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Research Article

Comparison of Purified Anthocyanin Isolated from *In Vitro* Cell Suspension Culture of *Begonia malabarica*, *Begonia rex-cultorum* 'Baby Rainbow' and its Antioxidant Activity

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ABSTRACT

Anthocyanins are the most common flavonoid molecules of vegetables and fruits, especially berries. Human consumption of anthocyanins represents the highest among the flavonoids. Epidemiological studies have suggested that the consumption of anthocyanins lowers the risk of life style disorders like cardiovascular disease, diabetes, arthritis and cancer. *Begonia malabarica* Lam. of Begoniaceae, is used traditionally as anti-hypoglycemic, antimicrobial, wound healing and in the treatment of anemia. *Begonia rex-cultorum* 'Baby rainbow' an ornamental species was also substituted. Experimentation of *in vitro* cell suspension culture, isolation, purification of anthocyanin and its antioxidant potential are targeted in the present study. Explants such as leaves and nodes were cultured on MS medium with various phytohormones for callus induction. Leaf explants of *Begonia* cultured on MS medium fortified with 2, 4-D and BAP showed significant callus induction and also in terms of fresh and dry weights. Significant reddish coloured callus was achieved in cultures initiated from nodal explants in MS medium supplemented with 2, 4-D. Cell suspension cultures were also established in liquid MS medium. After 14 days of culture, cell suspension was obtained with optimal biomass accumulation. Subsequently, bioactive anthocyanin was isolated, purified and fractionated from *B. malabarica* and *B. rex-cultorum*'Baby rainbow' using amberlite column chromatography and LC-MS/MS analysis. The major anthocyanins eluted from *Begonia* species at 4.7-5.4 min. Tandem MS of the *m/z* 655.3 peak was identified as anthocyanidin Malvidin-3 –diglucoside as the major compound. The other peaks identified were (584.3) Malvidin or Peonidin, (459.2) Delphinidin + Glucose, (403.2) may be Cynanidin, (287.1) Cyanindin Aglycone and other m/z 242.3, 195.1 & 144.1 were sugar derivatives or fragments. Purified anthocyanin exhibited remarkable inhibition of linoleic acid oxidation and also a concentration dependent free-radical scavenging activities were noticed against DPPH·, hydroxyl radicals and superoxide anions. The degradation of deoxyribose by hydroxyl radicals was also inhibited via iron ion chelators, rather than by directly scavenging the radicals. The results are comparable with reducing power activity of ascorbate and catechin.

Keywords: *Begonia*; purification, fractionation; anthocyanin; antioxidant activity; reducing power; free radicals; lipid peroxidation.

INTRODUCTION

Anthocyanins are flavonoid group of polyphenolics, which are responsible for the red to bluish colours of fruits, flowers, and leaves. Due to their frequent presence in fruits, vegetables and seeds, they are key molecules of the human diet. Awareness in anthocyanins has increased during the past decade. Many studies have revealed that anthocyanins have wide application in health-promoting characters. Therefore, these molecules are considered to be a functional food factor, which may minimize chronic diseases. Seventeen different anthocyanidins are reported. Out of that six were common like cyanidin, delphinidin, petunidin, peonidin, pelargonidin, and malvidin[1]. Differences between individual anthocyanidins are due to (a) number and the position of OH groups, (b) degree of methylation of OH groups, (c) nature, number, and location of sugars attached to the molecule, (d) the nature and number of aliphatic or aromatic acids attached to the sugar[2] (Mazza, 2007). Major therapeutic values of anthocyanins are antidiabetic, antiobesity and as neuroprotective agents[1,3]. Similarly, they are known to mitigate inflammation and exert cardiovascular protection[2,4] and further, inhibiting cancer growth[5]. The antioxidant potential of anthocyanins depends on the chemical structure of phenolics. This is further influenced by number of hydroxyl groups; the catechol moiety in the B ring; the oxonium ion in the C ring; the hydroxylation and methylation pattern; acylation; and glycosylation[6]. Glycosylation often decreases radical scavenger activity as compared with aglycone, as it reduces the ability of anthocyanin radicals to delocalize electrons. The contribution of the B ring substitutes to the efficiency of

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antioxidant activity is −OH > −OCH[3] >> −H, and thus the potency is in the order of dp > pt > mv = cy > peo > pl. Furthermore, the positively charged oxygen atom makes it potent and hydrogen−donating antioxidant, as compared to oligomeric proanthocyanidins and other flavonoids.

