African Journal of Pure and Applied Chemistry Vol. 5(7), pp. 181-193, July 2011
Available online at http://www.academicjournals.org/AJPAC
ISSN 1996 - 0840 ©2011 Academic Journals

Full Length Research Paper

Identification of 3-deoxyanthocyanins from red sorghum (*Sorghum bicolor*) bran and its biological properties

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Accepted 18 March, 2010

Anthocyanins known as potent antioxidant provides attractive colours and used as an ingredient in food systems. This study aims to evaluate a potential anthocyanin extraction method from red sorghum bran and to investigate its antioxidant and cytotoxic properties. The anthocyanin content was found to be higher in acidified methanol and on separation was identified as apigeninidin and leuteolinidin, which obviously could attribute to its antioxidant properties and cytotoxic activity. Anthocyanins are natural pigments used as food additives and known for its potent antioxidant and antiproliferative property. In addition to their potential as food colorants, anthocyanins are nowadays regarded as an important nutraceuticals mainly due to their possible antioxidant effects and they have been given a potential therapeutic role related to some cardiovascular diseases, cancer treatment, inhibition of certain types of virus including Human Immunodeficiency Virus type 1 (HIV-1) and improvement of visual acuity (Stintzing et al., 2002; Talavera et al., 2006; Sandvik et al., 2004; Beattie et al., 2005; Jang et al., 2005; Cooke et al., 2005; Andersen et al., 1997; Nakaishi et al., 2000). This study aims to evaluate anthocyanin content, to analyze antioxidant and antipropliferative activity from red sorghum bran. When acidified methanol was used as a solvent for extraction, maximum amount of anthocyanin was obtained as compared to methanol alone. On separation by Thin Layer Chromatography and High Pressure Liquid Chromatography, the compounds were identified as apigenindin and luteolindin. The antioxidant activity was found to be higher in red sorghum bran. Anthocyanin from red sorghum bran also showed moderate cytotoxic activity against HT 29 and HEP G2 cell lines. Is assumed that the antioxidant and antiproliferative activity of the extract from red sorghum bran was due to apigenindin and luteolindin. So the anthocyanins extracted from easily available red sorghum bran would be a valuable source for antioxidant and antiproliferative activity in food industry.

Key words: Bran, sorghum, anthocyanins, antioxidant activity, high pressure liquid chromatography, DPPH, HT 29 cancer cell line, HEP G2 cancer cell line.

INTRODUCTION

Sorghum grain is an important staple food in developing countries of the semi-arid tropics and is used as an animal feed in both developed and developing countries where people depend on it as the main source of energy and protein (Oria, 2000). Sorghum seeds are provided with antioxidant substances that are able to scavenge radical products. These compounds include lipid-soluble products, such as tocopherols and water-soluble substances such as ascorbic acid and thiols. The chemical components of the sorghum grain cuticle are flavonoids, anthocyanidins and tannins (Kaluza, 1988). Anthocyanins are becoming increasingly important not only as food colorants, but also as antioxidants.

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Anthocyanins are reported to have therapeutic benefits including vasoprotective and anti-inflammatory (Lietti et al., 1976), anti-cancer and chemoprotective (Karaivanova et al., 1990), as well as anti-neoplastic properties (Kamei et al., 1995). Anthocyanins are considered to contribute significantly to the beneficial effects of consuming fruits and vegetables (Wang et al., 1997). There is a rising demand for natural sources of food colorants with nutraceutical benefits (Boyd, 2000) and alternative sources of natural anthocyanins are becoming increasingly important.

Phenolic compounds are the principal antioxidant constituents of natural products and are composed of phenolic acids and flavonoids, which are potent radical terminators acting by donating hydrogen radicals (Kahkonen et al., 1999). Afaq et al. (1991) reported that flavonoids are well known anti-oxidants and free radical scanvengers. High potential of polyphenols to scavenge free radials are because of their many phenolic hydroxyl groups (Sawa et al., 1999). Phenolic compound plays a major role in antioxidant activity. Potterat (1997) reported that natural antioxidant phenolic compound in S*echium edule* with better performance than BHT, known as a very efficient synthetic antioxidant agent and widely used in Food Technology. Ordonez et al. (2003) reported that the qualitative and quantitative analysis of phenolic compounds in active, extracts showed the presence of flavanoids, flavonol and the chemical composition responsible for the antioxidant effects.

Sorghum contains significant levels of anthocyanins and other phenols in their brans (Awika, 2000; Gous, 1989) than other cereal brans like wheat, barley, buck wheat and rice, among others, are promoted as good source of antioxidants (Emmons and Peterson, 1999; Haber, 2002; Quettier-Deleu et al., 2000; Yu et al., 2002; Zielinski and Kozlowska, 2000). The chemical components of the sorghum grain cuticle are flavonoids, anthocyanidins and tannins (Kaluza, 1988). The sorghum bran can be used as a high value source of antioxidants at lower quantities than other cereal brans, or used at similar quantities to provide higher antioxidant activities in products. Anthocyanin content of sorghum brans are found to be closely related to anthocyanin from fruits and vegetables.

Rey et al. (1993) have identified apigeninidin as a major anthocyanidin present in sorghum. Black sorghum was reported to have significantly more anthocyanin pigments than other sorghums (Awika, 2000). Gous (1989) also reported luteolinidin and apigeninidin as the major anthocyanidins from a black sorghum variety. Cyanidin and pelargonidin were also reported in corn (Francis, 1989) and in sorghum (Yasumutsa et al., 1965). The most common anthocyanins in sorghum are the 3-deoxyanthocyanidins (Gous, 1989; Sweeny and Lacobucci, 1981), which comprise luteolinidin and apigeninidin. These anthocyanins have a small distribution in nature (Clifford, 2000) and are distinct from the more widely distributed anthocyanidins. These 3-deoxyanthocyanidins were reportedly very stable in acidic solutions relative to the anthocyanidins commonly found in fruits and vegetables (Sweeny and Lacobucci, 1981).

The lack of oxygen at C-3 is believed to improve their stability. These points to the potential advantage of sorghum over fruits and vegetables as a viable comercial source for anthocyanins regarding its physiological function, apigeninidin showed a high fungicidal activity in sorghum (Aldia, 1996) and it was reported that apigeninidin effectively quenched ascorbyl radical and lipid radicals when supplemented with doses up to 200 µg/ml (Boveris, 2001). Fratianni et al., (2007) found that apigenindin have diverse pharmacological activities and has demonstrated antioxidant and anticarcinogenic properties. In addition, Raj and Shalini (1999) and Badami et al. (2003) also reported a large number of flavanoids including apigenin and luteolin are known to possess strong antioxidant properties.

