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Original Article

Purified Anthocyanin, its Elicitation from Cell Cultures of *Begonia malabarica* and *Begonia rex-cultorum* 'Baby Rainbow'and its *In vitro* Cytotoxicity Analysis by MTT Assay

Aswathy Jayasree Madanakumar, Murugan Kumaraswamy*

ABSTRACT

Background: According to recent statistics, cancer accounts about marked percentage of total deaths in the world, although there are many therapeutic approaches. Unfortunately, the cytotoxicity properties of most chemotherapy drug are nonspecific and therefore do not distinguish between normal healthy cells and tumor cells, these events have led to inappropriate and toxic therapeutic agents with a wide range of side effects. However, several experimental and epidemiological studies have suggested that fruits and vegetables are associated with low risk of various types of cancer. Anthocyanins are natural pigments that provide intense purple to red color in plants. Anthocyanin possess the ability to inhibit oxidative stress and to induce apoptosis in malignant cells, thus may prevent carcinogenesis. **Methods:** Antiproliferative properties of purified anthocyanin extract from elicited cell suspension cultures of *Begonia malabarica* and *Begonia rex-cultorum* 'Baby rainbow' was investigated in terms of MTT assay. Anthocyanin extracts were tested for their ability to inhibit the growth of HT29 (colon cancer cells), MG63 (Osteosarcoma), HeLa (Cervical cancer cells) and L929 (Mouse Fibroblast L929) cell lines. **Results:** Cell viability decreased in a dose dependent manner in all the considered cell lines treated with anthocyanin extracts. The extract of *Begonia rex-cultorum* 'Baby rainbow' exhibited significant cytotoxic activity against all tumor cell lines than *Begonia malabarica* extract. *Begonia malabarica* and *Begonia rex-cultorum* 'Baby rainbow' anthocyanin extract exhibited the highest cytotoxicity towards HT29 and HeLa cell lines respectively. But, MG63 resulted in comparatively higher percentage of viability of cell lines at the same concentrations. The anthocyanin extract produced significant morphological alterations on cell lines in culture. Meanwhile, the extracts showed poor cytotoxicity against the normal cell line. **Conclusion:** The morphological alteration of the treated cancer cells presented clear evidence of significant cytotoxicity of anthocyanin extracts of both *Begonias* in all the three cell lines. Thus, anthocyanin may act as chemopreventive agents for various cancer cell lines.

Key words: Cytotoxicity, Cancer, Begonia, MTT assay, Anthocyanin, Cell suspension.

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INTRODUCTION

Cancers present a serious life style disorder and pose significant social and economic impacts on the health care system. Although there has been considerable improvement in the treatment of cancer, the overall prognosis remains unsatisfied.[1] More than 40% of the drugs identified were natural products and have not been chemically synthesized. Natural products and their derivatives including vinblastine, paclitaxel, and etoposide already proved critical roles in cancer chemotherapy. Anthocyanins are versatile group of polyphenolic compounds identified in plants responsible for vivid blue, purple, and red colour of fruits, vegetables and flowers.[2] Currently, anthocyanins have received much attention as a natural source of food and textile colourants. There is an increasing demand for natural food colourants that can be used as substitutes for synthetic colours. Moreover, anthocyanins possess putative health benefits as dietary antioxidants. The roles of anthocyanin as medicinal agents have been well-accepted dogma in folk therapy throughout the world. Secondary metabolites with bioactivity were isolated and used either directly or after chemical modification. Their pharmacological values are increasing due to the constant discoveries of their potential role in healthcare and as lead molecule for new drug designing. *Begonia*, a perennial flowering plant belongs to the family Begoniaceae, are rich source of anthocyanin content. Synthesis of anthocyanins often requires elicitors, which act as molecular signals in plant stress responses. Elicitation of cell cultures (using chemical or environmental) is a recognized and efficient means of maximizing anthocyanin pigment towards commercialization of product recovery. In both *in vitro* and *in vivo* research trials have demonstrated that the marked ability of anthocyanin to reduce malignant cell proliferation and to inhibit tumor formation.[3]

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Phytochemicals capable of retarding the cell cycle and/or activating the cellular apoptotic response in the cancerous cells is an attractive trend in antimetastatic studies. Thus, in the present study, a comparison of preliminary antiproliferative potential of the purified anthocyanin from the cell suspension cultures of *Begonia malabarica* and *Begonia rex - cultorum* 'Baby rainbow' were carried out on selected cell lines.

