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Anti-proliferative potentiality of purified anthocyanin from *in vitro* culture of *Clerodendron infortunatum* L. against human cervical cancer cells (HeLa)

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ABSTRACT

Anthocyanins are polyphenolic compounds possessing anti-carcinogenic effects through multiple ways. The proper mechanism for the effect of the anthocyanins againt proliferation of cancer cells is unknown. The present study aims to analyze the pro-apoptotic potential of purified anthocyanin from the *in vitro* cultures of *Clerodendron infortunatum* L. The cytotoxic activity was evaluated using MTT cell viability assay. Fluorescence microscopic, DNA fragmentation analysis, cell cycle phase distribution and Annexin V binding assay/Quantification of apoptotic cell death were also carried. Cellular morphological changes by fluorescence microscopy and flow cytometry were used to analyze the effect of anthocyanin on cell cycle and apoptosis. Anthocyanin extracted from the *in vitro* cultures of *C. infortunatum* was purified by column chromatography. The mean purity values obtained by HPLC were 90.9% ± 1.9, 80.60% ±2.3 for Oasis MCX, Amberlite XAD-7 +Sephadex LH-20 column respectively. However, the purity by molar absorptivity was found to be less. HPLC chromatogram revealed 12 fractions of anthocyanin. MTT assay revealed that anthocyanin inhibited *in vitro* cancer cell growth of human cervical cancer cells (HeLa) in a dose- and time-dependent manner as compared to other cell lines. The IC_{50} values of anthocyanin were found to be 47.2 and 32.5 µM at 24 and 48 h time intervals respectively. Anthocyanin treated cells exhibited apoptotic bodies, which inferred an early apoptotic event. DNA ladder was more apparent and increased with the anthocyanin dose as compared to the control group. Anthocyanin also induced sub-G1 cell cycle arrest and increased fraction of HeLa apoptotic cells. *Conclusion*: Purified anthocyanin from *in vitro* culture of *Clerodendron infortunatum* showed anti proliferative activity against HeLa cells.

INTRODUCTION

Anthocyanins are water soluble, flavonoid molecules, responsible for the attractive colors and are widely distributed in plants. Ontogeny of cancer includes initiation, promotion, progression, invasion and metastasis. Tumor initiation starts when DNA, in a cell is damaged by exposure to carcinogens, which are derived through cigarette smoking, infection/inflammation and nutrition/diet. Anthocyanin shows anti-carcinogenic effects through multiple ways like scavenging reactive oxygen species (ROS), increasing the oxygen-radical absorbing capacity of cells, stimulating the expression of Phase II detoxification enzymes, reducing the formation of oxidative adducts in DNA, decreasing lipid peroxidation, inhibiting mutagenesis by environmental toxins and carcinogens, and reducing cellular proliferation by modulating signal transduction pathways. Although most of the protective effects of anthocyanins are attributed by their ability to scavenge ROS, or chelating metals and by direct binding to proteins due to the presence of hydroxyl groups in position 3 of ring C and also in the 3′, 4′ and 5′ positions in B-ring of anthocyanin.

Shih *et al* revealed the role of anthocyanins as inducer of

phase II antioxidant and detoxifying enzymes in cultured cells[1]. Treatment of rat liver clone cells and also non-cancerous breast cells with anthocyanins enhanced the antioxidant capacity by activating glutathione-related enzymes as well as the activity of NAD(P)H: quinone reductase[1,2]. The mechanism exhibited was through the activation of the genes related with antioxidant enzymes[1].

Pure anthocyanins and anthocyanin-rich extracts from fruits and vegetables have exhibited anti-proliferative activity against multiple cancer cell types under *in vitro* conditions [3,4,5,6,7,8] via arresting the various stages of the cell cycle i.e., effects on cell cycle regulator proteins (p53, p21, p27, cyclin D1, cyclin A, etc.) [8,9,10] . The exact mechanism(s) for the selective effect of the anthocyanins on the growth of cancer cells vs. normal cells is/are not known. However, ethanol extract of black raspberries selectively inhibits the growth and stimulates apoptosis of a highly tumorigenic rat oesophageal epithelial cell lines (RE-149-DHD) when compared to its low tumorigenic precursor line, RE-149 cells after 2 h. Anthocyanins also induce the expression of FAS and FASL (FAS ligand) in cancer cells resulting in apoptosis. Anthocyanins have been shown to suppress angiogenesis through several mechanisms such as: inhibition of H_2O_2 - and tumor necrosis factor alpha (TNF-α)-induced VEGF expression in epidermal keratinocytes, and by reducing VEGF and VEGF receptor expression in the endothelial cells. Similarly, it was noticed that anthocyanin inhibit cancer cell invasion through reducing the expression of MMP and urokinase-plasminogen activator (u-PA), both of which degrade extracellular matrix as part of the invasive process and, by stimulating the expression of a tissue inhibitor of matrix metalloproteinase-2 (TIMP-2) and of an inhibitor of plasminogen activator (PAI), both of which counteract the action of MMP and uPA [11]. Sannigrahi *et al* documented the anticancer effect of terpenoids of methanol extract of *Clerodendrum infortunatum* against Ehrlich's ascites carcinoma in mice [12]. Similarly, Das *et al* reviewed the plusible biological potentialities and medicinal features of *Clerodendrum infortunatum*[*13]*. In this scenario, present study targets purification of anthocyanin elucidated from *in vitro* cultures of *C. infortunatum* and its antimetastatic potential.

