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Anti-metastatic Potentiality of Purified Anthocyanin from *Osbeckia aspera* (L.) Blume. and *O. reticulata* Bedd. Against Selected Human Cancer Cell Lines

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ABSTRACT

To evaluate the anti-metastatic potentialities of purified anthocyanin from *Osbeckia aspera* (L.) Blume. and *O. reticulata* Bedd. against selected human cancer cell lines such as HT29 colon, MG63 bone and HeLa cervical by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, apoptosis and DNA fragmentation test. Anthocyanin was extracted from the *in vitro* callus culture of the *Osbeckia* species, purified using amberlite column chromatography and fractionated by LC-MS/MS. Anthocyanin producing callus cultures were trialed on MS medium fortified with various combinations of phytohormones and sucrose. Significant callus formation in *O. aspera* was initiated in cultures containing 0.5 mg/L of 2, 4-D and 0.5 mg/L BA, while that in *O. reticulata* was initiated with 1.2 mg/L BA and 1.4 mg/L NAA. The same hormonal combinations on sub-culturing turned white friable callus into red compact callus. Purified anthocyanins obtained from *O. aspera* and *O. reticulata* contained Malvidin-3 -diglucoside, delphinidin, cyanindin aglycone and Peonidin. *Osbeckia* species displayed differential responses against the HT29 colon, MG63 bone and HeLa cervical cancer cell lines in terms of IC_{50} values of toxicity. *O. aspera* was more effective against HeLa cervical cell lines (23.7µg/ml) followed by HT29 colon (64.7µg/ml) as compared to *O. reticulata*. Poor selectivity index was noticed with bone cancer cell lines. The results were substantiated by apoptotic analysis and DNA fragmentation results. The overall results suggest that the purified anthocyanin of *O. aspera* and *O. reticulata* was excellent as antimetastatic and warrant further studies to isolate novel compounds for chemotherapeutic use.

Keywords: *Osbeckia*, anthocyanin, cytotoxic activity, human cancer cell lines, apoptosis.

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INTRODUCTION

Currently, cancer is documented as one of the major causes of mortality worldwide. Despite modern

techniques and therapy, it affects lakhs of people around the world. A novel lead phytochemical to treat cancer is need of the hour and researchers are involved in designing a new drug is progressing. However, herbals were long been shown an excellent and reliable source of novel anticancer drugs. The mechanism of interaction between the secondary metabolites and cancer cell lines has been studied extensively. [1]

The use of crude extracts and purified lead molecule can be of immense importance in therapeutic treatments of many human disorders. [2] However, herbals in general, produce toxic molecules as a defense strategy against pathogenic infections, insects and herbivores. Thus, an assessment of their cytotoxic capability is necessary to ensure relatively safe use of the herbals.

Anthocyanins are the water-soluble flavonoid pigments yield brilliant colors in flowers and fruits. In plants, anthocyanins were widespread reported among 27 families and a multitude of species. It is reasonable to state that humans are well acclimatized to ingesting anthocyanins. Further, it may be considered that the present day diet with its increasing dependence on processed foods has become deficient in anthocyanins. The role of anthocyanins as food colorant is becoming increasingly important. They contribute the food in terms of aesthetic value and for quality judgment, but also they tend to yield nutraceutical effects.

Researchers demonstrated the ability of anthocyanins to induce phase II antioxidant and detoxifying enzymes in cultured cells. Cell viability was inhibited by these molecules by blocking the cancer cell lines at various stages of the cell cycle via regulator proteins such as p53, p21, p27, cyclin D1, cyclin A, etc. Interestingly, researchers have compared the viability effects of anthocyanins on normal cells vs. cancer cell lines and found that they selectively function as antiproliferative with marginal or no impact on the growth and development of normal cell lines. Similarly, anthocyanins induce marked apoptosis through intrinsic or extrinsic pathways. In the intrinsic pathway, they regulate an increase in mitochondrial membrane potential, cytochrome *c* release and modulation of caspase-dependent anti- and pro-apoptotic proteins. In the second pathway, they modulate the expression of FAS and FAS ligand in cancer cells resulting in apoptosis. [3] In this juncture, the present study was undertaken to isolate anthocyanin from *in vitro* callus culture of selected *Osbeckia* species, its purification, and fractionation and antiproliferative potentials against HT29 colon, MG63 bone and HeLa cervical cell lines.

