Pharmacogn J. 2018; 10(3):559-566.
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
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Original Article

Purified Anthocyanin from *in vitro* Culture of *Bridelia retusa* (L.) Spreng. capable of Inhibiting the Growth of Human Oral Squamous Cell Carcinoma Cells

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ABSTRACT

The present study aims *in vitro* cell suspension culture of *Bridelia retusa,* isolation of anthocyanin, purification, fractionation and its anti-metastatic potential against oral squamous carcinoma cells. Experimental results reveal that 2, 4-D either alone or in combination with kinetin supplemented in MS medium showed significant initiation of callus from leaf explants than stem. Growth hormones, pH, light, and carbon source influence anthocyanin synthesis. Maximum callus induction was noticed with 2.5 mg/L N6-benzyladenine (BA) + 2 mg/L 2, 4-dichlorophenoxyacetic acid (2, 4-D) (98.9%). Fresh and dry weight of the calli were i.e., 1.9 ± 0.04 and 0.45 ± 0. 03 g respectively. Optimal response was seen with light on MS medium contain 4% glucose + 2.5 mg/L BA and 2 mg/L 2, 4-D at pH 3.5 yielded 2.8 mg /g of anthocyanins. Suspension culture medium fortified with 2, 4-D (2.5 mg/L) + BA (2 mg/L) at pH 5.0 induced anthocyanin production at pH 4.4 – 4.6. HCl-ethanol extraction for 90 min yielded the maximum anthocyanin content. Fractionation of anthocyanin using HPLC coupled with mass spectrometry revealed 07 fractions such as acylated cyanidins, two peonidins, cyanidin 3-p-coumaroyl and feruloyl diglucoside-5-glucosides. In the search of novel therapeutic drugs against cancer, cytotoxicity effect of *B.retusa* anthocyanin extracts on human oral squamous cell carcinoma (SCC4, SCC9 and SCC25) cells using cell adhesion and cell viability assay was carried. The morphological alterations in SCCs cells after treatment with *B.retusa* anthocyanin includes nuclear condensation, fragmentation and apoptotic cells as revealed by Hoechst stain. Flow cytometry showed arresting of SCC25 cells mostly in the G0/G1 and S-G2/M stages with a concomitant up regulation of sub-G1 fraction, indicating cell death by apoptosis. Apoptosis was further substantiated by the activation of caspase-3 expression in the SCC25 cells treated with *B.retusa* anthocyanin. Thus, it is possible to suggest that *B.retusa* anthocyanin cause apoptosis of SCCs and warrant further investigation using animal models.

Key words: *Bridelia retusa*, Anthocyanin, *in vitro* culture, Cell suspension, Purification, apoptosis, Anti-metastatic potential.

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History
- Submission Date: 30-12-2017;
- Review completed: 14-02-2018;
- Accepted Date: 20-02-2018

DOI : 10.5530/pj.2018.3.91

Article Available online
http://www.phcogj.com/v10/i3

INTRODUCTION

Herbal products contain a wide variety of phytochemical with potent biological features, including anti-metastatic activity. Screening of active molecules from the medicinal plants and their mode of action fascinate researchers in pharmaceutical field. Natural products as chemo-preventive agents may serve as alternative and safe for cancer treatments when compared to synthetic anti-cancer therapies, which offer modest benefits with a wide variety of side effects. 80% of the populations living in the developing countries rely mostly on traditional herbal medicine for their primary health needs according to the survey of WHO.[1] Phytonutrients have various health benefits.[2] Carcinogenesis was a multistage (initiation, promotion and progression) process that encompasses multiple genetic and epigenetic events.[3,4] Reactive oxygen species (ROS) were involved in a variety of pathophysiological conditions including mutagenesis and carcinogenesis. Free radicals play significant role in tumor promotion by direct chemical reaction or alteration of cellular metabolic processes, and their scavengers (SOD, CAT, etc) represent inhibitors at different stages of carcinogenesis. The continuing severity and magnitude of the cancer problems make it imperative to develop chemopreventive strategies utilizing natural antioxidants to block the initiation, or arrest, or reverse the progression of pre-malignant cells. Antioxidants protect the cells against the toxicity of ROS by preventing their formation and neutralization of oxygen-free radicals.[5] Environmental factors of either biologic or chemical origin, may act as initiators or promoters, of carcinogenesis. Oral squamous cell carcinoma (OSCC) was the most common of the head and neck cancers with a poor prognosis. This may be further explained by the frequent lymph node metastasis and local invasion. Tumor metastasis and invasion involve degradation of the subepithelial basement

Cite this article: Madanakumar AJ, Lawarence B, Manoj GS and Kumaraswamy M. Purified Anthocyanin from in vitro Culture of *Bridelia retusa* (L.) Spreng. Capable of Inhibiting the Growth of Human Oral Squamous Cell Carcinoma Cells. Pharmacog J. 2018;10(3):559-66.