MATERIALS AND METHODS

Plant materials

The fresh healthy plants of *Begonia malabarica* and *B. rex-cultorum* 'Baby rainbow' were collected from the wild habitats. Identity was confirmed by referring floras and authenticated with herbaria of Jawaharlal Nehru Tropical Botanical Garden and Research Institute, Palode, Kerala. The voucher specimen was deposited in the herbarium of University College, Trivandrum (UCB 1207, UCB 1208)

Establishment of cell suspension culture

Fresh callus of *Begonias* species was cultured in a 250 mL flask with 40 mL of modified liquid MS culture medium at (25 ± 1)°C on a rotary shaker with a speed of 100 rotations per min (rpm) under 16 h illumination with 80 µmol m^{-2} s^{-1}. The medium was autoclaved at 121°C for 20 min.

The cell suspension derived from the 1 g calli tissue from leaf explants of *B. malabarica* were subcultured in liquid MS culture medium containing 2,4-D (0.1 mg L^{-1}), NAA (0.5 mg L^{-1}), and BAP (0.5 mg L^{-1}) every week until the cells showed continuous and stable accumulation of biomass. Meanwhile, in *B. rex-cultorum* 'Baby rainbow' (leaf explant) cell suspension was established in liquid MS medium supplemented with the growth regulators such as BAP (1 mg L^{-1}) + 2,4-D (0.5 mg L^{-1}), BAP (2.0 mg L^{-1}) + IAA (1.0 mg L^{-1}), KN (2 mg L^{-1}) + IAA (1 mg L^{-1}) and KN (2 mg L^{-1}) + 2,4-D (1 mg L^{-1}).

After cell culture for one cycle, the cells were harvested by filtration via a Buchner funnel, washed with distilled water to remove residual medium, and filtered again. Then, the weighed fresh cells (FW) were dried at 50°C to constant dry weight (DW). Cell growth was measured based on the FW and DW. Every 20 days, cells were subcultured to fresh media [with 5.0 g fresh weight (FW)] in 250-mL flasks.

Elicitation of anthocyanin in cell suspension culture

Different concentrations of peptone water, yeast extracts, phenylalanine, abscisic acid (ABA), salicylic acid (SA), zinc sulphate and methyl jasmonate (MeJA) were used to study the elicitation effect on anthocyanin production on the cell suspension cultures of *B.malabarica* (BM) and *B. rex-cultorum* 'Baby rainbow' (BR). MeJA and SA were dissolved in ethanol and all others were dissolved in water and were filtered before adding into the suspension cultures. Based on the results of preliminary experiments, the concentration employed were peptone water (0.015%), yeast extracts (1%), phenylalanine (10 µM), abscisic acid (0.25mg/L), salicylic acid (60 µM), zinc sulphate (50 µM)) and methyl jasmonate (1 µM). All experiments were performed in triplicate.

Quantification and purification of anthocyanin

Anthocyanin was isolated and quantified from the *in vitro* cell suspension and *in vivo* plants. The anthocyanin elicited cell suspension culture and fresh leaves were homogenized in 3 ml methanol with 1% HCl and the extract was used for quantification of anthocyanin by the protocol of Sutharut and Sudarat.[4] The absorbance was read at 510 and 700 nm against distilled water as blank.

The combined aqueous concentrates of anthocyanin after evaporation were purified by partition method against ethyl acetate and further purified by Amberlite XAD-7 column chromatography. Then, the adsorbed anthocyanins were eluted using ethanol containing 7% acetic acid (v/v) as mobile phase.

