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HEPATOPROTECTIVE ACTIVITIES OF PURIFIED ANTHOCYANIN FROM *IN VITRO* CULTURES OF *BRIDELIA RETUSA* (L.) SPRENG. IN EXPERIMENTAL ANIMALS

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ABSTRACT

Bridelia retusa is traditionally used as an astringent and also for rheumatic pains. Present study reveals that 2, 4-D either alone or in combination with kinetin in MS medium showed good response in terms of callus induction from leaf explants of *B. retusa*. Significant amount of callus was induced with 2.5 mg/L N6-benzyladenine (BA) + 2 mg/L 2, 4-dichlorophenoxyacetic acid (2, 4-D) i.e., 98.9%. The optimal fresh and dry weights of the calli were i.e., 1.9 ± 0.04 and 0.45 ± 0. 03 g respectively. The calli incubated in light on MS medium with 4% glucose containing 2.5 mg/L BA and 2 mg/L 2, 4-D at pH 3.5 yielded 2.8 mg /g of anthocyanin. MS medium with glucose was optimal compared with sucrose for inducing anthocyanin synthesis. However addition of KIN inhibited anthocyanin accumulation. Pigmented calli transferred to half strength MS medium containing NH^{+}/NO^{3} (1:4 ratio), 70g/L sucrose with BA + 2, 4-D yielded remarkable biomass with anthocyanin content. Suspension cultures in half MS medium containing 2.5 mg/L 2, 4-D + 2 mg/L BA at pH 5.8 enhanced anthocyanin synthesis. pH and temperature factors against anthocyanin extraction and stability were analyzed. Enhancement in the degradation rate constant with a corresponding decline in the t1/2 values was seen with the increasing temperature at pH 1 and 4. Fractionation of anthocyanin was carried out by HPLC coupled mass spectrometry revealed 9 fractions comprising acylated cyanidins, two peonidins, cyanidin 3-p-coumaroyl and feruloyl diglucoside-5-glucosides. Hepatoprotective experimental results revealed that the anthocyanin extract at 200 and 400 mg/kg of body weight remarkably reversed the

changes in serum biochemical parameters and also histology of liver caused by CCl_4 indicating their hepatoprotective efficacy. Results were comparable with silymarin. The data was further supported by antioxidant enzyme profiles.

KEYWORDS: *Bridelia retusa*, *in vitro* culture, phytohormones, anthocyanin, hepatoprotective, antioxidant.

INTRODUCTION

Many kinds of xenobiotics like alcohol, CCl_4 and allelopathic agents influence liver for the biotransformation of the complex molecules with the risk of detrimental physiological and pathological alterations leading to liver diseases. The common liver prone disorders include cirrhosis, jaundice, cancer, metabolic and degenerative lesion, liver cell necrosis and hepatitis. The hepatotoxins trigger liver injury characterized by different degrees of hepatocyte degeneration and cell death. The major physiological derailments sparkles the formation of reactive oxygen species (ROS) including superoxide and hydroxyl radicals.[1] The chain of reactions leads to oxidative stress which leads to secondary disorders like cancer, inflammatory, cardiovascular and neurodegenerative diseases as well as drastic aging. Endogenous protection against these is achieved by antioxidant enzymes that catalytically remove free radicals and other reactive species.[2] There is a close relationship between nutrition and the antioxidant defense system, as exogenous low molecular weight antioxidants may be supplied by the diet.[3] Synthetic drugs have been employed as a therapy for liver disorders and have potential adverse side effects especially when administered for long term.[4] Harmful stimuli lead to activation of nociceptors through the release of variety of chemical mediators, such as excitatory amino acids, vasoactive amines (histamine, serotonin), proteins, peptides, nitric oxide (NO), arachidonic acids (prostaglandins E2, leukotrienes) and cytokines [TNF- α and interleukin-1], which act on specific receptors and ion channels, contributing to the induction of pain and inflammation. In this scenario, hepato protective agents of plant based molecules have attracted interest and numerous medicinal plants and their formulations have been used as liver tonics in the Ayurvedic system of medicine. These medicinal plants have been screened for their influence on liver dysfunction.

Bridelia retusa spreng. (Euphorbiaceae) is a small sized spinous deciduous tree, with grey bark, found throughout India up to an altitude of 1000 m except in dry zones. Pharmacologically *Bridelia retusa* exhibited antiviral, hypoglycemic and hypotensive properties. According to Ayurveda the species is good for removal of urinary issues. The bark

along with gingelly was used in rheumatism. The plant also promotes antifertility and liver protective activities.[5] Despite the traditional use of this plant in the treatment of jaundice and splenic issues, no scientific studies have been carried out to delineate its influence on experimentally induced hepatotoxicity. Further, the literature survey showed that there is no validation work has been made on this plant so it is the need of the hour to investigate the polyphenolic content and hepato protective potential of the species.

MATERIALS AND METHODS

In vitro callus culture

Fresh leaves of *Bridelia retusa* was collected from deciduous forests of Ponmudi hills as source of as explants. Cultures were initiated on MS medium[6] with 30 g/L sucrose and fortified with different phytohormones applied singly or in combinations such as naphthalene-acetic acid, 2,4-dichloro-phenoxyacetic acid (NAA) 0.5 - 4 mg/L, N6-benzyladenine (BA- 0.5 - 3 mg/L). pH of the media were adjusted to 5.6-5.8 prior to adding 8 g/L agar, autoclaved (121°C, 104 K Pa) for 15 min and dispensed into 8 x 7 cm flasks (30 mL of culture medium per flask) closed with polypropylene caps. 05 flasks containing 04 explants each were subjected to pre treatment and each experiment was repeated twice. Cultures were incubated in a growth chamber at 26 ± 2°C under 16 h photoperiod provided by cool-white fluorescent tubes (45 µmol m^{-2} s^{-1}). Subcultures to media with the same composition were performed after 30 days of culture. Callus biomass accumulation was estimated after 60 days of culture based on fresh (FW) and dry (DW) weight measurements. Dry mass was obtained after drying at 45°C to constant weight. Stock callus cultures were maintained under the same physical conditions described above with subcultures at 20-day intervals.

Cell suspension culture

Cell suspension culture was initiated by transferring 3 g of friable calli into 250 ml Erlenmeyer flasks containing 100 ml of fresh half strength MS liquid medium supplemented with different concentrations of 2,4-D (0.5 -2.5 mg/l) + BA (3 mg/l) and sucrose (30 g/l) pH 5.8. The suspension cultures were regularly sub-cultured in the MS liquid medium at 20 – 60 day intervals agitated on a rotary shaker (110 rpm, 25°C) and kept in darkness. For evaluation of growth curve, the cells were separated from the stock by filtration under suction. 1 ± 0.1 g cells were further inoculated into 50 ml of fresh MS liquid medium in a flask. Growth of cell suspension culture, cell viability and anthocyanin content were determined with sets of flasks

harvested at regular intervals from the 0 day of subculture up to 60 days. Cells were isolated from the medium by filtration using nylon mesh and weighed as fresh weight. Cell viability was determined by incubating 2 ml samples in 0.25% Evan's blue stain for 5 min and then at least 500 cells were counted, and this was repeated thrice.