membrane and extracellular matrix (ECM), mediated by multiple proteolytic enzymes, and angiogenesis. The genus *Bridelia* Willd. includes approximately 60–70 species distributed, from Africa to Asia. Stem bark and roots were used by natives to treat rheumatism and as astringent agents.[2] The Kani of the Kouthalai region in Southern India use a paste prepared from leaves along with the leaves of *Curculigo orchioides* and the oils of castor, coconut and gingelly applied externally to cure wounds. *Bridelia retusa* Spreng is a moderate sized tree or a shrub belonging to Euphorbiaceae found growing throughout India.[6] In Southeast Asia, this species is usually a part of the primary and secondary forest vegetation. Decoction of stem bark with country liquor was used against diarrhea, ear ache and pregnancy. Pounded bark was mixed with gum of *Steroulia urines* and the mixture was prescribed orally 2-3 days after menstruation for complete infertility. Extract from the stem bark has antiviral, anticancer and hypotensive properties. Paste of the stem bark was applied to wounds and bark juice taken internally in case of snake bite.[6] Despite the wide range of biological activities exhibited, the plant was not properly exploited for the development of novel phytopharmaceuticals, related with diseases. Thus, the objective of this study was to evaluate the anticancer activity of *Bridelia retusa* anthocyanin against human oral squamous cell carcinoma cells.

MATERIALS AND METHODS

In vitro callus culture

Fresh leaves and shoot tips of *Bridelia retusa* as explants were collected from the Ponmudi hills for *in vitro* culturing. Cultures were initiated on MS medium with 30 g/L sucrose + different growth hormones applied singly or in combinations like 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). 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 poured 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. Stock callus cultures were maintained under the same physical conditions described above with subculturing regularly 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 for a period of 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.

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 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.

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-3-3glucoside.

Anticancer analysis

Cell lines

Human oral SCCs cells (moderate differentiation as SCC4, poor differentiation as SCC9 and well differentiation as SCC25) were cultured in Dulbecco's Modified Eagle Medium (DMEM)/F12 medium supplemented with 0.4 µg/ml hydrocortisone. All culture cells were purchased from American Type Culture Collection and maintained in medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin at 37°C in 5% CO_2 humidified atmosphere. At various concentrations after the treatment, the cells were processed for the anti-metastatic analyses.

Measurement of cell adhesion

Cells (1.5×10^5 cells/well) were sub-cultured into 24-well plates and incubated. After 24 h of incubation, the medium was changed by adding medium containing 1% bovine serum albumin (BSA) and with or without serial concentrations of anthocyanin for 18 h. Attached cell number was estimated using a DNA carmine-based colorimetric method. Briefly, cells were fixed with 100% methanol, dried, and stained with alcoholic/HCl carmine. Colorant was extracted with 0.01 N NaOH, and absorbance was determined at 540 nm. The cell number was estimated using a titration curve of cell density, and results were given as a percentage of the cell number with respect to control cells. For the titration curve, cells were plated at densities ranging form 1×10^3 to 7×10^5 cells/well in 24-well plates using serial dilutions of concentrated cell suspensions. After adhesion, some wells of each density were harvested with trypsin and cells were counted in a hemacytometer; meanwhile, parallel cultures were fixed and stained as described above. A relationship between the cell number and resultant absorbance after the colorant extraction, for each cell density, was accomplished and cell-density titration-curve construction, which measured cell adhesion.

Determination of cell viability

Cells (1.5×10^4 cells/well) were seeded in each 100 µl of 96-well multidishes for at least 24 h and then treatment with serial concentrations of extracts for 18 h. After replacing new medium, cellular cytotoxicity

were determined by MTS [3-(4, 5-di-methyl-thiazol-2-yl)-5-(3-carboxy-methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay. The absorbance at 490 nm was measured by a multi-mode microplate reader. Values are expressed as the percentage of mean cell viability relative to the untreated cultures. The IC_{50} and IC_{80} values were calculated from the drug concentration that induced 50% and 80% of cell viability rate.

Assessment of cell morphological changes

Cells (1.5×10^5 cells/well) were plated in 24-well plates then treated with IC_{50} concentrations of anthocyanin for 18 h. After incubation, the medium was removed and cells were fixed in 4% paraformaldehyde and permeabilized in saponin (0.1% v/v in PBS-BSA). To assess specific apoptosis, Hoechst 33342 (1 µg/ml) was added to each well and further incubated at 37°C for 30 min in the dark. Living and apoptotic cells were visualized through blue filter of fluorescence inverted microscope (Nikon, TE2000-U, Japan) at 200× magnification.

Assessment of cell cycle distribution and apoptotic cells

Cells (1.5×10^5 cells/well) were seeded in 24-well plates and incubated with or without IC_{50} concentration of anthocyanin for 18 h. Cells were then fixed in 70% ethanol/PBS, pelleted and resuspended in buffer containing 200 µg/ml RNase A and 0.01 mg/ml propidium iodide (PI). The cells were incubated in the dark for 15 min at room temperature and then analyzed by FACScan flow cytometer. The cell distribution in each phase (sub-G1, G0/G1, S and G2/M phases) of the cell cycle was determined using Windows Multiple Document Interface software (WinMDI), including subG1-peak of apoptotic cells.

Determination of the activation of caspase-3 expression

Cells were treated with IC80 concentration of anthocyanin for 18 h and the expressions of caspase-3 were studied. Cells were stained with mouse anti-cleaved caspasae-3 monoclonal antibodies (1:300) in 1× PBS containing 0.5% BSA (PBS-BSA) and 0.1% sodium azide for 45 min at 4°C. Cells were then washed twice with cold PBS and incubated with FITC-conjugated anti-mouse IgG (1:500) at 4°C for 30 min. Cells were washed with cold PBS and fixed in 4% paraformaldehyde. The cell nuclei were stained with 0.1 µg/ml of Hoechst 33342 (Promega, USA) and inspected using a fluorescent microscope (Nikon, TE2000-U, Japan).