Medicinal plant wealth in South India has led to an escalating curiosity in the exploration of ethnomedicinal herbals as potential source of drugs. The herbal products symbolize safety in contrast to the synthetics. Dried aerial parts of *Begonia malabarica* has been made into paste with coconut oil and is taken to treat rheumatic pain or plant juice along with honey for blood purification or reduce fever and also taken as a general health tonic. The leaf juice mixed with ginger is taken for treating anemia. Kanikkar tribals of Agasthiarmalai Biosphere Reserve used the fresh leaf of *B. malabarica* with salt to treat giddiness[7]. The fresh leaf juice has been taken orally in empty stomach to relieve stomach pain by the tribals[8-10]. Leaf juice has been used for head ache and to cure wounds. *B. malabarica* is an important medicinal plant and the major phytochemicals reported were luteolin, quercetin and β-sitosterol. The leaves have been used for the treatment of respiratory infections, diarrhea, blood cancer and skin diseases. No reports on cultivation, breeding and improvement programmes including *in vitro* studies are available despite its commercial importance. In this juncture, the present study is aimed at *in vitro* cell suspension of *B. malabarica* and *Begonia rex-cultorum*'Baby rainbow', extraction, purification, fractionation of anthocyanin and its antioxidant potentialities.

MATERIALS AND METHODS

Plant material

The fresh healthy plants of *Begonia malabarica* and *Begonia rex-cultorum* 'Baby rainbow' were collected from the green house of Department garden. Identity was confirmed by referring floras and confirmed by authenticating with herbaria of Jawaharlal Nehru Tropical Botanical Garden, Palode, Kerala. The voucher specimens were deposited in the herbarium of University College, Trivandrum (UCB 1207, UCB 1208).

Surface sterilization

Various surface sterilization protocols were adapted using sterilants such as 0.01-0.1% mercuric chloride for 3-8 min, sodium hypochlorite - 5-15% for 5-10 min or calcium chloride 10-15% for 10 - 20 min. Invariably, Tween 20 the surfactant was used with all the sterilants. The explants used for *in vitro* callus induction were leaf and nodal regions. 0.1% mercuric chloride for 3-8 min was found to be the most effective sterilant. Subsequently, the sterilized explants were cultured on different culture media such as MS medium[11], Whites medium, Knops medium fortified with various combinations of hormones. MS media supplemented with 3% sucrose and 0.5% agar was yielding better. The cultures were incubated at 25 ± 2^{0}C with 12/8 h light/dark photoperiod (1000 lux intensity).

Induction of Callus

The sterilized leaf and nodal explants of *Begonia* species were inoculated in MS medium supplemented with various concentrations and combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) (0.1- 1 mg/l) alone and in combination with Benzylaminopurine (BAP) (0.5-2.0 mg L^{-1}), IAA (0.5- 2.0 mg L^{-1}) and Kinetin (0.5- 2.0 mg L^{-1}) for callus induction.

The cultures were maintained in continuous light/dark photoperiod with temperature 25 ± 2 ^{0}C. Data were recorded after 4 weeks on fresh weight of callus.

Establishment of cell suspension culture

Friable callus of *Begonia* were 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 80 rotations per minute (rpm) under 16 h illumination with 60 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 of *Begonia malabarica* were subcultured in liquid MS culture medium containing 2,4-D (0.1 mg L^{-1}), NAA (0.5 mg L^{-1}), and 6-BA (0.5 mg L^{-1}) every week until the cells showed continuous and stable accumulation of biomass.

Meanwhile, in *B. rex-cultorum* 'Baby rainbow' 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}), Kin (2 mg L^{-1}) + IAA (1 mg L^{-1}) and BAP (2 mg L^{-1}) + IAA (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 DW.

Extraction and purification

Anthocyanins were extracted with methanol containing 0.5% HCl (v/v) the extraction and purification was done according to the standard protocol of Andersen[12].

The aqueous extracts obtained after the liquid-liquid partition was purified through 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. Then the adsorbed anthocyanins and other flavonoids were eluted using methanol containing 0.5% HCl (v/v) as mobile phase[12].

LC-MS/MS analysis

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.

Antioxidant assay (AOX) analysis

Linoleic acid system

AOX activity of purified anthocyanin against lipid peroxidation was evaluated using peroxidation of linoleic acid of the ferric thiocyanate method (FTC), as described by Takao et al.[13] with minor modifications. The principle is peroxides formed can oxidize Fe^{2+} to Fe^{3+}. The Fe^{3+} ions form a complex with SCN- display a maximum OD at 500 nm. Thus, significant OD value result a high peroxide formation during the emulsion incubation. Catechin was used as a positive control.

α,α-diphenyl-β-picrylhydrazy (DPPH·) radicals

DPPH· radicals scavenging potential was analyzed according to the method of Larrauri et al. [14] with slight modifications. L-ascorbate and catechin were used as positive controls.

Superoxide radical ($O_2^{\cdot-}$) scavenging power assay

This was evaluated as per the method of Siddhurajua et al.[15]. The $O_2^{\cdot-}$ scavenging activity was quantified by the formulae (1−Asample/Acontrol)·100, where A is the absorbance using sample or control.