MATERIALS AND METHODS

Plant material

Osbeckia sps. for the present study were obtained from various parts of Idukki district of Kerala such as Munnar hills, Wagamon and Peerumed. *O. aspera,* is a perennial shrub distributed along the tropics and sub tropics. The plant was propagated naturally by vegetative and seeds. Seed derived plantlets were found to be less frequent in the natural habitat and distributed as fragmented patches derived from root stocks. *O. reticulata*, is a small tree frequently seen in temperate high altitude habitats i.e., 3000 feet in Western Ghats. *O. aspera* was established in the green house, but *O. reticulata* failed to acclimatize in the greenhouse conditions and remained live up to six months. Identity of the plants were confirmed by referring floras and authenticated by herbaria of Jawaharlal Nehru Tropical Botanical Garden and Research Institute, Palode, Trivandrum.

In vitro culture

The collected plantlets were reared in green house of the college Botanical garden. Stem and leaf cuttings from healthy, disease free plants were used as explants. The explants were subjected to surface sterilisation using 10% teepol solution followed by rinsing in tap water for 60 min. Then, the stem cuttings were immersed in ethanol 70% (v/v) for 30 s followed by mercuric chloride or sodium hypochlorate. Ethanol treatment was found hazardous in the case of leaves. Finally, the explants were washed in autoclaved distilled water.

The sterilized nodal segments (2-3 cm) were used for multiple shoot induction whereas inter nodal fragments and leaf cuttings for direct organogenesis and callus induction. The explants were transferred to MS culture medium supplemented with various phytohormones, besides the control group (without growth regulators). During the entire process of *in vitro* culture, the plantlets were kept in 2.5 × 15 cm test tubes. The MS culture media was supplemented with vitamins, sucrose (30 g /L), and agar (7 g /L). The culture media pH was adjusted to 5.7 ± 0.1. Media were sterilized by autoclaving for 15 min at 120°C and 15 lbs of pressure. Clonal fidelity of the regenerated plants was ascertained by morphological and histological evaluation. All experiments were conducted in a completely randomized design.

Estimation of anthocyanin content

Protocol of Sutharut and Sudarat [4] was used for the estimation of anthocyanin content from the flowers. The absorbance was read at 510 and 700 nm against distilled water as blank.

Silica gel, Amberlite IRC 80, Amberlite IR 120, DOWEX 50WX8, Amberlite XAD4 and Amberlite XAD7 were trialed for the effective purification of anthocyanins. Aqueous acidified methanol and ethanol were used for the extraction of anthocyanins.

In vitro assay for cytotoxic activity

For testing, HT29 colon, MG63 bone and HeLa cervical cells were washed by phosphate buffer saline (PBS) and harvested by tripsinization and were plated in 96 well plates (one cell line/well) and incubated under 5% CO_2 and 95% air at 37°C for 24 h. The cells were treated with different concentrations of purified anthocyanins (6.25, 12.5, 25, 50, 100µg/ml). Dilution of stock solutions was made in culture medium yielding DMSO

(0.1% concentration). DMSO was used as negative control to check the cell viability. Control cells were incubated in culture medium only. All concentrations of anthocyanins were in 6 replicates with the same cell batch.

MTT assay

Growth of cancer cells ability to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-terazolium bromide (MTT) to a blue formazan product. [5] At the end of 72 h incubation, the medium in each well was replaced by MTT solution (20 cell/well, 5 mg/ml in phosphate-buffered saline). The plates were incubated for 4 h under 5% CO_2 and 95% air at 37°C. MTT reagent was removed and the formazan crystals produced by viable cells were dissolved in 100 DMSO and gently shaken. The absorbance was then determined by ELISA reader at 492 nm.

The percentage growth inhibition was calculated using following formula,

$$\% \text{ cell inhibition} = 100 - [(A_t - A_b)/ (A_c - A_b)] \times 100$$

Where, A_t = absorbance value of test compound, A_b = Absorbance value of blank and A_c = Absorbance value of control.