Quantitative analysis of the activation of caspase-3 expressions

Cells were treated with IC_{50} and IC_{80} concentration of anthocyanin for 18 h and the expressions of caspase-3 were studied. Cells were stained with mouse anti-cleaved caspasae-3 monoclonal antibodies (1:300) in 1 × PBS containing 0.5% BSA (PBS-BSA) and 0.1% sodium azide for 45 min at 4°C. Cells were then washed twice with cold PBS and incubated with FITC-conjugated anti-mouse IgG (1:500) at 4°C for 30 min. Cells were washed with cold PBS and fixed in 4% paraformaldehyde. The cell nuclei were stained with 0.1 µg/ml of Hoechst 33342. For % of fluorescent staining analysis, caspase-3 expressions (Ex, 495 nm; Em, 525 nm) and the cell nuclei (Ex, 346 nm; Em, 460 nm) were measured from three independent experiments by Multi-Detection Microplate Reader (SynergyTM2, BioTek Instruments, Inc., USA) and calculated using Gene5TM software.

DNA fragmentation analysis

The SCC25 cells (3×10^5cells/dish) were plated in 6-cm dish and then subjected to the treatment of 0, 50, 100, 200 and 300 µM concentrations of anthocyanin for 48 h. After the treatment, the cells were washed with ice-cold PBS and resuspended in lysis buffer (25 mM Tris-HCl, pH 7.4, 5 mM EDTA and 0.6% SDS) with 1.0 mg/mL RNase A for 20 min at 50°C. Then proteinase K was added and the cells were incubated overnight. Separation of DNA was done using 2% agarose gel and detected under UV light after staining with ethidium bromide.

Statistical analysis

To evaluate the statistical significance of the difference of all the values, statistical analysis was performed on the means of the triplicates of at least three independent experiments using a two-tailed Student's t-test. *P* values less than 0.05 was considered significant for all tests.

RESULTS AND DISCUSSION

Callus induction of *B.retusa* was seen 6th week after inoculation. The calli formed from different combinations of hormones were generally creamy. The % of callus formed was 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% (Figure 1). Meanwhile, 50, 45.4, 42% induction were noticed with different concentrations of BA+ 2,4-D 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 increased profusely (Figure 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. The optimal conditions required for maximum callus induction were pH 4.2, light intensity 80µmol$m^{-2}s^{-1}$, 30^{0}C temperature, 4.1 glucose, 60mM nitrogen and 1:4 NH_4:NO_3 ratio.

Anthocyanin formation was initiated in the leaf calli from 25 to 30 days on MS medium with 2, 4-D. Higher concentrations of 2, 4-D (> 2.5 mg/g) reduced anthocyanin synthesis. Mean while, 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 fortified with 2 mg/L 2,4-D + 2.5 mg/L BA yielded 1.7±0.02 g of callus from the leaf explant (60 days) and also with the highest amount of anthocyanin content (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 (Figure 3). The 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 from 15 to 20 days of incubation i.e., the end of exponential growth phase. This may be due to the fact that the medium get saturated with toxic metabolites secreted by the cells. The cell viability was 80 % on 22nd day of culture (Figure 3) and then marginally declined.

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

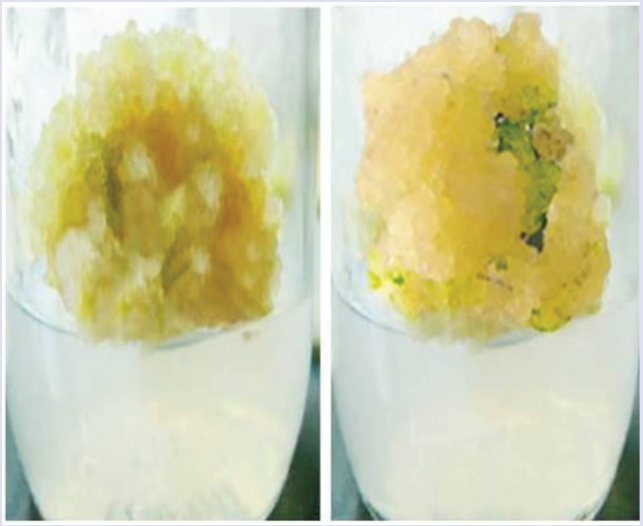

Figure 1: Calli from leaf explants from *B. retusa* using half strength MS medium.

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

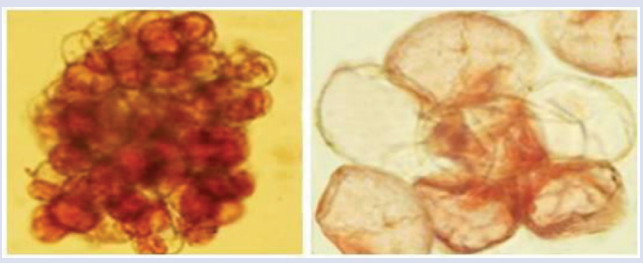

Figure 3: Viable cells from cell suspension culture of *B. retusa*

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) (Figure 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 28.8 and 26 mg/g respectively (Figure 5).