The column eluted fraction with highest anthocyanin content was used for the LC- MS analysis. The experiment was performed on a Thermo Scientific Dionex UltiMate® 3000 RSLC system with chromatographic separation achieved on a Thermo Scientific Acclaim® RSLC 120 C18 reversed-phase column (2.1 × 100 mm, 2.2 µm) operated at 40°C with gradient elution at 0.5 mL/min. The mobile phase consisted of three components: A) acetonitrile, B) deionized (DI) water, and C) 20% formic acid. Mobile phase C was held constant at 10% to provide 2% total formic acid in the mobile phase throughout the run. Mobile phase A was ramped from 0% to 8% from 11 to 42 min, then held for 13 min before returning to the initial composition from 55 to 60 min. Electrospray ionization (ESI) was used as the interface and was operated in positive selected ion monitoring (SIM) mode. The probe temperature was set at 500°C and needle voltage was set at 2000 V. The cone voltage was set at 50 V for all SIM scans with a span of 0.3 amu for each SIM.

In vitro antiproliferative effect determination by MTT assay

HT29 (colon cancer cells), MG63 (Osteosarcoma), HeLa (Cervical cancer cells) and L929 (Mouse Fibroblast L929) cell lines were cultured in 25 cm^2 DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate and antibiotic solution containing: Penicillin (100 U ml^{-1}), Streptomycin (100 µg ml^{-1}), and Amphoteracin B (2.5 µg ml^{-1}). Cultured cell lines were kept at 37°C in a humidified 5% CO_2 incubator. The viability of cells was evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method. Two days old confluent monolayers of cells were trypsinized and the cells were suspended in 10% growth medium. 100µl cell suspension ($5x10^4$ cells/well) was seeded in 96 well tissue culture plates and incubated at 37°C in a humidified 5% CO2 incubator. After 24 h the growth medium was removed, freshly prepared anthocyanin extracts in 5% DMEM were five times serially diluted by two-fold dilution (100, 50, 25, 12.5, 6.25 µg in 1000 µl of 5% MEM) and each concentration of 100 µl were added in triplicates to the respective wells and incubated at 37°C in a humidified 5% CO_2 incubator.

Detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity. 15 mg of MTT was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization. MTT assay was carried as per the protocol of Laura *et al.*[5] The absorbance was measured by using microplate reader at 570 nm.

The percentage of growth inhibition was calculated using the formula:

$$\% \text{ of viability} = \frac{\text{Mean OD Samples} \times 100}{\text{Mean OD of control group}}$$

Statistical analysis

Analysis of variance (ANOVA) was used to determine significant differences between treatments, using the statistical package STATISTICA. All the values were mean of 12 replicates ± SE. Significance level was noticed 5%.

RESULTS AND DISCUSSION

Cell suspension culture of B. malabarica and B. rex-cultorum 'Baby rainbow'

Cell suspension culture was established by culturing fresh friable calli from the leaf explants of *B. malabarica* in liquid MS media supplemented with definite combinations and doses of BAP, 2, 4-D and NAA with concentrations 0.5, 0.1, 0.5 mg L^{-1} respectively. MS liquid medium supplemented with 2, 4-D (0.1 mg L^{-1}) and BAP (0.5 mg L^{-1}) combinations showed well established suspension cultures i.e., suspensions without any aggregation or clumps of cells (Figure 1a). The *in vitro* suspension of

Figure 1 A: Cell suspension culture of Begonia malabarica from friable callus in MS medium fortified with 2, 4-D (0.1 mg L–1) and BAP (0.5 mg L–1) Figure 1 b: Cell suspension culture of Begonia rex - cultorum 'Baby rainbow' from friable callus in MS medium fortified with 2, 4-D (0.5 mg L–1) and BAP (1.0 mg L–1).

cells from *B. malabarica* revealed optimal and steady biomass accumulation on day 14. The same medium and growth hormone combinations were used for further growth kinetics studies. After 16th day, cells in the suspension exhibited a reduction in fresh weight as well as dry weight of cells (Table 1). The maximum fresh weight (8.0 g) and dry weight (0.83 g) was noticed at 14th day of culture in liquid MS medium complimented with 2, 4-D (0.1 mg L^{-1}) + BAP (0.5 mg L^{-1}). The time course of biomass accumulation was the typical sigmoid growth curves.