The effects of extracts were expressed by IC_{50} values (the drug concentration reducing the absorbance of treated cells by 50% with respect to untreated cells).

Apoptosis assay by acridine orange/propidium iodide staining

The morphology of cells was monitored during cell growth after treatment with the anthocyanin extract under an inverted microscope (Axiovert100, Zeitz, Germany). The cell morphology was also evaluated by adding 10 mlof acridine orange followed by 10 ml of propidium iodide staining before checking under the fluorescence microscope (BX51, Olympus). Pictures were taken at 400x magnification with excitation filter 480/30 nm, dichromatic mirror cut-on 505 nm LP and barrier filter 535/40 nm.

DNA ladder analysis

The anthocyanin treated cells of each concentration were pooled. The cells were plated and washed twice with cold PBS. Cell pallets were incubated in lysis buffer (1 ml) for 30 min at 60°C. The clear lysates were separated by centrifugation and re-incubated with RNase (3µl) for 30 min at 37°C. A mixture of solvents consisted of phenol, chloroform and isoamyl alcohol was added and vigorously vortex for a few seconds before centrifugation. This procedure was repeated twice. The layer of clear lysates was transferred into 100% ethanol (1 ml) and kept at 4°C. The mixture was re-centrifuged to discard the supernatant. The remaining pallet was washed with 70% ethanol and dried before dissolved in Tris-EDTA (TE) for DNA electrophoresis

Statistical analysis

Experimental results are expressed as mean ± SD. All measurements were replicated six times. The data were analyzed by an analysis of variance ($p < 0.05$). The IC_{50} values were calculated from linear regression analysis.

RESULTS AND DISCUSSION

In vitro culture and elucidation of anthocyanin

In vitro callus cultures were established for the efficient production of anthocyanin from the species *O. aspera* and *O. reticulata.* Tissue culture environments showed variations among the two species but callusing in both species was observed with auxin and cytokinin combinations. Combinations of BA along with NAA and IBA produced compact calli with morphogenesis, meanwhile combination of BA with 2, 4-D produced white friable callus. Combination of either of the cytokinins along with NAA or IBA and higher concentrations of sucrose on the medium (2 to 2.5 fold) induced synthesis of anthocyanin from the friable callus. However, darkening of the callus and subsequent death was noticed within 5-7 days after the anthocyanin production. The highest anthocyanin content in *O. aspera*, was obtained on MS medium containing 8% sucrose with 0.5 mg/L BA and 0.5mg/L 2, 4- D combination (*i.e*, 10.7 mg/g). Meanwhile, in *O. reticulata* the maximal anthocyanin content producing callus was obtained from half strength MS medium supplemented with 8% sucrose, 1.2 mg/L BA and 1.42 mg/L 2,4-D (13.3 mg/g). Anthocyanin content of the callus was found to be higher than the respective flowers of the species.

