Int J Pharma Res Health Sci. 2018; 6 (1): 2250-57
DOI:10.21276/ijprhs.2018.01.28
Murugan *et al*
CODEN (USA)-IJPRUR, e-ISSN: 2348-6465

PHS Scientific House
PHARMAHEALTHSCIENCES

International Journal of Pharma Research and Health Sciences

Available online at www.pharmahealthsciences.net

Original Article

Purified Anthocyanin from *Bridelia retusa* (L.) Spreng. as Antioxidant and Antimicrobial: A Medicinal Plant from South Western Ghats, Kerala

Greeshma Murukan, Kavitha CH, Bosco Lawarence, Aswathy JM, K Murugan*
Plant Biochemistry and Molecular biology Laboratory, Department of Botany, University College, Thiruvananthapuram, Kerala- 695 034, India.

ARTICLE INFO

Received: 01 Feb 2018
Accepted: 22 Feb 2018

ABSTRACT

Background: *Bridelia retusa* is traditionally used as astringent, stress reliever and for rheumatic pains. **Methodology**: *In vitro* cell culture, elicitation of anthocyanin and its evaluation of medicinal potentialities using standard protocols. **Results**: Present study reveals that 2, 4-D either alone or in combination with kinetin supplemented in MS medium showed significant callus induction from leaf explants than stem. Growth hormones, pH, light, and carbon source influence anthocyanin pathways. Remarkable callus induction was seen with 2.5 mg/L N6-benzyladenine (BA) + 2 mg/L 2, 4-dichlorophenoxyacetic acid (2, 4-D) (98.9%). Fresh and dry mass of the calli were i.e., 1.9 ± 0.04 and 0.45 ± 0. 03 g respectively. Good response was seen with light on MS medium with glucose (4%) containing BA (2.5 mg/L) and 2, 4-D (2 mg/L) at pH 3.5 yielded 2.8 mg /g of anthocyanins. Suspension cultures of medium containing 2, 4-D (2.5 mg/L) and BA (2 mg/L) at pH 5.0 triggered anthocyanin production into the medium with pH 4.4 – 4.6. HCl-ethanol extraction for 90 min yielded the maximum anthocyanin content. pH and temperature stability of anthocyanin was also analyzed. Rate constant with a corresponding decline in the t1/2 value was seen with the higher temperature at pH 1 and 4. Fractionation of anthocyanin using HPLC coupled with mass spectrometry revealed 09 fractions comprising acylated cyanidins and two peonidins. Cyanidin 3-p-coumaroyl and feruloyl diglucoside-5-glucosides were the major components. The antimicrobial activity was performed by disc diffusion and broth microdilution assays against bacterial strains including Gram-negative, Gram-positive bacteria and fungal strain. The anthocyanin displayed strong antioxidant activity in terms of scavenging DPPH and ABTS radicals (significant IC_{50} values). Antimicrobial activity showed variation among the bacterial and fungal strains. The largest diameter of inhibition zone (DIZ) (24.5 mm) and remarkable minimal inhibitory concentration (MIC) values were recorded with *Enterococcus faecalis* and *Pseudomonas aeruginosa.* **Conclusion**: This study validates the scientific base on the use of this plant in traditional health care system.

Keywords: *Bridelia retusa, in vitro* culture, phytohormones, anthocyanin, antioxidant, folk medicine, antibacterial activity, antioxidant.

1. INTRODUCTION

Polyphenols or phenolics comprises a large group of molecules found in vegetables, fruits, cereals, tea, coffee, cocoa, wine, fruit juice and soy. In plants, they have photoprotection function, defense against pathogens and

Corresponding author *
K Murugan
Plant Biochemistry and Molecular biology Laboratory, Department of Botany, University College, Thiruvananthapuram, Kerala
E-Mail: harimurukan@gmail.com

insects, responsible for pigmentation and provide some organoleptic characteristics for food. The polyphenols are antioxidants and partially respond for the taste and flavor of many plant products, and fruits such as apple and grape. The polyphenols are effective hydrogen donors and thereby contributing as potent antioxidant phytochemicals.

This antioxidant ability can be related to the position of the hydroxyl groups in their chemical structure. Antimicrobial activity of polyphenols in medicine plants has been extensively investigated against a wide range of microorganisms.[1] Among polyphenols, flavonols, tannins and flavan-3-ol (sometimes referred to as flavanols) received more attention due to its broad spectrum and antimicrobial activity when compared with other polyphenols and the fact that most of them are able to suppress microbial virulence factors (such as inhibition of biofilm formation, reduction of receptor ligands adhesion, and bacterial toxins neutralization) and show synergism with antibiotics.[1,2] Interestingly all these biological activities have an indirect impact in general and oral health. The glycosylation of polyphenols in nature helps to protect these molecules from oxidation, extending their half-life.

