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Research Article

Anthocyanin analysis and its Anticancer Property from Sugarcane (*Saccharum Officinarum* L) Peel

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ABSTRACT

Sugarcane peel is a renewable raw material rich indifferent molecular species of antioxidant. To utilize this source as a raw material an environmentally sustainable procedure has been developed for the extraction of anthocyanin and to analyze its anticancer properties .sugarcane peel (*Saccharum Officinarum)*were studied for their total phenol content ,total flavanoid content, total anthocyanin content content, antioxidant and antiproliferative activities. The anthocyanin and phenolic content were found to be high in sugarcane peel. The data represented in this study demonstrated that the amount of phenolic compound and anthocyanin content of sugarcane peel, and detemined the antiprolifertive activity of sugarcane peel by cytotoxicity assay which shows 51.2% inhibition HT29 cell line at concentration of 0.625µg/ml .these results suggest thet sugarcane peel anthocyanins may reduce the risk of colon cancer.

Keywords: Sugarcane peel, Anthocyanin, Antioxidants, Anticancer & Colon HT29 Cell Line.

1.INTRODUCTION

Epidemiological and experimental evidence has consistently shown that diets rich in fruits and vegetables decrease risk of colon cancer, and there is considerable interest in identifying the compounds in fruits and vegetables responsible for this protection. Sugarcane is one of the world's most important sugar crops', providing over 76% of sugar for human consumption. India is the second largest producer of sugar in the world (Hemlatha Chauhan *et al*., 2010). Brazil accounts for a large part of the world production, with current national figures exceeding 375 million ton annually, most of it intended for sugar and alcohol production (Agrianual, 2006). Sugarcane has flavonoids like apigenin and luteoledin. Both the roots and stems of sugarcane are used in ayurvedic medicine to treat skin and urinary tract infections as well as for bronchitis, heart conditions, and loss of milk production, cough and anemia. Phenolic compounds in sugarcane juice are partially responsible for its color. The major flavonoids in sugarcane are flavones, such as naringenin, tricin, apigenin and luteolin derivatives (William *et al*., 1974, Smith and Paton, 1985).

Anthocyanins are glycosides of anthocyanidins universally associated with attractive, colorful, and flavorful fruits. Recently, there has been a resurgence of interest in anthocyanins due to their potential biological and pharmacology benefit, such as antioxidant(Moyer et al., 2002), Anti-inflammatory (Subarnas and Wagner, 2000), reducing the risk of cardiovascular diseases (Wang et al.,1999; Seeram et al., 2001a,b). The antioxidant property of anthocyanins and anthocyanidins suggested that they played an important role in the prevention and carcinogenesis (Omenn, 1995). The anthocyanins in purple colored sweet potato and red cabbage suppressed Colon Carcinogenesis induced by 1,2 dimethylhydrzine (DMH) and 2-amino-1-methyl-6-phenylimidazo(4,5-b)pyridine in rats (Hagiwara et al.,2002). Although anthocyanins present in fruits and vegetables are known for their health benefits. Anthocyanins are naturally occurring flavonoids responsible for the intense red to purple to blue color of most flowers, fruits and vegetables they are popular as colorants in the food industry and used as a natural alternative to the use of artificial dyes (Giusti and Wrolstad, 2003). Anthocyanins from various sources have demonstrated anticancer and antitumor activity .growth inhibition of human colon cancer cell lines HT-29(Zhoa et al., 2004),(Kamei et al., 1998)&(Kang, et al., 2003).

2. OBJECTIVE

The aim of the study is to determine the anthocyanin content in sugarcane (Saccharum officinarum) peel using solvent system and analyze and to evaluate the anticancer activity of anthocyanins from sugarcane peel against human colon cancer.

3. MATERIALS AND METHODS

3.1. SAMPLE COLLECTION

Sugarcane (*Saccharum officinarum* L.) was collected from the field at Coimbatore in Tamil Nadu, India and stored at -20°C.

