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Antiproliferative Properties of Anthocyanin from Indian Cassava (*Manihot Esculenta,* Crantz) on Hep-2 And Mcf-7 Cell Lines

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ABSTRACT

Anthocyanins are multifunctional bioactive compounds implicated with beneficial health effects due to their strong antioxidant activity for scavenging free radicals that are involved in cell damage and tumour promotion. Evidence has accumulated to suggest that flavonoids potently suppress tumour cell proliferation and may effectively work as chemopreventive agents against carcinogenesis in humans. In the study, we detected the antiproliferative effects of anthocyanin on human epithelial cells and the Breast cancer cells by MTT assay. The HEp-2 and MCF-7 cells were seeded in 96-well culture plates in different concentrations of cassava leaf stalk extracts of Indian Cassava (*Manihot esculenta,* Crantz) to determine their anticancer effects using the MTT assay. Anthocyanin extracted from cassava leaf stalk extracts have 79% inhibition on HEp-2cells at 1000 µg/ml (Table1). The anthocyanin extracted from cassava leaf extracts was tested against MCF-7 cell lines shows 78% inhibition at 1000 µg/ml (Table 2). So we confirm that, the anthocyanin compounds present in cassava leaf extracts are inhibiting the proliferation of the cancer cells.

Keywords: Cassava (Manihot esculenta), Anthocyanin, Hep 2, MCF 7, Antiproliferative.

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INTRODUCTION

Cassava (Manihot *esculenta* Crantz) belongs to Euphorbiaceae family is a storage root crop. It is widely cultivated in the tropics, primarily for the storage roots, although the young leaves are also eaten (Lancaster *et al.,* 1982)[1]. The strong ability of cassava to survive adverse environmental conditions makes it an important crop for food security in many of the developing countries. Cassava is a readily available source of food or diet across the globe most especially to developing nations. In spite of the enormous biomedical potentials of this class of compound inherent in cassava, recent reviews have only dealt with the distribution, biosynthesis and toxicology of these compounds, rather than the practicality of isolation and identification of biochemical compounds. Crude cassava extract has been used to control one form of cancer and the other in time past (Iyuke *et al.*, 2004)[2]. Linamarin is the major cyanogenic component (93%) present in cassava relative to lotaustralin (7%) (Liang cheng *et al.,* 1995)[3]. There was no work has been reported on the use of cassava leaf stalk extract on anti proliferative activity. Anthocyanins have been found to significantly suppress the growth of cultured tumour cells and have been shown to have greater inhibitory effect than other flavonoids. Anthocyanins are regarded as important nuetraceuticals properties mainly due to their possible antioxidant effects and they have potential therapeutic role related to some cardiovascular diseases and cancer diseases. They were effective in reversing age related deficits in several nerve disorders and behavioural parameters (Joseph *et.al*., 1999)[4]. Flavonoids are polyphenolic compounds found as essential components of the human dietary system (Ewelina Szliszka, 2008)[5]. Flavonoids are present as constituents of flowering plants, particularly of food plants (Miean and Mohammed, 2001)[6]. Flavonoid derivatives have potential application as diseases preventive and therapeutic agents in traditional medicine in Asian countries for more than thousands of years (Nakatani, 2000)[7.] Several studies showed that the selection of a particular plant resources for its potential health benefits appears to mirror its flavonoid composition. Humans have consumed flavonoids and other dietary phenolics since the arrival of human life on earth. Flavonoids have been known as plant pigments for over a century and belong to a vast group of phenolic compound that are widely distributed in all foods of plant origin. In the normal North American diet, flavonoid glycosides are unavoidably consumed daily, with an estimated total consumption of 1g/day (Formica *et.al.,* 1995)[8], which could be much higher if dietary supplements are also consumed. Recent work is beginning to highlight the potential health-beneficial properties of flavonoids, known to be powerful antioxidants. The human clinical trials indicate that flavonoids have important effects on cancer chemoprevention and therapy (Pannala