Besides, herbal-based therapies, once used in traditional medical systems are now recommended for treatment of several degenerative disorders and chronic conditions where modern pharmaceutical agents have proved inadequate. Natural products with their potential to act as antioxidants play a major role in the prevention of various pathological conditions such as cancer, cardiovascular and neurodegenerative troubles. Furthermore, in developing countries, particularly in Asian countries, effective drugs are not often affordable.

Bridelia is a tropical small sized tree belongs to Euphorbiacea. This plant is extensively used in herbal medicine in Nigeria.[3] In phytomedicine, the bark infusion is used as a purgative and vermifuge. The latex from the bark and the leaves are used to prepare soup used to aid lactation by nursing mothers. The leaf decoction has been used extensively as a wash for fever. The bark, leaves and root extracts are ingredients used in the preparation of a popular mouth wash and as a remedy for thrush in children. Extracts from the roots and leaves are used to cure piles, diarrhea and dysentery.[4] In this juncture, the present study aims to isolate anthocyanin from *in vitro* cell suspension culture of *Bridelia retusa*, its purification, fractionation and its evaluation of biological potentialities.

2. MATERIALS AND METHODS

In vitro callus culture

Explants such as fresh leaves and shoot tips of *Bridelia retusa* were collected from natural habitat of deciduous forests of Ponmudi hills was used for *in vitro* culturing. Cultures were initiated on MS medium with 30 g/L sucrose + different phytohormones applied singly or in combinations such as naphthalene-acetic acid (NAA), 2,4-dichlor-phenoxyacetic acid (2,4-D) 0.5 - 4 mg/L, N6-benzyladenine (BA- 0.5 - 3 mg/L).[5] pH of the media were adjusted to 4 prior to adding 8 g/L agar, autoclaved (121 °C and 15 lb) 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 cultured per treatment and each experiment was repeated thrice. Cultures were maintained in a growth chamber at 26 ± 2 °C under 16 h photoperiod provided by cool-white fluorescent tubes (45 µmol m^{-2} s^{-1}). Sub culturing was carried in fresh media with the same composition after 30 days. Callus biomass accumulation was recorded 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- 30 days interval.

Cell suspension culture

Cell suspension culture was carried by transferring 3 g of friable calli into 250 ml Erlenmeyer flasks containing 100 ml of fresh half strength liquid MS medium + different concentrations of 2,4-D (0.5 -2.5 mg/l) + BA (3 mg/l) and sucrose (30 g/l) at pH 4.8. 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 to 60 days. Cells were isolated from the medium by filtration using nylon mesh and weighed as fresh weight. Cells 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

2 g of cell suspension were subjected to different temperatures 22°C or 36°C; light intensities i.e., 45, 67 and 80 µmol m^{-2} s^{-1}; different carbon source: sucrose, glucose, maltose, fructose concentrations (1,2,3,4%); different total nitrogen concentrations (50; 60; 70; 80 mM) and 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 harvested with 25 ml of two different extraction solvents: 0.01% (v/v) HCl-acidified water and HCl-acidified ethanol. The extraction was done at room temperature with constant shaking at 100 rpm for 60 min. The crude extract was filtered through Whatman 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 according to 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 Whatman No.1 paper, and then dried by rotary evaporator at 40°C under vaccum 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.

HPLC-MS analysis

The purified anthocyanins were mixed with 0.5% HCl, then filtered (0.45 µm) and injected (10 µL). The flow rate was 0.8 mL/min and maintained at 28°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 - cyanidin-3-glucoside.

Antioxidant activity power

Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant capacity of anthocyanin extract was estimated by the method described by Pulido *et al*.[6] FRAP reagent (900 µL), prepared freshly and incubated at 37℃, was mixed with 90 µL of distilled water and 50 µL test sample (1 mg/mL). The test samples and the reagent blank were incubated at 37℃ for 30 min in a water bath. At the end of incubation, the absorbance was taken immediately at 593 nm using a spectrophotometer. Methanolic solutions with known Fe (II) concentration, ranging from 100 to 2000 µmol/L (as $FeSO_4 \cdot 7H_2O$), were used for the preparation of the calibration curve.