Induction of anthocyanin synthesis

80 ml cell suspension was subjected to i). different temperatures (22°C or 36°C), ii) light intensities (45, 67 and 80 µmol $m^{-2} s^{-1}$) iii) different carbon source: sucrose, glucose, maltose, fructose concentrations (1,2,3,4%) iv) different total nitrogen concentrations (50; 60; 70; 80 mM) and v) different ratios of NH_4^+ to NO_3^-(1:1; 1:2; 1:3; 1:4). Cell biomass and anthocyanin content were quantified and each experiment was repeated thrice.

Purification of Anthocyanins

50 g fresh cells were extracted with 25 ml of two different extraction solvents: 0.01% (v/v) HCl-acidified water and 0.01% (v/v) HCl-acidified ethanol. The extraction was done at room temperature with constant shaking at 100 rpm for 60 min. The extract was filtered through What man No.1 paper, and the residue was subjected to extraction until it becomes colorless. Filtrates were mixed and used for anthocyanins purification. Suitable extraction solvent was identified as per the highest amount of anthocyanin content obtained. Similarly, the extraction ratios (1:10, 1:15 and 1:20 sample: solvent) and extraction periods 30 to 120 min were also studied.

After optimal extraction, the sample was filtered through What man No.1 paper, and then dried by rotary evaporator at 40^{o}C under vacuum conditions. The concentrated sample was loaded onto a C-18 open chromatographic column of silica. Elution was performed using three solutions with specific properties geared to optimal anthocyanin purification. The sample was initially eluted with 0.01% HCl acidified distilled water to eliminate organic acid and sugar, followed by ethyl acetate to remove phenolics and finally by acidified ethanol (Ethanol:1% w/v citric acid, pH 2.9). The purified anthocyanin fractions were collected for further analysis.

pH and temperature stability

The purified anthocyanin solution was adjusted to pH 1 and 4 by 0.1M HCl and pH 7.0 by 0.1 M NaOH at 25°C. The absorbance of all samples was measured at 400 nm to 700 nm. The assay was based on the color absorbance within 3 min.

The 5 mL purified anthocyanin (100 µg/ml) at the stable pH as determined previously was incubated in a water bath at the controlled temperatures of 20, 40, 60 and 80°C. Samples were periodically collected during 1 – 8 h. Each sample was cooled in an ice bath prior to absorbance measurement for evaluating the anthocyanin degradation.

$$\text{Anthocyanin (\% remaining)} = \frac{\text{A1or A2 or A3} \quad \text{x} \quad 100}{\text{A0h}}$$

Aoh= the absorbance at time 0 h A1, A2 or A3 = the absorbance at time 1 h, 1.5 h or 2 h at different temperatures.

HPLC-MS analysis

The purified anthocyanins were mixed with 0.5% HCl and then syringe filtered (0.45 µm). The injection volume was 10 µL. The flow rate was 0.8 mL/min and maintained at 35^{o}C. The mobile phases comprise 0.05% (v/v) trifluoroacetic acid (TFA, solvent A) in distilled water and 100% acetonitrile (solvent B). The gradient elution program was performed as follows: solvent A at 95-80% from 0 to 20 min, at 80-60% from 20 to 50 min. The chromatogram was then compared with the standard chromatogram using cyanidin-3-3glucoside.

Experimental animals

Male albino rats of Wistar strain weighing between 100 and 130 g (8 weeks old) were housed in polypropylene cages and maintained at 25 ± 2°C under 14/10 h dark and light cycle. They were allowed free access to standard pellet diet and water *ad libitum.* The animals were acclimatized for one week under laboratory conditions. Ethical clearance for handling the animals was obtained from the ethical committee constituted for the purpose.

Animal dose

Based on the earlier oral studies of acute and short term toxicities the appropriate dose for preclinical study was calculated and found to be 100 mg/kg body weight (low dose) and 200 mg/kg body weight (high dose).

Experimental design

Rats of either sex were divided into 5 groups, each with 6 animals:

Group I Control: Received distilled water (5ml/kg b.w.) daily dose for 6 consecutive days.

Group II (Administered with paracetamol (3g/kg) in water): single dose daily for 6 consecutive days.

Group III (standard): Received standard drug Silymarin (100 mg/kg body weight p.o.) once daily for 6 consecutive days.
Group IV (Low dose): Received purified anthocyanin (100 mg/kg body weight, p.o.) once daily for 6 consecutive days.
Group V (High dose): Received purified anthocyanin (200 mg/kg body weight, p.o.) once daily for 6 consecutive days.

Groups from II to V have received paracetamol (3g/kg) single dose on the 7th day. The quantity of food and water consumed was recorded for each group of animals during the course of the experiment. The body weight of each rat was recorded on the 1st day and on 8th day, mean body weights were calculated. Liver weight and kidney weight were also determined to know the effect of drug on normal morphology and physiology of rats. Organ index was calculated according to the formula: (liver or kidney weight/body weight) x 100%.

Sample collection

All experimental albino rats of Wistar were sacrificed by cervical decapitation separately after 24 h of the last treatment on 8^{th} day. Blood samples of each group were collected into dry sterilized tubes and centrifuged at 3000 r/min. for 10 min to obtain clear serum. Liver and Kidney were quickly excised, rinsed in cold saline, blotted, and weighed. One portion of liver was freshly used for malondialdehyde (MDA) assay and other part is used for histopathological evaluation.

Hepatotoxicity assessment

The hepatic enzymes SGOT and SGPT were used as the biochemical indicators for the acute liver injury. The serum SGOT, SGPT, ALP, TB and TP activities were determined by using a commercial diagnostic kit Biocompare, BioVision, Thermo scientific and Bioos-cientific Max discovery.

Antioxidant enzyme activity assay

Preparation of erythrocyte lysate

The packed cells, after separation of serum, were washed with a physiological saline 3 times and lysed with hypotonic phosphate buffer, pH 7.4. The hemolysate was separated by centrifuging at 2500 r/min for 15 min at 4°C and used for assay of erythrocyte superoxide dismutase (SOD) and catalase (CAT) activities.

Superoxide dismutase (SOD)

McCord and Fridovich[7] protocol was used for antioxidant activity assay of a sample. It is estimated in the erythrocyte lysate prepared from the 5% RBC suspension. To 50 µl of the lysate, 75 mM of Tris-HCl buffer (pH 8.2), 30 mM EDTA and 2 mM of pyrogallol are added. An increase in absorbance is recorded at 420 nm for 3 min by spectrophotometer. One unit of enzyme activity is 50% inhibition of the rate of autooxidation of pyrogallol as determined by change in absorbance/min at 420 nm. SOD activity is expressed as units/mg protein.

Catalase (CAT)

Aebi's protocol[8] was employed for catalase assay. Fifty micro liter of the lysate is added to a cuvette containing 2 mL of phosphate buffer (pH 7.0) and 1 mL of 30 mM H_2O_2. Catalase activity is measured at 240 nm for 1 min using spectrophotometer. The molar extinction coefficient of H_2O_2, 43.6 M cm^{-1} was used to determine the CAT activity. One unit of CAT activity is equal to1 mmol of H_2O_2degraded / min and is expressed as units / mg protein.