Cancer cells usually exist under heavy oxidative stress state, Shinkai et al. (1986) have explained that heavy oxidative stresses have induced mutation in the cancer cells, because of this the survival potential of the cancer cells is increased. Mild levels of Reactive Oxygen Species (ROS) in food have been shown to induce proliferation in cancer cells (Arora et al., 1999; Del Bello et al., 1999). Therefore, foods rich in antioxidant phytochemicals are important for the prevention of diseases related to oxidative stress such as heart disease and cancer.

Commercially prepared anthocyanin-rich extracts (AREs) from grape (*Vitis vinifera*), bilberry (*Vaccinium myrtillus* L.) and chokeberry (*Aronia meloncarpa* E.), have inhibited the growth of HT29 cells as compared to non tumorigenic colon cells. Thus there is the need to find a new and effective curative method for hepatocellular and colon cellular cancer treatment. Frantianni et al. (2007) found that apigenindin have diverse pharmacological activities and has demonstrated antioxidant and anticarcinogenic properties. The antiproliferative activities of commonly consumed, pigmented fruits such as grapes, raspberries, cranberries or strawberries and vegetables were reported to have potent inhibitory effects on HepG2 cell proliferation. (Chu et al., 2002; Liu et al., 2002; Meyers et al., 2003; Sun and Oberley, 1996). In this study our main aim is to identify the potential of anthocyanin extraction from red sorghum for its anticancer property against cancer cell lines. This property is evaluated through investigations on antioxidants and viable cell count.

Ferguston et al. (2004) reported that flavanoid rich extracts from cranberry showed *in vitro* antiproliferative activity against HT 29, SW 620 colon cancer cell lines and also implicated that pro anthocyanidin contributing to this activity.

Maria et al. (2004) reported that anthocyanins are promising substances for reducing cancer risk

because of their anti proliferative potential and their apoptotic effects specifically in cancer cells.

Sorghum has a good potential for commercial exploitation. To effectively characterize and quantify the sorghum anthocyanins, it is important to extract them in an efficient manner in which their original form is preserved as much as possible. The efficiency of several solvents to extract anthocyanins and other phenols from fruits, vegetables (Garcia et al., 1998; Kallithraka et al., 1995; Lu and Foo, 2001) and cereals (Gous, 1989; Hahn, 1984) have been reported but a limited data exists on the types and levels of anthocyanins in cereals, probably because they have never been regarded as a comercially significant source. Nip and Burns (1969, 1971) were able to isolate and identify apigeninidin, apigeninidin-5-glucoside, luteolinidin and luteolinidin-5-glucoside in red and white sorghum varieties by paper chromatography. However, there is no agreement on which solvents can extract anthocyanins better. The solvents that stand out as most efficient are acidified methanol and aqueous acetone (70%). Consequently, these two solvents were compared in this study in terms of their extracting potential on red sorghum anthocyanins.

The objective of this study was to assess the anthocyanin content, antioxidant potential and cytotoxity activity from bran of a red sorghum variety.

EXPERIMENTAL PROCEDURES

Samples

The bran of *Sorghum bicolor* (L.) red sorghum were collected from farmers field in Tamil Nadu and were stored at -20°C.

Sample extraction

Two extraction solvents, namely methanol and 1% HCl in methanol were used for extraction procedure. The extraction protocol involved the addition of 10 ml of solvent to 0.5 g of sample in 50 ml centrifuge tubes and shaking the samples for 2 h at low speed (75 rpm) in an orbitory shaker (Neolab). Samples were then stored at -20°C for overnight in the dark to allow for maximum diffusion of phenolics from the cellular matrix. Samples were then equilibrated to room temperature and centrifuged at 7,000 rpm for 10 min and taken for analysis. Residues were rinsed with 10 ml volumes of solvent for two times with shaking for 5 min, then centrifuging at 7000 rpm for 10 min and taken for analysis. Finally, the extracts were mixed well and stored at -20°C in the dark until further biochemical analysis (Joseph et al., 2004).

Analytical procedures

Flavanoid confirmation test

A small amount of extracted sample was treated with ferric chloride and the results were observed for the presence of flavanoid.

Total phenolics assay

Total phenolic compounds in red sorghum bran were quantified using Folin-Ciocalteu's method (Ronald et al., 1998). 25 µl of Folin-Ciocalteau's reagent (50%, v/v) was added to 10 µl of extract using calibrated micropipette (Eppendorf). After 5 min incubation at room temperature, 25 µl of 20% (w/v) Sodium carbonate and water were added to a final volume of 200 µl using micropipette. Blanks were prepared by replacing the reagent by water to correct for interfering compounds. After 30 min of incubation, the absorbance was read at 760 nm using UV / VIS spectrophotometer (Genesys 5 Technical trade links pvt.ltd).

Stability at variable pH

The stability of the compound was tested by treating about 1 ml of sample with 1 ml of sodium acetate at pH 1.0 and with potassium chloride at pH 4.5. The colour change was observed (Strack, 1989).

Determination of total anthocyanins

The pH differential method as reported by Fuleki and Francis (1968), Guisti (2003) and Wrolstad (1976) was used for quantitative determination with minor modifications. Each of two 0.2 ml aliquots was diluted with 2.8 ml of pH 1.0 buffer (125 ml of 0.2 N KCl and 385 ml of 0.2 N HCl) or pH 4.5 buffer (400 ml of 1 N sodium acetate, 240 ml of 1 N HCl and 360 ml distilled water) solutions, respectively. The absorbance was measured by scanning through a UV / VIS spectrophotometer between 210 – 750 nm ranges. Total anthocyanin pigments were determined from absorbance in pH 1.0 buffer, while monomeric anthocyanins were determined from the differences between absorbance in pH 1.0 and 4.5 buffers. Extinction coefficients for anthocyanin standards were determined using the formula described by Fuleki and Francis (1968). The total anthocyanin content and their absorbance maxima were determined by using UV / VIS spectrophotometer.