DPPH radical scavenging activity

Radical scavenging activity of anthocyanin extract was measured by DPPH radical scavenging method, described by Blois.[7] Plant extracts at various concentrations were taken and the volume was adjusted to 100 µL with ethanol. 5 mL of 0.1 mmol/L ethanolic solution of DPPH was added and shaken vigorously. The tubes were allowed to stand for 20 min at 27 °C. The absorbance of the sample was measured at 517 nm. IC_{50} values of the extract, i.e., the concentration of extract necessary to decrease the initial concentration of DPPH by 50%, was calculated.

ABTS cation radical scavenging activity

ABTS cation radical decolorization assay was measured according to the method described by Re *et al*.[8] ABTS▪+ was produced by reacting 7 mmol/L ABTS aqueous solution with 2.4 mmol/L potassium persulfate in the dark for 12–16 h at room temperature. Prior to the assay, this solution was diluted in ethanol (about 1:89, V/V) and equilibrated at 30 °C to give an absorbance at 734 nm of 0.700 ± 0.02. After the addition of 1 mL diluted ABTS solution to 10 µL sample or trolox standards (final concentration 0–15 µmol/L) in ethanol, absorbance was measured at 30 °C exactly 30 min after the initial mixing. Solvent blanks were also run in each assay. The total antioxidant activity (TAA) was expressed as the concentration of trolox having equivalent antioxidant activity in terms of µmol/L/g anthocyanin.

Antimicrobial assays

Disc diffusion method

The *in vitro* antibacterial activity of anthocyanin was studied by the paper disc diffusion method of Bauer *et al* as described previously by Zongo *et al*.[9,10] Bacteria grown on Mueller-Hinton Broth (MHB) at 37°C for 18 to 24 h were suspended in saline solution (0.9% NaCl) and adjusted to a turbidity of 0.5 McFarland standard corresponding to 10^8 colony forming units (cfu) mL^{-1}.[11,12] Bacterial suspensions were used to inundate sterile Petri dishes (90 mm diameter) containing 15 mL Mueller-Hinton Agar (MHA) (Liofilchem, Italy). Anthocyanin was dissolved in DMSO at 100 mg mL^{-1} and filtered with a Millipore filter. 10 µl was pipetted to impregnate sterile paper discs (6 mm diameter). The discs were then placed onto the surface of inoculated Petri dishes. Gentamicin (10 µg) and Ciprofloxacin (5 µg) were used as the positive control. Paper discs soaked in DMSO without anthocyanin were used as negative control. Petri dishes were incubated aerobically at 37°C for 18 to 24 h. The results were recorded by measuring the zones of growth inhibition (mm) surrounding the disc indicating the presence of microbicidal activity. All tests were performed in duplicate.

Broth microdilution assay

A broth microdilution method was performed to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) as recommended by NCCLS.[13] The inocula of the bacterial strains were prepared from 18 to 24 h broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity. The anthocyanin was first dissolved in 10% DMSO and then sterilized as described previously. 100 µl of this stock anthocyanin was transferred into the first well of a 96-well sterile plate previously filled with 100 µL of nutrient broth. Serial twofold dilutions were made in to 11 consecutive wells. Into each well, 95 µL of nutrient broth and 5 µL of the inocula were added to achieve concentrations of extracts ranging from 3 to 0.0049 mg mL^{-1}. The final volume in each well was 200 µL. On the same plate, some wells with specific medium and microorganism were used as control of the growth and other inoculated wells containing only 200 µL of the media were used for sterility control. Each plate was mixed on a plate shaker at 300 rpm for 20 sec and then incubated at 37°C for 24 h. Bacterial growth was indicated by the presence of turbidity and a pellet on the well bottom. The MIC is the lowest concentration demonstrating no visible growth in the broth.

The MBC which is the lowest concentration at which 99.99% or more of the initial inoculum was killed and was determinate as described previously.[13,14] 100µl from each well demonstrating no visible growth were removed to spread onto petri dishes filed with sterilized plate count agar (PCA) medium. Petri dishes were incubated at 37°C for a total period of 48 h.

Antifungal screening

The antifungal assay was performed by disc diffusion method and microdilution technique as described above with minor modifications.[13,15] Potato dextrose broth (PDB) and Potato dextrose agar (PDA) were used. The plates were incubated at 28°C for 48 to 96 h. Standard disc of nystatin (100 IU) was used as positive control.

Potassium (K+) leakage

The K+ leakage was determined using a flame emission and atomic absorption spectroscopy used for titration in solution.[16] The solution was filtrated after contact with the test compounds. The samples were analyzed in a GBC AAS 932 plus device using GBC Avante 1.33 software.