Lipid Peroxidation (LPX) assay

The liver tissues were homogenized in 10% ice-cold phosphate buffered saline (0.1 M, pH 7.4, w/v) and centrifuged at 2000 r/min for 30 min; the supernatant was used for the assay of MDA.

LPX is an autocatalytic process leads to peroxidative tissue damage. Malondialdehyde (MDA) is known end products in the lipid peroxidation process. MDA is formed during oxidative degeneration as a product of free oxygen radicals, which is accepted as an indicator of lipid peroxidation. This method described by Ohkawa[9] was used for the analysis. The levels of lipid peroxides can be expressed as moles of thiobarbituric acid reactive substances (TBARS)/mg protein using an extinction coefficient of 1.56· 10 5ML cm−1.

Histopathological studies

The liver tissues were removed from the animals and immediately fixed in 10% formalin. Subsequent processing included dehydrating in increasing ethanol solutions (50-100%), clearing in xylene and embedding in paraffin. Sections (4-5 µm) were prepared and then stained with Haematoxylin/Eosin dye for photo microscopic observation.

Statistical analysis

Statistical analysis was performed using Graph pad (version 6.04). All experiments were carried out in triplicates and presented as mean ± standard deviation (SD). The data were statistically analyzed by one-way ANOVA. The level of statistical significance was determined at $p < 0.05$.

RESULTS AND DISCUSSION

Remarkable callus induction in *Bridelia retusa* was noticed after 6th week of inoculation. Generally, the calli formed were creamy. The % of callus induction ranged from 17.8 to 98.9%. The highest callus induction i.e., 98.9% was found with the leaf explants cultured on MS medium supplemented with 2 mg/L 2,4-D + 2.5 mg/L BA (Table 1). The resultant calli from such treatments were found to be completely friable. NAA also induced calli optimally in combinations with BA. The calli yielded were found to be slightly compact. 2, 4-D + BA recorded the optimal callus fresh and dry weight i.e., 1.7 ± 0.02 and 0.65 ± 0.03 g respectively with leaf as explant (Fig.1 a and b). Meanwhile, in the case of stem (internodal segment) the respective values were 1.2 ± 0.04 and 0.59 ± 0.02. 4th week after sub-culturing, the calli were proliferated and enlarged profusely (Fig.2).

Table 1. Effects of NAA, BA and 2, 4-D growth hormones on callus induction (%) from leaf and stem explants

NAA (mg L-1)	BA (mg L-1)	2,4-D	Callus induction	
			Leaf (%)	Stem (%)
0.5	0.2		17.8±0.8	12±0.88
0.5	1		27±0.3	20.5±0.32
0.5	5		31±0.05	22±0.16
1	0.2		24.7±0.2	20±0.28
1	1		45.4±0.13	35±0.33
1	5		50±0.9	40±0.27
2	0.2		24±0.4	19±0.54
2	1		39±0.5	25.6±0.16
2	5		42±0.25	30±0.63
-	0.5	0.25	21±0.44	15.7±0.05
-	1	0.5	50.7±0.26	41±0.22
-	1.5	1	71.4±0.77	65±0.17
-	2	1.5	88±0.83	72±0.94
-	2.5	2	98.9±0.17	92±0.27
-	3	2.5	98±0.29	90±0.19

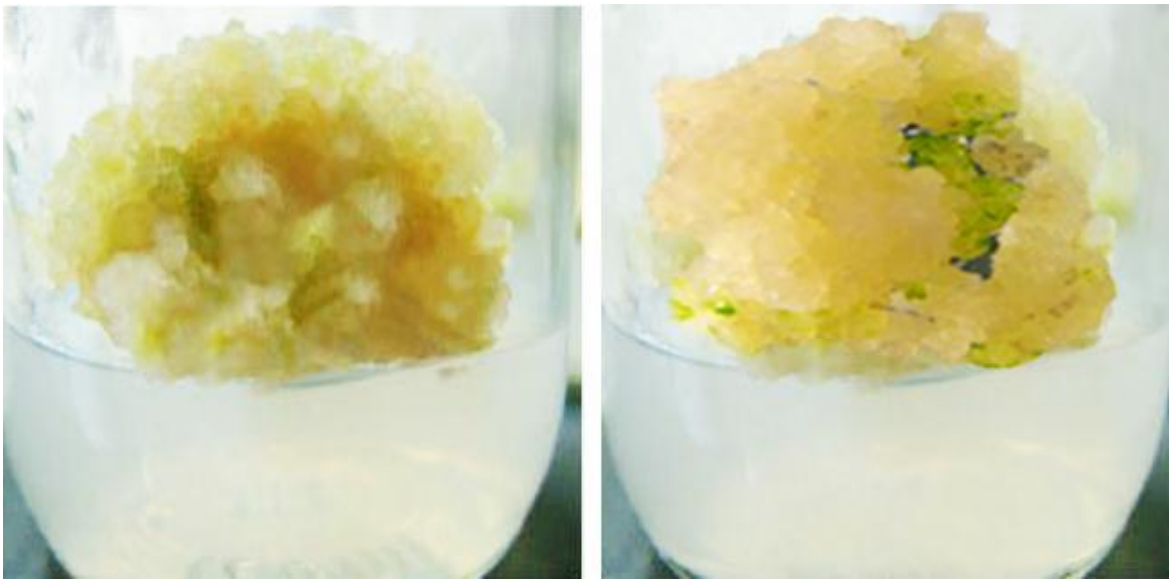

Fig. 1 a and b. Calli from leaf culture of half strength MS medium from *B. retusa*

Fig. 2. Compact calli from leaf culture of half strength MS medium from *B. retusa*

Anthocyanin formation was initiated in the leaf calli from day 25 to 30 in the MS medium with 2, 4-D. Higher concentrations of 2, 4-D (> 2.5 mg/g) reduced anthocyanin synthesis. MS medium with BA induced optimal callus formation with poor anthocyanin content. Similarly, NAA + BA treatments also showed poor anthocyanin synthesis. 2, 4-D or NAA in combination with BA showed varied anthocyanin synthesis when compared to 2, 4-D or NAA alone containing MS medium. Medium containing 2 mg/L 2,4-D + 2.5 mg/L BA which developed 1.7±0.02 g callus from leaf (60 days) with the highest amount of anthocyanin (3.4 mg/g;)(Table 2).

Table 2. Impact of growth hormones in the fresh and dry weight of calli of *B. retusa* in the MS medium.