MATERIALS & METHODS

Plant material and in vitro culture

Fresh excised leaves and nodes of *C. infortunatum* were employed as explants for culturing in the MS medium. The explants were surface sterilized, disinfected with teepol (5% v/v) for 20 min followed by mercuric chloride treatments (0.01 - 0.2%, for 1-5 min). Subsequently, the explants were thoroughly washed with deionized water for thrice. The sterilized explants were dissected out into suitable size and inoculated on MS medium fortified by sucrose (3%) in 0.6% agar for solidification. pH was maintained to 5.8, prior to autoclaving at 15 lb pressure or 121^0 C for 20 min. The cultures were kept at 25^{0}C with a photoperiod of 12 h.

The hormonal combinations in the MS medium supplemented were 2,4-dichlorophenoxyacetic acid (2,4-D) + Benzylaminopurine (BAP) (0.5- 3.0 mg/L) and BAP + NAA (0.5-3.0 mg/L) for induction of callus. Callus obtained were subcultured in fresh MS medium with 2,4-D + kinetin (0.5-3.0mg/L) at different concentrations to generate pigmented callus.

Cell suspension cultures were derived from friable callus in 250 mL Erlenmeyer flasks containing 100 ml of liquid MS medium fortified with various concentrations of 2,4-D + Kin (0.5 to 3 mg/L) in triplicates. The cell suspensions were kept at 110 rpm on a rotary shaker at 25±1ºC, 3000 lux and 16/8 h photoperiod. Cultures were retained for one month and their growth was determined by loss of weight by dissimilation.

Analysis was continued from 10th to 25th days using different types of elicitors such as salicylic acid, ethephon and precursors like phenylalanine and shikimic acid into the suspension cultures. Chemicals were sterilized through 0.22 µm millipore filters and added at the time of inoculation to make the concentration of 50 µL/25 mL suspension culture. The cultures were maintained at 25±1°C under continuous white fluorescent light on a rotary shaker.

Estimation of anthocyanin content

1 g of *in vitro* pigmented cell mass was used for isolation and estimation of anthocyanin. The OD was recorded at 510 and 700 nm against distilled water as blank [14]

Purification

Crude anthocyanin extract was purified using three different protocols and was fractionated by HPLC - PDA. *(a) Sepharose packs solid-phase extraction (SPE. C18)* was used [12]. Giusti et al. [13] method was employed to yield maximum anthocyanin fraction. (b) *Oasis- MCX SPE* is a mixed mode between cation exchange and reversed-phase interactions. (c) *Amberlite XAD-7 and Sephadex LH-20 SPE* i.e., purification through 2 different cartridges. Initial purification was done with Amberlite XAD 7, and the resulted fraction was loaded in to Sephadex LH 20 cartridge to separate anthocyanins from proanthocyanidins. (d) HPLC-PDA and MS analysis was carried out using Shimadzu HPLC-Photodiode array (PDA) system equipped with a SPD-M20A PDA detector and also Shimadzu LCMS-2010 EV liquid chromatography. The mobile phase used was solvent A: 4.5% formic acid in LC/MS grade water, and B: 0.1% formic acid in LC/MS grade acetonitrile.

Cell viability testing by MTT assay

The cytotoxic effects of anthocyanin on the cell viability were screened against SW 480, MCF 7, HeLa cell lines by MTT assay. The cell line was seeded in a 96- well plate at 3×10^3 cells per well and then treated with 0, 25, 50, 100, 200, 300 and 400 µM dose of anthocyanin for 24 and 72 h. The cell plates were then treated with MTT solution (10 µL) in PBS for 2h. The formazan crystals then formed were dissolved in DMSO and the absorbance was measured on a microplate reader. Data were expressed as a percentage of control measured in the absence of anthocyanin.

Fluorescence microscopic study

HeLa cells were seeded on a chamber slide at a cell density of 3×10^5 cells per chamber. The cells were treated with 0, 200, 300 and 400 µM anthocyanin for 48 h. After wards, 20 µg/mL of acridine orange and 20 µg/mL of propidium iodide were added to each chamber. It was then observed under Fluoview 1000 laser scanning confocal microscope (Olympus IX 81 motorized inverted microsope).