Reducing power assay

The reducing power was analyzed as per the method of Oyaizu[16]. The higher OD indicated a higher reducing power.

Inhibitory effect on deoxyribose degradation

The inhibitory effect of anthocyanin on deoxyribose degradation was measured by the reaction activity between either antioxidants or hydroxyl radicals (referred as non-site-specific scavenging assay) or antioxidants and iron ions (referred as site-specific scavenging assay), described by Lee et al.[17]. The OD was measured at 532 nm. Site-specific scavenging activity, which refers to the ability to chelate iron ions and interfere with hydroxyl radical generation, was measured using the same reaction buffer without EDTA. The control contained methanol instead of anthocyanin solution. Percentage inhibition of deoxyribose degradation was calculated as (1−Asample/Acontrol)·100, where A is the absorbance using sample or control.

ABTS radical scavenging activity

The total antioxidant capacity was determined using the horseradish peroxidase catalyzed oxidation of 2,2-azino-*bis*-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS). The reaction mixture contained 0.5 mL of 1000 μM ABTS (in ddH_2O) and 3.5 mL of 100 μM H_2O_2 (in 0.1 M PBS). The reaction was started by the addition of 0.5 mL of 44 UmL^{-1} peroxidase (in 0.1 M PBS). After 1 h, 0.05 mL of different concentrations of anthocyanin (for lipophilic and hydrophilic TEAC quantification, respectively) was added to the mixture. Absorbance was measured at 730 nm after 10 min. Trolox was used as a standard and the total antioxidant capacity of the sample extract was measured. The TEAC value was expressed as μmol Trolox per gram dry weight.

FRAP assay

The FRAP (Ferric reducing antioxidant power assay) procedure described by Benzie and Strain[18]. The principle of this method is based on the reduction of a ferric-tripyridyl triazine complex to ferrous colored form in the presence of antioxidants.

Statistical analysis

All experiments were carried out 12 replicates. Data were statistically analyzed for significance by analysis of variance with mean by means ± SD using the Statistica software (release 7.5).

RESULTS AND DISCUSSION

In vitro response varied greatly depending upon the medium, explant, age of explant and hormonal combinations as well as on the culture maintenance conditions. MS medium was found to be the best for the growth and regeneration of the *in vitro* cultured plantlets and also for callus induction.

Callus induction

In vitro cultured plantlets were used for initiating aseptic cultures and callus induction. Young leaf and nodal regions from the *in vitro* plantlets were used as sources of explants. The explants of *B. malabarica* were cultured for callus induction in MS medium supplemented with various concentrations of 2, 4-D alone (0.1-1.0mg L^{-1}) and also in combination with BAP (0.5-2.0mg L^{-1}). Callusing initiated within 16 days of inoculation and subsequently, calli were subcultured once in 2 weeks (Fig.1). Leaf and nodal explants showed high friable light yellowish coloured callus with embryogenic cells on MS medium fortified with 2, 4-D (0.3 mg L^{-1}) (Fig1). Interestingly, significant callus yield was noticed with leaf explants (0.3 mg L^{-1} 2, 4-D+ 0.5 mg L^{-1} BAP produced 2.5g of callus). Optimal yield of compact yellowish green callus was obtained on MS medium with both 2,4-D (0.5mg L^{-1}) and BAP (0.5mg L^{-1}) which was comparatively higher than MS medium supplemented with 2,4-D alone. Callus yield ranged from 1.0- 2.5g fresh weight after 3 months.

Meanwhile callusing was induced in explants of *Begonia rex-cultorum*'Baby rainbow' only in the combination of cytokinin and auxins. Cytokinins like benzyl amino purine (BAP) and kinetin (Kin) were tried. Auxins used for the induction of callus were (2,4-D) and indole acetic acid (IAA). Callus from different explants showed varied response when cultured under different growth regulator regimes. Percentage of callusing was significant for leaf explants. Maximum percentage of callus was observed in MS medium with BAP (1mg L^{-1}) + 2,4-D (0.5 mg L^{-1}) (86%) which gave creamy, embryogenic and fast growing callus for leaf explants (Fig 2 and b). Callusing response was also good in the case of leaf explants inoculated on media with BAP (2.0 mg L^{-1}) + IAA (1.0 mg L^{-1}) (69.6%) and was compact (Figure 2a and b). Callusing response in Kin (2 mg L^{-1}) + IAA (1 mg L^{-1}) and BAP (2 mg L^{-1}) + IAA (1 mg L^{-1}) showed variation but was

Figure 1: Friable callus of *B. malabarica* obtained on MS medium fortified with 2,4-D (0.3 mg L^{-1}) in leaf explants.

compact i.e. not ideal for initiating suspension culture. The duration for callusing was 4-5 weeks over the entire explants.