3.2. CHEMICALS

Sodium hydroxide (NaOH), hydrochloric acid (HCl), sodium carbonate (Na_2CO_3), Aluminium chloride ($AlCl_3$), Ferric chloride ($FeCl_3$), Butylatedhydroxy toluene (BHT), methanol, MTT (3-(4,5- Dimethyl thiazo-2yl)-2,5-Diphenyl Tetrazolium bromide), phosphate buffer saline (PBS), Di methyl sulfoxide (DMSO).

3.3. EXTRACTION OF ANTHOCYANIN

The peel of sugarcane (*Saccharum Officinarum* L) were extracted by an incubation with 1% HCl in methanol over night at room temperature followed by a filtration through What man filter paper no.4. Acidified methanol was removed by a rotary evaporation under 45 □ C and the pigmented fraction extracts were stored for further study. (Lachman *et al.,* 2003).

3.4. Analytical procedures

3.4.1 Flavanoid confirmation test (Harbone-1998)

A. $FeCl_3$

1 ml of sample extraction was added with a small amount of FeCl3**,** and results were observed.

B. $AlCl_3$

1 ml of sample extraction was added with 5% of $AlCl_3$ solution, and results were observed.

3.4.2 Confirmative test for anthocyanin

A. 2M HCl

1 ml of sample extraction was added with 2ml of HCl for 5 mins at 100 □ C, and results were observed.

B.2M NaOH

1 ml of sample extraction was added with 2ml of NaOH, and results were observed.

3.4.3. Total Phenolics assay

Total phenolics compounds in anthocyanin samples were quantified by using Folin-ciocalteu's method described by Ronald *et. al* (1998). 50 µl of Folin-ciocalteu's reagent (50% v/v) were added to 10µl of sample extract. It was incubated for 5 min. After incubation 50µl of 20 % (w/v) sodium carbonate and water was added to final volume of 400 µl. Blank was prepared by replacing the reagent by water to correct for interfering compounds. After 30 min of incubation, the absorbance was measured using spectrophotometer at 760 nm.

3.4.4. Total Flavonoid content

The flavonoid content was determined according as the aluminum chloride colorimetric method described by Chang, Yang and Chern (2002). Briefly, aliquots of 0.1g of sugarcane peel sample were dissolved in 1 ml of deionized water. This solution (0.5 ml) was mixed with 1.5 ml of 95% alcohol, 0.1 ml of 10 % aluminium chloride hex hydrate ($AlCl_3$), 0.1 ml of 1 M Potassium acetate and 2.8 ml of deionized water. After incubation at room temperature for 40 min, the reaction mixture absorbance was measured at 415 nm against a deionized water blank on a spectrophotometer. Quercetin was used as a standard. Using a seven point standard curve (0-50mg/l), the levels of total flavonoid contents in onion peel was determined in triplicate, respectively. The data was expressed as milligram Quercetin equivalents (QE)/100 g fresh matter from fresh the sugarcane peel.

3.4.5. Determination of total anthocyanin.

The total amount of anthocyanin content was determined by using p^H differential method. A spectrophotometer was used for the spectral measurements at 210 nm and 750 nm. (Fuleki & Francis, 1968). The absorbance of the samples (A) was calculated as follows:

A= (Absorbance λ vis-max-A750) pH 1.0-(Absorbance λ vis-max-A750) p^H 4.5 Anthocyanin pigment content (mg/liter) = (A X MW X DF X 1000)/ (ε X 1).

Where, Molecular weight of anthocyanin (cyd-3-glu) = 449 Extraction coefficient (ε) =29,600 DF=Diluted factor

3.4.6. Stability at variable p^H

The anthocyanin stability was tested by treating 1 ml of sample with 1 ml of p^H 1.0 and 4.5 solutions. The color change was observed. (Strack, 1909).

3.5. Anticancer assay
***In-vitro* studies**

Extracted sample were taken for cytotoxicity screening and MTT assay.