DNA fragmentation analysis

The HeLa cells (3×10^5 cells/dish) were plated in 6-cm dish and then subjected to the treatment of various concentrations (0, 200, 300 and 400 µM) of the anthocyanin for 48 h. After drug treatment, the cells were washed with ice-cold PBS and

resuspended in lysis buffer (25 mM Tris-HCl, pH 7.4, 5 mM EDTA and 0.6% SDS) with 1.0 mg/mL RNase A for 20 min at 50°C. Then proteinase K was added and the cells were incubated overnight. Separation of DNA was done using 2% agarose gel and detected under UV light after staining with ethidium bromide (EB).

Effect of anthocyanin on cell cycle phase distribution

Cell cycle was analyzed by FACSC alibur instrument equipped with Cell Quest 3.3 software. Briefly, HeLa cells (3 x 10^5) cells were treated with various concentrations of anthocyanin (0, 200, 300 and 400 µM) for 48 h. After this, the cells were collected, washed with ice-cold PBS buffer, fixed with 70% alcohol at 4°C for 12 h, and stained with propidium iodide in the presence of 1% RNAase A at 37°C for 30 min before analysis by flow cytometry.

Annexin V binding assay/Quantification of apoptotic cell death

To establish and confirm cells undergoing apoptosis, we performed annexin V binding assay through flow cytometry. Briefly, HeLa cancer cells were treated with the anthocyanin (0, 200, 300 and 400 µM) for 48 h, and then treated and untreated cells were harvested by trypsinization. Harvested cells were then incubated in annexin V-FITC (25 ng/mL) and propidium iodide (25 µg/mL), at room temperature for 30 min in the dark, and analyzed using a FACS Calibur flow cytometer (BD Bioscience) taking a minimum 10,000 cells in each sample.

Statistical analysis

All data were derived from at least six independent experiments. The results were expressed as the mean ± SD. Differences between groups were analyzed using the Student's t-test. $p<0.05$ was considered statistically significant.

RESULTS

In vitro culture

Pre-treatment with 0.1% $HgCl_2$ (2 min) was optimal to establish explants with reduced rate of contamination with maximum establishment (95%) on the medium. The leaf and nodal explants were inoculated on MS medium fortified with various combinations of 2,4-D and BAP for callus induction. After 30 days, leaf explants proliferated into green compact callus (Fig.1) remarkably. Callogenesis revealed that the fresh weight of all the calli increased in MS media combinations of BAP + 2,4 D (0.5, 3 mg/L) compared to BAP + NAA (0.5, 3 mg/L). The MS medium supplemented with 2 mg/L BAP + 0.5 mg/L 2,4 D yielded the highest (2. 7 ± 0.45 g) callus fresh weight compared with other treatments. All the media combinations when fortified with BAP remarkably improved the callus fresh weight indicating its importance for callus growth in *Clerodendron* comparing to 2, 4-D and NAA. The resulted callus was further subjected to sub-culturing to induce anthocyanin synthesis.

The sub-culturing of leaf callus was carried on MS medium supplemented with 2,4-D and kinetin at various concentrations. Luxuriant coloured callui were achieved on MS medium fortified with 1 mg/L 2,4-D + 2 mg/L kinetin after 60 days of subculture i.e., 2.68±0.66%. Increase in the concentration of 2,4-D and kinetin up to 5 mg/L decreased callogenic potentiality of the explants and in turn the pigmentation (Fig. 2). Interestingly, light is a critical factor influencing the green callus formation with protocorm like proliferation at 25^0C and 12 h photoperiod with an

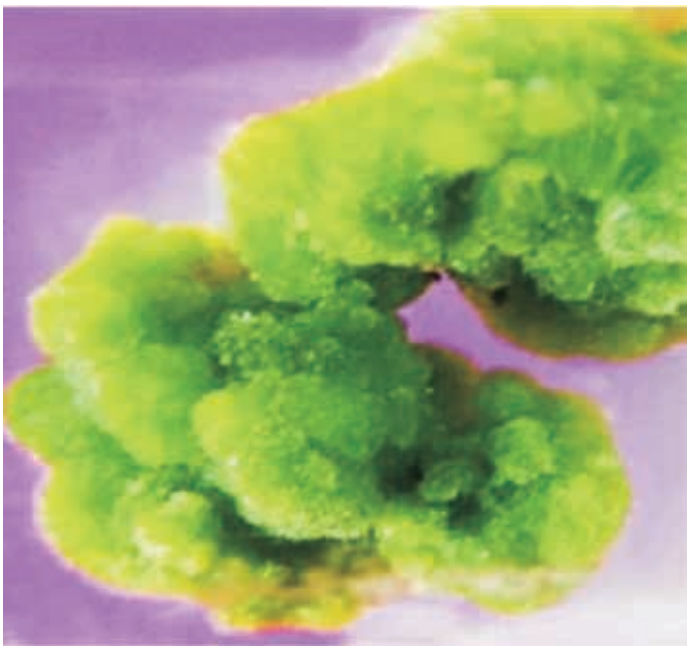

Fig. 1 : Green compact callus on MS medium supplemented with 2.0 mg/L BAP + 0.5 mg/L 2,4-D (30 days) of *C.infortunatum*

irradiance of 60 ± 2 µmol/m^2/sec.