Similarly, the cell suspension culture was evaluated by culturing calli of *B.malabarica* in the liquid MS medium fortified with the combinations of BAP (0.5, 1, 1.5) and NAA (0.1, 0.5, 1.0 mg L^{-1}). MS liquid medium supplemented with NAA and BAP showed less significant suspension cultures with aggregated or clumped cells. Generally, the cell growth was slow during the initial 5 days of culturing. Thereafter, biomass accumulated rapidly, and reached the highest value on the 14th day. Then the culture entered the stationary phase and declined marginally. Some cultures continued to grow even up to 30th day.

Suspension cultures were also initiated for *B. rex-cultorum* 'Baby rainbow' with 2 g of friable callus as an inoculum in the liquid MS medium supplemented with the different combinations of the growth regulators ie., BAP (1 mg L^{-1}) + 2,4-D (0.5 mg L^{-1}), BAP (2.0 mg L^{-1}) + IAA (1.0 mg L^{-1}), KN (2 mg L^{-1}) + IAA (1 mg L^{-1}) and KN (2 mg L^{-1}) + 2,4-D (1 mg L^{-1}). Maximum growth was achieved in suspension culture supplemented with BAP (1 mg L^{-1}) + 2,4-D (0.5 mg L^{-1}), followed by KN (2 mg L^{-1}) + IAA (1 mg L^{-1}). BAP (2.0 mg L^{-1}) + IAA (1.0 mg L^{-1}), KN (2 mg L^{-1}) + 2,4-D (1 mg L^{-1}) combinations showed only minimum outputs. The maximum fresh weight (7.37 g) and dry weight (0.70 g) was observed at 14th day of culture in liquid MS medium supplemented with 2, 4-D (0.5 mg L^{-1}) and BAP (1 mg L^{-1}). Here also, the cell suspension growth was sigmoidal (Figure 1 b, Table 2).

Quantification of anthocyanin

Anthocyanin was quantified from the *in vitro* cell suspension culture and *in vivo* plants. The *in vitro* cells showed remarkable level of anthocyanin ie., 10.4 mg and 20.6 mg / 100 ml for *Begonia malabarica* and *B. rex-cultorum* 'Baby rainbow' respectively. However, in the *in vivo* plants anthocyanin content noticed was 5.7mg g^{-1} for *Begonia malabarica* and for *B. rex-cultorum* 'Baby rainbow' 9.8 mg g^{-1}. From the given results it can be speculated that anthocyanin content may be effectively induced through *in vitro* culture by changing the culture parameters. The present results seem to be more effective and supported by other *in vitro* cultures of phytochemicals from medicinal plants.

Elicitation by chemicals on anthocyanin production

B. malabarica and *B. rex-cultorum* 'Baby rainbow' cell suspension cultures showed poor accumulation of anthocyanin under elicitation using peptone water and yeast extracts. Similarly, the addition of phenylalanine (10 µM), the precursor of secondary metabolite synthesis at lower doses enhanced the anthocyanin synthesis in the cells. In fact, abscisic acid (ABA) (0.25 mg/L) [18.6 g/100 mL (*B. malabarica*) and 30.8 g/100 mL (*B. rex-cultorum* 'Baby rainbow')] and salicylic acid (SA) (60 µM) [19.2 g/100 mL (*B. malabarica*) and 34.4 g/100 mL (*B. rex-cultorum* 'Baby rainbow')] elicited anthocyanin synthesis remarkably with anthocyanin content (i.e., promising effect on anthocyanin induction in cell suspension cultures). Zinc sulphate at lower dose showed slight rise in anthocyanin production (50 µM), but the content was lower than that of SA or ABA treatments. MeJ did not showed remarkable increase in anthocyanin production in cell cultures. Meanwhile, Saniewski *et al.*[6] succeeded in inducing anthocyanin synthesis by methyl jasmonate in shoots of *Crassula multicava*. Cetin and Baydar [7] elicited effectively cell suspension culture of phenolic compound synthesis in grapevine using physical parameters.