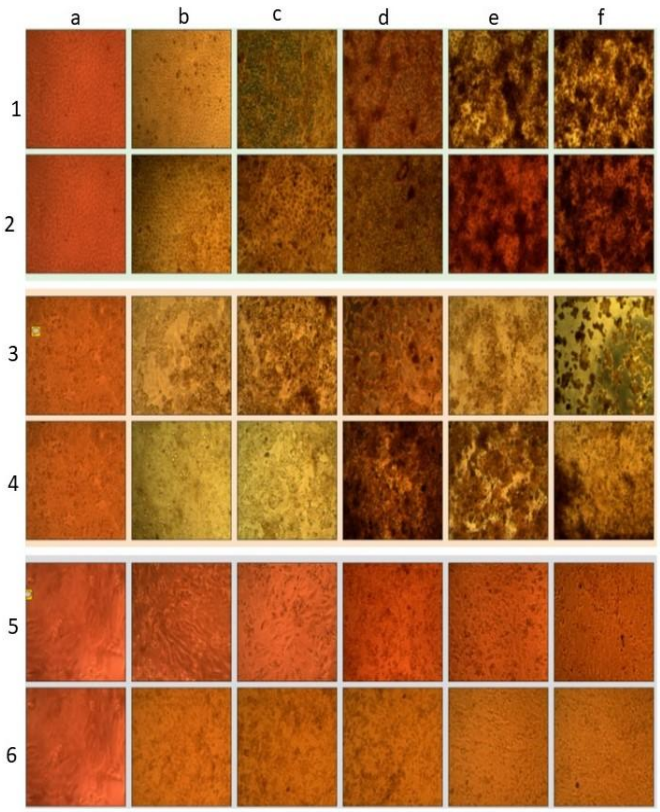

Fig. 1: *In vitro* cytotoxic effect of anthocyanin of *osbeckia aspera* and *osbeckia reticulata* by MTT assay. 1a-f HeLa cervical cell lines treated with *Osbeckia aspera* anthocyanin; 2 a-f HeLa cervical cell lines treated with *Osbeckia reticulata* anthocyanin; 3a-f HT 29 cell lines treated with *Osbeckia aspera* anthocyanin; 4 a-f HT 29 a cervical cell lines treated with *Osbeckia reticulata* anthocyanin. 5a-f MG 63 cell lines treated with *Osbeckia aspera* anthocyanin; 6 a-f MG 63 cell lines treated with *Osbeckia reticulata* anthocyanin. (Series a- 6.25µg/ml , b-12.5µg/ml , c-25µg/ml , d-50µg/ml , e-100µg/ml)

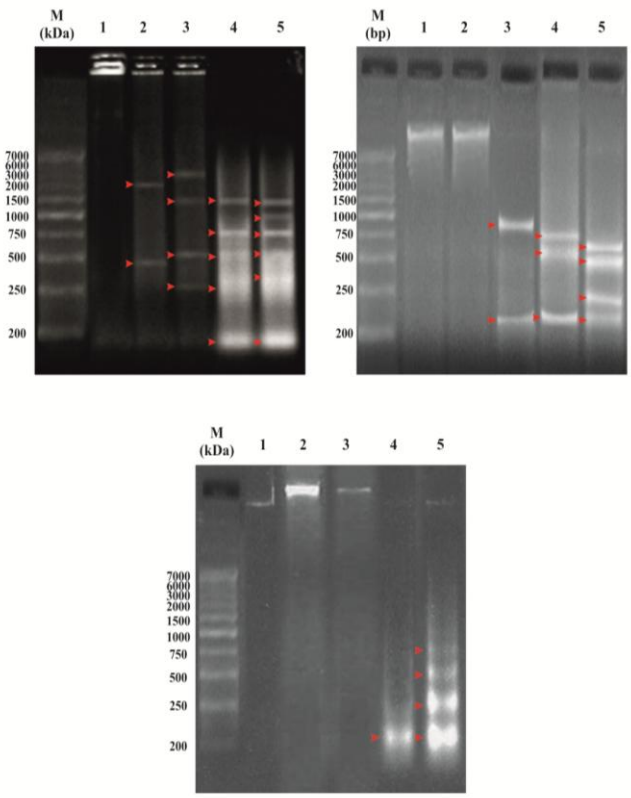

Fig. 2: Analysis of genomic DNA fragmentation in HeLa, HT29 and MG63 cells after treatment for 24 h with purified anthocyanin from *Osbeckia aspera* (L.) Blume. (12.5, 25, 50, 100µg/mL). DNA Fragmentation was assessed on agarose gel electrophoresis. M= DNA ladder used as marker; lane 1 = control; lane 2 = 12.5µg/mL; lane 3 = 25µg/mL; lane 4 = 50µg/mL, lane 5 = 100µg/mL.