Membrane integrity analysis using propidium iodide uptake

The Live/Dead BacLight kit (Invitrogen) assesses membrane integrity by selective stain exclusion method was used for the evaluation.[17,18]

Statistical analysis

The results of analysis were expressed as the means of three independent analyses. The results of antimicrobial activity was analyzed by one-way analysis of variance (ANOVA) followed by Tukey test for multiple comparison. The level of significance was set at 95%. The entire analysis
was performed with statistical software SPSS 17 (SPSS for Windows; SPSS Inc, Chicago, IL).

3. RESULTS AND DISCUSSION

Callus induction in *B.retusa* was seen after 6th week of inoculation. The calli formed from different combinations of hormones were generally creamy. The % of callus formed ranged from 17.8 to 98.9%. The callus induction was maximum with the leaf explants cultured on MS medium supplemented with 2 mg/L 2,4-D + 2.5 mg/L BA i.e.,98.9% (Fig.1). Meanwhile, 50, 45.4, 42% with BA + 2,4-D combinations with the concentrations 1+2, 1+1, 2+5 mg/L respectively. The calli yielded from such treatments were found to be completely friable. NAA also induced calli optimally in combinations with BA. The calli yielded from such treatments were found to be loose. 2, 4-D (2 mg/L) + BA (2.5 mg/L) recorded the optimal callus fresh and dry weight i.e., 1.7 ± 0.02 and 0.65 ± 0.03 g respectively with leaf as explants followed by 2.5 mg/L 2, 4-D and 3 mg/L BA (1.55 ± 0.61, 0.61±0.09 g). Meanwhile, in the case of stem 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). Most of the calli formed during this phase were found to be compact. Insignificant callus induction was noticed with stem under various hormonal conditions compared to leaf explants.

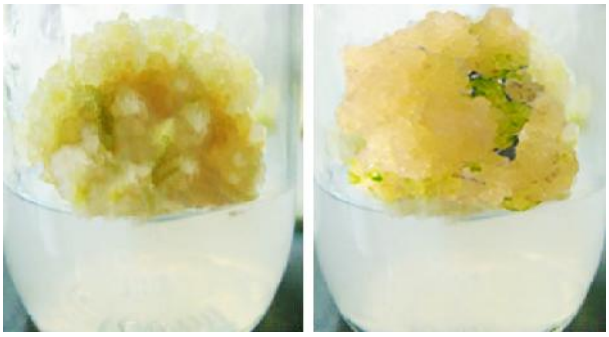

Fig 1: Calli from leaf explants from *B. retusa* using half strength MS medium.

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

Anthocyanin formation was initiated in the leaf calli from 25 to 30 days 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 synthesis (3.4 mg/g).

Cell suspension culture

Fresh friable calli clumps grown on 2.5 mg/l BA + 2.0 mg/l 2,4-D were used for initiating cell suspension culture. Cell growth was measured between 2 day intervals 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 (Fig.3). The growth of suspension cultures revealed that the growth rate of cells was slow initially (3 days -lag phase). However, a marked increase was seen from 6th day onwards in terms of mass (exponential phase). Maximum fresh weight was reached on 20th day and was about 20 fold higher than the initial mass. Subsequently, the growth rate 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 of toxic metabolites were

accumulated by the cells. The cell viability was 80 % on 22nd day of culture (Fig.3) and then marginally declined.[19]

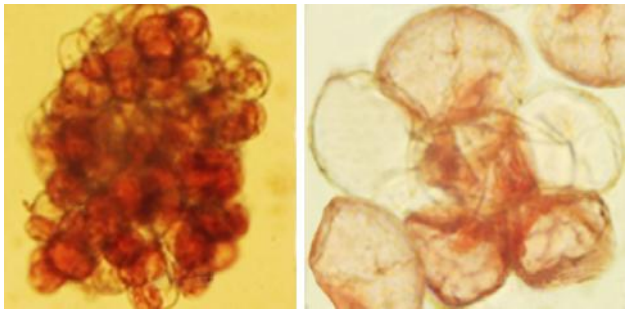

Fig 3: Viable cells from cell suspension culture of *B. retusa*

Purification of anthocyanin

Anthocyanin was extracted by using acidified water (1:20) and acidified ethanol(1:100) for 90 min and 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 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) (Fig.4) and acidified ethanol (1:100) by comparing with the standard with the retention time of 10.562 min . The amount of cyanidin-3-glucoside by acidified water (1:20) and acidified ethanol (1:20) were 288 and 260 mg/g respectively (Fig.5).

