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Antiproliferative and antioxidant activity of anthocyanins from red sorghum (*Sorghum bicolor*) bran

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ABSTRACT

Anthocyanins are natural pigments used as food additives and known for its potent antioxidant and antiproliferative property. So this study aims to evaluate anthocyanin content, to analyse antioxidant activity and antipropliferative from red sorghum bran. When acidified methanol was used as a solvent for extraction maximum amount of anthocyanin was obtained when compared with methanol alone as a solvent. On separation by TLC and HPLC, the compounds were identified as apigenindin and luteolindin. The antioxidant activity was also found to be higher in red sorgam bran. Anthocyanin from red sorgam bran also showed moderate cytotoxic activity against HT 29 and HEP G2 cell lines. However, it is assumed that the antioxidant and antiproliferative activity of the extract from red sorgam 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.

KEYWORDS

Husk;
Sorghum;
Anthocyanins;
Antioxidant activity;
HPLC;
DPPH;
HT 29 cancer cell line;
HEP G2 cancer cell line.

INTRODUCTION

Anthocyanins are becoming increasingly important not only as food colorants, but also as antioxidants. Anthocyanins are reported to have some therapeutic benefits including vasoprotective and anti-inflammatory properties, anti-cancer and chemoprotective properties, as well as anti-neoplastic properties. Anthocyanins are therefore, considered to contribute significantly to the beneficial effects of consuming fruits and vegetables. There is a rising demand for natural sources of food colorants with nutraceutical benefits and alternative sources of natural anthocyanins are becoming increasingly important.

Specially sorghums contain significant levels of anthocyanins and other phenols concentrated in their brans[5]. Black sorghum was reported to have significantly more anthocyanin pigments than other sorghums[5]. Therefore, this sorghum has a good potential for commercial exploitation. Anthocyanins have been extensively studied in fruits and vegetables. Limited data exists on the types and levels of anthocyanins in cereals, probably because they have never been regarded as a commercially significant source. Nip and Burns were able to isolate and identify apigeninidin, apigeninidin-5-glucoside, luteolinidin and luteolinidin-5-glucoside in red and white sorghum varieties by paper chromatography.

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Sorghum grain is an important staple food in developing countries of the semiarid 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[41]. Besides the antioxidant enzymes, sorghum seeds are provided with antioxidant substances that are able to scavenger radical products. These compounds include lipid-soluble products, such as tocopherols and, water-soluble substances such as ascorbic acid and thiols. On the other hand, the chemical components of the sorghum grain cuticle are flavonoids, anthocyanidins and tannins[31]. Rey *et al.*[45] have identified apigeninidin as a major anthocyanidin present in sorghum. Regarding its physiological function, apigeninidin showed a high fungicidal activity in sorghum[1], and it was reported that apigeninidin effectively quenched ascorbyl radical and lipid radicals when supplemented with doses up to 200 µg/ ml[8].

Gous also reported luteolinidin and apigeninidin as the major anthocyanidins from a black sorghum variety. Cyanidin and pelargonidin were also reported in corn[16], and sorghum. The most common anthocyanins in sorghum are the 3-deoxyanthocyanidins[33], which comprise luteolinidin and apigeninidin.These anthocyanins have a small distribution in nature, 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[33]. 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 commercial source for anthocyanins. 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[22,32,35] and cereals have been reported. However, there is no agreement on which solvent extracts 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.

Commercially prepared grape (*Vitis vinifera*), bilberry (*Vaccinium myrtillus* L.), and chokeberry (*Aronia meloncarpa* E.), anthocyanin-rich extracts (AREs) inhibited the growth of HT29 cells as compared to non tumorigenic colon cells.

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

EXPERIMENTAL

Samples

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

Sample extraction

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

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 anthocyanin samples were quantified using Folin-Ciocalteu's method[48]. 25 µl of Folin- Ciocalteau's reagent (50%, v/v) was added to 10 µl of extract. 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. Blanks were prepared by replacing the reagent by water to correct for interfering compounds. After 30 min of incubation, the absorbance read at 760 nm using UV /

VIS spectrophotometer (Genesys 5).