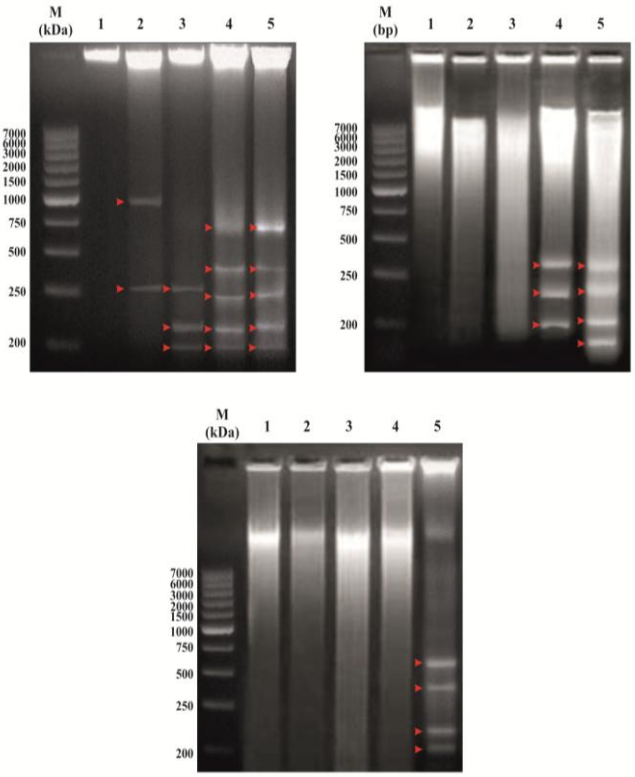

Fig. 3: Analysis of genomic DNA fragmentation in HeLa, HT29 and MG63 cells after treatment for 24 h with purified anthocyanin from *O. reticulata* Bedd. (12.5, 25, 50, 100µg/mL). DNA Fragmentation was assessed on agarose gel electrophoresis. M= DNA ladder used as marker; lane 1 = control; lane 2 = 12.5µg/mL; lane 3 = 25µg/mL; lane 4 = 50µg/mL, lane 5 = 100µg/mL.

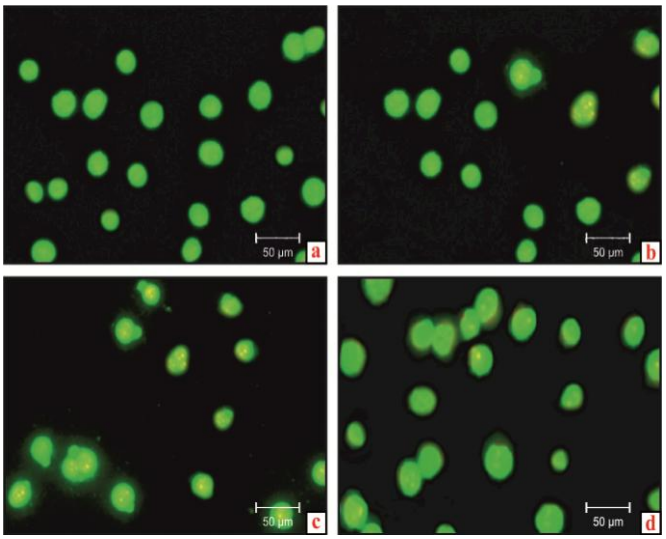

Fig. 4: Acridine orange/ propidium iodide, AO/PI-stained and tunnel assay of HeLa cancer cells treated with purified anthocyanin from *Osbeckia aspera* (L.) Blume. 48 h (a) Control; (b) 25µg/ml (c) 50µg/ml (d) 100µg/ml.

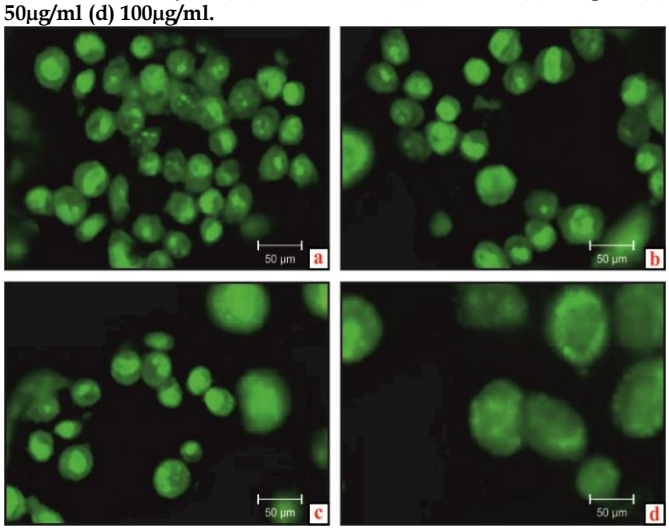

Fig. 5: Acridine orange/ propidium iodide, AO/PI-stained and tunnel assay of HT 29 cancer cells treated with purified anthocyanin from *Osbeckia aspera* (L.) Blume. 48 h (a) Control; (b) 25µg/ml (c) 50µg/ml (d) 100µg/ml.