Chromatographic analyses

The sample extracts were separated by Thin Layer Chromatography (TLC) on silica gel plates (Himedia). Samples were spotted and air-dried. Separation of compounds could be accomplished by spotting up to the equivalent of 0.04 g sample extract. The solvent system was the upper phase of a mixture of ethyl acetate/water/formic acid/HCl (85: 8: 6: 1 vol/vol) (Stafford, 1965). Compounds were detected under ultraviolet light (366 nm) before and after exposing the plates to ammonia fumes (Markham, 1982) and by spraying the plates with aqueous 2% $FeCl_3$ (Harbone, 1984). The silica gel containing individual bands of compounds was scraped from plates. Compounds were eluted from the gel with the original Thin Layer Chromatography solvent by centrifugation (Bioanalytical Systems, West Lafayette, IN). The samples were then reduced to dryness under vacuum and resuspended in High Pressure Liquid Chroamtography-grade methanol for use in bioassays and for compound identification. For further characterization some samples were spotted again on silica gel Thin Layer Chroamtography plates and the plates were sprayed with 1% Aluminium Chloride in ethanol (Gage, 1951) and Benedict's reagent (Reznick, 1951).

HPLC analysis

For High Pressure Liquid Chromatography analysis of anthocyanins, separation was carried out on reverse phase C-18 column (Shimadzhu). The method was done by Wang et al. (2000) Solvent A was Water: Acetic acid: Methanol (8:1:1) and Solvent B was methanol: Acetic acid (9:1). Crude extract (20 µl) were injected

and eluted 40% solvent B at a flow rate of 1 ml min^{-1}. Twenty micro liters of crude extract from sorghum mesocotyls (generous gift from Dr. R.L. Nicholson) was used as the phytoalexin standard. Compounds were detected at 480 nm for the presence of 3 deoxy anthocyanidin.

Antioxidant properties

The antioxidant property of methanol extract of red sorghum bran was analysed by DPPH method (Smith et al., 1987; Yamaguchi, et al., 1998)

DPPH free radical-scavenging assay

The procedure described by Smith et al. (1987) Yamaguchi et al. (1998) were exactly followed. Ethanolic DPPH (400 mM) was used in the reaction mixture. Serial dilutions of the test sample were combined with the DPPH solution. Methanol was used as a negative control and ascorbic acid and a-tocopherol was used as positive controls. The reaction mixtures were incubated for 30 min at 37°C and the change in absorbance at 517 nm was measured. Mean values were obtained from triplicate experiments. Inhibition percent was:

$$\% \text{ Inhibition} = [(C - S/C)] \times 100$$

where C is the net absorbance of the control and S is the net absorbance of the sample. Percent inhibition was plotted against concentration and the equation for the line was used to obtain the IC_{50} value. A lower IC_{50} value indicates greater antioxidant activity.

In vitro studies

Cytotoxicity screening

The cytotoxicity screening was done by MTT assay (Mosmann, 1983) and SRB assay (Mitry et al., 2000). The HT 29 cell line (Human Colon carcinoma) and HepG2 (Human liver carcinoma) were cultured in McCoy's 5A and DMEM (Dulbecco's modified eagles medium) medium respectively containing 10% fetal calf serum, penicillin (100 U) and streptomycin (100 µg). 10 ml of DMEM or McCoy's 5A containing 10% serum was added to the flask and pipetted to breakdown the clumps of cells. Total cell count was taken using a haemocytometer and calculated the total number of cells. The medium was added according to the cell population needed. Required amount of medium containing the required number of cells (0.5 - 1.0 x 10^5 cells/ml) was transferred into bottles according to the cell count and the volume was made up with medium and required amount of serum (10% growth medium and 2% maintenance medium) was added. The flasks were incubated at 37°C and the cells were periodically checked for any morphological changes and contamination. After the formation of monolayer, the cells were further utilized.

RESULTS AND DISCUSSION

Anthocyanin extraction and quantification

The extraction of anthocyanin from red sorghum bran was done in methanol and acidified methanol. In the spectrum of the extracts, a peak in the visible region was recorded at 400 nm. A single peak was observed in methanol and in acidified methanol peaks were observed and the absorbance was also high in acidified methanol extract (3.742) of sorghum bran compared with methanol extract (0.085) (Figure 1). Joseph et al. (2004) have already stated that the acidified methanol preserves the extracted anthocyanins in their original form for long duration so that the acidified methanol is most preferred solvent system used in this study for quantification and analysis of anthocyanins.

Flavanoid confirmation test

The extracted anthocyanin from the red sorghum bran using acidified methanol and methanol solvents were shown brown colour. The formation of brown color had indicated the presence of flavanoid.

Total phenolic assay

The total content of phenols in the methanol extract from the bran of red sorghum was 38 mg/ml and it was observed as 97 mg/ml in acidified methanol extract (Figure 2). Sene et al. (2001) reported that enhanced phenol synthesis in sorghum mainly due to nutrition (nitrogen) and other environmental factors which helped to promote the growth and grain yield.

Stability at variable pH

The samples appeared red in color at pH 1.0. When the pH was increased to 4.5, the red color disappeared and the solutions become transparent. The same results were observed in methanol extract as well as in acidified methanol extract of red sorghum bran. From the results obtained in stability test at variable pH, it was identified that anthocyanin was found to be more stable in low pH. Giusti (2003) has explained about the same observations in fruits and vegetables.

Determination of total anthocyanins

The total anthocyanin content in methanol extract of red sorghum bran was 98.59 mg/l and in acidified methanol extract it was 242.7 mg/l (Figure 2). Acidified methanol extract resulted, significantly higher values for the total anthocyanins than the methanol extract. The reason is that the acidified methanol preserves the extracted anthocyanins in their original form for long duration (Joseph et al., 2004). At low pH level (1.0) the stability of anthocyanin was more (Guisti, 2001). So in our study it was identified that the acidified methanol with low pH is most preferred solvent system for extraction, quantification and analysis of anthocyanins from red sorghum bran.

