Swazi), obtained from an open air market in Bulawayo, Zimbabwe, were used. The red sorghum originated from the agricultural area called Rio Tinto in Zimbabwe. The sorghum samples were stored in a cereal store room at 9-10 ºC until needed for analyses.

2.3.2 Reagents

Sodium carbonate, hexane, acetone, Folin-Ciocalteu phenol reagent, tannic acid, methanol, vanillin, hydrochloric acid and catechin were obtained from Merck in Johannesburg, South Africa.

2.3.3 Testing for the presence of pigmented testa in sorghum grains

The chlorox bleach test developed by Waniska *et al*. (1992) was used to test for the presence of pigmented testa in the two sorghum varieties. The chlorox bleach test is based on the assumption that if the kernels contain a pigmented testa layer then condensed tannins are present in the kernels and this distinguishes Type I sorghums (without tannins) from Types II and III sorghums (with tannins) (Waniska *et al*., 1992).

The bleach test causes the pericarp to dissolve, revealing the presence or absence of a pigmented testa layer (Price and Butler, 1977; Waniska *et al*., 1992).

Chlorox reagent [5 % caustic soda (NaOH) in domestic bleach (3.5 % sodium hypochlorite)] was added to 100 whole sorghum kernels just to cover the kernels, in 100 ml beakers. The beakers were then covered with aluminum foil. The containers were incubated for 20 min at room temperature (20-25 °C) with swirling every 5 min. The kernels were then tipped into small strainers, rinsed with tap water, blotted dry on a paper towel and the number of completely black kernels counted. The experiment was carried out in triplicate for both the red and the white sorghum varieties.

2.3.4 Preparation of sorghum bran fractions

Whole grain sorghum (4000 g) for both white and red sorghum varieties was decorticated by passing through a dehuller (Rural Industries Innovation Centre, Kanye (Botswana) twice, which operates on the principle of abrasive decortication. The bran fraction produced from the white and the red sorghum types (approximately 7.1 and 12.3 % yield respectively) was collected and milled to pass through a laboratory hammer mill (Falling Number AB, Huddinge, Sweden) fitted with a 250 µm sieve. The sample from the white and the red sorghum types, that went through the sieve was collected, vacuum packed in laminated plastic bags and stored at 4 °C in a cold room until required for further analyses.

Extracts for determination of total phenols and condensed tannins were prepared by suspending sample (0.125 g) of sorghum bran fractions from both condensed tannin and condensed tannin-free sorghums in 6.25 ml of 75 % aqueous acetone and acidified methanol (1 % HCl in 100 ml of methanol) extractants for total phenols and condensed tannins respectively in clean 10 ml test tubes. The contents of the test tubes were vortex mixed at 5 minutes intervals for 2 hrs and 20 minutes using a vortex mixer and centrifuged for 6 minutes at 3500 rpm (Selecta Medifridge, UK) for the determination of

total phenols and condensed tannins respectively. After centrifugation, extracts were assayed for total phenols and condensed tannins as outlined in sections 2.3.5 and 2.3.6.

2.3.5 Determination of total phenol content of sorghum bran fractions

The Folin-Ciocalteu method (Singleton and Rossi, 1965) as described by Waterman and Mole (1994) was used to determine total phenols.

Aliquots (1 ml) of each of the extracts were mixed with 5 ml Folin-Ciocalteu reagent in 100 ml volumetric flasks that contained 70 ml of deionised water. Sodium carbonate solution (15 ml of 20 % m/v anhydrous sodium carbonate in deionised water) was added after 1 min but before 8 min. The volumetric flasks were then made up to volume with deionised water. After standing for 2 h at room temperature, the absorbance was read at a wavelength of 760 nm in the visible range of the spectrum using a UV/Vis-spectrophotometer (Perkin Elmer, New York, USA). The estimation of total phenols in the extracts was carried out in triplicate for both the condensed tannin and the condensed tannin-free sorghum varieties. Tannic acid was used as a standard and the results obtained were expressed as mg tannic acid equivalent/g of sample, on a dry weight basis.