Stability at variable pH

The stability of the compound was tested by treating about 1ml of sample with 1ml of Sodium acetate at pH 1.0 and Potassium chloride at pH 4.5. The colour change was observed[52].

Determination of total anthocyanins

The pH differential method as reported by Fuleki and Francis[19], Guisti[24] and Wrolstad[60] 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 (Genesys 5, Technical trade links pvt.ltd) 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[19]. The total Anthocyanin content and their absorbance maxima was 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/HCI (85:8:6:1,vol/vol)[54]. Compounds were detected under ultraviolet light (366 nm) before and after exposing the plates to ammonia fumes[36] and by spraying the plates with aqueous 2% $FeCl_3$[25]. The silica gel containing individual bands of compounds was scraped from plates. Compounds were eluted from the gel with the original TLC solvent by centrifugation (Bioanalytical Systems, West Lafayette, IN). The samples were then reduced to dryness under vacuum and resuspended in HPLC-grade methanol for use in bioassays and for compound identification. For further characterization some samples were spotted again on silica gel TLC plates and the plates were sprayed with 1% $AlCl_3$ in ethanol[21] and Benedict's reagent[46].

HPLC analysis

For HPLC analysis of anthocyanins, separation was carried out on reverse phase C-18 column (Shimadzhu). 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}. 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 480nm for the presence of 3- deoxy anthocyanidin.

Antioxidant properties

The antioxidant properties were analysed using DPPH method. The DPPH is a stable free radical with an absorption band at 515 nm. It loses this absorption when reduced by an antioxidant or a free radical species. The DPPH method is widely used to determine antiradical /antioxidant activity of purified phenolic compounds as well as natural plant extracts[9,20,37,43,53]. Bondet *et al*.,[7] found that most phenolic antioxidants react slowly with DPPH, reaching a steady state in 1-6h or longer. This suggests that antioxidant activity using DPPH should be evaluated over time. The method also has good repeatability and is used frequently. Also, color interference of DPPH with samples that contain anthocyanins leads to underestimation of antioxidant activity[2].

DPPH free radical-scavenging assay

The extracted sample were assayed for free radical scavenging activity by the DPPH assay. The procedure used is an adaptation of those previously described[51,61]. 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 were 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 % Inhibition = $[(C - S/C)] \times$

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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

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). 10ml 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 ($0.5\text{-}1.0x10^5$ 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.

Determination of mitochondrial synthesis by microculture tetrazolium (MTT) assay[39]

This is a colorimetric assay that reduction of yellow 3-(4,5 – dimethylthiazol – 2 – yl) – 2,5 – diphenyl tetrazolium bromide (MTT) by succinate dehydrogenase. The MTT enters into the cells and passes into the mitochondria where it is reduced to an insoluble, coloured (dark purple) formazan product. The cells were then solubilised with an organic product (eg isopropanol) and solubilised formazan product is measured spectrophotometrically. The reduction of MTT level in the assay can occur only if the cells are viable. So the viability of the cells indicates the level of activity is measured based on the viability of the cells. In the MTT assay the number of viable cells was found to be proportional to the extent of formazan production.