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 (Figure 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 and 287), pelargonidin-3-glucoside (433 and 271), peonidin-3-glucoside (463 and 301) and malonyl derivatives or ethylmalonyl derivatives (acylated forms) includes cyanidin- 3-(6-malonylglucoside) (353 and 287), pelargonidin-3-(6-malonylglucoside) (519 and 271), peonidin-3-(6-malonylglucoside) (549 and 301), and cyanidin-3-(6-ethylmalonylglucoside) (563 and 287) respectively.

Effect of anthocyanin on cell adhesion

To investigate the influence of anthocyanin on cell density, SCC4, SCC9 and SCC25 cells were treated with fixed amount of anthocyanin extracts (0-200 μM) for 18 h and then the cell adhesion was analyzed. Carmine, a natural stain was widely used for chromosome staining in cytological studies, and chromosome-specific stain method was highly accurate in a broad spectrum of cell types and cell densities. As shown in Table 1, the addition of different concentrations (0–200 μM) of anthocyanin to cancer cells for 18 h inhibited cell attachment from the culture dish surface. Although the inhibition of three oral squamous cell lines generally increases with concentration, the increase is not a linear function of concentration. *Bridelia* anthocyanin extract showed potent inhibition of cell adhesion. These experimental results indicate that *Bridelia* anthocyanin may influence cell connection on collagen fibers, thus probably raising cell cytotoxicity. To determine further the effects of anthocyanin on cell viability, an enzymatic tetrazolium combined with high-sensitivity test, like MTS assay, is required.

Table 1: Inhibition of cell adhesion by anthocyanin extracts of SCCs cells. Percentage of viable in SCC4, SCC9 and SCC25 cells treated with serial concentrations (0-200 g/ml) of extracts for 18 h as determined by cell adhesion assay. Data are means ± S.D.

Concentrations (μM)	SCC 4	SCC 9	SCC 25
0	84 ± 4.6	87 ± 6.3	84±4.4
25	78 ±2.7	75 ±5.1	71 ±5.9
50	65 ±3.8	61 ±3.87	57±2.7
75	57 ±7.1	53 ±2.99	46±3.6
100	50 ±3.7	47±5	40 ± 2.9
200	47 ±1.8	41 ±1.6	35 ± 6.8

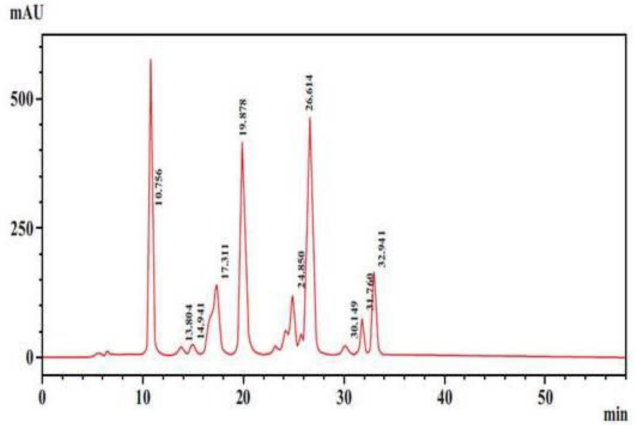

Figure 4: Chromatogram of HPLC at 520 mm of anthocyamins extracted by 0.01% HCl-acidified water of *B. retusa*

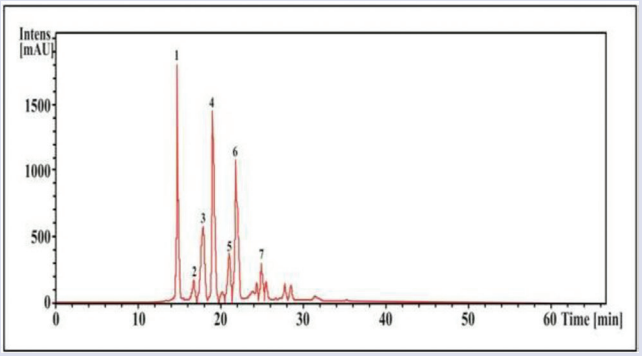

Figure 5: Chromatogram of HPLC of cyamidin-3-glucoside and [M]+ and fragment [M+H]+ extracted from *B. retusa*.

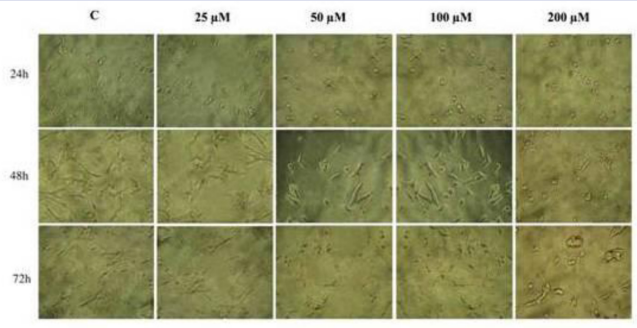

Figure 6: Morphological changed among SCC 25 cell lines density of 8×10^3 per well against different concentrations (25, 50, 100 and 200 µM) of anthocyamins and duration (24, 48, 72 h).