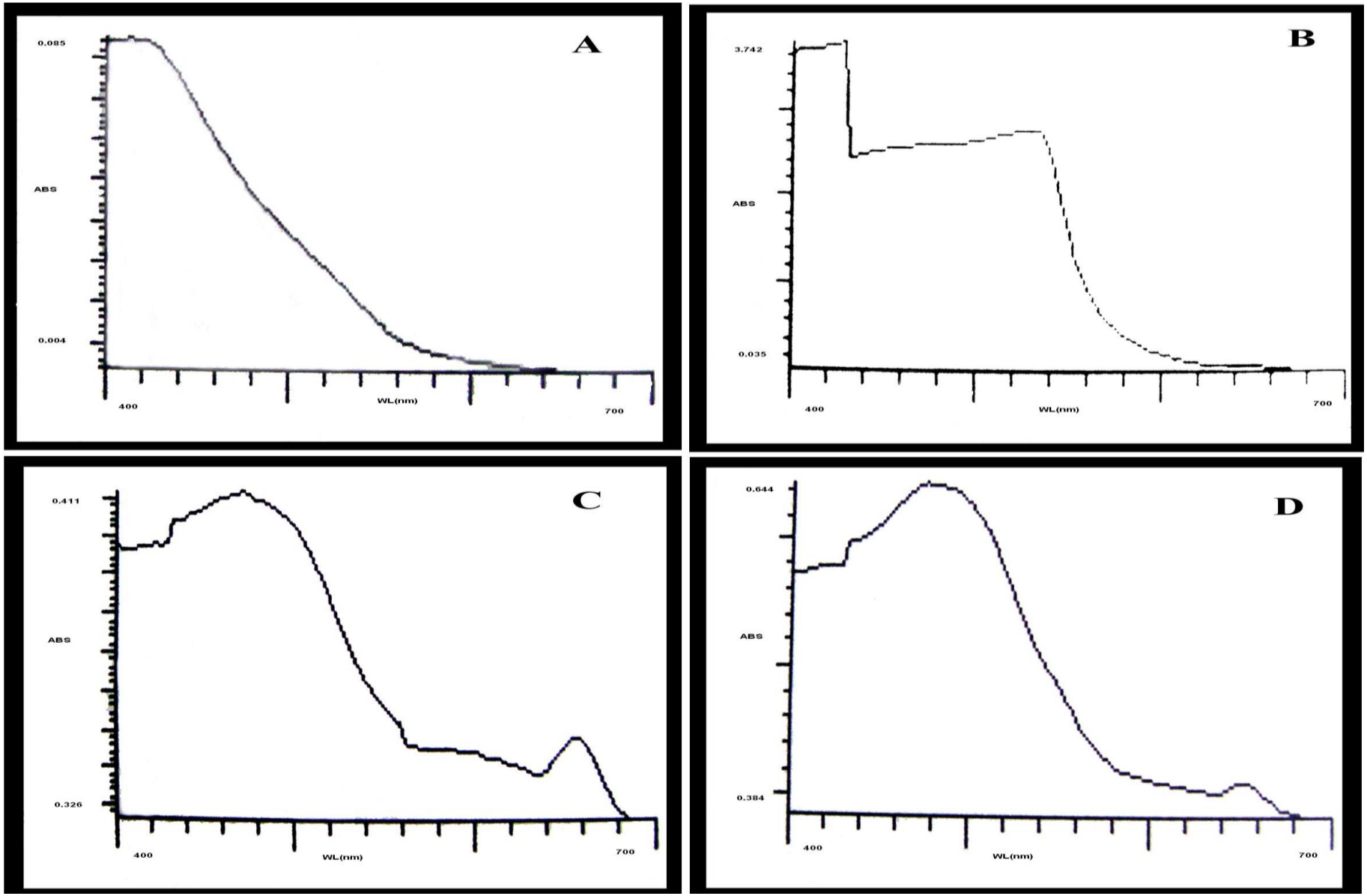


Figure 1. Spectral characteristics of peaks corresponding to Methanol and Acidified methanol extraction, luteolinidin and apigeninidin isolated from red sorghum bran by UV – Visible Spectrophotometer. A – Methanol extraction of Anthocyanin from husk of red sorghum. B – Acidified methanol extraction of Anthocyanin from husk of red sorghum. C – Luteolinidin. D - Apigenindin

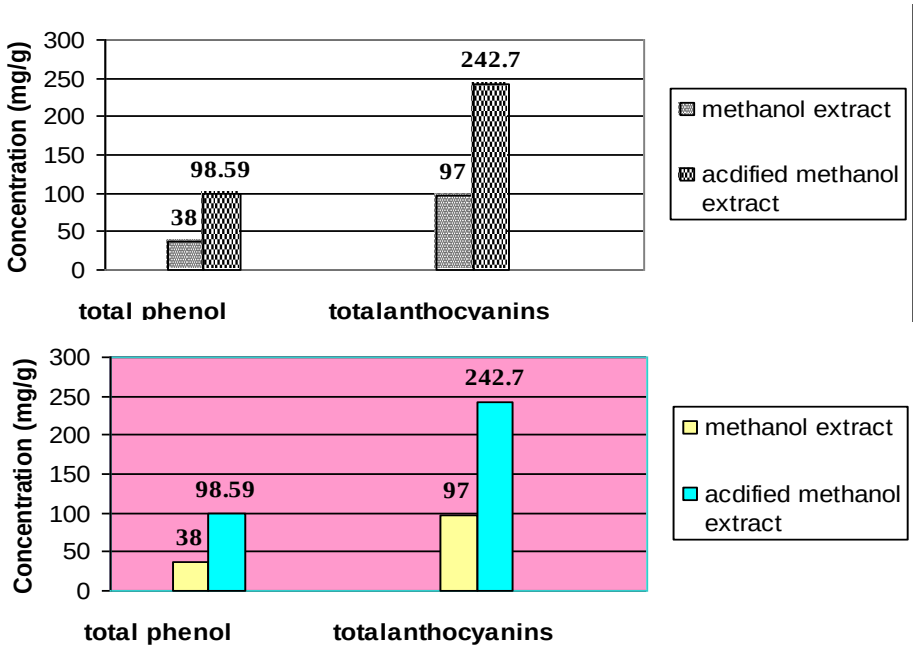
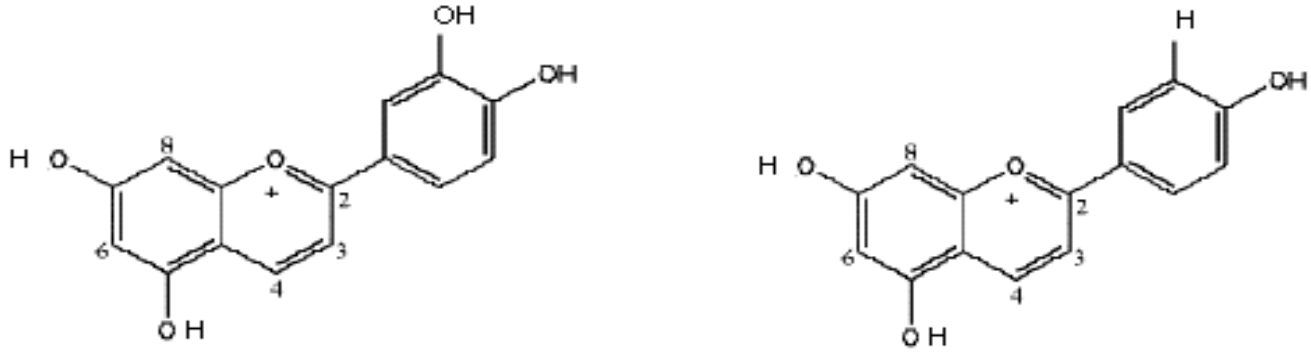


Figure 2. Anthocyanin content from the bran of red sorghum.