The percentage growth inhibition of the cell was calculated using the formula below:

$$\text{\% Growth Inhibition} = 100 - \left[\frac{\text{Mean OD of Individual Test Group}}{\text{Mean OD of Control Group}}\right] \times 100$$

Determination of total cell protein content by sulphorhodamine B (SRB) assay

SRB assay is used to determine the total cell protein content. The principle is based on the ability of the protein protein dye sulforhodamine B to bind electrostatically with basic aminacids residues of the protein of TCA fixed cells. Since the assay is pH dependent, under mild acidic conditions it binds to protein and under mild basic conditions it can be extracted from cells and solubilized for measurements. Results of the SRB assay were linear with cell number and cellular protein measured at cellular densities. The SRB posses a colorimetric end point and is nondestructive and indefinitely stable. This practical point and is nondestructive and indefinitely stable. These practical advances make the SRB assay an appropriate and sensitive assay to determine the total cell content of the cell lines. Colour development in SRB assay is rapid, stable and visible. The developed colour can be measured over a broad range of visible wavelength in either a spectrophotometer or a 96 well plate reader. When TCA-fixed and SRB stained samples are air-dried, they can be stored indefinitely without deterioration.

The percentage growth inhibition was calculated using the formula below:

$$\text{\% Growth Inhibition} = 100 - \left[\frac{\text{Mean OD of Individual Test Group}}{\text{Mean OD of Control Group}}\right] \times 100$$

RESULTS AND DISCUSSION

Anthocyanin extraction and quantification

The extraction of anthocyanin from red sorgam bran was done in methanol and acidified methanol. A Spectrum of the extracts, the peak in the visible region was recorded at 400 nm in Spectrophotometer (Genesys 5), where a single peak was observed in methanol extract but in acidified methanol extract three peaks were observed and the Absorbance was also high in acidified methanol extract (3.742) of sorgam bran compared with methanol extract (0.085)(Figure 1). .[59]. Because Joseph *et al.*, in 2004 have already stated that the acidified methanol preserves the ex-

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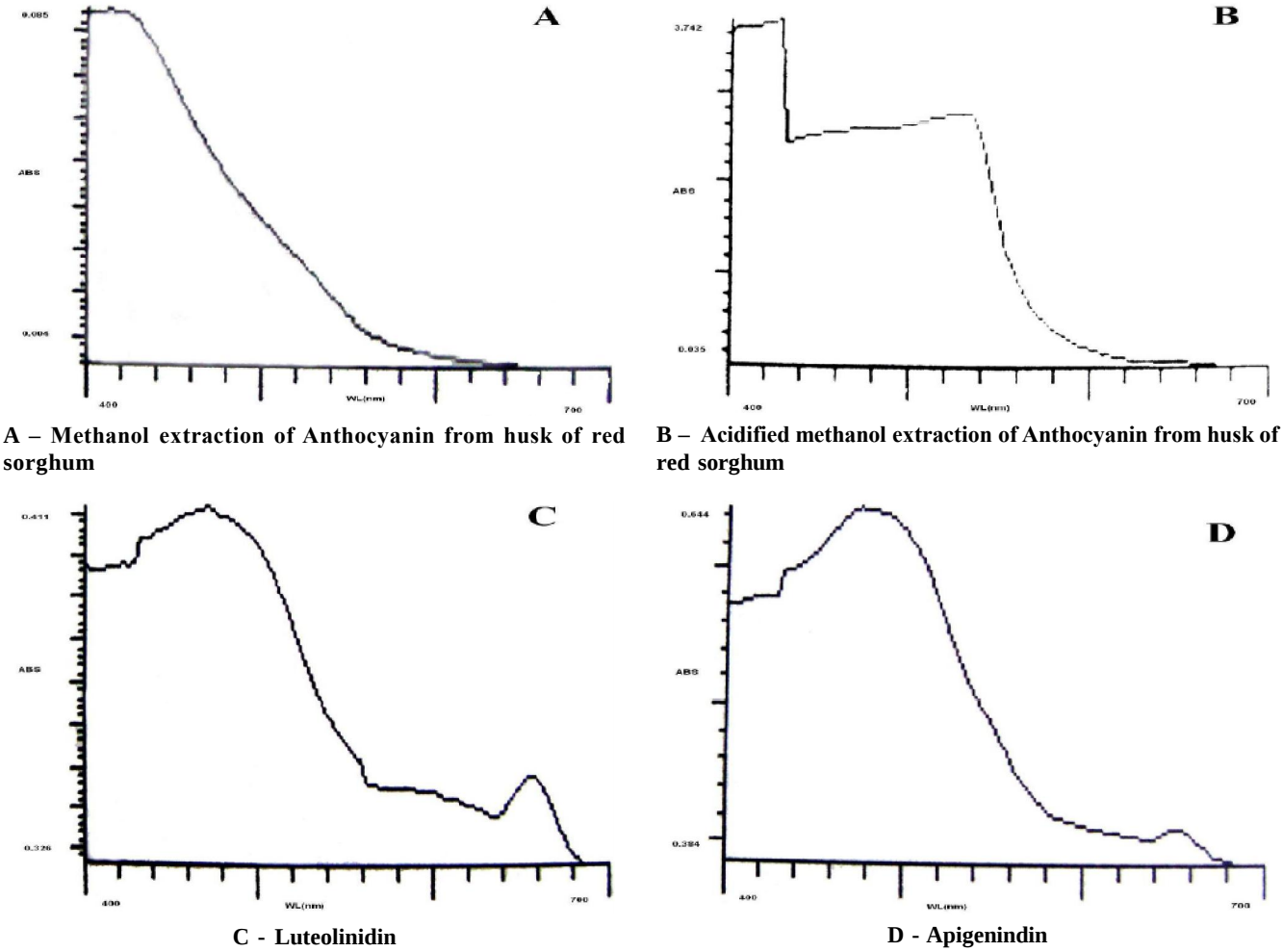
tracted anthocyanins in their original form for long duration so that the acidified methanol is most preffered solvent system used in this study for quantification and analysis of anthocyanins.