3.5.1.Cytotoxicity Screening

HT 29 cell line (Human Colon carcinoma) was 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 (0.5-1.0x105 cells/ml) were 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 37ºC for 48h in 5% CO2 and the cells were periodically checked for any morphological changes and contamination. After the formation of monolayer, the cells were further utilized.

3.5.2. Determination of Mitochondrial Synthesis by Micro culture Tetrazolium (MTT) Assay (Mosmann , 1983)

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, colored (dark purple) formazan product. The cells were then solubilised with an organic product (e.g. 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:

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

4.1. RESULTS AND DISCUSSION
4.1.1. ANTHOCYANIN EXTRACTION

The extraction of anthocyanin from sugarcane peel was done by using acidified methanol as a solvent. A spectrum of the extract, especially a peak in visible region was recorded at 418 nm. Joaquim *et al,* 2011 reported the presence of phenolic compounds in sugarcane products.

5.2. ANALYTICAL PROCEDURES
5.2.1. FLAVONOID CONFIRMATION TEST
A. $FeCl_3$

In the presence of $FeCl_3$, the acidified methanol extracts showed brown color (Plate1) which confirms the presence of Flavonoids (Harbone, 1969).

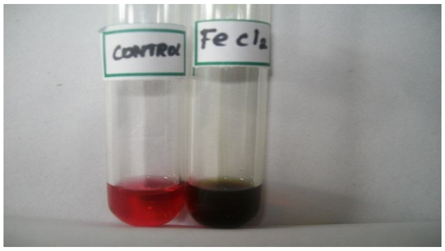

PLATE 1

B. $AlCl_3$
In the presence of $AlCl_3$, the acidified methanol extracts showed dark color (Plate2) which confirms the presence of Flavonoids (Harbone, 1969).

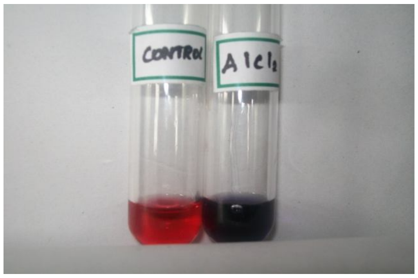

PLATE 2

5.2.2. CONFIRMATORY TEST FOR ANTHOCYANIN
A. 2M HCl In the presence of anthocyanin was again confirmed with the presence of 2M HCl. The red color was found to be stable when allowed to heat at 100°C (Plate 3). This confirms the presence of anthocyanin (Harbone, 1969)

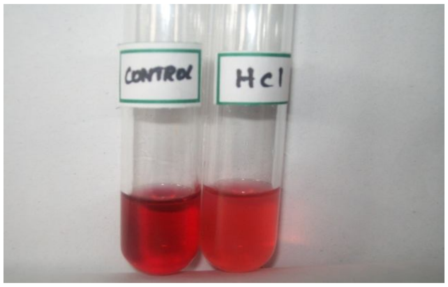

PLATE 3

B. 2M NaOH
The extract to be analyzed was again confirmed with the presence of anthocyanin with 2M NaOH. The with the addition of NaOH, the initial red color was changed to blue and gradually faded (Plate 4) (Harbone, 1969).

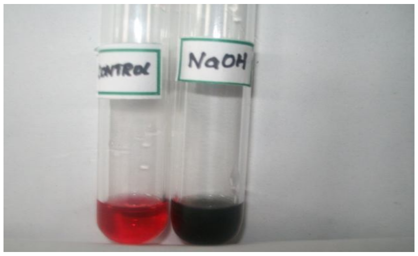

PLATE 4

5.3. stability at variable pH
EFFECT OF pH ON SUGARCANE PEEL ANTHOCYANIN
Anthocyanin may exhibit different color depending on their structure (glyposylation ,acylation) pH concentration of co pigments at a given pH an equilibrium exist between anthocyanin /aglycone structures. pH dependent.e.g., lowering the pH in the range of 5.0-1.0 several in a significant retention of anthocyanin in sugarcane peel. The stability of these pigments at low pH is largely attributed to the higher concentration of the flavylium cation. Stabilization of the colored species especially quinoidal base (A) could be further confer through intermolecular co pigmentation (Brouillard and Dangles, 1994, Jackmanet *et al*., 1987).(PLATE 5& 6).