et.al., 1998)[9]. Flavonoids may interfere in several of the steps that lead to the development of malignant tumors, including protecting DNA from oxidative damage, inhibiting carcinogen activation, and activating carcinogen detoxifying systems (Kerry *et.al*., 1999)[10]. The search for anticancer compounds from natural resources has received much attention and efforts have been put into identify compounds that can act as a suitable antitumor compounds to replace synthetic ones. However the use of these synthetic antitumor compounds has been questioned due to their potential health risks and toxicity (Kalt & Kappus 1993)[11]. In addition these naturally occurring anticancer can be formulated to give nuetraceuticals that can help to prevent oxidative damage from occurring in the body. Cassava leaf stalks as also suspected to have bioactive compounds that exhibit antioxidant and anti-proliferative effects. It will also unlock the potentials of other medical, bio-chemical applications and research, wealth, social and job creation to nations because cassava is readily available across the globe and is a crop that can tolerate adverse environmental conditions.

MATERIALS AND METHOD

Analytical Procedures

Sample collection and Extraction

Cassava (*Manihot esculenta,* Crantz) leaf stalk were collected from field and stored in sealed polyethylene bags at -20˚C. 0.5 gm of cassava leaf stalk were extracted with 10 ml of acidified methanol solvent. The mixture was centrifuged at 10,000 rpm for 10 min and supernatant was subjected to antiproliferative analysis.

Flavanoid conformation test

1 ml of sample extraction was added with a small amount of $FeCl_3$**,** and results were observed and

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

Materials Required in Mem

i) Monolayer culture bottle of Hep2 cell lines.

ii) 5ml, 10ml serological pipette

iii) Minimal essential media (MEM) with 10%, 2% fetal calf serum

iv) TPVG (Trypsin PBS versene glucose)

v) Discarding jar, inverted microscope, desiccators

vi) Gloves, spirit, cotton, label pad, marker pen

Materials Required In Cytotoxicity Assay

1. Monolayer culture in log phase

2. Drug extracts (different concentrations)

3. MEM without FCS

4. 0.45µ filter

5. 5ml sterile storage vial

6. Tissue paper, spirit, cotton, marker pen and gloves

7. Micropipette and tips

Materials Required In Mtt Assay

1. MTT (3-(4, 5-dimethyl thiazol-2yl)-2,5-diphenyl tetrazolium bromide) stock solution 5mg/ml

2. DMSO-dimethyl sulfoxide

3. Micropipette and 200µl of sterile tips

4. Spectrophotometer with 1ml cuvette holder.

Minimal Essential Media Preparation

Media is defined as a complex source of nutritional supplementation vital for the growth proliferation and maintenance of cells *in vitro*. The MEM dissolved in the pre sterilized Millipore distilled water and mixed well, closed and sterilized at 15lbs 121ºc for 15mins. Allow ingredients in the quantity, depending on the concentration of fetal calf serum (2% or 10%) mix well by shaking. Take care avoid spills pass CO2 using sterile pipette, Shake the bottle, check Ph and adjust to 7.2 to 7.4. The MEM bottles are kept for 2 days at 37ºc and checked for sterility, PH drop and floating particles they are then transferred to the refrigerator Essays, UK. (November 2013)[12].

Media Preparation

Ingredients	10% Growth Media	2%Growth Media	Maintance Media Without FCS
MEM	857ml	937ml	957ml
Penicillin and streptomycin	1ml	1ml	1ml
Phenol red	1ml	1ml	1ml
Amphotericin B	1ml	1ml	1ml
3% L-glutamine	10ml	10ml	10ml
Foetal calf serum	100ml	20ml	nil
7.5%NaHCo3	30ml	30ml	30ml
Total volume	1000ml	1000ml	1000ml

Preparation of Ingredient

1. Penicillin and streptomycin: (concentration 100IU of penicillin and 100 µg 0f streptomycin)

Dissolve both antibiotics in sterile Millipore distilled water, so as to give a final concentration 100 IU of penicillin and 100µg of streptomycin/ml. Mix well and distribute in 1ml aliquots. Store at -20º C Check sterility.

2. Fungi zone (amphotericin B): (conc.: 20µg/ml)

Dissolve in sterile Millipore distilled water so as to give a final concentration of 20µg/ml and distribute in 1ml aliquots in vials. Store at -20ºc. Check sterility.