PLATE I



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 1 : Spectral characteristics of peaks corresponding to methanol and acidified methanol extraction, luteolinidin and apigeninidin isolated from red sorghum bran by UV – Visible spectrophotometer.

Flavanoid confirmation test

The anthocyanin was extracted from the red sorgam bran using acidified methanol and methanol solvent extracts systems respectively, shows brown colour in the presence of flavanoid. Recent studies have shown that many flavonoids and related polyphenols contribute significantly to the total antioxidant activity of many fruits and vegetables.

Total phenolic assay

The total content of phenols in the methanol extract from the bran of red sorgam was 38 mg/ml and the phenol content in acidified methanol extract was 97 mg/ml (Figure 2). Sene *et al.*, 2001[49] reported that nitrogen nutrition and environmental factors that promote

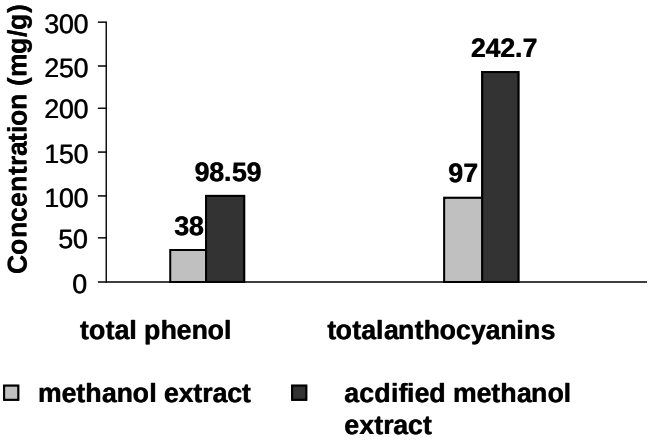


Figure 2 : Anthocyanin content from the bran of red sorghum

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growth and grain yield also enhances phenol synthesis in sorghum. Polyphenols are used to represent anticarcinogenic food components in a human diet rich in fruits and vegetables.

Stability at variable pH

The sample appeared red colored at pH 1.0 and colour disappeared at a pH 4.5. The results were found to be same in both the solvents used for extraction. Anthocyanin was found to be stable in low pH[24].