2.3.6 Determination of condensed tannin content of sorghum bran fractions

The vanillin-HCL method of Price, Scoyoc and Buttler, (1978) as described by Waterman and Mole (1994) was used to quantify condensed tannins. Vanillin reacts with proanthocyanidins and leucoanthocyanidins or catechins in the presence of HCL giving rise to a bright red colour. The method is preferred because of its sensitivity, specificity and simplicity and is quite specific to a narrow range of flavanols (monomers and polymers) (Sun *et al*., 1998).

Aliquots (1 ml) of each of the extracts were mixed with 5 ml vanillin-HCL reagent in clean 10 ml test tubes. The test tubes were incubated for 20 min at room temperature. The absorbance was then read at 500 nm in the visible range of the spectrum using a UV/Vis-

spectrophotometer, zeroing the spectrophotometer with deionised water. The estimation of condensed tannins was carried out in triplicate. Catechin was used as a standard and the results obtained were expressed as mg catechin equivalent/g of sample, on a dry weight basis.

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2.3.7 Statistical analysis

The student's t-test was used to compare the data and all the tests were considered significantly different at $p \leq 0.05$.

2.4 Results and discussion

2.4.1 Chlorox bleach test for the sorghum grains

The results of the chlorox bleach test are shown in Figure 11. The red sorghum (Rs) kernels turned black after the chlorox bleach test while the white sorghum (Ws) kernels remained light in colour.

The chlorox reagent detaches the pericarp and clearly shows the testa (Beta *et al*., 1999). According to Hahn *et al*. (1984) and Waniska *et al*. (1992), sorghum kernels with a pigmented testa turn black and those lacking the pigmented testa remain light in colour or become a light yellow or white (or slightly coloured) when treated with chlorox bleach. Hahn *et al*. (1984) also mentioned that sorghums with a pigmented testa contain condensed tannins while sorghums without a pigmented testa do not have condensed tannins.

As shown in Figure 11, the red sorghum gave a positive chlorox bleach test indicating that it possesses a pigmented testa and hence is a condensed tannin variety while the white sorghum variety gave a negative chlorox bleach test through lack of pigmented testa and hence is a condensed tannin-free variety.

Figure 11. Appearance of sorghum grain samples before and after the chlorox bleach test.

2.4.2 Total phenol and condensed tannin content of sorghum bran fractions

The contents of total phenols and condensed tannins in the condensed tannin and condensed tannin-free sorghum bran fractions are given in Table 3. The amount of total phenols and condensed tannins was significantly higher in the condensed tannin sorghum bran fractions compared to the amounts obtained from the condensed tannin-free sorghum bran fractions. This was an expected result as the chlorox bleach test confirmed the red sorghum to be a condensed tannin type and the white sorghum, condensed tannin-free. Ratnavathi and Sashidhar (1998) reported amounts of total phenols using the Folin-Dennis assay ranging from 2.25 to 3.8 mg tannic acid equivalents/g of defatted grain flour for a red sorghum variety compared to a range of 0.52 to 1.94 mg tannic acid equivalents/g of defatted grain flour for a white variety. Using the vanillin-HCl method Beta *et al*. (1999) reported condensed tannin content of 54.8 mg catechin equivalent/g whole grain flour for DC 75 (a condensed tannin sorghum variety) compared to 0.8 mg catechin equivalent/g for Mukadzidzoka (a condensed tannin-free sorghum variety). Similar results have been reported for red and white sorghum bran fractions (Awika *et al*., 2003).