Effect of anthocyanin on cells growth

In order to examine cytotoxic effects of anthocyanin, the MTS assay were carried out using various doses of anthocyanin on SCC4, SCC9 and SCC25 cells. As shown in Table 2 and Figure 6, the anthocyanin

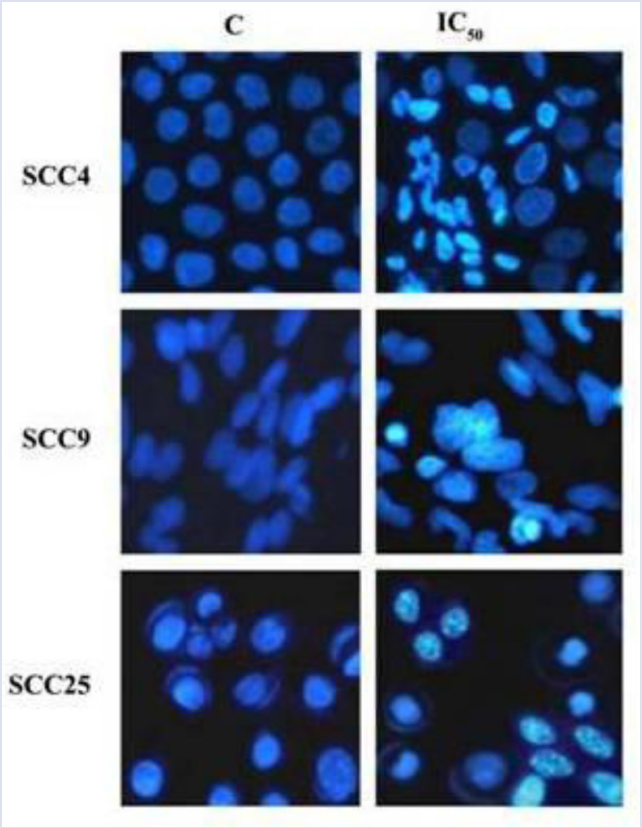

Figure 7: Morphological changes in SCCs cells after treatment with anthocyamin extract. The SCC 25 cells were seeded in 24-well plates and then treated with or without the extracts (IC_{50}) for 18 h. The cells were then fixed in 4% formaldehyde and DNA stained with Hoechst. The nuclei of the cells were visualized using an inverted fluorescent microscope (200x) and photographed.

Table 2: Cell viability by anthocyanin extracts against SCCs cells. Data are Mean ± S.D.

IC (µM)	IC_{50}	IC_{80}
SCC	4 52.7 ± 2.5	139.9 ± 1.3
SCC	9 49 ±5.8	128 ±9.8
SCC	25 37.8 ±0.98	118 ±8.7

concentrations that caused 50% cell growth inhibition (IC_{50}) were approximately 52.7 for SCC4, 49 for SCC9, and 37.8 µM for SCC25 cells. The inhibitory 80% concentration (IC_{80}) of the *Bridelia* anthocyanin was 139.9 for SCC4, 128 for SCC9, and 118 µM for SCC25. Concentration based morphological variations were also noticed in the SCC 25 cell lines i.e., morphometric changes in the cells as compared to control (Figure 6).

Effect of anthocyanin on cell morphological changes

Further, viability decrease due to a specific death type, cellular shape and nuclear morphology of exposed and anthocyanin treated SCC4, SCC9 and SCC25 cells were analyzed. As shown in Figure 7, the carcinoma cells treated with anthocyanin (IC_{50}) presented morphological characteristics of apoptosis, including chromatin condensation and nuclear fragmentation, were noticed under fluorescence inverted microscope. The

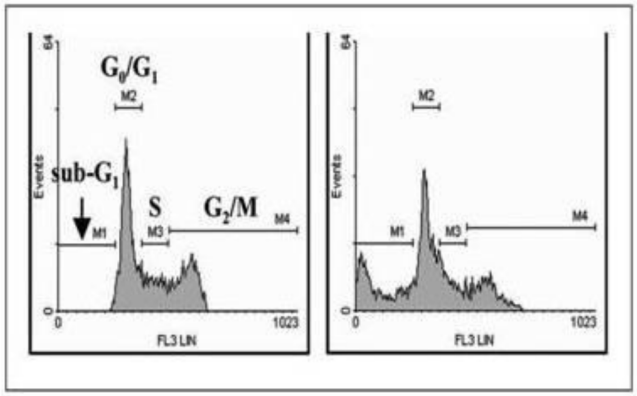

Figure 8A and B: Effects of anthocyamin extract on the cell cycles of SCC25 cells. Flow cytometric analysis of the cell cycle of SCC25 cells after three extracts (IC_{50}) treatment for 18 h. (B) The percentage of cell population in the cell cycle of SCC25 cells after three extracts (IC_{50}) treatments of 18 h. The cell populations were determined by WmMDI software. Data are means ± SD from three independent experiments.