Batch suspension cultures in triplicate were initiated with 2-3 g of friable callus as an inoculum at temperature 25±1°C having photoperiod of 16-8 µmol/m^2/sec at 110 rpm in liquid MS medium supplemented with the same combinations of the growth regulators as those used in callus culture yielded transparent, homogeneous and nonchlorophyllous cultures which were used to evaluate the growth of biomass and anthocyanin content. Growth pattern of each culture was determined by loss of weight dissimilation method.

Interestingly, the cultures yielded a sigmoid mode of curve with five growth phases i.e. lag, exponential, linear, stationary and progressive decline. The growth rate of cells were initially slow (lag phase) but as the cultures proceeded, the growth increased significantly and accumulated a great amount of fresh weight (4 fold) over a period of 20-25 days then the growth of cells became

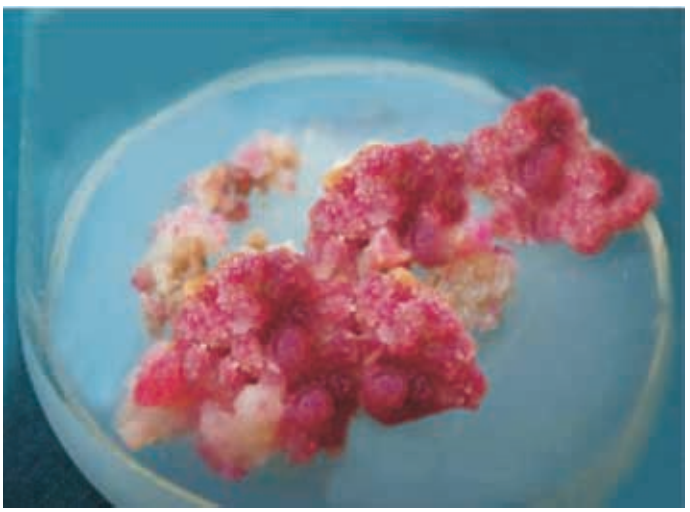

Fig. 2 : Production of pigment callus of *C.infortunatum* in MS medium fortified with 2,4-D (1mg/L) and kinetin (2mg/L) (Kn)

Table 1 : Cytotoxicity by different concentrations (µM) of anthocyanin at 24 and 72 h against HeLa cell lines

Conc (µM)	Cytotoxic (%)					
	HeLa cells		SW 480 cells		MCF 7 cells	
	24 h	72 h	24 h	72 h	24 h	72 h
Control	3.1 ±0.05	5.3 ±0.07	1.5 ±0.01	2.2±0.11	1.3±0.01	1.8 ±0.02
25	7.5 ±0.91	10.3±0.32	1.9 ±0.06	3.1 ±0.03	2.6±0.12	4 ±0.077
50	11.4±0.12	19.1 ±0.6	3.4 ±0.02	4.8 ±0.2	3.4±0.09	7.5 ±0.26
100	18.8±0.20	27 ±0.82	5.8 ±0.09	7.5 ±0.1	4.6±0.15	9.4 ±0.18
200	50 ±0.45	63 ±0.93	7.4 ±0.07	11 ±0.34	6.2±0.03	11.3 ±0.59
300	68 ± 0.56	70 ±0.15	10 ± 0.06	13 ±0.04	7.4±0.14	12 ±0.43
400	79.4 ± 0.7	86.2±0.23	10.5±0.08	13. 6±0.4	8.7±0.04	12.64 ±0.08

stable and started declining from 30th day. Maximum growth was achieved in suspension culture supplemented with Kin (2.5 mg/L) + 2, 4-D (0.5 mg/L) i.e., 3.86±0.01

Fresh cell weight (FCW) showed marginal increase initially with salicylic acid (SA) and then declined. The maximum FW noticed was at 20th day and subsequently declined i.e., 9.8 ± 0.32. Meanwhile, the anthocyanin content increased and attained a maximum value of 4.5 ± 0.22. Similarly, no sound variations were noticed with ethephon on the fresh cell weight and anthocyanin content. Further, phenylalanine and shikimic acid did not showed remarkable values on the FCW and anthocyanin content.

Quantification of anthocyanin

The anthocyanin content was quantified from the *in vitro* elicited cells and pigmented calli which showed a range from 1.7 to 4.5 mg/g monomeric anthocyanin i.e., approximately 5 fold increase compared with the *in vivo* seeds (0.89 mg/g).