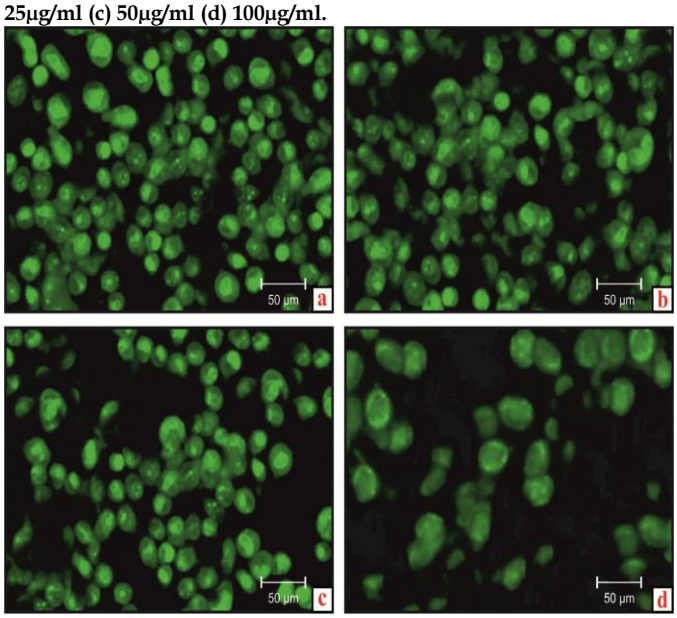

Fig. 6: Acridine orange/ propidium iodide, AO/PI-stained and tunnel assay of MG 63 cancer cells treated with purified anthocyanin from *Osbeckia aspera* (L.) Blume. 48 h (a) Control; (b) 25µg/ml (c) 50µg/ml (d) 100µg/ml.

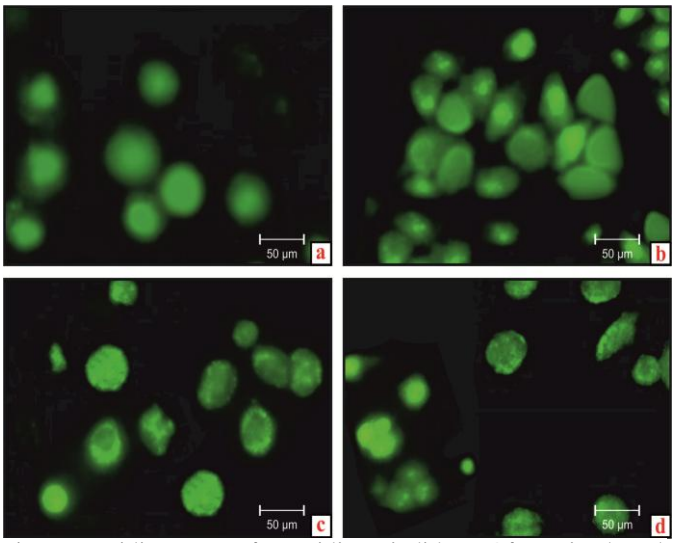

Fig. 7: Acridine orange/ propidium iodide, AO/PI-stained and tunnel assay of HeLa cancer cells treated with purified anthocyanin from *O. reticulata* Bedd. 48 h (a) Control; (b) 25µg/ml (c) 50µg/ml (d) 100µg/ml.