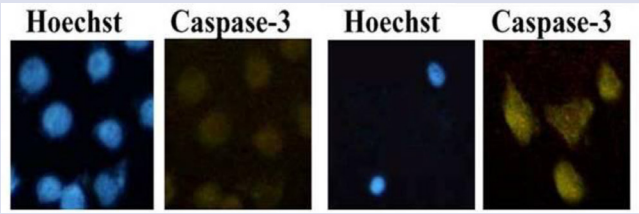

Figure 9: Expression of caspase-3 in SCC25 cells after treatment with anthocyamin extract. (A) An IC_{80} concentration of extracts was added to SCC25 cells and the expression of caspase-3 were determined by immunofluorescence

experimental results substantiate that *Bridelia* anthocyanin may cause apoptosis of human SCCs cells at varied levels.

Effect of anthocyanin on cell cycle distribution and apoptosis assay

For further analysis only SCC25 carcinoma cell lines were used. To evaluate anthocyanin mediated SCC25 cell apoptosis, the cell cycle distribution and specific DNA content in the sub-G1 peak were investigated using flow cytometry. Treatment of SCC25 cells with IC_{50} concentration of the anthocyanin revealed the characteristic of apoptotic events such as the cleavage of nuclear DNA into multiple fragments which in turn leads to increase in the sub-G1 phase (Figure 8 A). Incubation with anthocyanin showed the typical DNA pattern that reflected in the percentage of G0/G1 and S-G2/M phases of the cell cycle together with a marked apoptotic sub-G1 phase in SCC25 cells as showed in the Figure 8B.

Effect of anthocyanin on the expression of caspase-3

To analyze the involvement of caspase-3 activities in SCC25 cells apoptosis induced by anthocyanin, immunofluorescence analysis was carried. Caspase-3 up-regulation upon exposure to IC_{80} concentration of anthocyanin in SCC25 was showed in Figure 9A. Remarkable decrease in the % of DNA content in SCC25 cells with IC_{50} anthocyanin concentration establishes the cell cytotoxicity. The % of caspase-3 expression level in anthocyanin (IC_{50} and IC_{80}) treated SCC25 cells were approximately between 1.5 - 3 folds compared with control cells i.e., 89.6 (control), 134(IC_{50}), 267.5%((IC_{80}). Hence, the overall results suggest the over expression of caspase-3 by the anthocyanin may partially account for the cell apoptosis in SCC25 cells.

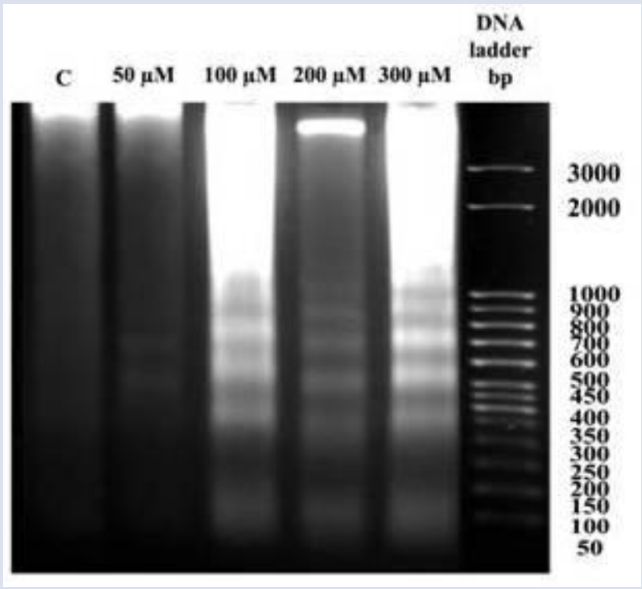

Figure 10: Analysis of DNA fragmentation induced by amthocyamins in SCC25 cell lines. Treated cells were analyzed for DNA fragmentation by 1.5% agarose gel electrophoresis and ethidium bromide staining.

Effect of flavonoid on DNA fragmentation

Flavonoid treated SCC 25 cells showed morphological changes of apoptosis and also DNA fragmentation i.e., the formation of DNA ladder. DNA ladder appeared to be more conspicuous with the increasing concentration of flavonoids as compared to control group (Figure 10). However, 50, 100, 200 and 300 µM doses of anthocyanin extract after 48 h exposure led to a considerable remarkable in DNA fragmentation (Figure 10). The DNA fragmentation is a symbol of apoptotic events, further confirming that the anthocyanin triggered cell death through apoptosis.

Anthocyanin is a major pigment belongs to the group of polyphenols. They have shown potential as drugs for the treatment of diverse human diseases. Currently, attention has been focused on the antimetastatic potentials of plant products and their derivatives. Most of the herbals are deposits of anthocyanin, in which most common were malvidin and cynidins. In addition, polyphenols, have fascinated because of their novel structural features and significant antileukemic and antiviral potentialities. Additionally, the *Bridelia* species were rich in phytochemicals and were used by the tribals for curing any ailments.