Cell suspension culture

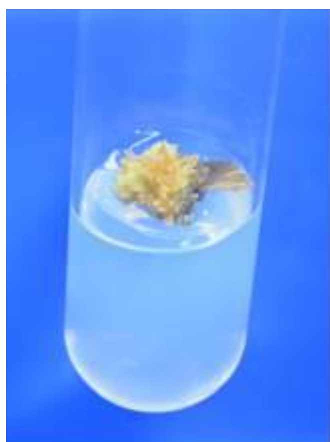
The cell suspension cultures showed continuous and stable accumulation of biomass in 14 days at temperature 25±2°C having photoperiod of 16-8 h at 80 rpm in liquid MS medium supplemented with the same combinations of the growth regulators as those used in callus culture for both the species of *Begonia*. Cell suspension culture was established by culturing calli of *Begonia malabarica* in liquid MS medium fortified with different concentrations and combinations of BAP, 2, 4-D and NAA (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}) showed well established suspension cultures with no aggregation or clumps of cells. The time courses of biomass accumulation described a sigmoid growth curve in culture cycle. Cells growth was slow during the initial 5 days of cultivation. Thereafter, biomass accumulated rapidly, and reached the highest value on the 14th day. Then the culture entered the stationary phase. Some cultures continued to grow up to 30th day. The maximum fresh weight (2.9 g) and dry weight (0.64 g) were observed at 14th day of culture in liquid MS medium supplemented with 2, 4-D (0.1 mg L^{-1}) and BAP (0.5mg L^{-1}).

Similarly, suspension cultures were initiated for *Begonia rex-cultorum* 'Baby rainbow' with 2g of friable callus as an inoculum in liquid MS medium supplemented with the same combinations of the growth regulators used in callus culture BAP (1mg L^{-1}) + 2,4-D (0.5 mg L^{-1}), BAP (2.0 mg L^{-1}) + IAA (1.0 mg L^{-1}), Kin (2 mg L^{-1}) + IAA (1 mg L^{-1}) and BAP (2 mg L^{-1}) + IAA (1 mg L^{-1}). Maximum growth was achieved in suspension culture supplemented with BAP (1mg L^{-1}) + 2,4-D (0.5 mg L^{-1}),followed by Kin (2 mg L^{-1}) + IAA (1 mg L^{-1}) and then by BAP (2.0 mg L^{-1}) + IAA (1.0 mg L^{-1}), BAP (2 mg L^{-1}) + IAA (1 mg L^{-1}), with Kin (2 mg L^{-1}) + IAA (1 mg L^{-1}). The maximum fresh weight (2.96 g) and dry weight (0.55 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}).

Quantification and fractionation of anthocyanin content

Anthocyanin content was quantified from the *in vitro* cell suspension and *in vivo* plants. The *in vitro* cells showed remarkable level of anthocyanin ie., 15 mg/ 100ml for *Begonia malabarica* and for *B. rex-cultorum* 'Baby rainbow' 30 mg/ 100ml where as *in vivo* plants anthocyanin content noticed was 56.76±0.64 mgg^{-1} for *Begonia malabarica* and for *B. rex-cultorum* 'Baby rainbow' 87.5±0.64 mgg^{-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 including elicitors. The present results seem to be more effective and parallel to other *in vitro* culture of medicinal plants protocols. Subsequently, the amberlite column eluted fraction with highest anthocyanin content from cell suspension culture of *Begonia malabarica* & *Begonia rex-cultorum* 'Baby rainbow' was used for the by LC-MS/ MS analysis.

LC- MS was successful in identifying the major anthocyanins in *B. malabarica* and *B. rex-cultorum* 'Baby



a

b

Figure 2: a and b: Friable callus of *Begonia rex-cultorum* 'Baby rainbow' obtained on MS medium fortified with BAP (1mg L–1) + 2,4-D (0.5 mg L–1) in leaf explants.