The total anthocyanins extracted by acidified methano were as an average of 57% higher than the methanol extracts. Several authors reported that aqueous acetone was better than various alcoholic solvents for fruit procyanidins, anthocyanins and other phenols (Garcia-Viguera et al., 1998; Kallithraka et al., 1995). However, more recently Lu and Foo (2001) observed significant anthocyanins interaction with aqueous acetone to form pyrano-anthocyanidins significantly lowered quantities of detectable anthocyanins. However, since acidified methanol preserves the extracted anthocyanins in their original form better, it should be the solvent of choice for quantification and analysis of anthocyanins. Sorghum brans had on average three to four times the levels of anthocyanins than in grains. Joseph et al. (2004) also reported that sorghum brans were a good source of anthocyanins as sorghum anthocyanins are readily concentrated by decortication. Bruneton (2006) also reported that methanol and acetone and to a lesser extent water and ethanol and their mixture are frequently used for phenolic extraction.

Compound identification

Separation of the methanol and acidified methanol soluble extracts by silica gel in TLC demonstrated the presence of several compounds. Six bands were visualized after the separation in TLC. These pigments represented by bands D and F were the most prominent components of the extracts and the compounds associated with these bands that we attempted to identify. Pigments D and F were eluted from silica gel and subjected to acid hydrolysis with 2 M HCl for up to 2 h at 100°C. Hydrolysates were dried under vacuum and the residue was dissolved in acidified methanol with 0.01% HCl. TLC on silica gel plates demonstrated that hydrolysis did not affect the Rf, indicating that neither D nor F was a glycoside. TLC of eluants of bands D and F with solvents of ethyl acetate/water/formicacid/HCl (85: 8: 6: 1, vol/vol, upper phase) and butanol/acetic acid/water (6:1:2, vol/vol) indicated that only a single compound was present in each band. Fluorescence of compound F but not compound D was quenched when sprayed with Benedict's reagent, indicating the presence of an ortho dihydroxylation (Reznick, 1961). The Rf value of apigeninjdin was found to be 0.76 and the band shows orange – yellow and under UV light the band shows orange only. The Rf value of luteolinidin was found to be 0.19 and the band shows dark – rose and under UV light the band shows dark red. These two anthocyanidins (luteolinidin and apigeninidin) are structurally different from the rest of the anthocyanidins which are commonly found in fruits and vegetables. The common anthocyanidins lack an oxygen molecule at the C-3 position (Figure 3). But the 3-deoxyanthocyanidins, (luteolinidin and apigeninidin) had absorption maximum that were particularly different from those of the other anthocyanidins. The absorption maximum of apigeninidin is 468 nm and luteolinidin is 482 nm in pH 1 buffer solution which also exhibits yellow and orange colour, respectively. This was in contrast with the other anthocyanidins which were all reddish at pH 1. At near neutral pH (in methanol), apigeninidin appeared yellowish



Chemical structure of luteoli **Chemical structure of apigenidin**

Figure 3. Chemical structure of 3-deoxyanthocyanidins, luteolinidin and apigeninidin.

orange in colour and luteolinidin appeared reddish orange in colour. The rest of the anthocyanins ranged from red to dark blue in color at neutral pH. Compounds A, D, E and F exhibited yellow fluorescence when sprayed with alcoholic AICl3, indicating that these compounds were flavonoids (Gauge, 1951).Pigment D exhibited an absorption maximum of 480 nm and pigment F a maximum of 495 - 498 nm in acidified methanol. These absorption maxima and the shapes of the spectra suggested that D and F were the 3-deoxyanocyanidins apigeninidin and luteolinidin, respectively (Staffold, 1965; Harbone, 1958). Compounds D and F exhibited the same spectra and TLC mobilities as authentic apigeninidin and luteolinidin. Also, they exhibited the same retention times as the apigeninidin and luteolinidin standards (26.43 and 23.45 min, respectively) when separated by HPLC on a reversed-phase C18 column (Figure 4). Compound F exhibited a bathochromic shift of 44 - 47 nm in the presence of $AICl_3$ whereas compound D did not, which again indicated ortho dihydroxylation in compound F and that D and F were apigeninidin and leuteolinidin, respectively (Harbone, 1958).

Dependent upon the time when samples were taken, HPLC separations of extracts also gave several minor peaks indicating the presence of other pigments that absorb in the visible range in addition to apigeninidin and luteolinidin (Figure 4). Two of the compounds were identified as the 3-deoxyanthocyanidins apigeninidin (I) and luteolinidin (II). These rare anthocyanidins differ from the common anthocyanidins because they lack the hydroxyl group at carbon-3 of the oxygen heterocyle (C ring) of the flavonoid nucleus. In addition to apigeninidin and luteolinidin there were several as yet unidentified compounds in the husk of red sorghum with the accumulation of apigeninidin and luteolinidin, suggested that the other compounds were precursors of apigeninidin and luteolinidin. Regardless of their origin or route of synthesis it is important to note that compounds A, B, C and E also showed fungitoxicity to *Helminthosporium maydis* and *Collectotrichum graminicola* (Ralph et al., 1987).

At neutral pH, apigeninidin shows yellowish orange and leuteolinidin shows reddish orange. The absorption maximum of apigeninidin in pH 1 was found to be 468 nm and the colour was yellow. The absorption maximum of leuteolinidin was found to be 482 nm and the colour was orange at pH 1. The other compounds were red to dark blue at pH 1. (Table 1)

HPLC analysis

For HPLC analysis of 3 deoxy anthocyanins, separation was carried out on reverse phase C-18 column (Shimadzhu). As a standard the extracts from sorghum mesocotyl (Nicholson et al., 1987) were used. Only one compound was seemed to present in both acidified methanol and methanol extracts of sorghum bran and the compound was identified as luteolinidin, based on their retention time and spectral characteristics relative to the standard compounds (Figure 5).