NAA	BA	2,4-D	Leaf		Stem	
			Fresh weight	Dry weight	Fresh weight	Dry weight
(mg L^{-1})	(mg L^{-1})	(mg L^{-1})	(g)	(g)	(g)	(g)
0.5	0.2		1.3 ±0.05	0.66±0.01	1.08 ±0.25	0.56±0.3
0.5	1		1.1±0.04	0.49±0.06	1.03±0.01	0.5±0.08
0.5	5		0.93±0.02	0.32±0.2	0.99±0.03	0.39±0.09
1	0.2		0.78±0.09	0.31±0.05	0.61±0.1	0.29±0.06
1	1		0.79±0.05	0.28±0.06	0.59±0.12	0.25±0.04
1	5		0.78±0.32	0.26±0.1	0.58±0.13	0.24±0.01
2	0.2		0.74±0.16	0.21±0.2	0.54±0.06	0.24±0.001
2	1		0.63±0.09	0.13±0.08	0.43±0.09	0.12±0.05
2	5		0.58±0.03	0.11±0.07	0.38±0.03	0.10±0.007
	0.5	0.25	0.97±0.04	0.43±0.03	0.67±0.04	0.4±0.008
	1	0.5	1.08±0.01	0.5±0.07	0.9±0.08	0.45±0.05
	1.5	1	1.2±0.08	0.56±0.01	1±0.02	0.46±0.002
	2	1.5	1.3±0.03	0.59±0.02	1.02±0.07	0.52±0.005
	2.5	2	1.7±0.02	0.65±0.03	1.2±0.04	0.59±0.02
	3	2.5	1.55±0.01	0.61±0.09	1.1±0.05	0.57±0.009

Cell suspension culture

Friable and fresh calli clumps grown on 2.5 mg/l BA + 2.0 mg/l 2, 4-D media were used for initiating cell suspension culture. Cell growth was measured in liquid MS medium supplemented with 2.5 mg/l BA + 2.0 mg/l 2, 4-D by recording the fresh mass of the cells once in 2 days. The growth of suspension cultures indicated that the growth rate of cells was slow initially (3 days -lag phase). However, a marked increase was seen from 6^{th} day onwards in terms of mass (exponential phase). Maximum fresh weight was reached on 20^{th} day and was about 20 fold higher than the initial mass. Subsequently, the rate of growth was stable (Stationary phase). Later, a gradual reduction in cell density was noticed. Based on the results, sub-culturing to new fresh media was carried between days 15 and 20 days of incubation i.e., the end of exponential growth phase. This may be due to the fact that the medium became exhausted and toxic metabolites were accumulated by the cells. The cell viability was 80% on 22 days of culture (Fig.3 a and b)and then marginally declined.[10]

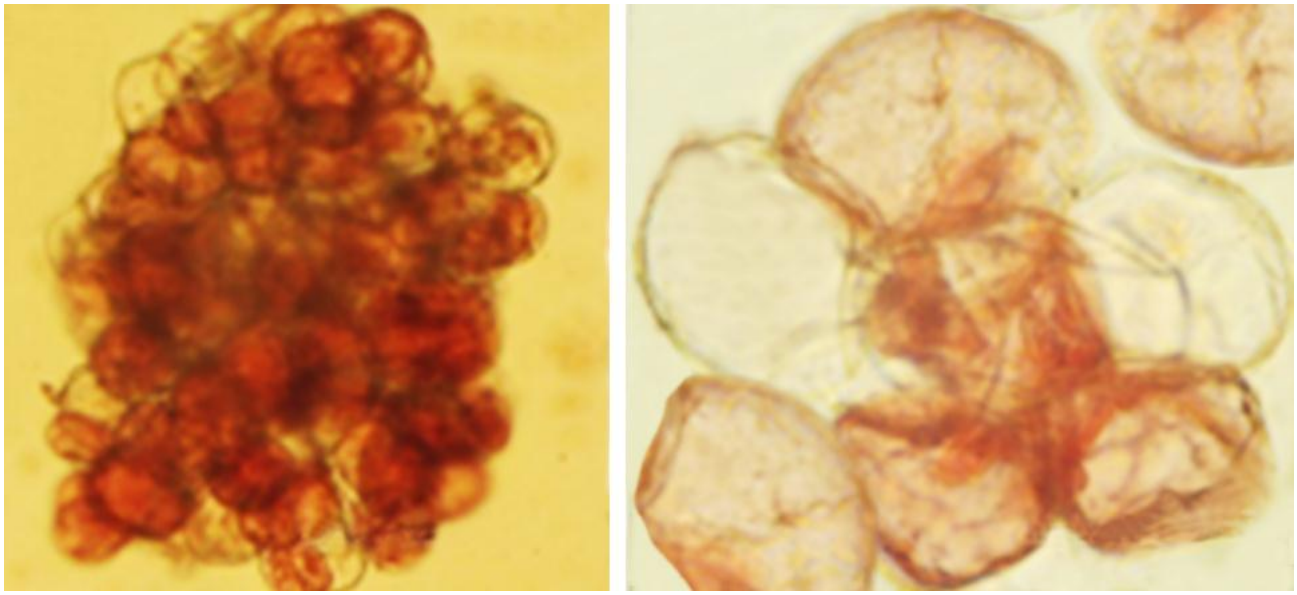

Fig. 3 a and b. Viable cells from cell suspension culture of *B. retusa.*

Effect of pH, light and carbon source

Liquid MS medium containing 2, 4-D + BA with pH 4.2 showed the highest anthocyanin formation followed by pH 4.4 i.e., 7.9mg/g and 6.6mg/g tissue respectively. Similarly, cells were analyzed for anthocyanin level and biomass against different regimes of light irradiation and temperature. Initially anthocyanin content increased gradually with temperature and reached the peak at 30°C i.e. 8.1±0.78mg/g f.w. and 22.7 mg/g respectively. Higher temperature showed a negative impact on pigment accumulation irrespective of the light irradiation. This may be due to the hydrolysis of glycosidic bonds of glucosidases, resulting in pigment degradation and formation of other degraded products.[11] Similarly, Narayan *et al*., (2005) studied temperature induction versus anthocyanin synthesis in carrot and revealed that it was species-dependent i.e., cultures of *Daucus carota*, the peak anthocyanin content was noticed at 30°C when compared to lower and higher temperatures.[12]

Meanwhile, increasing on light irradiation had a positive impact in anthocyanin synthesis (Table 4). Cells at 30°C and 80 µmol m^{-2} s^{-1} achieved the highest anthocyanin content (8.2 ± 0.78 mg/g FW) and biomass accumulation (22.7 ± 0.093) i.e., room temperatures connected with high light irradiation are the best physical parameters to anthocyanin synthesis.[13]

Liquid MS medium with BA and 2, 4-D at pH 4.2 were further analyzed for different carbohydrate sources and concentrations required for anthocyanin synthesis. Of the various carbon sources tested, MS medium having 4% glucose produced the maximum anthocyanin (8.4 ±0.13mg/g) and was followed by 3% sucrose (7.4±0.72). Subsequent sub-culturing

displayed a stable yield of anthocyanins. The anthocyanins formation was accelerated significantly with glucose and sucrose. Addition of sugars in the culture medium plays multiple roles like source of energy, structural components and also metabolic signals regulating the gene expression.[14]

To evaluate, the ideal period of cell suspension culture for anthocyanin, cells were collected regularly at 2 day intervals throughout the experimental periods. Quantity of anthocyanin increased proportionally with days and reached maximum level at 22nd day (7.1 ± 0.04 mg /g FW), after which the levels declined. Subsequently, the cells became brownish when retained on the same medium without sub-culturing, probably due to degradation of anthocyanin. Zhou *et al*., noticed similar observation in *Prunus incisa* due to nutrient depletion or oxidative stress by toxic byproducts in the culture medium or phytochemicals synthesized by stressed cells.[15]

60 mM nitrogen concentration yielded an optimal anthocyanin synthesis when compared with other concentrations, although the biomass was not altered remarkably i.e.8.5±1:4. 1:4 ratio of $NH4^{+}$: $NO3^{"}$ enhanced the anthocyanin content as compared to other ratios i.e.8.7±0.19 mg/g.