The embryonic nature of agitated cells was analysed using acetocarmine staining method. Pinkish coloured cells were noticed indicate the active embryonic cells. Subsequently, the anthocyanin extracted from the callus was subjected to TLC. 3 prominent bands were obtained in the chromatogram with yellow, dark violet and green yellow colours having Rf values 0.77, 0.65 and 0.511 respectively. Based on the Rf values, anthocyanin were predicted as cyanidin, delphinidin and malvidin based compounds. Further, the crude anthocyanin extract was subjected

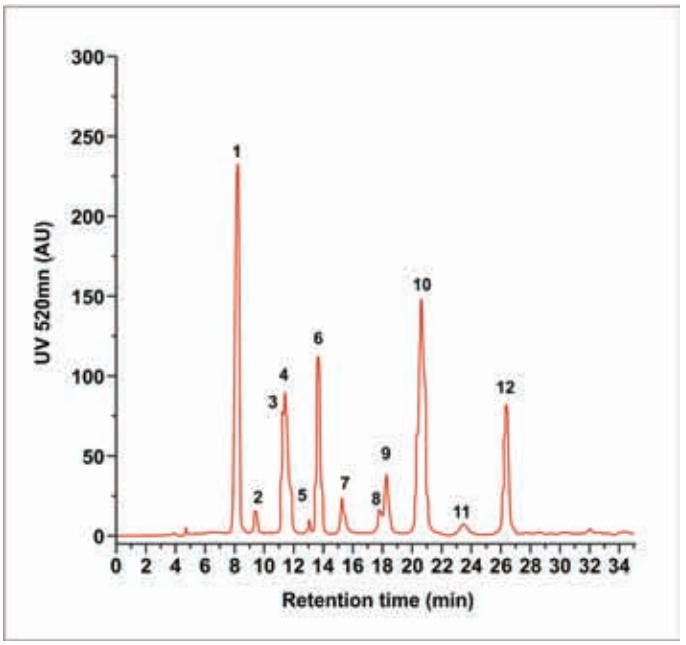

Fig. 3 : HPLC-PDA chromatogram of purified anthocyanin of *C.infortunatum* L.

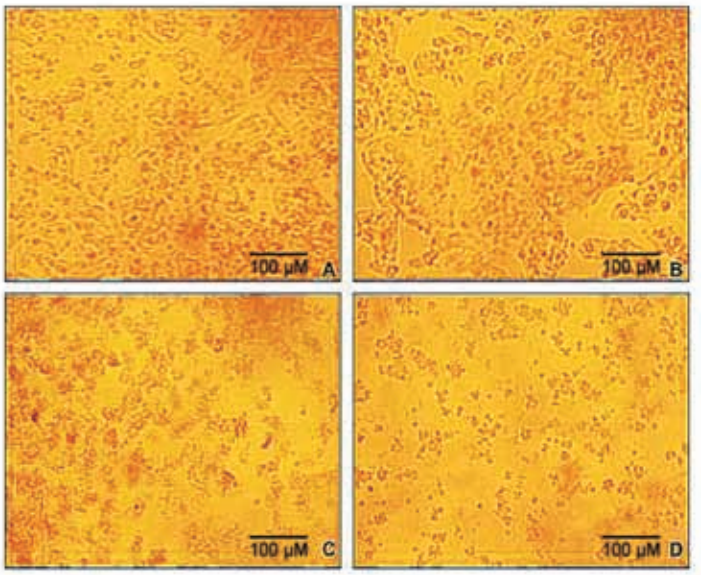

Fig. 4 : Cells treated with different concentrations of anthocyanin. Magnification=×400

a. Normal HeLa cell lines; b. 400µM; c. 300 µM; d. 200 µM

to purification with 3 different columns and further fractionated by HPLC- PDA analysis.

Purity evaluation by HPLC-PDA and molar absorptivity

The highest purity was found to be with Oasis MCX (90.9% ± 1.9) with molar absorptivity 90.9 ± 1.9%.

Identification and quantification of anthocyanins

The HPLC chromatogram of purified fractionated anthocyanin comprises 12 peaks (Fig. 3). Based on reference literature data, the elution order reported for anthocyanin derivatives were galactosides, glucosides and arabinosides. The fragmentation of delphinidin derivatives in MS2 yielded the formation of delphinidin aglycone *(m/z* = 302 [M+H] +), after the characteristic release at 160 indicating the galactoside or glucoside in case of compounds 1 and 2, respectively and the release of 131 indicating the arabinoside unit in case of compound 4. Using a similar procedure, the identification of all other compounds was carried. Peaks were identified as 1-Delphinidin-3-O-galactoside, 2-Delphinidin-3-O-glucoside, 3-Cyanidin-3-O-galactoside, 4-Delphinidin-3-O-arabinoside, 5-Cyanidin-3-O-glucoside, 6-Petunidin-3-O-galactoside, 7-Cyanidin-3-O-arabinoside, 8- Paeonidin-3-O-galactoside, 9-Petunidin-3-O-arabinoside, 10-Malvidin-3-O-galactoside, 11-Malvidin-3-O-glucoside, 12-Malvidin-3-O-arabinoside.