Purification and identification of anthocyanin

25 g of fresh, homogenized cell suspension sample was extracted out from the cultures of *Begonia malabarica* and *B. rex-cultorum* 'Baby rainbow'. The combined aqueous concentrates after evaporation were purified by partition method against ethyl acetate to remove chlorophylls, stilbenoids, less polar flavonoids and other non-polar compounds from the mixture. The non-aromatic compounds were removed with the use of Amberlite XAD-7 column chromatography. Amberlite XAD-7 adsorbs the aromatic compounds including anthocyanins and other flavonoids in aqueous solutions, whereas free sugars and other polar non-aromatic compounds were removed by washing with distilled water until the eluted water has a neutral pH. Therefore, aqueous acidified ethanol with the concentration of 75% was used for the desorption of anthocyanins from the Amberlite XAD-7HP column. Subsequently, purified amberlite column eluted fraction was used for the antimetastatic analysis.

LC- MS/MS was successful in identifying the major anthocyanin fractions of *B. malabarica* and *B. rex-cultorum* 'Baby rainbow'. The major anthocyanin fractions from the two species were eluted between 4.7 and 5.4 min. Tandem MS of the *m/z* 655.3 peak was identified as the major anthocyanidin fraction namely, malvidin-3,5–diglucoside. The other peaks identified were malvidin or peonidin (584.3), delphinidin + glucose (459.2), cyanidin (403.2) and cyanidin aglycone (287.1). The others were sugar derivatives or minor fragments (m/z 242.3, 195.1 and 144.1) (Figure 2 a). Meanwhile, the major anthocyanin of *B. rex-cultorum* 'Baby rainbow' were similar to the anthocyanins of *B.malabarica* and were identified as (655.3) malvidin-3 -diglucoside, (584.3) malvidin or peonidin, (468.4) delphinidin + glucose, (403.2) cyanidin, (286.2) cyanidin aglycone and others (242.3, 195.1 and 144.1) may be sugar derivatives or insignificant fragments (Figure 2 b).

Cytotoxicity analysis by MTT assay

Many vegetables and fruits in the human diet have been proved as potential neutraceutical agents and consuming optimal amounts can inhibit the development of life style diseases. Cancer is one of the major issues

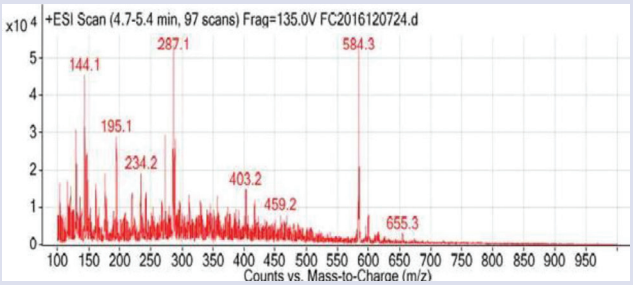

Figure 2 a: LC- MS analysis of *B. malabarica*.

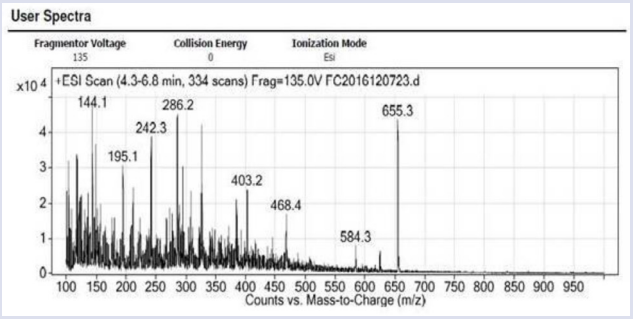

Figure 2 b: LC- MS analysis of *B. rex-cultorum* 'Baby rainbow'.