The flavanoids have been suggested to display anti-metaststic potentials in many types of human tumors. Newly isolated phenols showed inducing of Fas clustering, enhancing of endoplasmic reticulum stress and promising anti-cancer activity and apoptosis in human hormone-resistant prostate cancers and human hepatocellular carcinomas. Reports have shown that crude phenolic extract of herbals showed cytotoxicity against MCF-7, MDA-MB-231 breast cancer cell lines and liver cell lines (HepaG2/DMEM-12). However, no report of cytotoxicity has been performed on *Bridelia* species and also the mechanisms underlying their cancer efficiency of anthocyanins and their derivatives are poorly documented. The present study shows the mode of action of *Bridelia* anthocyanin in the human oral SCCs cell lines. Cell density and cell proliferation analysis were usually carried during *in vitro* studies regarding the pharmacological effects of specific compounds. In this study, cell adhesion and MTT assay revealed that *Bridelia* anthocyanin inhibited SCC4, SCC9 and SCC25 cell lines growth in a dose dependent manner. Results revealed *Bridelia* anthocyanin was most potent inhibitor of SCC25 cells adhesion and exhibited the activity in inhibiting cell growth than other cell lines. This study was a preliminary attempt for cytotoxic activity of soft antho-

cyanin and a few correlated researches could be noticed. Although synthetic compounds like 5-fluorouracil, 5% imiquimod cream, and 3% diclofenac gel were available as agents for topical skin cancer therapies. However, reports have shown that application of topical 5-fluorouracil cream was inevitably accompanied by inflammation, pain, pruritus, irritation, erythema, erosion, and scar, in diseased skin and also peripheral normal skin. Similarly, 5-fluorouracil and imiquimod compounds incubated with cancer cell lines exhibited cytotoxicity after 72 h only. The IC_{50} value of 5-fluorouracil were approximately 44.5 µg/ml for SCC25 cells. So no significantly cytotoxic difference was seen between *Bridelia* anthocyanin and 5-fluorouracil and additionaly the interaction time was only 18 h. Cancer was featured by deregulated cell proliferation combined with suppressed apoptosis. Most chemotherapeutic drugs exert their cytotoxic action via inhibiting cancer cell growth and inducing apoptosis. Documented anticancer studies have revealed that crude herbal extracts exhibited potent cytotoxicity against many cancer cells and induced apoptotic DNA fragmentation and condensation of chromatin. Morphologic anomalies, nuclear chromatin condensation and apoptotic bodies formation noticed in the present study confirms apoptosis induced by *Bridelia* anthocyanin against SCCs cells at varied levels. Cells adapt different pathways for safeguarding of DNA from the toxicity that can result in genomic instability. Regulatory proteins that control normal passage of cells through the cell cycle play a crucial role of this response. During cancer, tumor cells loose such controls, which induce malignancy. Therefore, the possible approach for cancer chemotherapy is to regulate cell-cycle events. In this study, the cell cycle data indicates that *Bridelia* anthocyanin regulate cells in the G0/G1 and S-G2/M phases with a concomitant remarkable increase in the sub-G1 phase. Similarly, the caspases class can be subdivided based on their specificity of substrate, sequence homology and biochemical functions. Caspase-3 plays a checkpoint role in the terminal phase of apoptotic cycle. The present has revealed that *Bridelia* anthocyanin may induce caspase-3 activation, leading to apoptosis of SCC25 cells. This study was the first evidence that human oral SCCs cells were sensitive to *Bridelia* anthocyanin.

Studies showed that the crude combination of flavonols, proanthocyanidin oligomers, and triterpenoids present in *Vaccinium oxycoccos* fruit may produce synergistic health benefits. *In vitro* studies proved that fruit extract reduced tumor cell growth against breast, colon, prostate and lung. It has also been reported that quercetin was effective against growth of MCF-7 human breast adenocarcinoma, HT-29 human colon adenocarcinoma, and K562 human chronic myelogenous leukemia cell lines.[7,8]

Carica papaya was rich in lycopene potentially ideal against antitumor and free-radical scavenging activities. Rahmat *et al*.[9] proved that papaya juice display anticancer property against liver cancer cell line (HepG2).[9]

Polyphenols of *Prunus avium* and perilly alcohol were effective anticancer molecules.[10] Lee *et al*.[11] showed that the cherry blossom methanolic extract arrest the growth of human colon cancer cell line HT-29. In animal models perillyl alcohol has been proved to induce pancreatic, mammary and liver tumours regression and thereby chemopreventive agent for colon, skin, and lung cancer, as well as for neuroblastoma, prostate and colon cancer.[11]

In vitro and *in vivo* antitumor properties of bromelain from *Ananas comosus* have been proved as an effective anticancer agent for malignant peritoneal mesothelioma (MPM).The anti-cancer activity of bromelain was attributed mainly by their protease molecules and it has been reported that bromelain was shown to upregulate the expression of p53 as well as Bax, in mouse skin papillomas.[12,13]

The crude leaf hexane extract of *Psidium guajava* suppresses AKT/mTOR/S6K kinase signaling and thereby apoptosis through the down-regulation of proteins that mediate tumor cell survival, proliferation, metastasis, and angiogenesis in human prostate cancer cells. Lupeol, a triterpene, of guava has been proved as an anticancer molecule. It has also been demonstrated that acetone extracts of guava had cytotoxic effects on HT-29 cells. [14,15]

Malus domestica peels have been reported to inhibit the cell proliferation of HepG2 human liver cancer cells, MCF-7 human breast cancer, cells and Caco-2 colon cancer cells. The lead molecules in the peels were quercetin and quercetin- 3-O-beta-D-glucopyranoside. Many reports indicate that apples play an important role in the prevention and treatment of prostate cancer.[16]

Similarly consumption of grape could be associated with the reduced risk of cancers like breast cancer and colon cancer. The antitumor activity of grape antioxidants have been proved in *in vitro* and *in vivo* models. It has been reported that grape seed extract contain proanthocyanidins and was active against colorectal cancer, A549 and H1299 and oral squamous cell carcinoma.[17]

Lupeol, a triterpene reported from mango has shown to possess antitumor properties *in in vivo* and *in vitro* studies. The apoptogenic activity in mouse prostate by lupeol and mango pulp extract has been documented. Noratto *et al*. [18] correlated the anticancer potentialities of polyphenolics from mango varieties against Molt-4 leukemia, A-549 lung, MDA-MB-231 breast, LnCap prostate, and SW-480 colon cancer cells and non-cancer colon cell line like CCD- 18Co.