Another important observation from Table 3 is the fact that the amount of condensed tannins and total phenols obtained in the bran fractions of the two sorghum varieties are significantly higher than values obtained in literature for whole grain sorghums. This is because phenolic compounds are mainly concentrated in the outer layers of the kernel (pericarp, testa and aleurone), which form the main components of the bran fraction. Awika *et al*. (2003) reported total phenol contents (using the Folin-Ciocalteu method) of 1 mg gallic acid equivalent/g for whole grain white sorghum and 5 mg gallic acid equivalent/g for the bran fraction of the white sorghum. They reported similar trends for red sorghum whole grain and bran and for condensed tannin content (whole grain and bran) of sumac, a brown sorghum variety.

The Folin-Ciocalteu method is used to quantify the total concentration of phenolic hydroxyl groups present in a sample. The conceptual basis of the assay is to quantify the total concentration of phenolic hydroxyl groups present in the extract being assayed. The method does not provide any data on the particular phenolic compounds present in the extract. According to Hahn *et al*. (1984) and Sun, Ricardo-da-Silva and Spranger (1998) the Folin-Ciocalteu method is based on the reducing power of phenolic hydroxyl groups and is not very specific but detects all phenols with varying sensitivity. A reduction-oxidation reaction of phenolate occurs under alkaline conditions reducing the phosphotungstic-phosphomolybdic complex in the reagent to a blue colour (Waterman and Mole, 1994). Under alkaline conditions, phenolate ions reduce the phosphotungstic-phosphomolybdic complex in the reagent to a blue colour (Waterman and Mole, 1994). The method does not distinguish between different types of phenolic compounds. The greater the amount of phenolic hydroxyl groups (as found in condensed tannin type compounds), the greater the concentration of phenolic compounds detected by the assay. The high total phenol value in the condensed tannin sorghum is therefore attributed to the greater amount of phenolic hydroxyl groups in the condensed tannin type (red) sorghum confirmed to be a condensed tannin type by the chlorox bleach test as compared to the condensed tannin-free (white) sorghum.

The results from Table 3 indicate that even though the white sorghum was identified as a condensed tannin-free variety, some components of the bran fraction gave a positive vanillin reaction. The vanillin reagent reacts with proanthocyanidins in the presence of hydrochloric acid (Sun *et al*., 1998). The method is quiet specific for condensed tannins. Nevertheless, a number of compounds other than condensed tannins in sorghum may give a positive vanillin reaction (Earp, Akingbala, Ring and Rooney, 1981). In their studies, Yasumatsu, Nakayama and Chichester (1965) have reported the presence of eriodictyol, a flavanone in a commercial sorghum whilst Kambal and Bate-Smith have also reported the presence of the flavanone, eriodictyol and luteoforol (a pentahydroxy flavan compound) in a white and a red pericarp sorghum, respectively (Earp *et al*., 1981). These compounds have been previously reported as tannin (Earp *et al*., 1981). The presence of these other non-tannin compounds may account for the tannin levels reported for

condensed tannin-free sorghums (without a pigmented testa) as shown in this work (Table 3).

Table 3. Total phenol content (expressed as mg tannic acid equivalent/g) and condensed tannin content (expressed as mg catechin equivalent/g) of sorghum bran fractions on a dry matter basis

Sample	Total phenols (± SD)	Condensed tannins (± SD)
White sorghum bran fractions	6.81[a] (0.58)	8.52[a] (0.65)
Red sorghum bran fractions	33.18 [b] (3.17)	117.98 [b] (4.27)

Values in the same column with different superscripts are significantly different $p \leq 0.05$
SD Standard deviation

2.5 Conclusions

The red sorghum is a condensed tannin type whilst the white sorghum is a condensed tannin-free type as confirmed by the chlorox bleach test. The red sorghum variety which was found to be a condensed tannin variety has significantly higher amounts of total phenols and condensed tannins when compared to the white sorghum variety which was found to be a condensed tannin-free variety. The results obtained from this work show that sorghum bran from condensed tannin sorghum may be exploitable as a potential source of phenolic compounds for possible use as antimicrobial agents.