Based on the results, an ideal medium formulation was designed for anthocyanin synthesis was established by combining MS1/2 with 1:4 ratio of $NH4^{+}$ to $NO3^{"}$, 4% glucose and supplementation with 2 mg/L 2,4-D + 2 mg/L BA at 30°C and 80 µmol m^{-2} s^{-1} light irradiance. The culture conditions established in the present work induced the formation of friable fast-growing calli from leaf explants of *B. retusa.*

Maximum anthocyanins content was noticed with HCl-acidified ethanolic extraction (0.01%) i.e., 8.6 ± 1.3 mg/ g at 90 min in the sample-solvent ratio of 1:20. Further, in the present study the extraction time using acidified ethanol as solvent, the recovery of yield was high from first to third extraction (Table3 and 4). Generally, anthocyanins was extracted with various solvents like water, ethanol, methanol or acetone + marginal amount of acid to obtain the flavylium cationic form which is relatively stable in acid medium. Preferable solvents are water and ethanol because of less toxic, especially in food and consumable samples, rather than methanol or acetone.

Table 3. Extraction of anthocyanin by 0.01%-HCl-acidified ethanol (thrice) at different ratios and extraction times.

Ratio	Time (min)	Anthocyanins (mg/g)			
		1st extraction	2nd extraction	3rd extraction	Total amount
	30	2 ± 0.04	1 ± 0.07	0.89 ± 0.001	3.89 ± 0.5
1:20	60	2.8 ± 0.66	1.4 ± 0.03	0.81 ±0.01	5.01 ± 0.22
	90	5.2 ± 0.88	2.1 ± 0.13	1.3 ± 0.06	8.6 ± 1.3
1:40	30	1.82 ± 0.22	0.89 ± 0.03	0.75 ± 0.02	3.46 ± 0.07
	60	2.2 ± 0.05	0.9 ± 0.01	0.79 ±0.01	3.89 ± 0.042
	90	3 ± 0.62	1.4 ± 0.09	0.71 ± 0.03	5.11 ± 0.62
1:60	30	1.73 ± 0.35	0.76 ± 0.009	0.67 ± 0.03	3.16 ± 0.08
	60	2.3 ± 0.07	0.87± 0.004	0.68 ±0.005	3.85 ± 0.15
	90	2.78 ± 0.05	1.27 ± 0.07	0.85 ± 0.01	4.9 ± 0.68
1:100	30	1.61 ± 0.08	0.65 ± 0.003	0.60 ± 0.02	2.86 ± 0.08
	60	2.1 ± 0.03	0.80± 0.04	0.65 ±0.002	3.55 ± 0.06
	90	2.65 ± 0.04	1.18 ± 0.02	0.8 ± 0.08	4.63 ± 0.09

Table 4. Extraction of anthocyanin by 0.01%-HCl-acidified water (thrice) with different ratios and extraction times.

Ratio	Time (min)	Anthocyanins (mg/g)			
		1st extraction	2nd extraction	3rd extraction	Total amount
	30	1.7 ± 0.02	0.72 ± 0.05	0.59 ± 0.031	3.01 ± 0.03
1:20	60	1.7 ± 0.007	0.8 ± 0.04	0.61 ±0.003	3.11 ± 0.09
	90	3.3 ± 0.15	1.56 ± 0.003	1 ± 0.06	5.86 ± 0.63
1:40	30	1 ± 0.09	0.66 ± 0.002	0.54 ± 0.007	2.2 ± 0.005
	60	1.5 ± 0.01	0.77 ± 0.04	0.65 ±0.07	2.92 ± 0.022
	90	4.01 ± 0.064	1.1 ± 0.09	0.61 ± 0.008	5.31 ± 0.62
1:60	30	1.53 ± 0.05	0.65 ± 0.023	0.61 ± 0.009	2.79 ± 0.001
	60	1.88 ± 0.002	0.75± 0.023	0.6 ±0.012	3.23 ± 0.02
	90	2.55 ± 0.026	1.15 ± 0.033	0.6 ± 0.005	4.33 ± 0.056
1:100	30	1.54 ± 0.016	0.61 ± 0.045	0.560 ± 0.047	2.71 ± 0.014
	60	1.77 ± 0.016	0.67± 0.028	0.56 ±0.019	3 ± 0.018
	90	2.41 ± 0.012	1 ± 0.032	0.53 ± 0.029	3.94 ± 0.016

Purification of anthocyanin

Anthocyanin extracted through acidified water and acidified ethanol using 1:20 and 1:100 ratio for 90 mins was filtered through Whatman No.1 paper and then evaporated under vacuum. Crude concentrated anthocyanin was loaded to C-18 chains column of silica. Elution was performed using three solutions such as 0.01% HCL acidified distilled water (to eliminate organic acid and sugar compounds), followed by ethyl acetate (to exclude phenol

compounds) and finally by acidified ethanol (Ethanol:1% w/v citric acid, pH 2.9). The final purified anthocyanin fractions were collected for subsequent analysis.

Determination of cyanidin-3-glucoside content

The cyanidin-3-glucoside content of purified anthocyanin was analyzed by HPLC at 520 nm at the retention time 10.8 min was the peak of cyanidin-3-glucoside as extracted by acidified water (1:20) and acidified ethanol (1:100) (Fig. 4) by comparing with the standard with the retention time of 10.562 min (Fig. Fig. 5 and 6). The amount of cyanidin-3-glucoside by acidified water (1:20) and acidified ethanol (1:20) were 288 and 260 mg/g respectively.

Identification of anthocyanins from purified extracts by HPLC-MS

Reverse phase HPLC and MS analysis were used to identify the anthocyanins at 520 nm indicated the cyanidin-3-glucosides eluted at peak 1 as compared to the standard. This was confirmed by retention time, spectroscopic characteristic, and fragmentation pattern between sample extract and the standard solution (Fig. 4 a, b, c). Peak 1 was cyanidin-3-glucoside with molecular ion $[M+H]^+$ at m/z 449 and a fragment ion [M+H-162] at m/z 287 (Fig. 8a and b). The molecular ion (M+) and fragment $(M+H)^+$ from HPLC-MS analysis indicated 7 anthocyanin fractious. Non-acylated forms such as cyanidin-3-glucoside (449 & 287), pelargonidin-3-glucoside (433 & 271), peonidin-3-glucoside (463 & 301) and malonyl derivatives or ethylmalonyl derivatives (acylated forms) includes cyanidin- 3-(6-malonylglucoside) (353 & 287), pelargonidin-3-(6-malonylglucoside) (519 & 271), peonidin-3-(6-malonylglucoside) (549 & 301) and cyanidin-3-(6-ethylmalonylglucoside) (563 & 287) respectively.