Determination of total anthocyanins

The total content of Anthocyanin in methanol extract of red sorgam bran was 98.59 mg / L and 242.7 mg / L in methanol and acidified methanol extracts respectively (Figure 2). Acidified methanol extract resulted, insignificantly higher values for the total anthocyanins than methanol extract. The total anthocyanins extracted by acidified methanol extracts were on 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[22,32]. However, more recently Lu and Foo (2001)[35] observed significant anthocyanins interaction with aqueous acetone to form pyrano-anthocyanidins insignificantly 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 in grains. Joseph *et.al*., (2004)[4] also reported that sorgam brans were a good source of anthocyanins as sorghum anthocyanins are readily concentrated by decortication. This implies that acidified methanol is more powerful solvent than methanol for extracting red sorgam antioxidants. Bruneton, 2006[11] also reported that methanol and acetone, and to a lesser extent water and ethanol and their mixture are frequently used for phenolic extraction.

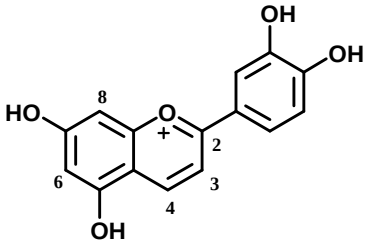
Red sorghum brans were a very good source of anthocyanins (1.9 – 4.8 mg/g) relative to the commercial sources currently available (0.2–10 mg/g). This coupled with the fact that they possess mostly the relatively stable 3-deoxyanthocyanidins gives them an edge over the fruits and vegetables as a source of natural anthocyanins. The sorghum bran anthocyanin has an additional advantage in terms of storage stability relative to the fruits and vegetables. Sorghum is harvested at low moisture (13 – 15%) compared to fruits and vegetables which normally have >80% moisture and require significant time and energy for drying to improve their storage stability. Because of this the red sorghum bran can be a competitive source of anthocyanins also.

Monomeric anthocyanins contributed an average of 30– 50% of the anthocyanins in the sorghums. This implies the larger part of sorghum anthocyanins are polymerized or complexed with other compounds and are not readily separated by the extraction conditions used. Such complexed anthocyanins usually have better color stability in solution than their monomeric constituents[12,16,57].

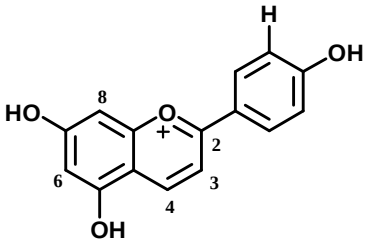
Compound identification

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 it was 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 hr 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/formic acid/HCI (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[46]. 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. 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 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[21].



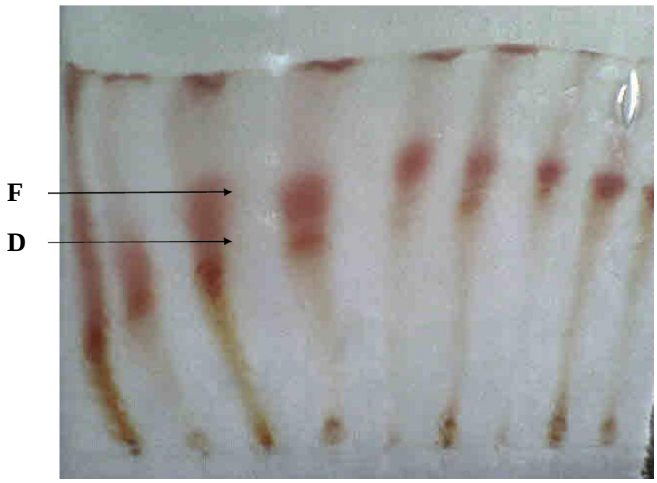
Chemical structure of luteolinidin



Chemical structure of apigenidin

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

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[26,54]. 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 min 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 $AICI_3$ whereas compound D did not, which again indicated ortho dihydroxylation in compound F and that D and F were apigeninidin and leuteolinidin, respectively[26].