3. L.glutamine: 3%

Weigh 3g of l-glutamine accurately and dissolve in 100ml sterile Millipore distilled water and mix well. Filter through Millipore membrane filter 0.22µ and distribute in 5ml aliquots in vials. Store at -20ºc. Check sterility.

4. 7.5% sodium-bi-carbonate

Weigh requisite quantity of sodium-bi-carbonate (to give 7.5% solution) accurately and dissolve in 100ml of sterile Millipore distilled water. Filter through what man filter paper No.1, distribute into bottles and at 121ºc, 15lbs, 15mins. Cool and store at +4ºc.

4. Foetal calf serum

Bring FCS at room temperature. Inactivated at 56ºc in water bath for 30 minutes and cool at room temperature. If floating particles are seen filter through Seitz filter. Distribute in 100ml, 50ml, and 20ml quantities in sterile bottles. Store at -20ºc.

5. Trypsin, PBS, versene, glucose solution: (TPVG)

2% trypsin: 100ml

Weigh 2g of trypsin accurately; dissolve in 100 ml sterile Millipore distilled water with magnetic stirrer for ½ hour. Filter through membrane filter. Store at -20ºc

0.2%EDTA (versene)

Weigh 200mg of EDTA accurately. Dissolve in 100 ml of sterile Millipore distilled water. Autoclave at 121ºC 15lbs/15mins.

10%glucose -100ml

Weigh 1g of glucose accurately. Dissolve in 100 ml of sterile Millipore distilled water and filter through what man filter paper and autoclave at 15lbs/15mins.

TPVG-100ml

PBS - 840ml

2%trypsin -50ml

0.2%EDTA -100ml

10%glucose -5ml

Penicillin & streptomycin -5ml

Mix all ingredients and adjust the pH to 7.4 with 0.1 N HCl or 0.1 N NaOH. Distribute in 100 ml aliquots. Store at -20ºc.

Maintenance of Cell Line

Maintenance of cells involves the following operations:

Dispersion and Sub culturing (seeding) of cells.

Preservation of cells in repository.

Revival of cells from repository

Subculturing and Maintenance of Cell Line

1. Bring the medium and TPVG to room temperature for thawing.

2. Observe the tissue culture bottles for growth, cell degeneration, pH and turbidity by seeing in inverted microscope.

3. If the cells become 80% confluent it goes for sub culturing process

4. Wipe the mouth of the bottle with cotton soaked in spirit to remove the adhering particles.

5. Discard the growth medium in a discarding jar keep distance between the jar and the flask.

6. Then add 4 – 5 ml of MEM without FCS and gently rinsed with tilting. The dead cells and excess FCS are washed out and then discard the medium.

7. TPVG was added over the cells. And incubate at 37º C for 5 minutes for disaggregation. The cells become individual and it's present as suspension.

8. Add 5ml of 10% MEM with FCS by using serological pipette.

9. Gently give passaging by using serological pipette. If any clumbs is present then repeat the process.

10. After passaging split the cells into 1:2, 1:3 ratio for cytotoxicity studies for plating method

"Seeding of cells"

After homogenize take one ml of suspension and pour in to 24 well plates. In each well add 1ml of the suspension and kept in a desiccators in 5% CO2 atmosphere. After 2 days incubation observe the cells in inverted microscope. If the cells became 80% confluent

Cytotoxicity assay

In order to study the antitumor activity of a new drug, it is important to determine the cytotoxicity concentration of the drug. Cytotoxicity tests define the upper limit of the extract concentration, which is non-toxic to the cell line. The concentration nontoxic to the cells is chosen for antiviral assay. After the addition of the drug, cell death and cell viability was estimated. The result is confirmed by additional metabolic intervention experiment such as MTT assay

Stock drug concentration

0.5ml of drug is dissolved in 4.5 ml of DMSO giving a working concentration of 1mg/ml. the working concentration is prepared fresh and filtered through 0.45µfilter before each assay.

1. To prepare 5 ml of extract and giving conc. (1mg/ml).

2. 500µl of MEM without FCS was taken in 9 eppendroff tubes. /each samples

3. Then 500µl of the working conc. was added to the first eppendroff tube and mixed well then 500µl of this volume was transferred from first to last tube by serial dilution to obtain the desired concentration of the drug.