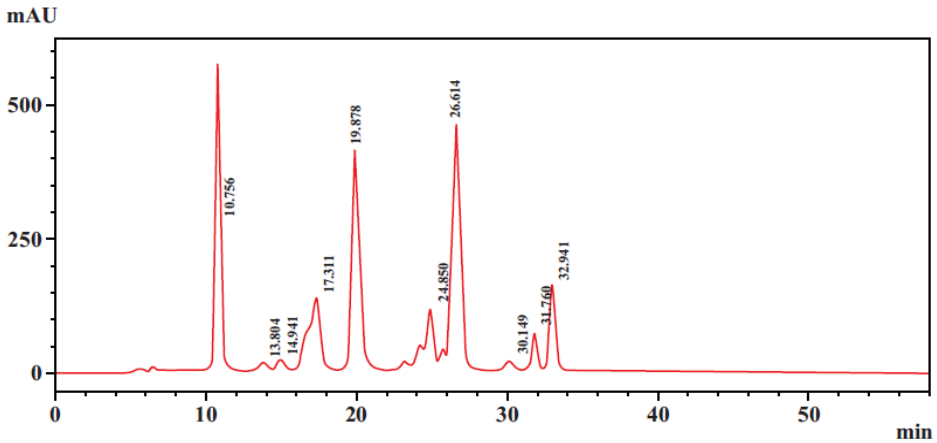

Fig. 4a Chromatogram of HPLC at 520 nm of anthocyanins extracted by 0.01%h HCl-acidified water of *B. retusa.*

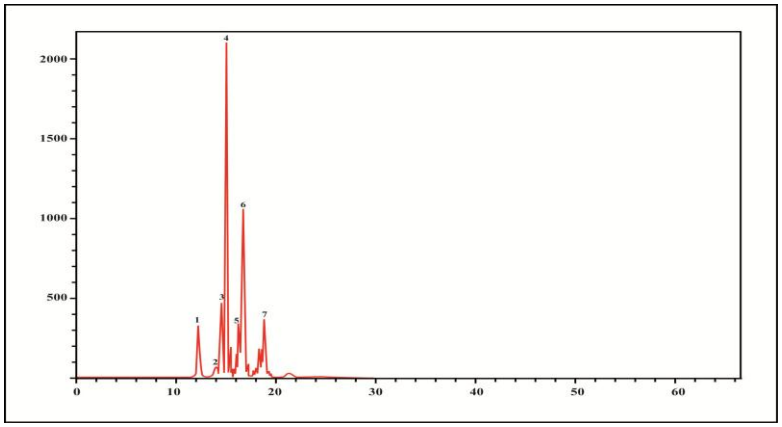

Fig. 4 b. Chromatogram of HPLC (a) Anthocyanin extracts from *B. retusa* (b) the standard cyanidin-3-glucoside.

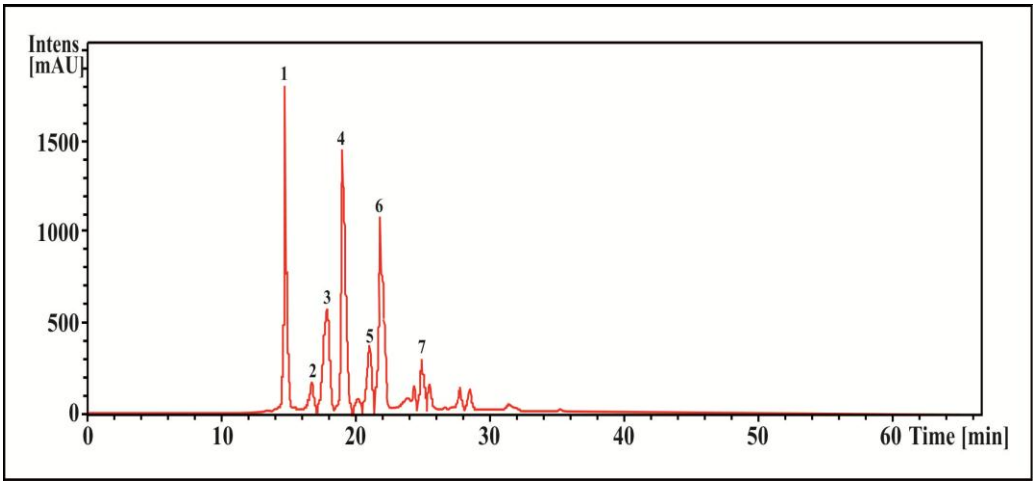

Fig. 4c. Chromatogram of HPLC of cyanidin-3-glucoside and [M]+ and fragment [M+ H]+ extracted from *B. retusa.*

Hepatoprotective activity

Body and organ weight

Decrease in organ and body weight is a sign of deformity in the organism. Changes of organ weight have been considered as a sensitive marker of xenobiotic compounds induced changes. In toxicological studies, comparison of organ weights between treated and untreated groups of animals have conventionally been used to evaluate the toxic effect of the test drug.[16] [17] The present analysis reveals that the body and the liver and kidney weight differed soundly between the paracetamol (PCM) treated group and the normal control group. However, pretreatment with anthocyanin of *B. retusa* has neutralised the effect of PCM on rat's body weight and organ weight (Table 5).

Table 5 Effect of anthocyanin of *B. retusa*, silymarin on body, liver and kidney weight compared to PCM treated rats.

Groups	Body weight		Relative organ weight		
	Before	After	Liver	Kidney	
				Right side	Left side
Gr. I Distilled water	110 ± 2.5	117 ± 0.64	4.8 ± 0.309	0.45 ± 0.02	0.44 ± 0.03
Gr. II PCM 3g/kg bw.i.p	123 ± 0.34	115 ± 6	3.8 ± 0.17	0.4 ± 0.03	0.39 ± 0.01
Gr. III Silymarin 100 mg/kgbw.p.o	111 ± 0.82	117 ± 0.99	4.7 ± 0.13	0.45 ± 0.038	0.45 ± 0.09
Gr. IV 100 mg/kg bw.p.o	102 ± 0.78	110 ± 0.55	4.5 ± 0.28	0.46 ± 0.009	0.43 ± 0.008
Gr. V 200 mg/kgbw.p.o	111 ± 0.26	116 ± 0.54	4.71 ± 0.04	0.45 ± 0.010	0.45 ± 0.02

Each value represents the mean of 6 rats ± SD. Significant at $P < 0.05$, as compared with the corresponding control group.

Food and water intake

The water and food intake capacity of the rats treated with water and silymarin were normal and there was no remarkable difference found between these groups over the period of treatment (Table 6). The animals administrated with PCM, failed to eat and drink when compared to water and silymarin treated groups. Meanwhile, pretreatment with anthocyanin of *B. retusa* normalised the effect of PCM on food and water consumption. These results were in agreement with the report of Ashok kumar *et al*, i.e. methanol extract of *Amaranthus caudatus* against paracetamol-induced hepatic injury in rats.[18]

Table 6. Effect of anthocyanin of *B. retusa* on water (ml) and food (g) consumption in PCM toxicity induced rats. Group I: (control), Group II: (PCM treated), Group III: (pretreatment with silymarin), Group IV: (pretreatment of anthocyanin at low dose of 100 mg/kgbw), Group V: (pretreatment of anthocyanin at low dose of 200 mg/kg b.w).