Marcela et al. (2003) reported the seeds of S*. bicolor* contain significantly high content of Apigenindin and there by suggest that an integrated antioxidant system is triggered during the early stages of sorghum seeds.

The lack of oxygen on C-3 of the 3-deoxyanthocyanidins is thought to give them greater stability in solution compared to the other anthocyanidins (Figure 6) (Iacobucci and Sweeny, 1983; Sweeny and Iacobucci, 1981; Timberlake and Bridle, 1980). For example, Timberlake and Bridle (1980) reported that apigeninidin was stable in pH 2.8 solution for up to 1 year at room temperature and laboratory light, whereas cyanidin degraded within a few hours under similar conditions. Hence the 3-deoxyanthocyanidins may have an advantage over the other anthocyanidins in food applications. Among the anthocyanin standards used, only the 3-deoxyanthocyanidins (apigeninidin and luteolinidin) were identified in acidified methanol extract from sorghum (Figure 3).

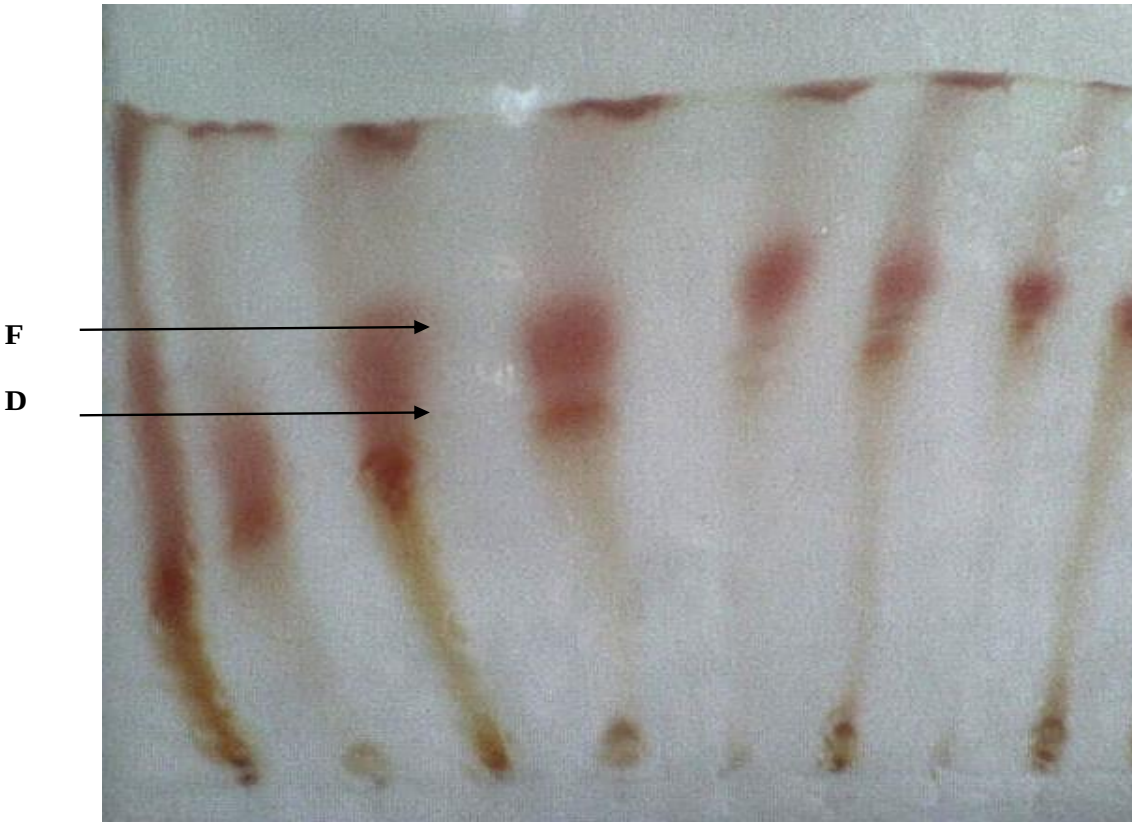


Figure 4. Thin Layer Chromatography of the Anthocyanin extracted from bran of sorghum. The pigment complex was separated on silica gel (2.5 mm) with the upper phase of a mixture of ethyl acetate/water/formic acid/HCl (85: 8: 6: 1,vol/vol). *Bands D and F were composed mainly of apigeninidin and luteolinidin, respectively. Other bands were not identified. Bands A - E fluoresced, whereas band F was a dark red-absorbing area. A,B,C,E – other Flavoinds.

Table 1. Anthocyanin levels in bran of red sorghum brans extracted with methanol and acidified methanol.

Parameters	Methanol extraction	Acidified methanol extraction
Total content (mg/ml)	38 ± 1.1	97 ± 3.4
Anthocyanin (mg/L)	98.59 ± 3.7	242.7 ± 4.5
Stability at variable pH	2.0	5.9
Antioxidant properties (DPPH) µg/ml	0.09 ± 0.01	0.2015 ± 0.01

Each value is expressed as the mean ± standard deviation (n = 6).

Antioxidant properties

The antioxidant activities of the sample extracted in acidified methanol and methanol were compared (Table 2). Samples extracted in acidified methanol had significantly higher antioxidant activity than those extracted in methanol. Even though there are so many methods like enzymatic and non enzymatic methods available to analyze the antioxidant properties, in our study it was analyzed using DPPH method. The DPPH method is widely used to determine antiradical /antioxidant activity of purified phenolic compounds as well as natural plant extracts (Brand-Williams, 1995; Sripriya, 1996; Mahinda, 2000; Peyrat-Maillard, 2000; Fukumoto, 2000). The study cannot be done in enzymatic method because, the samples used for the extraction was dried sorghum bran. The enzymes may be denatured in dried sorghum bran, so as an alternative the antioxidant activity was done by DPPH method.