In vitro cytotoxic effect of anthocyanin

Varied *in vitro* inhibitory effect of anthocyanin was revealed by the MTT viability assay against HeLa cells, SW 480 and MCF 7 cell lines with the tested concentrations such as 0, 25, 50, 100, 200,300 and 400 µM at 24 and 72 h. Further, it revealed a concentration as well as time-dependent growth inhibitory effects against the different malignant cells (Table 1). The efficacy of the compound was confirmed at two different time intervals by calculating the IC_{50} values, concentration that causes 50% growth inhibition. The IC_{50} values of anthocyanin at 24 and 72 h time intervals (HeLa cells) were found to be 200 and 164.5 µM respectively. Further studies are carried only with HeLa cell lines only.

Control HeLa cells were regular in shape and size (Fig. 4a). At 400 µg of anthocyanin, the cells become irregular with distorted changes on the cell surface (Fig. 4b). After treatment with 300 µg, the cells become round in shape and size with alter nuclear cytoplasm ratio (Fig. 4c). Most of the HeLa cells had relatively flattened appearance with long multiple cytoplasmic processes forming cross bridges with adjacent cells (Fig. 4d).

Analysis of anthocyanin induced cellular morphology by fluorescence microscopy

HeLa cells were stained and evaluated for nuclear shape using a fluorescence microscope with Hoechst 33258 staining (Fig.5). The results revealed that anthocyanin treated cells showed considerable chromatin condensation or dense staining fragmentation called apoptotic bodies, which suggests an early apoptotic event.

Effect of anthocyanin on DNA fragmentation

Besides the morphological changes of apoptosis in anthocyanin treated HeLa cells, DNA fragmentation was also studied by evaluation of DNA ladder formation. As shown in the fig. 6, DNA ladder appeared to be conspicuous with the increasing anthocyanin concentration, however, no DNA fragments were seen in the control groups (Fig.6). However, 200, 300 and 400 µM doses of anthocyanin after 48 h exposure led to a considerable upsurge in DNA fragmentation (Fig.6).

Effect of anthocyanin on cell cycle phase distribution

DNA content in cells was detected by propidium iodide staining and flow cytometry. Flow cytometry data revealed that anthocyanin arrest sub-G1 cell cycle and induces the fraction of apoptotic cells in HeLa cells in a dose-dependent manner (Table 2). At the same time, the fractions of G0/G1 cells were decreased with the increase in the concentration of anthocyanin. The percentage of cells in S and G2/ M phase were more or less remained unaffected.

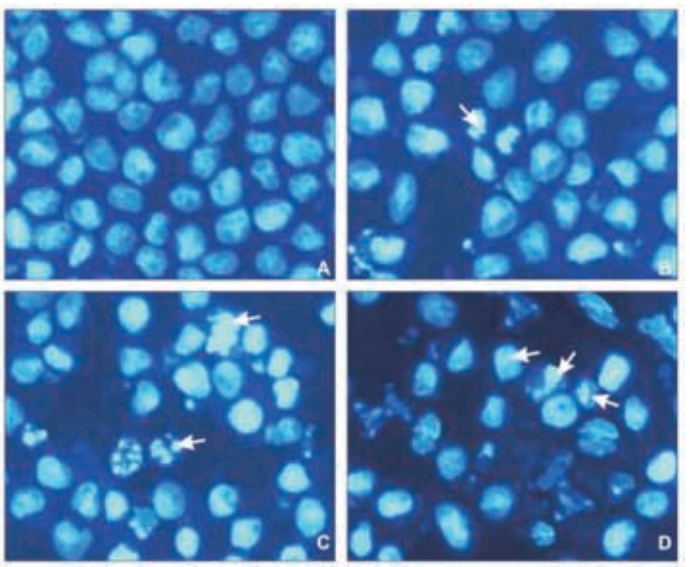

Fig. 5 : Effect of anthocyanin on the nuclear morphology of HeLa cells using Hoechst 33258 staining at actual magnification 200x. Control cells (A) and treated cells (B) 200 µM, (C) 300 µM and 400 µM (D) for 48 h. Arrows represent apoptotic cells exhibiting chromatin condensation and nuclear fragmentation

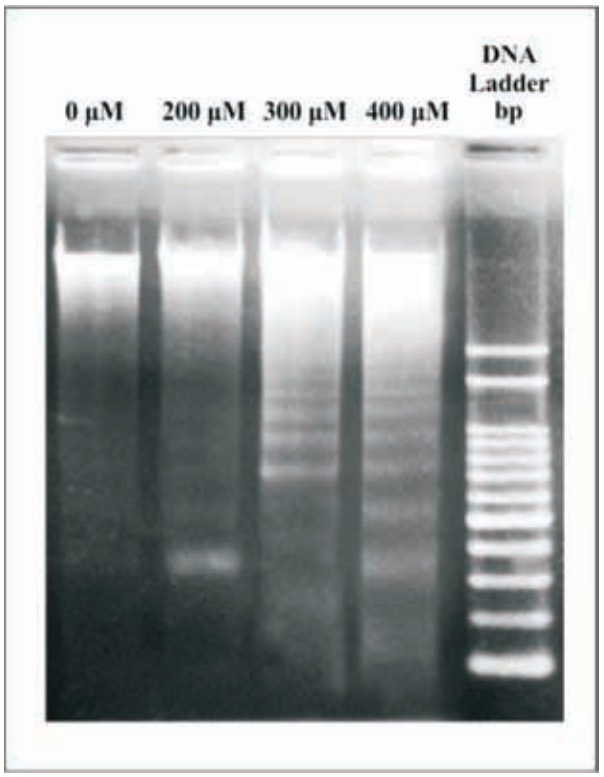

Fig. 6 : Anthocyanin induced DNA fragmentation in HeLa cell lines. The cells were treated with 0, 200, 300 and 400 µM of the compound for 48 h.