		1 d	2 d	3d	4d	5d	6d	7d	8d
Group I	Water (ml)	60	49	57	63	71	83	97	95
	Food (g)	9	20	42	60	55	54	54	55
Group II	Water (ml)	49	45	40	38	40	42	44	35
	Food (g)	11	22	28	24	17	16	16	15
Group III	Water (ml)	32	45	50	60	63	72	91	70
	Food (g)	10	18	40	49	58	55	55	55
Group IV	Water (ml)	30	41	55	67	73	75	90	76
	Food (g)	12	19	42	51	56	57	59	57
Group V	Water (ml)	29	38	52	64	70	74	88	74
	Food (g)	09	16	40	49	57	58	58	58

Hepatotoxicity assessment

Liver is the major organ regulating homeostasis in living organisms including detoxification and excretion of many xenobiotic compounds and its dysfunction may lead to many intrinsic human health issues.[19] It has been reported that hepatotoxicants induce stress leads to the release ROS, RNS or free radicals, which act on hepatic liver parenchyma cells.[20] These liver cells are the production site for many vital enzymes, bile salts and pigments etc. Undue damage caused by toxic chemicals, induces the level of liver enzymes. Liver damage induced by pain killers are commonly employed model for the screening of hepatoprotective drugs. Thus, in the present study PCM, a common drug used for curing many ailments like fever, body pain etc and is proved to induce acute liver damage. Alkaline phosphatase (ALP) is a mucosal membrane bound glycoprotein that line the bile system. The free flow of bile and down into the billary tract is responsible for maintaining the optimal level of ALP in the blood. When the liver is not functioning ideally this enzyme is secreted via bile into the bloodstream. ALP is the marker of the integrity of hepato billary system and flow of bile.[21] Similarly, SGOT and SGPT are aminotransferase enzymes present in many tissues and were released into the blood stream whenever these tissues were damaged.

The PCM induced serum levels of SGOT, SGPT, ALP, TB and TP suggests the possible damages observed in the structural integrity of the liver. The most of the above said proteins were directly released into the blood during cellular damages.[22] Thus it is possible to interpret that when rats were treated with PCM it induces hepatotoxicity by metabolic induction, in liver cells that leads to its dysfunction.

Overdose of paracetamol leads to saturation of the sulfate and glucuronide pathways, and more of these were shunted to the cytochrome P450 system to produce N-acetyl-p-benzoquinoneimine (NAPQI). This further reduces the hepatocellular supplies of glutathione i.e., the demand for glutathione increase than its regeneration. NAPQI remains in its toxic form in the liver and reacts with cellular membrane molecules, resulting in widespread hepatocyte damage and death thus leading to acute liver necrosis. In animal studies, the liver's stores of glutathione must be depleted to less than 70% of normal levels before liver toxicity occurs.[23]

The biochemical results of rats intoxicated with PCM induced a rise of the serum enzymes which are well known markers of hepatic injury. i.e, drastic increase in the levels of serum glutamic pyruvic transaminase (SGPT), serum glutamic oxaloacetic transaminase (SGOT),

alkaline phosphatase (ALP) (67.5 ± 0.43, 281 ± 5.7, 347 ± 11.8 U/L respectively). Total bilirubin (TB) was (1.5 ± 0.02 mg/dl) and with a significant decrease in total protein (TP) (4.1 ± 0.03 mg/dl) in PCM intoxicated rats as compared to the control group with the corresponding values were 43.15 ± 3.832, 120 ± 0.65, 442 ± 0.87 U/L, 6.5± 0.05, 0.80 ± 0.05 mg/dl for SGPT, SGOT, ALP, TP and TB respectively (Table -7).

Table 12 Effect of anthocyanin of *B. retusa* on plasma enzymes (SGOT, SGPT, ALP), Cholesterol, total bilirubin (TB) and total protein (TP) compared to silymarin in liver damaged rats. Each value represents the mean of 6 rats ± S.E. significant at *P*> 0.0001; as compared with the corresponding control group. SGOT- serum glutamic oxaloacetic transaminase, SGPT-serum glutamic pyruvic transaminase, ALP- Alkaline phosphatase, TP- Total protein TB -Total bilirubin.

Groups	SGOT (U/L)	SGPT (U/L)	ALP (U/L)	TP (mg/dL)	TB (mg/dL)
Gr. I 1% Tween 80 5 ml/kg bw.p.o	120 ± 0.65	43.15 ± 3.832	159 ± 0.87	6.5± 0.05	0.80 ± 0.05
Gr. II PCM 1g/kg bw.i.p	281 ± 5.7	67.5 ± 0.43	347 ± 11.8	4.1 ± 0.03	1.5 ± 0.02
Gr. III Silymarin 50 mg/kgbw.p.o	135 ± 0.98	43 ± 0.029	161.2 ± 0.51	5.6 ± 0.06	0.89 ± 0.05
Gr. IV 100 mg/kg bw.p.o	159 ± 0.097	53 ± 0.63	163 ± 0.843	5 ± 0.08	0.94 ± 0.09
Gr. V 200 mg/kgbw.p.o	137 ± 0.56	47 ± 0.05	168.2 ± 0.45	6 ± 0.09	0.98±0.04

However, pretreatment of anthocyanin from *B. retusa* (100 mg/kg bw and 200 mg/kg bw) and silymarin significantly regained the biochemical changes induced by PCM intoxification and confirmed their hepatoprotective potential to accelerate the regeneration of parenchyma cells. There is a reduction in the level of SGPT, SGOT, ALP and TB in the rats treated with 100 mg/kg bw, 200 mg/ kg bw of anthocyanin from *B. retusa* and silymarin. Foufelle and Fromenty reviewed that the elevated serum protein level suggests the stabilization of endoplasmic reticulum leading to an increase in protein synthesis that enhanced the hepatocyte regeneration.[24] Meanwhile, the reduction in the ALP level with concomitant depletion of raised bilirubin level reveals the stability of the biliary function.

Antioxidant enzyme activity assay

The cells possess an effective antioxidant system against free radicals and ROS induced damage via the endogenous enzymatic and non enzymatic antioxidants such as SOD, CAT and MDA content.[25] SOD and CAT enzymes constitute a mutually interlinked defense enzymes of Halliwell - Asada cycle against ROS. The elevated level of MDA reveals the excessive free radical generation and activation of lipid peroxidation (LPX) system resulting

in hepatic damage. MDA produced as byproduct by lipid peroxidation that occurs in hydrophobic core of cell membrane.

Intoxication with PCM caused an upset in the antioxidant defense systems results into oxidative stress as evident from a marked decline in the antioxidant enzyme activities of SOD and CAT along with boosting in the MDA level. The SOD and CAT values are found to be 1.89 ± 0.15 and 1.7 ± 0.11U/mg Hb compared to control with 3.45 ± 0.23 and 2.9 ± 0.23U/mg Hb respectively. The elevated level of MDA in PCM treated group was 25 ± 1.76 compared to control group with 8.7 ± 0.08 nmol/mg, indicates excessive formation of free radicals / ROSs and activation of lipid peroxidation system resulting into hepatic damage.