Stability of Anthocyanin at various pH

PLATE 5

PLATE 6

5.4. Determination of total phenolic content

Phenolic content in sugarcane peel was estimated as 3.69mg/gm (Table 1). Godshall *et al*., 2002, reported that very high phenolic contents were observed were observed for sugar products because they are highly colored materials and phenolic compounds are strongly involved in formation of this color.

Phenolic composition, flavonoid and anthocyanin content of sugarcane (*Saccharum Officinarum* L)

5.5. Evaluation of total flavonoid content

The flavonoid content in sugarcane peel was estimated as 28.5mg/g (Table1).
Hakkinen and Torronen (2000) reported that flavonoid and selected phenolic acid contents in strawberry and Vaccinium species influenced by cultivar, cultivation site and technique.

S No	*Saccharum Officinarum* L		
	Assays	**Solvent**	**Sample (mg/g)**
1	Total anthocyanin content	Acidified methanol	0.00253
2	Total flavanoid content	Acidified methanol	28.5
3	Total phenol content	Acidified methanol	136.12

5.6. Determination of total anthocyanin content

Total anthocyanin content was found to be 0.00253 mg/g (Table 1). Joaquim et al., 2010, reported apigenin and luteoledin has predominant flavonoid in sugar cane products

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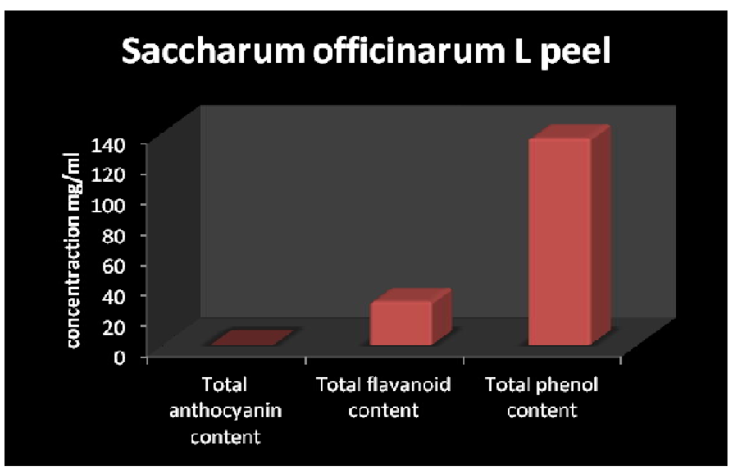


Fig. 1: Total Anthocyanin Flavanoid Phenolic Content of Sugarcane Peel

5.4 ANTICANCER ACTIVITY

Invitro antiproliferative activity of crude extract from sugarcane peel was performed by **MTT assay**. The crude extract of exhibited 51.2% inhibition HT29 cell line at concentration of 0.625µg/ml ,of the nine different concentration of crude extract .the highest concentration displayed a highest inhibition displayed a dose dependent antiproliferative activity on HT29 cell line (table 2).untreated HT29 cell line elongated shape,attached smoothy on the cell surface and some of the cells grouped together to form colonies(fig 2).