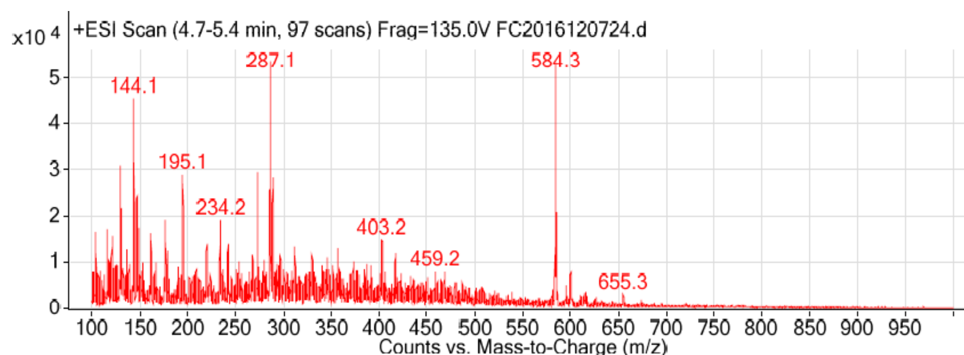


Figure 3: LC- MS analysis of *B. malabarica.*

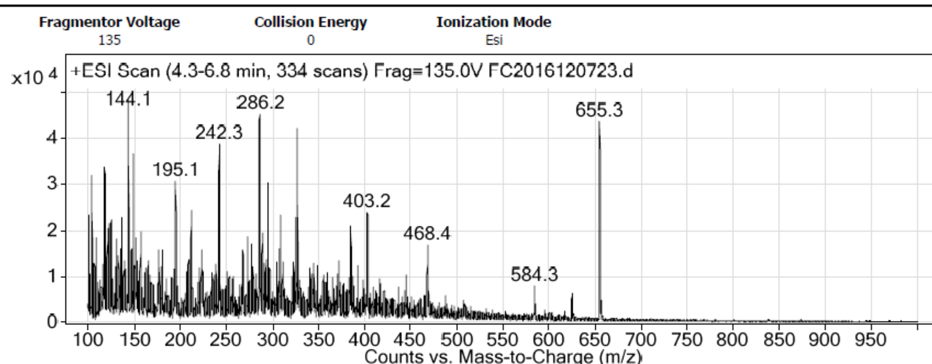


Figure 4: LC- MS analysis of *B. rex-cultorum* 'baby rainbow'.

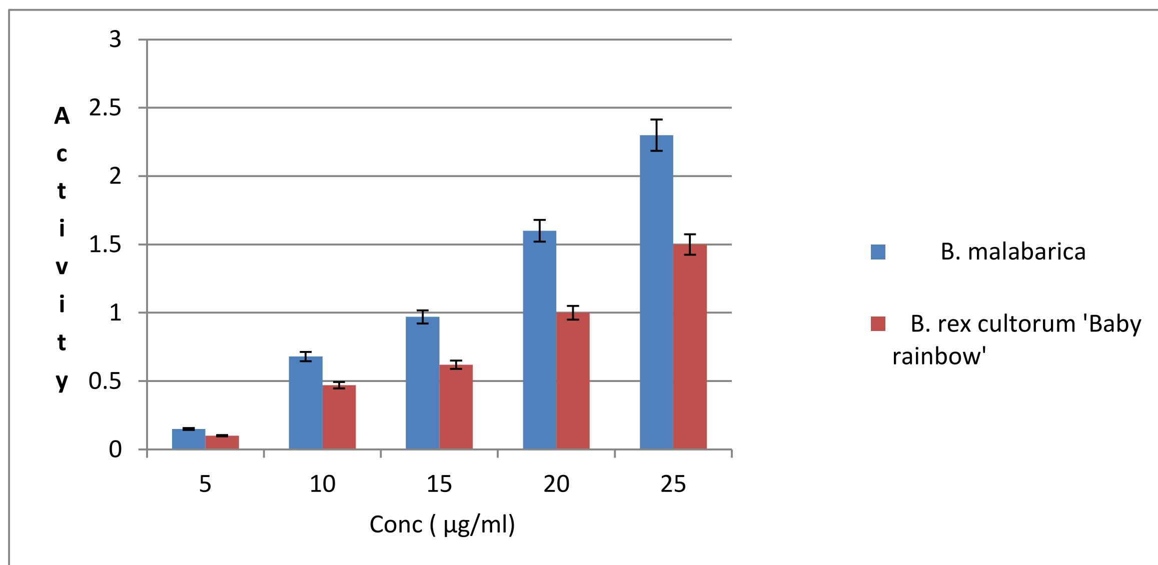


Figure 5: Reducing power of anthocyanin from *Begonia* species.

rainbow'. The major anthocyanin eluted from *B. malabarica* between 4.7 to 5.4 min. Tandem MS of the *m/z* 655.3 peak was identified as anthocyanidin Malvidin-3 –diglucoside which was the major compound. Because of known elution patterns of anthocyanins, the other peaks were identified as (584.3) Malvidin or Peonidin, (459.2) Delphinidin + Glucose, (403.2) may be Cyanidin, (287.1) Cyanidin Aglycone and other m/z 242.3, 195.1 & 144.1 were sugar derivatives or fragments (Fig 3). Meanwhile, the major anthocyanins in *B. rex-cultorum*'Baby rainbow' were similar to the anthocyanins in *B.malabarica* and were identified as (655.3) Malvidin-3 -diglucoside, (584.3) Malvidin or Peonidin, (468.4) Delphinidin + Glucose, (403.2) may be Cyanidin, (286.2) Cyanidin Aglycone and others (242.3, 195.1 & 144.1) may be sugar derivatives or fragments (Fig 4).