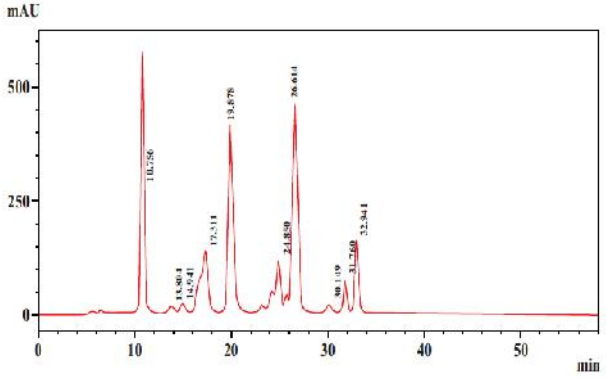

Fig 4: Chromatogram of HPLC at 520 nm of anthocyanins extracted by 0.01% HCl-acidified water of *B. retusa*

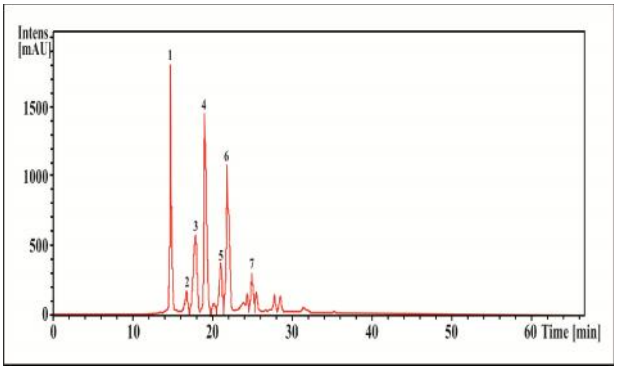

Fig 5: Chromatogram of HPLC of cyanidin-3-glucoside and [M]+ and fragment [M+ H]+ extracted from *B. retusa*.

Identification of anthocyanins from purified extracts by HPLC-MS

Reverse phase HPLC and MS analysis was 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 anthocyanin and the standard solution. 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. 5). The molecular ion (M+) and fragment $(M+H)^+$ from HPLC-MS analysis indicated 7 anthocyanin fractions. 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.

Antioxidant potentiality

The results of FRAP assay exhibited by the purified anthocyanin from *B. retusa* reveals its ferric reducing ability i.e., it showed the maximum degree of reduction (414.5 µmol Fe (II)/mg). Free radical scavenging activities was also investigated with the free radicals such as DPPH▪ and $ABTS^{▪+}$ (Table 1). The results suggest that the purified anthocyanin exhibited the remarkable DPPH scavenging activity i.e., with IC_{50} 0.31 mg/ml. The efficiency of ABTS cation radical scavenging activity also revealed that the anthocyanin displayed sound activity (IC_{50} 0.55 mg/ml). DPPH▪ and $ABTS^{▪+}$ scavenging activities involve hydrogen atoms transfer and electrons transfer. The results of the present investigation suggests that the anthocyanin of *Bridelia retusa* may contain enormous amount of hydrogen donor molecules which may scavenge the free radicals produced as indicated by the decolorization in the DPPH▪ and $ABTS^{▪+}$. Saravanan and Parimelazhagan reported that the fruit pulp of *Passiflora ligularis* exhibited effective DPPH▪ and $ABTS^{▪+}$ scavenging activities.[20]

Table 1: DPPH, ABTS radical-scavenging and FRAP activities of anthocyanin of *B. retusa*

	DPPH assay	ABTS radical scavenging	FRAP assay
Conc. (mg/ml)	% Inhibition ± SD	% Inhibition ± SD	µm/mg
0.1	13 ± 0.16	17.5±0.12	148±3.29
0.2	45 ± 0.62	30.6±0.13	217±5.02
0.4	58.5 ± 0.57	44±0.29	301±2.27
0.6	63 ± 0.42	53±0.60	370±6.05
0.8	75 ± 0.39	60±0.29	413.6±4.03
1	80.6 ± 0.12	69±0.09	414.5±1.27
Ascorbate (1mg)	96%	79%	481 ± 2.01
Quercetin (1mg)	94%	69.6%	512 ± 0.02