Table 2

S.No	Concentration of sample (µg/ml)	Percentage cell viability (MTT Assay)
1	10	17.9
2	5	33
3	2.5	43.5
4	1.25	48.7
5	0.625	51.2
6	0.312	58.9
7	0.156	61.5
8	0.078	69.2
9	0.039	79.4
10	Cell control	---

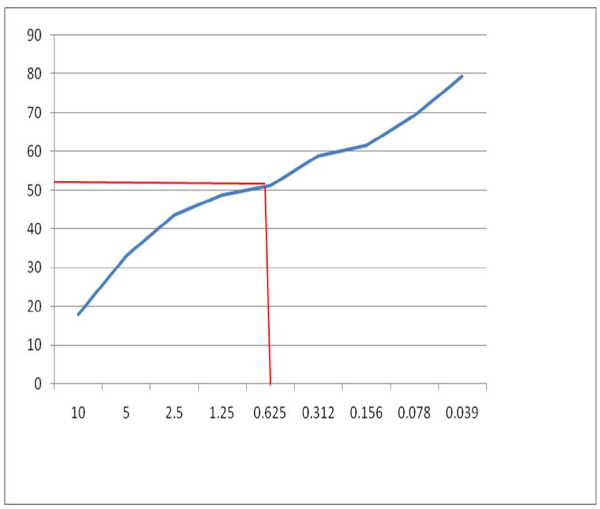
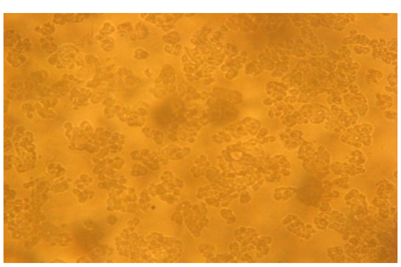
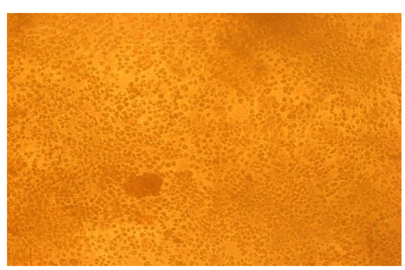


Fig. 2: Anticancer activity against colon cancer HT29 cell line

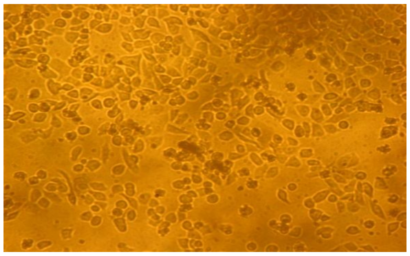


Control HT 29 Cell line showing oval or Rod shaped cells with cell to cell anchorage



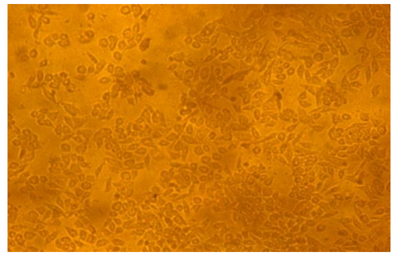
extracts were treated HT29 cells showing spherical shaped cells leading to loss of cell anchorage with concentration of 125µg/ml

Plate7



Extracts were treated HT29 cells showing spherical shaped cells leading to loss of cell anchorage with concentration of 625µg/ml

Plate 8



Extracts were treated HT29 cells showing spherical shaped cells leading to loss of cell anchorage with concentration of 156µg/ml

Following treatment with extracts for 24 hours, the cells changed round shape and lost contact (fig 3). In particular the cells lost their surface morphology and died at a concentration of 50%. The study confirms the invito antiproliferative activity of sugarcane peel extracts against HT29 cell line. Geeta Lala *et al.,* 2006 and Joaquim Mauricio *et al*., 2007 reported that the extract of sugarcane juice have reported antioxidant and antiproliferative activity .there is no previous work on anticancer activity of the sugarcane peel extract against HT29 cell line.

CONCLUSION

The sugarcane peel could be evaluated as a major source of anthocyanin ,flavanoids and polyphenol antioxidants. Anticancer activity of sugarcane peel is well known and reported in the literature.Sugarcane peel extracts inhibit HT-29 colon cancer cell growth even at lower concentration .therefore ,we conclude that sugarcane peel extracts can be a promising and inexpensive anthocyanin source for therapeutic purposes against human colon cancer.

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