Black pepper and cardamom exert immunomodulatory and antitumor activities. Capsaicin enhances the antitumor effect against bladder cancer. The potent *in vitro* and *in vivo* antitumor properties of capsaicin revealed that capsaicin may be used for the treatment of human colon cancer.[19]

Oral administration of *Crocus sativus* carotenoid extract rich extract inhibits the growth of mouse tumors from (sarcoma-180), Ehrlich ascit-escarcinoma (EAC), Dalton's lymphoma ascites (DLA) and significantly increased the life spans of treated tumor-bearing mice.[20] Crocetin, the carotenoid constituent of saffron, has shown significant potential as an anti-tumor agent in animal models and cell line systems. Crocetin affects the growth of cancer cells via inhibiting nucleic acid, anti-oxidative mode, inducing apoptosis and hindering growth factor signaling pathways.[20]

Curcumin form *Curcuma longa* has anticarcinogenic activity against colon cancer, breast cancer and leukaemia. Curcumin has been reported to reduce cell proliferation and apoptosis in prostate tumours. Curcumin and genistein have been reported as the most potent inhibitors against the growth of human breast tumour cells. It has been reported that the anti-cancer property of curcumin was mediated in part by its anti-angiogenic activity.[21]

Sweet potato protein (SPP) exerts anti-proliferative and anti-metastatic effects on human colorectal cancer cell lines, both *in vitro* and *in vivo* conditions. Anticancer activity of sweet potato green extract in prostate cancer has also been proved. It has been noticed that purified Kunitz-type trypsin inhibitor (KTI) from sweet potato inhibited proliferation and induced apoptosis of promyelocytic leukemia cells. KTIs isolated from other sources, such as human urine and soybeans, have also been shown to exert antiproliferative, anti-invasion and anti-metastatic activities in a variety of malignant cells, animal models and cancer patients.[22]

CONCLUSION

Anthocyanin was extracted and purified from cell suspension culture of *B.retusa*. Fractionation of anthocyanin from *B.retusa* revealed 07 fractions comprising acylated cyanidins, two peonidins, cyanidin 3-p-coumaroyl and feruloyl diglucoside-5-glucosides. Further, the study demonstrates the cytotoxicity effect of anthocyanin extracts on human oral squamous cell carcinoma (SCC4, SCC9 and SCC25) cells using cell adhesion and cell viability assay. The mode of action may via regulating cell cycle and

inducing apoptosis via up regulation caspase3 in SCC25 cell lines. Future works are warranted to confirm the data using animal models and also the receptor involved in the caspase3 activation.

ACKNOWLEDGEMENT

This work carried out with the financial support of Kerala state Council for science, technology and Environment, Govt. of Kerala.

CONFLICT OF INTEREST

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

ABBREVIATIONS USED

2,4-D: 2,4-Dichlorophenoxyacetic acid; **BA:** Benzyl Amino Purine; **Kin:** Kinetin; **NAA:** Naphtalic Acetic Acid; **SCC**: squamous cell carcinoma; TFA: trifluoroacetic acid**; HPLC**: High-Performance Liquid Chromatography; **EAC**: Ehrlich ascitescarcinoma; **DLA**: Dalton's lymphoma ascites; **KTI**: Kunitz-type trypsin inhibitor.

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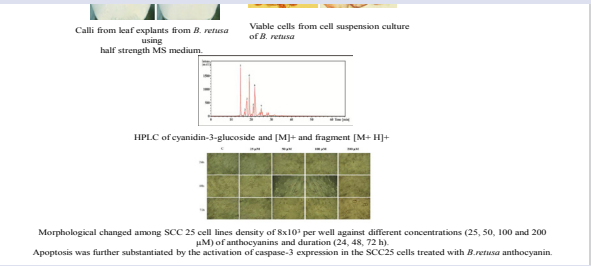
GRAPHICAL ABSTRACT



SUMMARY

- Anthocyanin from B.retusa. revealed 07 fractions The compounds identified were acylated cyanidins, two peonidins, cyanidin 3-p-coumaroyl and feruloyl diglucoside-5-glucosides. The cytotoxicity effect of anthocyanin extracts on human oral squamous cell carcinoma (SCC4, SCC9 and SCC25) cells was by regulating cell cycle and inducing apoptosis via up regulation caspase3 in SCC25 cell lines.

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Cite this article: Madanakumar AJ, Lawarence B, Manoj GS and Kumaraswamy M. Purified Anthocyanin from in vitro Culture of *Bridelia retusa* (L.) Spreng. Capable of Inhibiting the Growth of Human Oral Squamous Cell Carcinoma Cells. Pharmacog J. 2018;10(3):559-66.