Antioxidant activity

As shown in figure 5, purified anthocyanin of *B.rex-cultorum* 'Baby rainbow' and *B. malabarica* from cell suspension cultures exhibited reducing power at par with catechin and ascorbate i.e., suggesting its electron

Table 1: AOX potential of *B. malabarica* anthocyanin using various assays.

Concentration (µg/ml)	DPPH (%)	FRAP (%)	ABTS (Trolox µmol/g DW)	Superoxide anion (%)	Deoxyribose degradation	
					Non-site-specific	Site-specific
5	32.7 ± 0.87	53 .8± 0.2	98.7 ± 0.12	15 ± 0.05	34 ± 2.5	9 ± 0.02
10	58.4 ± 1.2	63 ± 2.6	250 ± 3.5	27.6 ± 0.15	48 ±4. 2	18 ± 0.09
15	65 ± 3.8	70.4 ± 1.1	365 ± 0.69	48.3 ± 0.84	57.5 ±3.2	27.5 ± 0.05
20	79.3 ± 4.7	74.5 ± 5.2	579 ± 1.8	60 ± 0.64	89 ± 3.2	33 ± 1.05
25	90.9 ± 0.99	77.2 ± 0.67	987.7 ± 3.1	77 ± 0.64	97.6 ± 7.4	39.6 ± 2
Ascorbate (25)	87.64 ± 0.27	80.7 ± 6.9	1017 ± 1.7	77 ± 0.53	90.6 ± 0.23	40 ± 0.92
Catechin (25)	79.65 ± 0.55	70± 0.12	1060 ±0.12	53.5 ± 0.24	78 ± 1.5	30 ± 2.2

Table 2: AOX potential of *B. rex cultorum* 'Baby rainbow' anthocyanin using various assays.

Concentration (µg/ml)	DPPH (%)	FRAP (%)	ABTS (Trolox µmol/g DW)	Superoxide anion (%)	Deoxyribose degradation	
					Non-site-specific	Site-specific
5	27 ± 0.07	33 ± 0.32	77 ± 0.56	9 ± 0.01	29 ± 0.5	6 ± 0.04
10	38.4 ± 0.2	46 ± 1.6	178.6 ± 0.5	16 ± 0.32	34 ± 0. 2	11 ± 0.02
15	45 ± 1.8	54 ± 0.1	334 ± 0.98	38 ± 0.04	45 ±0.65	18.5 ± 0.03
20	59.3 ± 1.7	65 ± 0.2	489 ± 0.8	49 ± 0.64	59 ± 0.76	23 ± 1
25	67.5 ± 2.6	70 ± 0.17	686.7 ± 3.1	62.4 ± 0.09	77 ± 0.4	29.6 ±0.08
Ascorbate (25)	87.64 ± 0.27	80.7 ± 6.9	1017 ± 1.7	77 ± 0.53	90.6 ±0.23	40 ± 0.92
Catechin (25)	79.65 ± 0.55	70± 0.12	1060 ± 0.12	53.5 ± 0.24	78 ± 1.5	30 ± 2.2

donating power. Similarly, Luximon-Ramma et al.[19] correlated the reducing power with the AOX activity of phenols and flavonoids. In addition, a linear relationship existed between the dose and reducing power of ascorbic acid, catechin and anthocyanin, with correlation coefficients of 0.9997 (y = 0.0036 x + 0.0412), 0.9958 (y = 0.0014 x + 0.025) and 0.9990 (y = 0.0038 x + 0.1039), 0.9875 (y = 0.0037 x + 0.1033 for ascorbic acid, catechin and the anthocyanin respectively. The values were statistically significant at 5% level. Similar apparent linear relationship between AOX capacity of phenol content of *Ribes, Rubus, Vaccinium*, fruit juices and red wine were noticed[20-22].

Antioxidants scavenge free radicals and there by inhibit lipid peroxidation. DPPH· radical AOX activity assay has been used extensively for screening antioxidants from fruits and leafy vegetables and juices or medicinal plant extracts[23]. Table 1 and 2 compares the DPPH· scavenging activities of *Begonia* species, ascorbic acid and catechin. *B. malabarica* anthocyanins exhibited a concentration dependent DPPH· scavenging potentiality. In addition, *Begonia* species displayed remarkable scavenging activity, followed by catechin and ascorbate.

At 25 µg/mL, the DPPH· radical scavenging activities of *B. malabarica*, ascorbate, catechin, *B. rex-cultorum* 'Baby rainbow' were 90.9 %, 87.64 %, 79.65 % and 67.5% respectively. Analysis of cranberry, apple, red grape, strawberry, pineapple, banana, peach, lemon, orange, pear, and grapefruits using total oxyradical scavenging capacity with phenolics showed a positive correlation[24]. The values were statistically significant at 5% level.