So, the sorghum brans can be used as a high value source at very lower quantities than other cereal brans as an alternative and can be used to provide higher antioxidant activity in products. Red sorghum bran imparts a natural dark red appealing color normally associated with ‘healthy’ baked goods and was shown to produce acceptable quality bread (Gordon, 2001) and

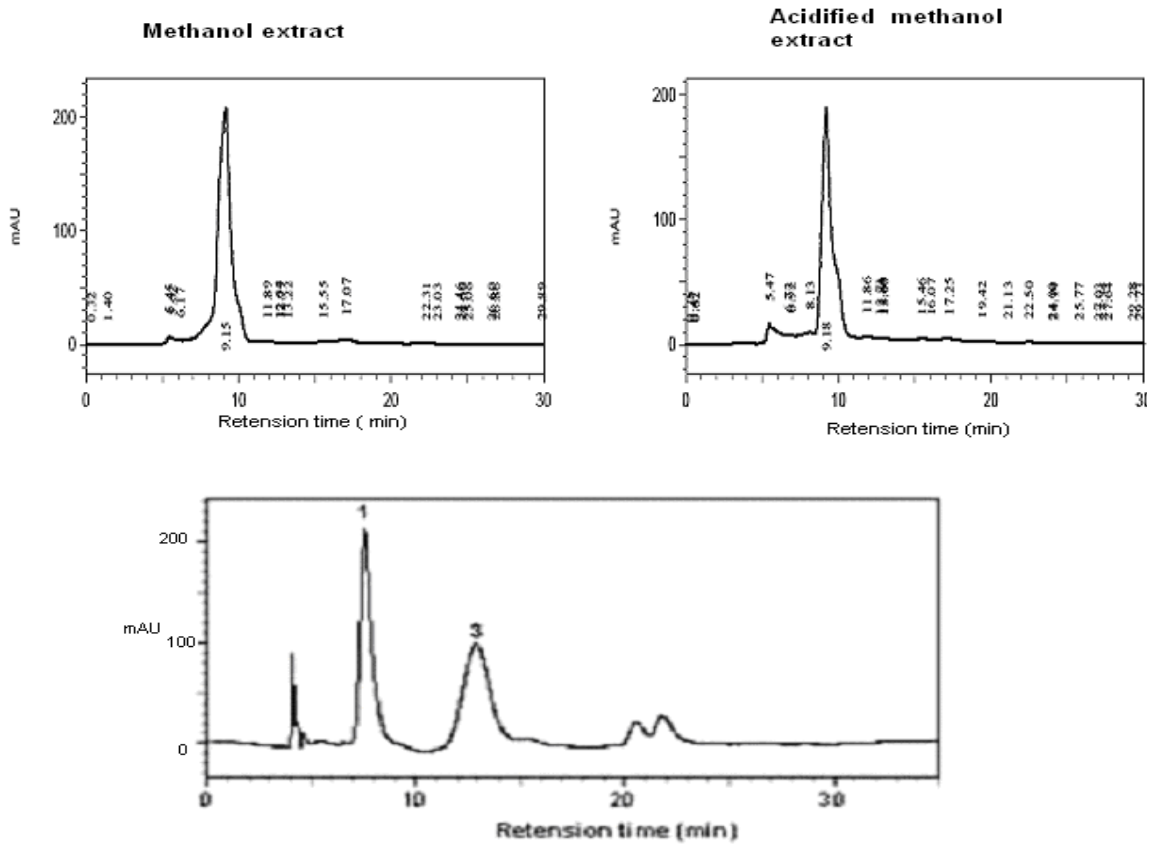
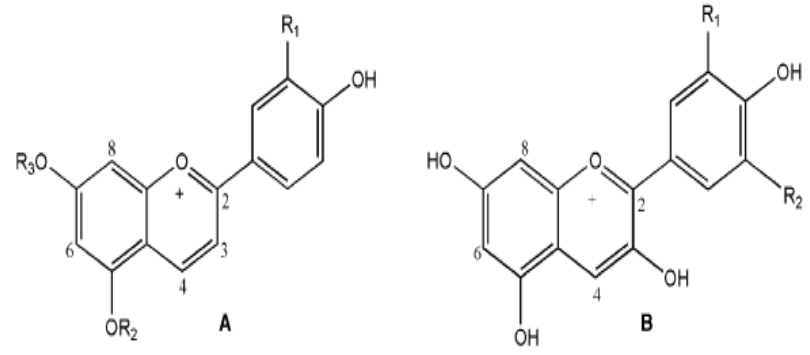


Figure 5. HPLC Profile of Anthocyanin extracted from red sorghum bran. HPLC profile of anthocyanidin pigments from Methanol and acidified methanol extracts, HPLC Chromatogram at 480 nm with a standard extracts from sorgam mesocotyl (R.L. Nicholson *et.al.,* 1987). Solvent A was water: Acetic acid: Methanol (8:1:1) and Solvent B was methanol: Acetic acid (9:1). Samples (20 µl) were injected and eluted isocratically with 40% solvent B at a flow rate of 1 ml min^{-1}. In the standard two peaks was identified and the compound (1 and 3) was identified as luteolinidin and apigeninidin. In the Methanol and Acidified methanol extracts only one compound was identified as luteolinidin, based on their retention time and spectral characteristics relative to the standard compounds.



R_1 = H, R_2 = H, R_3 = H: apigeninidin
R_1 = H, R_2 = Glc, R_3 = H: apigeninidin-5-glucoside
R_1 = H, R_2 = H, R_3 = CH_3: 7-*O*-methyl apigeninidin
R_1 = OH, R_2 = H, R_3 = H: luteolinidin
R_1 = OH, R_2 = Glc, R_3 = H: luteolinidin-5-glucoside
R_1 = OH, R_2 = CH_3, R_3 = H; 5-methoxyluteolinidin

R1 = OH, R2 = H: cyanidin
R_1 = R_2 = H: pelargonidin
R1 = OCH_3, R_2 = H: peonidin
R_1 = R_2 = OCH_3: malvidin
R_1 = R_2 = OH: delphinidin
R_1 = OCH_3, R_2 = OH: petunidin

Figure 6. Chemical structure of anthocyanidins and 3-deoxyanthocyanidins.
A: structure of 3 – deoxyanthocyanidins, B: structure of anthocyanidins

Table 2. Determination of CTC $_{50}$ by using MTT and SRB assay in HT 29 and HEPG2 cell cultures.

Extract	Concentration (µg/ml)	CTC $_{50}$ in (µg/ml)	
		MTT	SRB
Methanol extract of red sorgam on HT 29	500 250 125	384	360
Methanol extract of red sorgam on HEP G2	500 250 125	398	374

Table 3. Observations of apigenindin and leuteolinidin at different pH levels.