The pigment complex was separated on silica gel (2.5mm) with the upper phase of a mixture of ethyl acetate/water/formic acid/ HCI (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

Figure 4 : Thin layer chromatography of the anthocyanin extracted from bran of sorghum

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 sorgam 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 *H. maydis* and *C. graminicola*.[47].

At neutral pH, apigeninidin shows yellowish orange and leuteolinidin shows reddish orange. The absorp-

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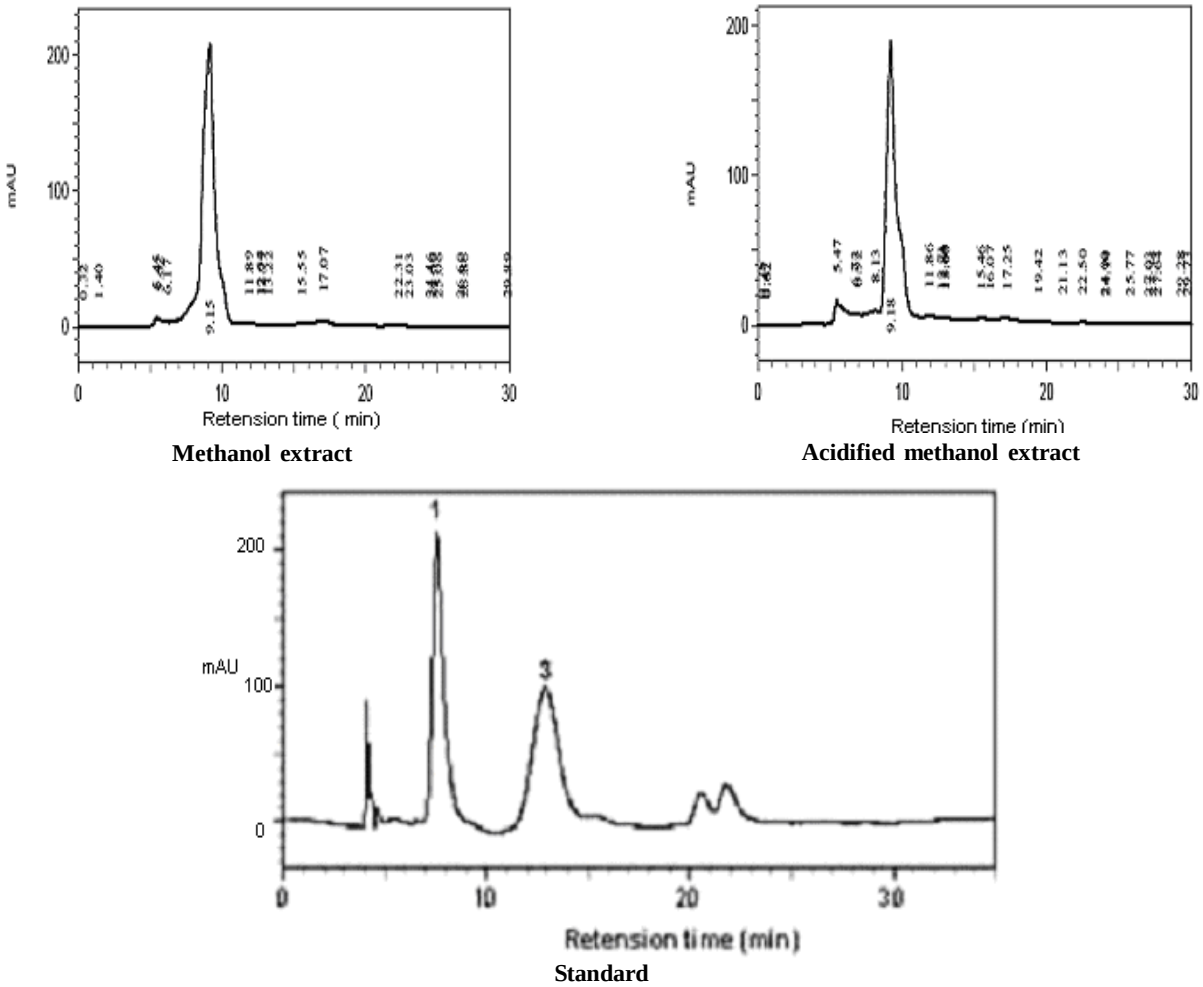
tion maximum of apigeninidin in pH 1 was found to be 468 nm and the colour was yellow. The absorption maximum of leuteolinidin was found to be 482 nm and the colour was orange at pH 1. The other compounds were red to dark blue at pH 1.