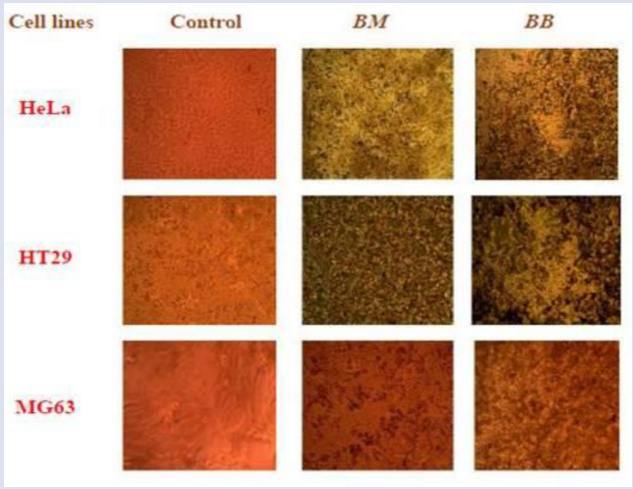

Figure 3: MTT assay of purified anthocyanin extract of *Begonia malabarica* and *B. rex-cultorum* 'Baby rainbow' on different cell lines (100 µg ml-1 concentration) along with their control.

Table 1: Fresh (FW) as well as dry weight (DW) of cells obtained in suspension culture for first 20 days of *B.malabarica* in liquid MS medium with 2, 4-D (0.1 mg L^{-1}) + BAP (0.5 mg L^{-1}).

Days	FW -Mean (g)	DW- Mean (g)
2	0.844	0.130
4	2.268	0.366
6	3.454	0.418
8	5.388	0.504
10	6.108	0.628
12	7.106	0.738
14	8.090	0.832
16	8.082	0.848
18	7.834	0.836
20	7.800	0.820
Mean	5.697	0.612
F- FW	1735.196	
F-DW	196.730	
SE-FW	1.51	
SE-DW	5.57	
CD-FW	4.953	
CD-DW	1.817	

Table 2: Fresh (FW) as well as dry weight (DW) of cells obtained in suspension culture for first 20 days *B.rex-cultorum* 'Baby rainbow' in liquid MS medium BAP (1mg L^{-1}) + 2,4-D (0.5 mg L^{-1}).

Days	Mean	Mean
2	0.740	0.136
4	1.814	0.366
6	2.598	0.4400
8	3.152	0.476
10	4.364	0.538
12	5.876	0.556
14	7.378	0.704
16	7.382	0.704
18	7.378	0.698
20	7.344	0.694
Mean	4.803	0.531
F-FW	1563.58	
F-DW	196.730	
SE-FW	6.57	
SE-DW	1.54	
CD-FW	0.185	
CD-DW	4.353	

worldwide. Therapeutic herbals have been utilized massively by the local populations as remedial for many disorders and hence, it is need of the hour to evaluate the efficacy in terms of toxicity in using them. Several studies have reported that herbal extracts can be used in the management of oxidative stress.

In vitro cytotoxicity activity analysis against selected cancer cell lines

In the present study, the cytotoxic effect of purified anthocyanin was analyzed using MTT assay on three cancer cell lines (HT29, MG63 and HeLa cell lines) and one normal cell line (Mouse Fibroblast L929). The concentration employed ranged from 6.25 to 50 µg ml^{-1}. IC_{50} level of

Table 3a: Percentage of viability of different cancer cell lines against purified anthocyanin from *Begonia malabarica*.

Concentration (µg ml^{-1})	24h	48h	72h
HELA Cervical			
6.25	88.15	70.2	61.5
12.5	65.48	59.3	53.6
25	52.02	47.4	41.7
50	31.38	29.5	28.2
HT29 Colon			
6.25	75.42	69.3	63.5
12.5	61.18	55.2	54.0
25	50.21	47.0	45.2
50	38.4	34.3	33.0
MG63 Bone			
6.25	82.38	74.4	61.0
12.5	70.62	63.2	54.5
25	60.08	53.0	43.0
50	51.8	47.3	30.1
F HELA	3254.69**	2815.2**	2459.6**
F HT29	1398.44**	1416.5**	1512.4**
FMG63	1668.28**	1715.3**	1800.6**
CD	0.410	0.312	0.504

Table 3b: Percentage of viability of different cancer cell lines against purified anthocyanin from *Begonia rex-cultorum* 'Baby rainbow'.