4. As a result the volume remains constant but there is a change in concentration.

Sampling

1. 48hr monolayer culture of Hep2cells at a concentration of one lakh /ml /well (10 cells / ml / well) seeded in 24 well titer plates.

2. The plates were microscopically examined for confluent monolayer, turbidity and toxicity if the cells become confluent.

3. The growth medium (MEM) was removed using micropipette. Care was taken so that the tip of the pipette did not touch the cell sheet.

4. The monolayer of cells was washed twice with MEM without FCS to remove the dead cells and excess FCS.

5. To the washed cell sheet, add 1ml of medium (without FCS) containing defined concentration of the drug in respective wells.

6. Each dilution of the drug ranges from 1:1 to 1:64 and they were added to the respective wells of the 24 well titer plates.

7. To the cell control wells add 1ml MEM (w/o) FCS.

8. The plates were incubated at 37ºc in 5% CO2 environment and observed for cytotoxicity using inverted microscope.

Mtt Assay

MTT assay is called as (3-(4, 5-dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide.MTT assay was first proposed by Mossman in 1982.

Principle

MTT is cleaved by mitochondrial dehydrogenase in viable cells, yielding a measurable purple product formazan. This formazan production is proportionate to the viable cell number and inversely proportional to the degree of Cytotoxicity

Procedure

After incubation, remove the medium from the wells carefully for MTT assay. In each well wash with MEM (w/o) FCS for 2- 3 times. And add 200µl of MTT conc of (5mg/ml). And incubate for 6-7hrs in 5% CO2 incubator for cytotoxicity. After incubation add 1ml of DMSO in each well and

mix by pipette and leave for 45sec. If any viable cells present formazan crystals after adding solublizing reagent (DMSO) it shows the purple color formation. The suspension is transferred in to the cuvette of spectrophotometer and an O.D values is read at 595nm by taking DMSO as a blank. Graph is plotted by taking concentration of the drug on X axis and relative cell viability on Y axis.

Cell viability (%) = Mean OD/Control OD x 100

RESULTS AND DISCUSSION

Several bioactive compounds from plant products found to exhibits chemo preventive activities against cell lines in both *in vitro* and *in vivo.* The anthocyanin extracted from leaf stalk of Indian cassava was tested against HEP-2 and MCF-7 cell lines for antiproliferative properties.

Anthocyanin extraction

The anthocyanin was extracted from leaf stalk of Indian Cassava was done by using acidified methanol. By observing colour of the extract confirmed the presence of anthocyanin in cassava leaf stalk and further assay procedures were done for antioxidant analysis.

Flavonoid confirmation test

In the presence of **$FeCl_3$** acidified methanol extracts of leaf stalk of Indian Cassava showed brown color which confirms the flavonoids presence. In the presence of **$AlCl_3$**, dark color was observed in acidified methanol extract of cassava leaf stalk which shows the presence of anthocyanin.

Antiproliferative Analysis

Cassava (*Manihot sp.*) has been used to control different forms of cancer or the other in the Chinese traditional medicine (*Yeoh et al.,* 1998)[13] without scientific validation. Similarly, research

based on both aqueous and methanol crude cassava extracts showed potential of anticancer activity (*lyuke et al.,* 2004)[13]. Several compounds particularly plant products and dilatory constituent found to exhibits chemo preventive activities both *in vitro* and *in vivo.* Our earlier research work (*Suganyadevi et al.,* 2011)[14] report that anthocyanin content is higher in cassava leaf stalk extracts and it have potential antioxidant analysis. Based on that research work we done the Antiproliferative analysis of flavonoids extracted from Cassava leaf stalks was tested against HEP-2 and MCF-7 cell lines.