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 sorgam mesocotyl[47] were used. Only one compound was seemed to present in both acidified methanol and methanol extracts of sorgam 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*orghum 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 anthocya-nidins[33,57]. For example, Timberlake and Bridle (1980)[57] reported that apigeninidin was stable in pH 2.8 solution for up to 1 year at room temperature and laboratory light, whereas



HPLC profile of anthocyanidin pigments from methanol and acidified methanol extracts, HPLC chromatogram at 480 nm with a standard extracts from sorgam mesocotyl[47]. 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.

Figure 5 : HPLC profile of anthocyanin extracted from red sorghum bran.

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).

Antioxidant properties

The antioxidant activities of the sample extracted in acidified methanol and methanol were compared (TABLE 1). Samples extracted in acidified methanol had significantly higher antioxidant activity than those extracted in methanol. So, the sorgam 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 sorgam bran imparts a natural dark red appealing color normally associated with 'healthy' baked goods, and was shown to produce acceptable quality bread[23] and cookies at levels of up to 15% and 50%, respectively in black sorgam 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.[13,28,29,40].

TABLE 1 : Anthocyanin levels in bran of red sorghum brans extracted with methanol and acidified methanol

Sr. No	Parameters	Methanol extraction	Acidified Methanol Extraction
1.	Total content (mg/ml)	38	97
2.	Anthocyanin (mg/L)	98.59	242.7
3.	Stability at variable pH	2.0	5.9
4.	Antioxidant properties (DPPH) μg/ml	0.09	0.2015

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[30]. Afaq *et.al.*, 1991 reported that flavoids are well - known anti oxidants and free radical scanvengers. High potential of polyphenols to scavenge free radials may be because of their many phenolic hydroxyl groups. Similarly in our results the high antioxidant activity was due the presence of high phenol content. Fratianni *et.al.* (2007)[17] found that apigenindin have diverse pharmacological activities and has demonstrated antioxidant and anticarcinogenic properties. In addition, Raj & shalini, 1999 and Badami *et. al.*, 2003[6] also reported a large number of flavanoids including apigenin and luteolin are known to possess strong antioxidant properties.

Phenolic compound plays a major role in antioxidant activity. Potterat 1997[44] 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[42] 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.

The sorgam grains and brans had significantly higher phenols and antioxidant activity than other cereal brans like wheat, barley, buck wheat and rice, among others, are promoted as good source of antioxidants.[15,27]. Such brans are sold in the market for use in fortified baked products. Black sorghum bran offers a major advantage in terms of antioxidant value per unit weight. 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.

Anti proliferative assays

Cancer cells usually exist under heavy oxidative stress state, Shinkai *et.al.*, (1986)[50] 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[3,14]. Therefore, foods rich in antioxidant phytochemicals are important for the prevention of diseases related to oxidative stress such as heart disease and cancer. In this study our main aim is to identify the potential of anthocyanin extraction from red sorgam for its anticancer property against cancer cell lines. This property is evaluated through investiga-

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tions on antioxidants and viable cell count. 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

Cytotoxicity was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide(MTT, Sigma) assay reported by Mosmann (1983)[39]. MTT analysis has been developed for evaluating cell-mediated cytotoxicity. The assay was performed in HT29 and HEP G2 and reduction of yellow tetrazolium salt to a purple intracellular formazan by active mitochondria, was spectrophotometrically detected. The MTT cell-viability assay produced a dosedependent 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 preincubated with different concentration of anthocyanin from red sorgam bran using methanol as a solvent (TABLE 2).