Anthocyanin induced both early and late apoptosis Annexin V/PI double staining was used to detect (HeLa cells) (Fig. 7). HeLa cells were treated with various concentrations (0, 200, 300 and 400 µM) of anthocyanin for 48 h. Anthocyanin induced both early and late apoptosis in a concentration-dependent manner (Fig. 7) as compared to the untreated control cells (Fig. 7). The different quadrants such as Q1, Q2, Q3 and Q4 reveal necrotic cells, late apoptotic cells, viable cells and early apoptotic cell population respectively. Percentage of apoptotic cells increases from 5.6% in control cells (A), to 29.3%, 37.1% and 45.2 % in 200 µM (B), 300 µM (C) and 400 µm (D) anthocyanin treated cells respectively.

DISCUSSION

MTT viability assay suggest that anthocyanin had potent cytotoxic effects on HeLa cancer cells as compared to others. Progressive inhibition noticed with concentration and time against the HeLa cell lines was remarkable. Morphometric anomali of HeLa cells further substantiates the MTT assay results.

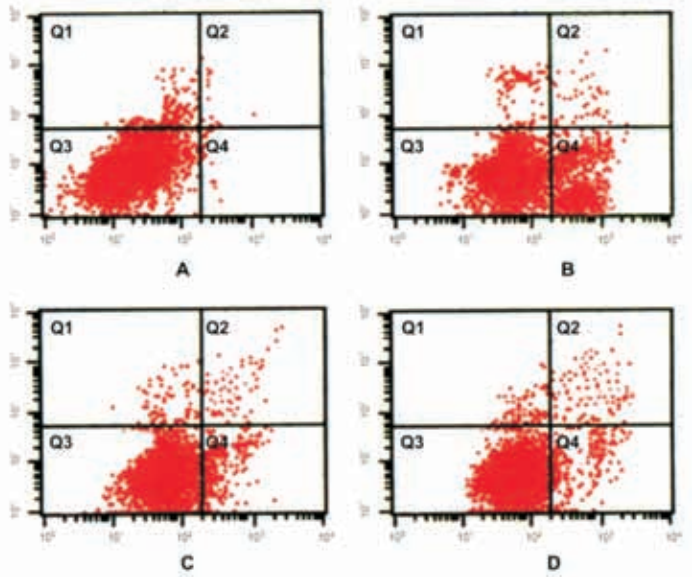

Fig. 6 : Quantification of anthocyanin induced apoptosis in HeLa cells. The cells were subjected to different doses (0, 200, 300 and 400 µM) for 48 h and analyzed by flow cytometry with annexin V-FITC/PI staining. The different quadrants Q1, Q2, Q3 and Q4 represent necrotic cells, late apoptotic cells, viable cells and early apoptotic cell population respectively.

Anthocyanin treated HeLa cells visualized by fluorescence microscope with Hoechst 33258 staining suggests the amount of apoptotic bodies was positively related to the concentration of anthocyanin. The morphologic changes revealed by HeLa cells were typical of apoptosis after treatment with the anthocyanin. Uniform spherical HeLa cells with normal morphology were seen in untreated cultures, whereas chromatin condensation, chromosomal DNA cleavage were noticed when HeLa cells were treated with anthocyanin at different concentrations.

DNA fragmentation evaluated by DNA ladder formation especially at 200, 300 and 400 µM doses of anthocyanin after 48 h exposure led to a considerable upsurge in DNA fragmentation (Fig.6). The DNA fragmentation is a marker of apoptosis, further confirming that the anthocyanin induced cell death through apoptosis.

Apoptosis and cell cycle are inter connected biochemical events, and any interference in cell cycle progression may eventually lead to apoptotic cell death. DNA content in cells was detected by propidium iodide staining and flow cytometry. In order to elucidate the mechanistic pathway of the growth inhibitory effect exerted by anthocyanin in HeLa cancer cells, flow cytometry analysis was carried out to confirm whether

Table 2 : Effect of anthocyanin on the cell cycle analysis of the HeLa cells. The cells were treated with 0, 200, 300 and 400 µM for 48 h and analyzed by flow cytometry

	Control	200 µM	300 µM	400 µM
Sub G0/G1	4.7 ±0.02	24.3 ±0.05	33.2 ±0.66	49.6 ±0.38
Go/G1	68.5 ±0.01	30.5±0.18	20.3 ±0.29	14.5 ±0.24
S	16 ±0.73	15.2 ±0.32	15 ±0.11	15.7 ±0.31
G2/M	12.4 ±0.21	11.4 ±0.02	10.9 ±0.09	10 ±0.07

anthocyanin triggers cell cycle arrest in the cell lines. Positive results were noticed and further establish the impact of anthocyanin in cell cycle arrest.