Antimicrobial activity

Antimicrobial activity of purified anthocyanin from *B. retusa* against selected bacterial and fungal strains recorded significantly by disc diffusion method. Present results show that the anthocyanin extract exhibited weak to strong inhibition against all microbes used. The highest DIZ (24.5 mm) has been recorded with *P. aeruginosa* followed by *C. albicans* and *E. faecalis.* The MICs and MBCs values obtained with microdilution assay were displayed in Table 2. The lowest MIC value was recorded for *Pseudomonas aeruginosa* (-) (0.0625 mg/l) followed by *E. faecalis*, *E.coli* and *S. aureus* (0.25, 0.5 and 0.75 mg ml^{-1} respectively) confirming their susceptibility in disc diffusion method. MIC values ranged from 0.625 to 1.5 mg/ ml. The order of fungicidal potential of anthocyanin is *Candida albicans*, *Aspergillus flavus* and *Trichophyton rubrum.* DIZ values for fungal species ranged from (9.3 to 21.9 mm).

Intracellular potassium leakage

The K^+ leakage was used to identify disryption of the cell membrane permeability. The impacts of anthocyanin on K^+ release from bacterial strains are shown in Table 2. 0.74 ± 0.01 loss of intracellular K^+ was observed for *Pseudomonas aeruginosa* cells with anthocyanin, at the tested concentration. For *Bacillus subtilis*, K^+ leakage was found as 0.18 ± 0.007 (Table 2).

Table 2: Minimum inhibitory concentration (MIC), minimum killing concentration (MKC), potassium leakage and cell membrane permeability of purified anthocyanin from *B. retusa* and standard antibiotics against pathogens.

Pathogens	MIC (mg/ml)	MBC (mg/ml)	Zone of inhibition (mm)	[illegible] (K^+ µg/mL)	Permeability to propidium iodide (%)
Pseudomonas aeruginosa (-)	0.0625	0.25	24.5	0.74 ± 0.01	94 ± 0.43
Candida albicans	0.125	0.25	21.9	-	-
Enterococcus faecalis (+)	0.25	0.5	19.5	0.59 ± 0.002	83 ± 0.55
Aspergillus flavus	0.5	1.0	16.0	-	-
Escherichia coli (-)	0.50	1.0	15.7	0.46 ± 0.003	72 ± 0.09
Staphylococcus aureus (+)	0.75	1.25	13.6	0.21 ± 0.001	61±2.5
Bacillus subtilis(+)	1.0	1.50	11.4	0.18 ± 0.007	56±2.7
Trichophyton rubrum	1.5	3.0	9.3	-	-
Tetracycline	0.0625	0.25	28.6	0.88 ± 0.01	97.8 ± 1.9
Gentamycin	0.125	0.25	29.4	-	-

Anthocyanin on bacterial membrane integrity

The functional nature of cell membranes can be evaluated based on the ability of PI to penetrate cell membrane. Generally, PI penetrates cells with cells showing damaged membrane. In this way, the potential of anthocyanin to interfere with membrane integrity after 1 h exposure was analyzed (Table 2). The PI uptake results suggest that anthocyanin compromise the integrity of the cytoplasmic membrane of both bacteria ($p < 0.05$). For *Staphylococcus aureus* the percentage of cells stained with PI after 1 h of treatment (at corresponding MIC) was 61±2.5%. For *Bacillus subtilis* exposed to anthocyanin, the damage in cytoplasmic membrane was about 56±2.7% of the total cells (Table 2).

The indiscriminate uses of synthetic antibiotics have lead to multiple drug resistant strains.[21] In addition to the increasing prevalence of drug resistance; certain synthetic antibiotics are producing adverse side effects. Therefore, it is the need for hour to screen new plant drugs particularly affordable for least developed countries. Hassawi and Kharma reviewed that herbals are potential as antibacterial, antifungal and antiviral. *B. retusa* is well known in ethnic medicine for its biological properties.[22] It has been reported that the bark of the species yield black coloured dye. The present results showed that anthocyanin is potential antimicrobial agents. The present results confirm that purified anthocyanin extracted from cell suspension culture revealed a pool of anthocyanin fractions. Similarly, the anthocyanin was also potent DPPH and ABTS radical scavenger. Antimicrobial activity of a molecule can be considerate when the diameter of inhibition zone (DIZ) observed was 9 mm or more around the paper disc.[23] Generally, DIZ obtained with Gram-positive bacteria are larger than those obtained with Gram-negative bacteria. This is in phase with several previous studies which demonstrated that Gram-positive bacteria are more sensitive to plant extracts or phytochemicals than Gram -negative bacteria.[10,24,25] Indeed, the Gram-negative bacteria which are responsible for a large number of infectious diseases have a unique outer membrane that contains lipopolysaccharides which render them impermeable to certain antibacterial substances including antibiotics.[26] In some cases the MIC was equivalent to the MBC, indicating a bactericidal action of the extracts. Obtained results are in agreement with what was reported in literature concerning the biological activities of polyphenols. Indeed, there are several data in literature demonstrating the antibacterial, antifungal and antioxidant activity of these compounds.[27,28] Plant extracts with antimicrobial activities are generally active on multi-drug resistant human pathogens.[29] Ignat *et al* reviewed antioxidant and