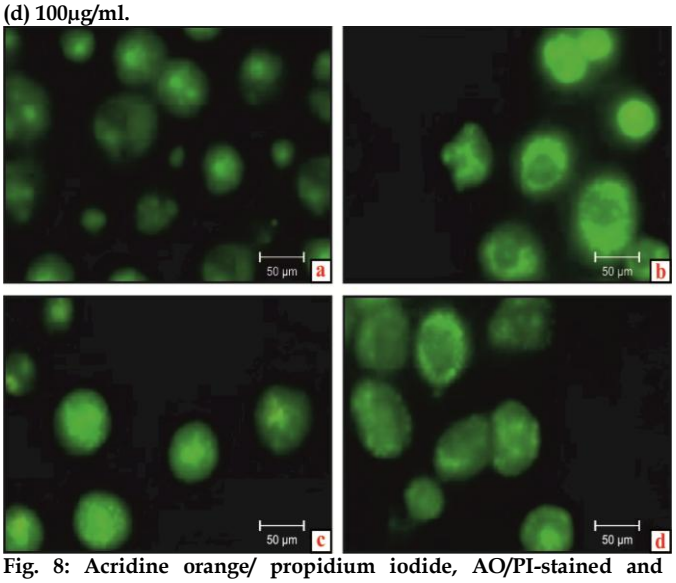

Fig. 8: Acridine orange/ propidium iodide, AO/PI-stained and tunnel assay of HG 29 cancer cells treated with purified anthocyanin from *O. reticulata* Bedd. 48 h (a) Control; (b) 25µg/ml (c) 50µg/ml (d) 100µg/ml.

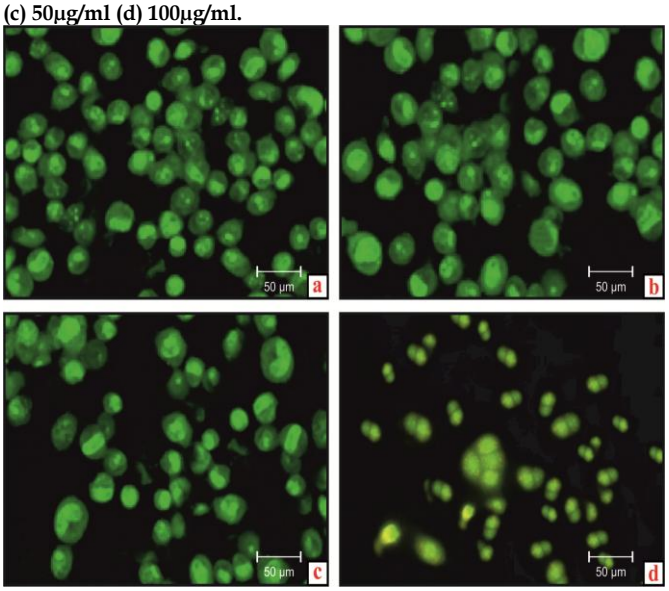

Fig. 9: Acridine orange/ propidium iodide, AO/PI-stained and tunnel assay of MG 63 cancer cells treated with purified anthocyanin from *O. reticulata* Bedd. 48 h (a) Control; (b) 25µg/ml (c) 50µg/ml (d) 100µg/ml.

Purification and fractionation using LC- MS/MS

Anthocyanin was extracted from the calli using a mixture of ethanol and water in the ratio 70:30 acidified with 1% HCl without any accelerator and energy sources. Initial purification was carried by solid-liquid separation method. Further, the anthocyanin was eluted and purified by column chromatography in silica gel followed by using amberlite column. Amberlite XAD7HP gel was unique in terms of capacity and desorption ratios compared to others. As free sugar may leads to degradation of anthocyanins, the level of free sugar concentration before and after purification were tested and it was found decreased significantly.

LC-MS/MS analysis of purified anthocyanin from *O. aspera* and *O. reticulata* contain significant levels of anthocyanins, such as malvidin-3 -diglucoside, peonidin, delphinidin and cyanindin.

