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 determined the antiproliferative activity of sugarcane peel by cytotoxicity assay which shows 51.2% inhibition HT29 cell line at concentration of 0.625µg/ml. these results suggest that 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 luteolin. 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 dimethylhydrazine (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 (Na₂CO₃), Aluminium chloride (AlCl₃), Ferric chloride (FeCl₃), Butylated hydroxy toluene (BHT), methanol, MTT (3-((4,5-Dimethyl thiazol-2-yl)-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 Flavonoid confirmation test (Harbone-1998)
A. FeCl₃
1 ml of sample extraction was added with a small amount of FeCl₃, and results were observed.
B. AlCl₃
1 ml of sample extraction was added with 5% of AlCl₃ 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 (AlCl₃), 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 pH 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) pH 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 pH
The anthocyanin stability was tested by
treating 1 ml of sample with 1 ml of
pH 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%
sodium bicarbonate, 10ml 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 cel
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:

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

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. FeCl₃
In the presence of FeCl₃, the acidified
methanol extracts showed brown color
(Plate1) which confirms the presence of
Flavonoids (Harbone, 1969).
B. AlCl₃
In the presence of AlCl₃, the acidified methanol extracts showed dark color (Plate 2) which confirms the presence of Flavonoids (Harbone, 1969).

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).

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).

5.3. stability at variable pH
EFFECT OF pH ON SUGARCANE PEEL ANTHOCYANIN
Anthocyanin may exhibit different color depending on their structure (glycosylation, 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, Jackman 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 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 (Table 1). Hakkinen and Torronen (2000) reported that flavonoid and selected phenolic acid contents in strawberry and Vaccinium species influenced by cultivar, cultivation site and technique.

<table>
<thead>
<tr>
<th>S No</th>
<th>Assays</th>
<th>Solvent</th>
<th>Sample (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Total anthocyanin content</td>
<td>Acidified methanol</td>
<td>0.00253</td>
</tr>
<tr>
<td>2</td>
<td>Total flavanoid content</td>
<td>Acidified methanol</td>
<td>28.5</td>
</tr>
<tr>
<td>3</td>
<td>Total phenol content</td>
<td>Acidified methanol</td>
<td>136.12</td>
</tr>
</tbody>
</table>

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.

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

*In vitro* 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 smoothly on the cell surface and some of the cells grouped together to form colonies (fig 2).

**Table 2**

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

![Fig. 2: Anticancer activity against colon cancer HT29 cell line](image)

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

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 invitro 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 extract 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 therapeautic purposes against human colon cancer.

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