Compounds	pH level	nm	Observations
Apigenindin	Neutral pH	-	Yellowish – orange
	pH 1	468	Yellow
Leuteolinidin	Netural pH	-	Reddish – orange
	pH 1	486	Orange

cookies (Mitre-Dieste et al., 2000) at levels of up to 15 and 50%, respectively in black sorghum brans. It was also reported that polyphenolic compounds, flavonoids have the ability to act as antioxidants by a free radical scavenging mechanism, since the sorghum bran had no condensed tannins (Cotelle, 1992; Hanasaki, 1994; Heilmann, 1995; Montensino, 1995).

Anti proliferative assays

The cytotoxity assays were carried out on two human tumor cell lines namely, HT 29 (Human Colon Carcinoma cell line) derived from Human intestinal epithelial cells and HEPG2 (Human Hepatocellular Liver Carcinoma cell line) derived from Hepatocytes.

Determination of CTC $_{50}$ by using MTT and SRB assay in HT 29 and HEPG2 cell cultures

MTT assay

The MTT cell-viability assay produced a dose dependent effect on HT29 and HEP G2 cell lines at 72 h. These absorbance values were converted to percentage cell viability using the formula percentage growth inhibition. The cells were pre-incubated with different concentration of anthocyanin from red sorghum bran using methanol as a solvent (Table 3).

The results are expressed as the percentage of viable cells with respect to the control. The CTC 50 value was calculated as 384 (µg/ml) in HT 29 and 360 (µg/ml) in HEP G2 cell-lines. It has been reported that different methods often yield considerably different values of cytotoxicity (Brenan and Parish, 1988). Because the MTT assay is based on the hydrolysis of MTT by mitochondrial dehydrogenases of living cells resulting in the production of highly colorimetric blue formazan (Mosmann, 1983).

Sulphorhodamine (SRB) cytotoxicity assay

The cells were preincubated with different concentration of anthocyanin from red sorghum bran using methanol as a solvent (Table 3). The results were expressed as the percentage of viable cells with respect to the control. The CTC 50 value was calculated as 398 (µg/ml) in HT 29 and 374(µg/ml) in HEP G2 cell-lines.

The MTT and SRB analysis of anthocyanin extracts showed a moderate effect when subjected to HT 29 and HEP G2 cell line. Thole et al. (2006) reported that the anticancer bioactivity of berries especially against the initiation and promotion stages of carcinogenesis containing phenols like proanthocyanidins and quercetin. So, the anthocyanin extracts from red sorghum bran can be used effectively at the initial stages and promotion stages of carcinogenesis.

The present study demonstrated that anthocyanin inhibits the growth of HT 29 colon cancer / HEP G2 in a concentration dependent manner. Maximum cytotoxity was observed at the concentration of 500 µg/ml after 72 h treatment. In concordance with our results a previous report by (Brenan and Parish, 1988) showed the same

type of inhibitory effect after a 72 h treatment.

It was confirmed that the red sorghum bran contains apigenindin and leuteolindin in the extracts, so the cytotoxity may be due the presence of both of this compounds in the extracts. But, Weiqun et al. (2004) reported that apigenindin has been shown to induce G2/M cell – cycle arrest in human colon cancer cell lines. In their study they also assessed seven selected apigenindin analogs including leuteolidin, on cell cycle, cell number and cell viability in human SW 480 and Calco – 2 colonic carcinoma cells were shown to have higher activity.

It was reported that apigenindin exhibited a significant growth inhibition against human haptoma cells namely Hep G2, Hep 3B cell lines but not in the normal murine live BNLCL 2 cells (Lien–chai et al., 2006). They also further investigated that the cellular mechanism of apigenin effect on Hep G2 cell death. It was shown that the apoptosis induced apigenindin in Hep G2 cells was possibly mediated through the P53 dependent pathway and the induction of P 21 expression, which was probably associated with the cell cycle arrest in G2/M phase.

In summary, the data's reported clearly indicated that the anthocyanin from red sorghum bran can exert significant modulatory effects on cell proliferation, cytotoxicity and oxidative reactions in cellular systems.

Conclusion

In conclusion, red sorghum brans were a very good source of anthocyanins (1.9 - 4.8 mg/g) compared to other anthocyanin sources currently available. The bran of red sorghum anthocyanins were composed largely of the 3-deoxyanthocyanidins,(apigenindin and luteolindin) which are more stable than the anthocyanins mostly found in fruits and vegetables used currently as commercial sources of anthocyanins.

The quantities of anthocyanin from red sorghum bran can be considered as a competitive natural food coloring agent. The antioxidant activity of the sorghum antho-cyanins were similar to those of the anthocyanins found in fruits and vegetables, hence they may offer similar health benefits. The red sorghum brans are superior to other cereal brans as a source of antioxidants. Anthocyanins from red sorghum bran also inhibit the growth of HT 29 and HEP G2 cell lines in a concentration dependent manner. They may provide more health benefits when used in cereal based foods than the current commercial brans. The results obtained have supported the efficacy of natural phenolics from red sorghum bran offering protection against oxidative stress and cytotoxity effects and highlighted the fact that phenolic-rich processed foods may provide health benefits. This may be the first report of anthocyanins from red sorghum bran, which acts as a source of anthocyanin with antioxidant and anticancer properties.

Relative oxygen species (ROS) have been associated with carcinogenesis, coronary heart disease and many other health issues related to advancing age (Steer et al., 2002; Uchida, 2000). To avoid the ROS associated problems in humans the intake of foods rich in antioxidant compounds can be recommended due to their well known healthy effects.

ACKNOWLEDGEMENTS

The author would like to thank Dr. Ganesh kumar, Research Associate, Sugarcane Breeding Institute, Coimbatore and also we like to thank our staff members Dr. A. Arunkumar, Dr. K.M. Aravinthan, R. Kavithakrishna, Mrs K. Latha and the Management of Nallamuthu Gounder Mahalingam College of Arts and Science, Pollachi, Tamilnadu for their kind cooperation to complete this work.

Abbreviations: HPLC, High pressure liquid chromatography; **TLC,** thin layer chromatography; **HT 29,** human colon carcinoma cell line; **HEP G2,** human liver carcinoma cell line, **DPPH,** 2,2-Diphenyl-1-picryhydrazyl; **DMEM,** Dulbecco's modified eagles medium.

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