antibacterial activities of some natural polyphenols.[30] Leaf extracts from *Passiflora nitida* show antioxidant, anti-inflammatory, and hypoglycemic effects.[31] Sasikala *et al* evaluated antioxidant potential of different parts of wild edible plant *Passiflora foetida*.[32] Kannat *et al* also confirmed antioxidant potential of mint (*Men-thaspicata*) in radiation-processed lamb meat.[33] Rao *et al* evaluated anti-oxidant activities and total phenolic content of *Chromolaena odorata*.[34] Nitiema *et al* analyzed i*n vitro* antimicrobial activity of some phenolic compounds (Coumarin and Quercetin) against gastroenteritis bacterial strains.[35]

4. CONCLUSION

In vitro culture of leaf expalnts from *B. retusa* with varied concentrations of phytohormones and culture media was carried for the induction and formation of calli. Production of calli and anthocyanin extraction from cell suspension culture was attempted by standardization of various parameters for enhancing anthocyanin synthesis. Subsequently, anthocyanin was purified, characterized and fractionated by HPLC-MS. Second part of the study justifies the uses of this plant to treat several infectious diseases in folk medicine. Investigations may continue in order to know the limit of toxicity of this plant and its *in vivo* potencies. The studies can lead to formulate new active molecules or lead drug to enhance the strength of traditional medicines (ETM) which are cheaper and disposable for population in developing countries.

5. REFERENCES

1. Daglia M. Polyphenols as antimicrobial agents. Curr Opin Biotechnol 2012; 23: 174–181.
2. Aron PM, Kennedy JA. Flavan-3-ols: Nature, occurrence and biological activity. Mol Nutr Food Res 2008; 52: 79-104.
3. Iwu MM. Handbook of Africa Medicinal Plants. Conservation International New York:1993.
4. Amanlou M, Ataie S, Farsam H. Xanthohumol from Humulus lupulus cultivated in Iran. Journal of Medicinal and Aromatic Plant Science 2005;27:469 - 475.
5. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 1962; 15:473-97.
6. Pulido R, Bravo L,Sauro-Calixto F. Antioxidant activity of dietarypolyphenols as determined by a modified ferric reducing/antioxidant power assay. J. Agric. Food Chem 2000;48: 3396–3402.
7. Blois MS. Antioxidants determination by the use of a stable free radical. Nature1958; 26:1199–1200.
8. Re R, Pellegrini N, Proteggente A, et al. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radic. Biol. Med. 2000; 26:1231–1237.
9. Bauer AW, Kirby WMM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardised single disc method. Am. J. Clin. Pathol, 1966; 45: 493-496.
10. Zongo C, Akomo EFO, Savadogo A, Obame LC, Koudou J , Traore AS. *In vitro* antibacterial properties of total alkaloids extract from *Mitragyna inermis* (Willd.) O. Kuntze, a West African traditional medicinal plant. Asian J. Plant Sci 2009; 8: 172-177.
11. McFarland J. The nephelometer: An instrument for estimating the number of bacteria in suspension used for calculating the opsonic index and for vaccines. J. Am. Med. Assoc 1907; 49: 1176-1178.
12. Sittiwet C, Puangpronpitag D. Anti-bacterial activity of *Cryptolepis buchanani* aqueous extract. Int. J. Biol. Chem 2009; 3: 90-94.
13. NCCLS. Reference Method for Broth Diluition Antifungical Susceptibility Testing of Yeasts: Approved Standard. 3rd Edn., National Committee for Clinical Laboratory Standards, Wayne, PA:2008.
14. Akomo EFO, Zongo C, Karou SD, Obame LC, Savadogo A, Atteke C , Traore AS. *In vitro* antiplasmodial and antibacterial activities of *Canthium multiflorum* schum and thonn (Rubiacea) extracts. Pak. J. Biol. Sci 2009; 12: 919-923.
15. Singh M, Singh N, Khare PB , Rawat AKS. Antimicrobial activity of some important *Adiantum* species used traditionally in indigenous systems of medicine. J. Ethnopharmacol 2008; 115: 327-329.
16. Borges A, Ferreira C, Saavedra MJ, Simões M. Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria. Micro Drug Resis 2013; 19(4): 256–265.
17. Simões M, Pereira MO, Vieira MJ. Validation of respirometry as a short-term method to assess the efficacy of biocides. Biofouling 2005; 21(1): 9–17.
18. Borges A, Simoes LC, Saavedra MJ, Simoes M. The action of selected isothiocyanates on bacterial biofilm prevention and control. Int Biodet Biodeg 2014; 86:25–33.
19. Mathur S, Shekhawat GS. Establishment and characterization of *Stevia rebaudiana* (Bertoni) cell suspension culture: an in vitro approach for production of stevioside. Acta Physiol Plant 2013; 35:931–939.
20. Saravanan S, Parimelazhagan T. *In vitro* antioxidant, antimicrobial and anti-diabetic properties of polyphenols of *Passiflora ligularis* Juss. fruit pulp. Food Science and Human Wellness 2014; 3: 56–64
21. Motamedi H, Darabpour E, Gholipour M , Seyyed Nejad SM. Antibacterial effect of ethanolic and methanolic extracts *of Plantago ovata* and *Oliveria decumbens* endemic in Iran against some pathogenic bacteria. Int. J. Pharmacol. 2010; 6: 117-122.
22. Hassawi D, Kharma A. Antimicrobial activity of some medicinal plants against *Candida albicans.* J. Biological Sci. 2006; 6: 109-114.