Present study reveals that anthocyanin exhibited potent proapoptotic potential in HeLa cell lines by inducing early, late apoptosis, cell cycle arrest in sub-G1 phase and DNA fragmentation. Cell viability assay using MTT showed that anthocyanin inhibited *in vitro* cancer cell growth in a concentration and time-dependent manner. The IC_{50} values of anthocyanin at 24 and 72 h time intervals were found to be 200 and 164.5 µM respectively. Fluorescence microscopy studies revealed that anthocyanin, treated cells showed remarkable chromatin condensation or dense staining fragmentation called apoptotic bodies, which inferred an early apoptotic event. Spherical HeLa cells with normal morphology were seen in untreated cells, whereas chromatin condensation and chromosomal DNA cleavage were seen when HeLa cells were treated with anthocyanin at various concentrations. Further, gel electrophoresis indicated that DNA ladder seemed to be more conspicuous with the increasing the anthocyanin concentration, however, no DNA fragments were noticed in the untreated groups. 200, 300 and 400 µM doses of the compound after 48 h exposure led to a considerable upsurge in DNA fragmentation. Flow cytometry studies indicated that anthocyanin induces sub-G1 cell cycle arrest and increases the fraction of HeLa apoptotic cells in a dose-dependent manner. However, the percentage of G0/G1 cells diminished with the increase in the dose of anthocyanin. Anthocyanin induced both early and late apoptosis in a concentration -dependent manner as compared to the untreated control cells. This study reports the effect of anthocyanin on cellular morphology, DNA fragmentation, cell cycle arrest, externalization of phosphatidylserine and activation of caspases 3/7. These results are of interest in view functional food components on potential cancer treatment and prevention.

Epidemiologic studies by Marcelia *et al* reported that major anthocyanins from purple-fleshed sweet potato (PFSP) Tainung 73 were cyanidin or/and peonidin and their acylated derivatives have potential anti-inflammatory and anticancer activities [15]. The anthocyanin extract could inhibit significantly the growth of some cancer cell lines, such as human breast cancer (MCF-7), gastric cancer (SNU-1), and colon adenocarcinoma (WiDr), in concentration- and time-dependent manner. The molecular mechanism demonstrated the ability to induce apoptosis in MFC-7 cancer cell line was through extrinsic and intrinsic pathways.

Anthocyanins have been evaluated for anti-cancer activity in reddish callus cultures of *Celosia cristata*. Remarkable amount of callus was obtained after 3 weeks in stem explants cultured on MS media supplemented with 0.51.0 mg/l 1-Naphthaleneacetic acid (NAA) and 0.5-1.0 mg/l 6- Benzylaminopurine (BAP). The cytotoxic effect of anthocyanin extract from pigmented callus was determined against HCT116 cancer cells line using MTT cell proliferation assay. Percentage of inhibition observed was 18% at 20 µg/ml of ethanolic extract solution [16].

In most of the studies related with anthocyanin reveals strong antioxidant activity such as the oxidative DNA damage prevention assay, hemolysis inhibition assay and liver mitochondria oxidative damage prevention assay. Similarly, apoptosis was another mechanism of melanoma cell death after anthocyanin treatment and confirmed morphologically by acridine orange/ethidium bromide double staining and TUNEL analysis.

Ratana *et al* evaluated the cytotoxic effects of purple rice methanolic (MeOH) and dichloromethane (DCM) extracts of three cultivars of purple rice in Thailand such as Doisaket (DSK), Nan and Payao (PYO), and was compared with white rice (KK6)[17]. Cytotoxicity by MTT assay in human hepatocellular carcinoma HepG2, prostate cancer LNCaP and murine normal fibroblast NIH3T3 cells. Interestingly, additive cytotoxic effects were reported on HepG2 and LNCaP cells, except with MeOH-extract which showed synergistic effects on HepG2 cells when combined with vinblastine. Thus, it is possible to confirm the antimetastatic potential showed by *C. infortunatum anthocyanin against SW480 cell lines.*

CONCLUSION

In the present study, remarkable pro-apoptotic activity was noticed with purified anthocyanin from *in vitro* culture of *Clerodendron infortunatum* against HeLa cells using MTT cell viability assay. Fluorescence microscopy revealed cellular morphological changes induced by anthocyanin. Anthocyanin treated cells exhibited significant apoptotic bodies, which inferred an early apoptotic event. Further work is warranted to isolate and characterize the individual bioactive fraction of anthocyanin and to evaluate its molecular antimetastatic mode of action.

Conflict of interest

No conflict of interest

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