CONCENTRATION (µg ml^{-1})	24h	48h	72h
HELA Cervical			
6.25	73.5	70.2	62.6
12.5	65.12	61.5	53.8
25	53.15	50.2	44.3
50	40.88	37.3	30.2
HT29 Colon			
6.25	67.68	60.7	51.3
12.5	57.65	49.3	41.6
25	38.3	31.2	27.5
50	26.62	24.3	19.6
MG63 Bone			
6.25	71.86	64.5	55.2
12.5	69.32	54.2	44.4
25	50.32	37.6	30.3
50	39.18	30.1	22.5
F HELA	1157.02**	1816.5**	1714.63**
F HT29	1967.18**	1188.5**	1316.54**
FMG63	1775.14**	1610.61**	1488.59**
CD	0.421	0.358	0.324

anthocyanin was further evaluated at different time intervals i.e., 24, 48 and 72 h against each cell lines. The *in vitro* cytotoxic activities of purified anthocyanin from *Begonia malabarica* and *B. rex-cultorum* 'Baby rainbow' were shown in the Table 3 a and b and IC_{50} values were determined from the dose response curves (Table 3 a and b). Comparison of purified anthocyanins from the two-species revealed that anthocyanin from *B. rex-cultorum* 'Baby rainbow' exhibited significant cytotoxic activity against all tumor cell lines with remarkable IC_{50} values. However, anthocyanin of *B. malabarica* showed tumor selective cytotoxic activity depending on the cell line type i.e., HeLa cervical cell were the most sensitive cell line and MG63 was the most resistant tumor cell line (in terms of IC_{50} values). Meanwhile, *B. rex-cultorum* 'Baby rainbow' anthocyanin extract was more cytotoxic against HT29 cell lines followed by MG63 bone cells (Table 3 a and b). The values obtained were statistically significant.

The cytotoxic impact of the anthocyanin was evaluated *via* MTT assay revealed the cell viability based on the reduction of yellow tetrazolium MTT to a purple formazan dye by the mitochondrial dehydrogenase enzyme. The quantity of formazan produced reflects indirectly the number of metabolically active viable cells. MTT results showed that anthocyanin of both the species displayed cytotoxic effect against cancer cell lines in a concentration dependent manner. Such anti-proliferative potential of anthocyanin was featured by its concentration and tumor-selective manner, as reflected by the comparatively low IC_{50} values. The IC_{50} concentrations of anthocyanin from *B. malabarica* and *B. rex-cultorum* 'Baby rainbow' were evaluated against different cell line for 24 h. The IC_{50} values of *B. malabarica* were 35.9, 26.2, 53.2 µg mL^{-1} against HeLa, HT 29, MG63 cell lines respectively. Meanwhile, *B. rex-cultorum* 'Baby rainbow' displayed 38.7, 20.4, 26.83 µg mL^{-1} against HeLa, HT 29, MG63 cell lines respectively. Anthocyanin extracts of *B. malabarica* and *B. rex-cultorum* 'Baby rainbow' have shown significant inhibition of HT29 cell proliferation as compared to other cell lines. Further, a duration (24, 48, 72 h) and concentration dependent analysis was carried. When the duration of treatment was prolonged to 48 and 72 h, the % of viability was found to decrease concentration and duration dependently. At the concentrations of 6.25, 12.5, 25 and 50 µg mL^{-1} of *B. malabarica*, the % of viability further decreased to 61.5, 53.6, 41.7, 28.2 % respectively in HeLa cells after 72h. A similar tendency was noticed with HT29 and MG63 cell lines. The results were found to be statistically significant. The concentration and duration dependency of anthocyanin extract may be due to the presence of different anthocyanin fractions that function synergistically together. *B. rex-cultorum* 'Baby rainbow' also showed a similar trend against HeLa, HT29 and MG63 cell lines (Table 3 a and b)

Cytotoxic effect on Mouse Fibroblast (L929) Cells

No significant cytotoxicity was noticed with the anthocyanin of *B. malabarica* and *B. rex-cultorum* 'Baby rainbow' by MTT assay on mouse fibroblast (L929) cells. The IC_{50} values were found to be 14.8 and 5.5 g L^{-1} respectively. Thus, revealing the selectivity mode of action of anthocyanin i.e. toxic towards malignant cells and safe against fibroblast cells.