23. Kitzberger CSG, Smania A, Pedrosa RC, Ferreira SRS. Antioxidant and antimicrobial activities of shiitake *(Lentinula edoles)* extracts obtained by organic solvents and supercritical fluids. J Food Eng 2006; 80: 631-638.
24. Masoodi MH, Ahmed B, Zargar IM, Khan SA, Khan S , Singh P. Antibacterial activity of whole plant extract of *Marrubium vulgare.* Afr. J. Biotechnol 2008; 7: 86-87.
25. Khanahmadi M, Rezazadeh SH, Taran M. *In vitro* antimicrobial and antioxidant properties of *Smyrnium cordifolium* Boiss. (Umbelliferae) extract. Asian J. Plant Sci 2010; 9: 99-103.
26. Clements JM, Coignard F, Johnson I, Chandler S, Palan S et al. Antibacterial activities and characterization of novel inhibitors of LpxC. Antimicrob. Agents Chemother 2002; 46: 1793-1799.
27. Mohsen S M, Ammar A S M. Total phenolic contents and antioxidant activity of corn tassel extracts. Food Chem 2009; 112: 595–598.
28. Jimoh FO, Adedapo AA, Aliero AA, Afolayan AJ. Polyphenolic contents and biological activities of *Rumex ecklonianus.* Pharm. Biol 2008; 46: 333-340.
29. Oskay M, Oskay D, Kalyoncu F. Activity of some plant extracts against multi-drug resistant human pathogens. Iran. J. Pharm. Res 2009; 8: 293-300.
30. Ignat I, Radu DG, Andreea IV, Pag, Popa V I. Antioxidant and antibacterial activities of some natural polyphenols, Cellulose Chem. Technol. 2013; 47: 387-399.
31. Montefusco-Pereira CV, de Carvalho MJ, de Araújo Boleti AP. Antioxidant, anti-inflammatory, and hypoglycemic effects of the leaf extract from *Passiflora nitida* Kunth, Appl. Biochem. Biotechnol. 2013; 170:1367–1378.
32. Sasikala V, Saravana S, Parimelazhagan T. Evaluation of antioxidant potential of different parts of wild edible plant *Passiflora foetida* L. J. Appl.Pharm. Sci. 2011; 1: 89–96.
33. Kannat SR, Chander R, Sharma A. Antioxidant potential of mint (*Men-thaspicata* L.) in radiation-processed lamb meat, Food Chem. 2007; 600: 451–458.
34. Rao KS, Chaudhury PK, Pradhan A. Evaluation of anti-oxidant activities and total phenolic content of *Chromolaena odorata*. Food. Chem. Toxicol. 2010; 48:729–732.
35. Nitiema LW, Savadogo A, Simpore J, Dianou D, Traore AS. *In vitro* antimicrobial activity of some phenolic compounds (Coumarin and Quercetin) against gastroenteritis bacterial strains Int J Micro Res 2012; 3 (3): 183-187.

Conflict of Interest: None

Source of Funding: Kerala State Council for Science, Technology and Environment (KSCSTE), Govt. of Kerala