Antiproliferative analysis of flavonoids against HEP-2 cell lines

The present study of the cytotoxic effects of anthocyanin extracted from cassava leaf stalks on HEP-2 cells were analyzed by conducting MTT assay. Cultures of HEP-2 cell were treated with the different concentration ranging from 15.6-1000 µg/ml and the cell viability was counted. Control assay were carried out for sample containing only the approximately volume of blank solution and those showed no effect on cell growth. *Shilpa et al.,* (2000), reported a significant decrease in Melanoma B16 F 10 cell population by the aqueous extract of *Allium cepa*. Flavonoids extracted from cassava leaf stalks showed approximately 79% inhibition on HEP-2 cells at 1000 µg/ml , 69% of inhibition on HEP-2 cells at 500µg/ml and 58% of inhibition on HEP-2 cells at 250 µg/ml (Table1). Untreated HEP-2 cells are appeared as elongated shape, attached smoothly on the culture cell face and some of the cells are grouped together to form colonies after treating with drugs for 24 hours, the cells changed to round shape and lost cell content. The OD values are read at 595nm in Spectrophotometer by using DMSO solution as an control cytotoxicity were been obsereved. Based on OD values graph are plotted to detect cell viability against the different drug concentrations towards the cancer cells (Figure 2).

Antiproliferative analysis of flavonoids against MCF-7 cell lines

The anthocyanin extracted from cassava leaf stalks was tested against MCF-7 cell lines showed 78% inhibition at 1000 µg/ml , 62% of inhibition on MCF-7 cells at 500µg/ml and 54% of inhibition on MCF-7 cells at 250 µg/ml (Table 2) .*Chun et al*., (2009)[15], demonstrated the antiproliferative activity for *Etlingera elatior* on human colorectal carcinoma cells and reported that the phenolics compounds may be responsible for its Antiproliferative activity.cell growth reduction can be attributed to a decreased proliferation rate or an enhanced cell death by apoptosis or necrosis. Apoptosis is a programmed cell death, which eliminates redundant or damaged cells. Cancer cells have deregulated proliferation and they are not able to undergo apoptosis naturally. To quantify and further support to the finding that flavonoids from plant sources causes apoptosis on MCF-7 cells. In apoptotic cells the DNA fragments intact within the cell membrane or

apoptopic body. Conversely, lysosomal enzymes in necrotic cells digest the cell membrane and cause the release of DNA fragments from the cells. In our results by using various concentration of anthocyanin extracted from cassava leaf stalks on MCF-7 cells showed apoptopic bodies when viewed under phase contrast microscope, where the cells are spherical or round shape when compared with normal MCF-7 cell lines (Fig 3). These results suggested that the anthocyanin compounds present in cassava leaf stalk extracts are inhibiting the proliferation of cells.

Table 1: Cell viability analysis of anthocyanin from cassava leaf stalk at different concentration against HEP-2 cell lines.

S.no	Concentration(µg/ml) of extract	DilutionRatio	Absorbance	Cell viability
1	1000	Neat	0.12	21.05
2	500	1:1	0.18	31.57
3	250	1:2	0.24	42.10
4	125	1:4	0.26	45.61
5	62.5	1:8	0.35	61.40
6	31.25	1:16	0.44	77.19
7	15.625	1:32	0.50	87.71
8	Cell control	-	0.53	100