Morphological studies

In this part of the study, morphological anomaly if any with anthocyanin treatments was analyzed. No visible morphological changes were noticed for L929 cells with anthocyanin treatments. In fact, HT29, MG63 and HeLa cell lines (Figure 3), showed an increased number of rounded cells and growth inhibition when compared with the untreated control cells. This data further substantiates the MTT results.

Shahneh *et al.*[8] reported varied *in vitro* cytotoxic activity by MTT assay of four herbals used in persian traditional medicine on six cancer cell lines such as non-Hodgkin's B-cell lymphoma, human leukemic monocyte lymphoma, human acute myelocytic leukemia, human breast carcinoma, human Prostate Cancer and mouse fibrosarcoma cell lines and one normal cell line; Human Umbilical Vein Endothelial Cells. Masuda *et al.*[9] screened medicinal and edible plants in Okinawa, Japan. Cytotoxic

activity of ethanol extracts from 36 species was evaluated against K562 human leukemia cells. *Rhodea japonica* and *Hypericum chinense* were cytotoxic at a concentration of 10 mg ml^{-1}. The main cytotoxic constituent of *Rhodea japonica* was isolated and identified as rhodexin A.

Nalbantsoy *et al.*[10] analyzed antimicrobial and cytotoxic activities of *Zingiber officinalis* extracts. The results of the morphological observation and MTT assay revealed that the cytotoxic activity was dose dependent. IC_{50} values versus L929 and HeLa cells were found to be 87.28 μg ml^{-1} and 74.32 μg ml^{-1}, respectively, for the chloroform extract, while the ethanol extract showed IC_{50} values at 101 μg ml^{-1} and 33.78 μg ml^{-1}, respectively.

Weisburg *et al.*[11] compared the *in vitro* cytotoxicity of epigallocatechin gallate and tea extracts against cancerous and normal cells from the human oral cavity. Negahdari *et al.*[12] compared wound healing activity of extracts and herbal formulations of *Aloe vera*, henna, *Adiantum capillus-veneris* on mouse dermal fibroblast cells. All the results substantiate that phytochemicals from plants showed selective toxicity against cells in a varied manner as revealed by the anthocyanin of the present study.

CONCLUSION

In the present study, significant antiproliferative activity was found, which varied among the Begonia species, but the values were acceptable comparing to the international standards. Further work is required to isolate and characterize the individual bioactive fraction of anthocyanin and to evaluate its potentiality.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest pertaining to this work.

ABBREVIATION USED

2,4-D: 2,4-Dichlorophenoxyacetic acid; **BAP:** Benzyl Amino Purine; **Kn:** Kinetin; **NAA:** Naphtalic Acetic Acid

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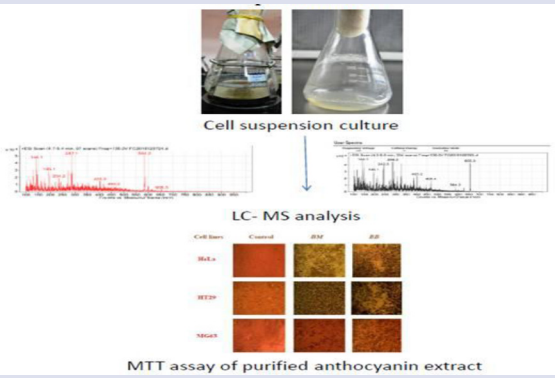
GRAPHICAL ABSTRACT



SUMMARY

- The present investigation reveals an optimal protocol for the synthesis of anthocyanin in *Begonia malabarica* and *B. rex-cultorum* 'Baby rainbow'.
- Anthocyanin content recorded in cell suspension culture was significantly higher compared with *in vivo* plants grown in fields.
- The anthocyanin showed significant antiproliferative activity, which varied among the Begonia species.

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