TABLE 2 : Determination of CTC_{50} by using MTT and SRB assay in HT 29 and HEPG2 cell cultures

Extract	Concentration in (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

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. 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[39].

Sulphorhodamine (SRB) cytotoxicity assay

SRB analysis has been performed according to Mitry *et al.* (2000), this assay is mainly to determine cell survival using HT29 and HEP G2. The cell-mediated cytotoxicity assay was performed in HT29 and HEP G2 cell lines at 72 h in a dosedependent manner. These absorbances were converted to percent cell viability. These absorbance values were converted to percent cell viability using the formula percentage growth inhibition. The cells were preincubated with different concentration of anthocyanin from red sorgam bran using methanol as a solvent (TABLE 2).

The results are 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 sorgam bran can be used effectively at the initial stages and promotion stages of carcinogenesis.

A very large number of plant extracts have been screened for cytotoxic effects against cancer cell lines over the last twenty-five years and have resulted in some significant drugs being introduced, paclitaxel probably taking pride of place. In addition, the traditional use of a considerable number of plants for cancer has been justified to some extent by the findings that have shown that their extracts are cytotoxic, especially if selectivity is demonstrated, either between different cancer cell lines or between cancer and non-cancer cell lines.

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. Besides in our studies it was confirmed by HPLC and TLC analysis that the anthocyanin extract contains apigenindin and leutolindinin in higher concentration. Frantianni *et.al.*, 2007[17] found that apigenindin have diverse pharmacological activities and has demonstrated antioxidant and anticarcinogenic properties.

So, the purified apigenindin and leutolindinin may give a good result for cytotoxity assay. 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.

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 showed the same type of inhibitory effect after a 72 h treatment.

Ferguston *et.al*., 2004[18] reported that flavanoid rich extracts from cranberry showed *invitro* antiproliferative activity against HT 29,SW 620 colon cancer cell lines and also implicated that pro anthocyanidin contributing for this activity. Simililary in our studies it was confirmed that red sorghum bran is also a flavonid rich source, so the cytotoxic activity may be due to the flavanoid.

In our studies, it is showed that the presence of phenol in the red sorghum bran extract, which also showed antiproliferative effects against cancer cell lines. Polyphenols are used to represent anticarcinogenic food components in a human diet rich in fruits and vegetables[3]. This indicates that anthocyanins were rapidly absorbed by cells and contributed to the cellular antioxidant defenses.

Anthocyanin consists of apigenindin and leuteolindin is a phenolic compound that moves into the cytosol through the plasma membrane. This phenolic compound inhibits carcinogensis, and has been demonstrated to inhibit the proliferation of Human cancer cell lines.

Maria Claudia *et. al*., 2004[38] reported that anthocyanins are promising substances for reducing cancer risk because of their antiproliferative potential and their apoptotic effects specifically in cancer cells. However, pre-clinical studies using appropriate *in vivo* animal models as well as carefully designed pharmacokinetics studies are needed before clinical testing of anthocyanins as cancer preventive or therapeutic agents.

It was confirmed that the red sorgam 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 was 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. And 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 datas we reported, have clearly indicated that the anthocyanin from red sorgam 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 & 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 anthocyanins 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 sorgam 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

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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.

A general recommendation to the public is to increase the intake of foods rich in antioxidant compounds due to their well known healthy effects. ROS have been associated with carcinogenesis, coronary heart disease and many other health issues related to advancing age[55,58].

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