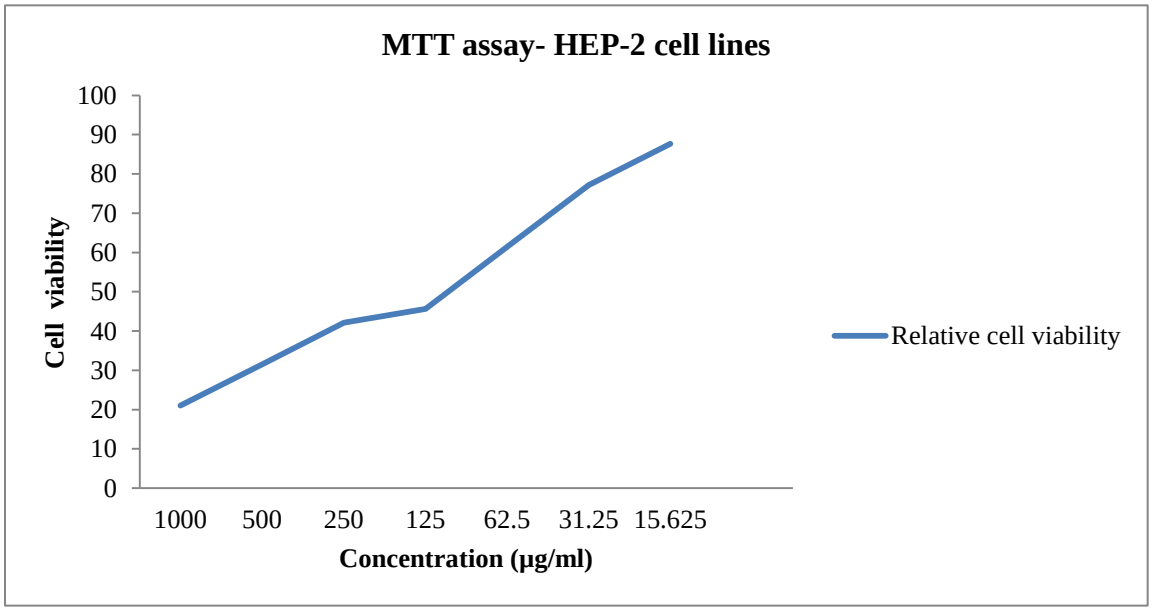

Figure 1:

Table 2: Cell viability analysis of anthocyanin from cassava leaf stalk at different concentration against MCF-7 cell lines.

S.no	Concentration(µg/ml) of Extract	Dilution Ratio	Absorbance	Cell viability

1	1000	Neat	0.11	22.44
2	500	1:1	0.19	38.77
3	250	1:2	0.23	46.93
4	125	1:4	0.31	63.26
5	62.5	1:8	0.39	79.59
6	31.25	1:16	0.44	89.79
7	15.625	1:32	0.47	95.91
8	Cell control	-	0.49	100

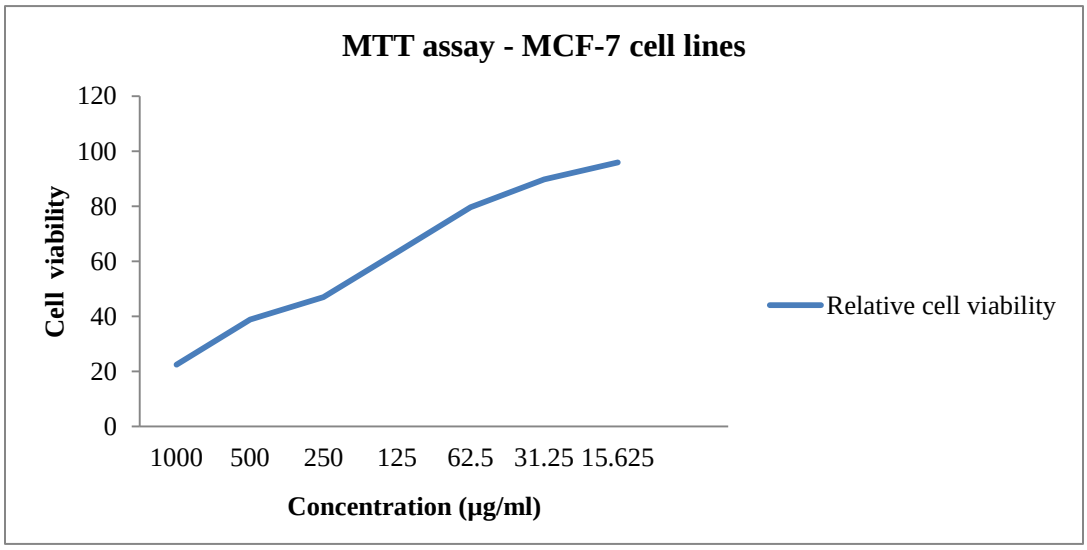

Figure 1:

CONCLUSION

This study represents the first report on the leaf stalk of cassava extract has a very good source of anthocyanins compared to other sources from plant products. From this research study the results demonstrated that the concentration of anthocyanin from Indian cassava *Manihot esculenta, Crantz* was significantly inhibiting the proliferation of cells. Finally it shows antiproliferative activity aganist HEP-2 and MCF-7 Cell lines by treating with different concentration of cassava leaf extracts was confirmed. The study on specific bioactive compounds in cassava leaf stalks are presently in progress.

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