The pre-treatment of anthocyanin from *B. retusa* at the dose of 100, 200 mg/kgbw and silymarin (at the dose of 100 mg/kg b.w. enhanced the activity of SOD with 3.9 ± 0.05, 4.7 ± 0.04, 4.8 ± 0.04 U/mg Hb and CAT with 3.4 ± 0.04, 4.1 ± 0.27, 3.5 ± 0.5 U/mg Hb respectively. Further, there was a decline in the concentration of MDA in the rats pretreated with anthocyanin of *B. retusa* of 100, 200 mg/kg bw and silymarin with 15.7 ± 0.03, 9.3 ± 0.04, 10± 0.97 nmol/mg respectively, indicates antilipid peroxidation effect of anthocyanin (Table 8), i.e., the pretreatment with cyanin of *B. retusa* effectively blocked PCM induced damages in the liver and regaining the SOD and CAT activities in the rats.

Table 8 Effect of anthocyanin of *B. retusa* on SOD, CAT, MDA compared to silymarin in liver damaged rats Each value represents the mean of 6 rats ± S.D. significant at *P*< 0.05; as compared with the corresponding control group. SOD: superoxide dismutase; CAT: catalase; MDA = malondialdehyde.

Groups	SOD (U/mg Hb)	CAT (U/mg Hb)	MDA (nmol/mg)
Gr. I Water	3.45 ± 0.23	2.9 ± 0.23	8.7 ± 0.08
Gr. II PCM 1g/kg bw.i.p	1.89 ± 0.15	1.7 ± 0.11	25 ± 1.76
Gr. III Silymarin 100 mg/kgbw.p.o	4.8 ± 0.04	3.5 ± 0.5	10 ± 0.97
Gr. IV 100 mg/kg bw.p.o	3.9 ± 0.05	3.4 ± 0.04	15.7 ± 0.03
Gr. V 200 mg/kgbw.p.o	4.3 ± 0.04	3.9 ± 0.27	9.3 ± 0.04

Histopathological studies

Histopathological analyses provide additional supportive for biochemical data. Histology of liver section of control rats exhibit normal hepatic cells each with well defined cytoplasm, prominent nucleus and nucleolus and well defind central vein (Fig.5a). The PCM intoxicated rats showed the total loss of hepatic architecture with centrilobular hepatic necrosis, fatty changes vacuolization and congestion of sinusoids, kupffer cell hyperplasia, crowding of

central vein and apoptosis (Fig. 5b). Pre-treatment with anthocyanin of *B. retusa* at the dose of 100 and 200 mg/kg b.w. and silymarin showed protecting activity (Fig. 5C and 5D). The anthocyanin of *B. retusa* at low dose of 100 mg/kg b.w. showed moderate hepatoprotective activity. However, at the high dose of 200 mg/kg b.w. and silymarin had shown significant hepatoprotective activity and reduced the degenerative changes in the liver. The result thus obtained was in agreement with the report of Ashok Kumar *et al*., *i.e.* methanol extract from *Amaranthus caudatus*, against paracetamol-induced hepatic injury in rats.[18] Selvanayaki and Ananthi also evaluated the hepoprotective activity of aqueous extract of *Lawsonia inermis* against paracetamol induced rats.[19] Asha Tukappa *et al*., proved the cytotoxic and hepatoprotective attributes of methanolic extract of *Rumex vesicarius.*[20] Naggayi *et al*., analyzed the protective effects of aqueous extract *of Carica papaya* seeds in paracetamol induced nephrotoxicity in male wistar rats.[21] Garba *et al*., also proved that the aqueous extract of *Kohautia grandiflora* has a protective effect on paracetamol induced liver damage in albino rats.[22] Thus, the safeguarding role of anthocyanin against hepatotoxicity may be justified.

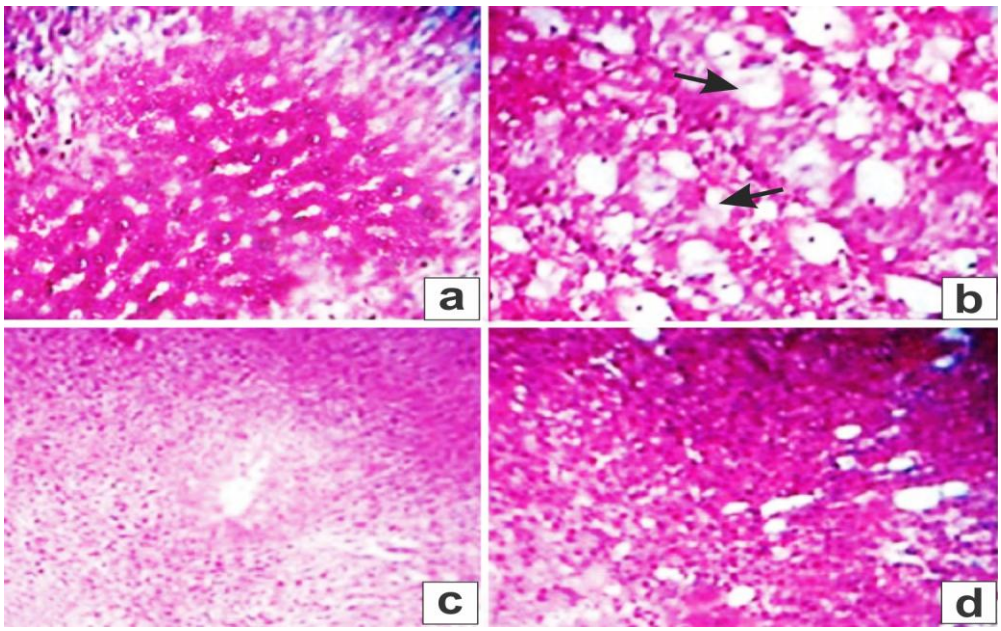

Figure 5 Effect of anthocyanin from *B. retusa* on acute liver injury induced by PCM. A: (control): liver section with normal structure and architecture. B: (PCM treated): showing extensive area of necrosis, profound inflammation and congestion. C: (pretreatment with silymarin): reduced inflammation and degenerative changes. D: (pretreatment of extract at low dose of 100 mg/kgbw): reduced inflammation, degenerative changes.

CONCLUSION

The effects of phytohormones and culture media on the induction and formation of calli from leaf explants of *B. retusa* had been proved. Production of calli, anthocyanin extraction from cell suspension culture and various parameters that enhance anthocyanin synthesis were standardized. Subsequently, anthocyanin was purified, characterized and fractionated by HPLC-MS. The protective activity of anthocyanin from *B retusa* against paracetamol induced hepatotoxicity was proved with its serum enzymes and antioxidant potential. The hepatoprotective role of the anthocyanin was found to be comparable with silymarin providing a new way to develop potential hepatoprotective drugs. Further study is warranted in analyzing the cytotoxicity analyzing the cytotoxicity and mutagenic properties of the anthocyanin.

Competing interests

The authors declare that they have no competing interests.

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