As shown in table 3, anthocyanin from *Begonia* species can effectively inhibit peroxidation of linoleic acid and inhibits the formation of hydroperoxides, implying that anthocyanin are powerful natural antioxidants. The measured antioxidant activity of the anthocyanin from *Begonia* species was less than that of ascorbate and catechin the commercial antioxidant.

$O_2^{\cdot -}$ radicals were produced through many cellular reactions, including enzyme like lipoxygenases, peroxidase, NADPH oxidase and xanthine oxidase. $O_2^{\cdot -}$ results oxidative stress in plant tissues via the formation of cell-damaging free radicals[25]. The scavenging effects of *B. malabarica* and *B.rex-cultorum* 'Baby rainbow' anthocyanin towards $O_2^{\cdot -}$ are compared with synthetic antioxidant in the table 1 and 2. At 25 µg/mL the *B. malabarica* and *B. rex-cultorum* 'Baby rainbow' anthocyanin exhibited the superoxide anion scavenging activity and was comparable to ascorbate and catechin values ie., 78.6 %, 62.4 %, 77% and 53.5 %, respectively ($p<0.05$) (Table 1 and 2).

Hydroxyl radicals ($^{\cdot}OH$) can be formed via the Fenton reaction in presence of reduced transition metals like Fe^{2+} and H_2O_2, which is considered as the most reactive of the reduced forms of dioxygen, capable of damaging all biomolecule like nucleotides of DNA strand, which contributes to tumors, mutagenesis and cytotoxicity including cellular organelles[26]. In addition, $^{\cdot}OH$ are drastic initiators of the lipid peroxidation, due to

Table 3: Antioxidant activity of anthocyanin measured by the ferric thiocyanate method.

	Incubation time				
	0	1	2	3	4
Control	0	0.87	1.8	2.7	3.6
B. malabarica 10 µg/ml	0	0.08	0.11	0.14	0.18
25 µg/ml	0	0.03	0.05	0.07	0.06
B. rex cultorum 'Baby rainbow' 10 µg/ml	0	0.18	0.23	0.47	0.69
25 µg/ml	0	0.09	0.1	0.14	0.16
Ascorbate (25)	0	0.02	0.04	0.06	0.07
Catechin (25)	0	0.04	0.09	0.13	0.15

withdrawal of hydrogen atoms from unsaturated fatty acids[26]. To evaluate whether *B. malabarica* and *B. rex-cultorum* 'Baby rainbow' anthocyanins could reduce ˙OH formation either by chelating metal ions or via directly scavenging ˙OH, were analyzed by determining the amount of deoxyribose degradation. Table 1 and 2 refers the dose-dependent inhibition of ˙OH by *B. malabarica* and *B.rex-cultorum* 'Baby rainbow' anthocyanin in both the site and non-site-specific assays. Employing the same doses, relatively optimal antioxidant activity was observed in the site-specific assay rather than in the non-site-specific, implying that the *B. malabarica* and *B. rex-cultorum* 'Baby rainbow' anthocyanin inhibited deoxyribose degradation mainly via chelating metal ions rather than directly degrading ˙OH. The values were statistically significant at 5% level (Table 1 and 2).

The relative antioxidant ability to scavenge the radical $ABTS^+$ has been compared with the standard catechin and ascorbate. ABTS radical cation was produced in the stable form using potassium persulphate. After getting the stable absorbance, the anthocyanin was added to the reaction medium and the antioxidant power of anthocyanin was measured by studying decolorization. Anthocyanin of both *Begonia* species shows an antioxidant capacity for scavenging the ABTS radical cation. *B. malabarica* had significantly higher values i.e., 987.7 ± 3.1 Trolox µmol/g DW) than the other species (Table 1 and 2).

FRAP assay measures the reducing potential of an antioxidant reacting with a ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex and producing a colored ferrous tripyridyltriazine (Fe^{2+}-TPTZ). The free radical chain breaking takes place through donating a hydrogen atom. At pH 3.6, reduction of Fe^{3+}-TPTZ complex to blue colored Fe^{2+}-TPTZ takes place, which has read at 593 nm. % of inhibition by FRAP assay at 25 µg/ml ranged from 53.8 to 77.2% in *B. malabarica* and 33 to 70% in *B.rex-cultorum* 'Baby rainbow' (Table 1 and 2). The results obtained are highly reproducible and related linearly with the molar concentration of the antioxidants present. This is in accordance with the results reported by Benzie et al.,[27] and Jeong et al.[28]. AOX potential of anthocyanins was through an indirect pathway i.e., enhance endogenous antioxidant defenses through restoring or increasing the activities of the antioxidant enzymes superoxide dismutase and glutathione peroxidase, consequently increasing glutathione content[29]; activation of genes that code for these enzymes[30]; and a reduction in the formation of oxidative abducts in DNA, reducing the formation of endogenous ROs by inhibiting NADPH oxidase and xanthine oxidase, or by modifying mitochondrial respiration and arachidonic metabolism[31].

Zhou et al.[32] reported similar results from the crude extract of *Opuntia ficus-indica* var. Saboten and *Hypericum perforatum*. Das et al.[33] attempted in evaluating *in vitro* antioxidant potentialities of purified polyphenols from four species used in rice beer preparation in Assam. Miguel et al.[34] screened phenols, flavonoids and antioxidant activity in terms of ABTS•+, DPPH•, and O2•- scavenging capacity, and metal chelating activity in the propolis samples. Methanol was more effective than water in extracting total phenols. Flavones and flavonols were also better extracted with methanol than with water. The free radical scavenging activity, ABTS (IC50=0.006-0.036 mg/mL), DPPH (IC_{50}=0.007-0.069 mg/mL) and superoxide (IC50=0.001-0.053 mg/mL) of the samples was also higher in methanolic extracts. Budak et al.[35] analyzed antioxidant activity and phenolic content of apple cider. Chlorogenic acid was the major phenolics in apple juice samples while chlorogenic acid increased during maceration. Chlorogenic acid, catechin, epicatechin, caffeic acid contents of macerated apple cider were in high concentrations correlated with AOX potentiality. Figueroa et al.[36] evaluated antioxidant activity, total phenolic and flavonoid contents and cytotoxicity of *Bougainvillea xbuttiana.* The phytochemical screening of ethanolic extracts from different colour revealed the antioxidant activity with ranking order for the antioxidant activity index. Siti Azima et al.[37]compared anthocyanin content in relation to the antioxidant activity and colour properties of *Garcinia mangostana*, *Syzigium cumini* and *Clitoria ternatea* extracts. *Garcinia mangostana* showed higher FRAP value and lower EC_{50} value which were 79.37 mmoles/g and 0.11 mg/ml, respectively, as compared to *Syzigium cumini* extract with FRAP value, 25.66 mmoles/g and EC_{50} value, 0.22 mg/ml. Total monomeric anthocyanin exhibited a strong correlation between FRAP assay and DPPH assay. Thaipong et al.[38] made a comparison of ABTS, DPPH, FRAP and ORAC assays from guava fruit extracts. EC_{50} of methanolic extract were 31.1, 25.2, 26.1, and 21.3 against ABTS, DPPH, FRAP, and ORAC assays, respectively. Averaged

dichloromethane extract were 0.44, 0.27, and 0.16 respectively. Akinmoladun et al.[39] quantified phytochemical constituent and antioxidant activity of extract from the leaves of *Ocimum gratissimum.* Here also, methanolic extract had a DPPH scavenging activity of 84.6% at 250 µg/ml and a reductive potential of 0.77 at 100 µg/ml. These values were comparable with those of gallic acid, 91.4% at 250 µg/ml and ascorbic acid, 0.79 at 60 µg/ml as standards for DPPH scavenging activity and reductive potential, respectively. Karamac et al.[40] evaluated antioxidant activity of phenolic compounds identified of sunflower seeds. HPLC–MS(ESI) analysis revealed a pool of fractions. The antioxidant activity of the fractions vs DPPH and ABTS and to reduce Fe3+/ferricyanide complex to the ferrous form showed variability. Al-Jassabi and Abdullah[41] extracted, purified and characterized the antioxidant fractions from *Zizyphus spina-christi* fruits. The % of DPPH radical inhibition and values ranged from 31.76% - 90.23% which indicated strongest capacity for neutralization of DPPH radicals. The total phenolic content ranged from 11.04 - 56.44 mg/g expressed as quercetin equivalent. The concentration of flavonoids varied from 16.66 - 58.32 mg/g expressed in terms of rutin equivalent. Methanolic extract of showed the highest phenolic and flavonoid concentration and strong antioxidant potential. The obtained results in *B. malabarica* and *B. rex-cultorum* 'Baby rainbow' anthocyanin were commendable compared to the above research out puts.

CONCLUSION

In vitro cell suspension culture, isolation of anthocyanin, its purification, and fractionation in *B. malabarica* and *B.rex-cultorum* 'Baby rainbow' were successfully established. Subsequently, antioxidant potential of purified anthocyanin were proved in terms of inhibiting the peroxidation of linoleic acid, acted as strong electron-donating agent in the Fe^{3+} to Fe^{2+} assay and hydrogen-donating agent in the DPPH· assay. Furthermore, the anthocyanin was effective in scavenging superoxide anion radicals and inhibiting deoxyribose degradation induced by ˙OH, mainly via chelation of iron ion. Future studies are warranted at *in vivo* levels using animal models.

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