Table1: *In vitro* cytotoxic effect of anthocyanin of *Osbeckia aspera* and *O. reticulata* by MTT assay against HeLa cervical, HT 29 and MG 63

Concentrations (µg/ml)	*O. aspera*			*O. reticulate*		
	He La	HT29	MG63	He La	HT29	MG63
6.25	60.1 ± 6.2	68.8 ± 1.5	79.1 ± 4.8	88.9 ± 3.4	70.4 ± 2.5	70.7 ± 10.3
12.5	54.3 ± 3.4	60.1 ± 1.5	70.7 ± 8.5	72.3 ± 4.6	61.2 ± 3.2	70.1 ± 8.6
25	45.5 ± 6.1	54.6 ± 2.2	66.4 ± 3.3	65.5 ± 2.9	59.5 ± 4.6	66.6 ± 9.2
50	40.2 ± 7.6	49.0 ± 2.9	56.6 ± 4.7	50.2 ± 1.8	49.4 ± 1.7	57.7 ± 3.5
100	35.7 ± 2.4	40.6 ± 6.8	49.4 ± 4.2	43.3 ± 5.4	40.6 ± 6.2	50.02 ± 4.4

Cytotoxicity activity

Purified anthocyanin extracts of *Osbeckia aspera* and *O. reticulata* were tested against HT29 colon, MG63 bone and HeLa cervical lines at 6.25, 12.5, 25, 50, 100µg/ml concentrations to determine the IC_{50} values (50% growth inhibition) using MTT assay. Results of various concentrations of *O. aspera* and *O. reticulata* were tabulated in Table 1. MTT assay of *O. aspera* shows significant effect on HeLa cell in concentrations ranged between 6.25µg/ml to 100µg/ml when compared with the control (Fig. 1). The highest cytotoxicity of anthocyanin extract against HeLa cell was found at 50 and 100µg/ml concentrations with 40.2 ± 7.6 and 35.7 ± 2.4% of cell growth inhibition. The percentage of growth inhibition was found to increase with increasing concentration of the tested compounds, and IC_{50} value was 23.7µg/ml.

Anthocyanin extract from *O. reticulata* also showed potential cytotoxicity on HeLa cell line with the concentrations ranging from 6.25 to 100µg/ml as compared with control. *O. reticulata* extract exerts high cytotoxicity against HeLa cell at 100µg/ml concentration with 56.7% of cell growth inhibition. IC_{50} value of the extract against HeLa cell was 51.4µg/ml (Table 1).

Similarly, the anthocyanin extract of *Osbeckia* species displayed cytotoxicity effect on HT29 colon and MG63 bone cell lines in the concentrations ranged from 6.25 to 100µg/ml. The IC_{50} value of anthocyanin extract of *Osbeckia aspera* and *O. reticulata* against HT29 colon was 64.7 and 49.8µg/ml respectively. Meanwhile, IC_{50} values of anthocyanin extract of *Osbeckia aspera* and *O. reticulata* against MG63 bone cells were 95.5 and 100µg/ml respectively (Table 1).

DNA fragmentation

The agarose gel electrophoresis was performed on the HeLa, HT 29 and MG 63bone cell lines treated with 12.5, 25, 50 and 100µg/ml of anthocyanin extract from *Osbeckia aspera* and *O. reticulata* for 48 h. Remarkable ladder pattern was visualized with the anthocyanin of *O. aspera* and *O. reticulata* treated HeLa and HT 29 cell lines (Fig. 2a & b and 2a & b) when compared to the control. However, MG63 bone cell lines showed DNA laddering effectively with 100 µg/ml concentrations of anthocyanin extract of *O. aspera* and *O. reticulata* (Fig 2a, b & c; 3a, b & c).

Generally, inter nucleosomal DNA cleavage results into ladder pattern for the anthocyanin treated cells. The untreated cells yielded a discrete band above 6000 kbp. Meanwhile, the treated cells indicate cleaved base pair that resulted in to DNA laddering. This was supported by the occurrence of apoptotic bodies in the cell morphological study. An increasing trend of DNA fragmentation could be detected with anthocyanin treated HeLa cells from 12.5-100 µg/ml concentrations. However, MG63 bone cell lines showed no fragmented DNA with the concentrations less than 100 µg/ml (Fig 2a, b & c; 3a, b & c).

Devika and Mohandass documented that the apoptosis hallmark is the cleavage of the nuclear DNA into ~ 200 base pair multiples. [6] This specific DNA cleavage is due to the activation of endogenous endonuclease that cleaves at the exposed linker regions between nucleosomes. Paul et al., reported similar cytotoxic and DNA damaging activities by three herbal extracts on cervical cancer cell lines. [7]

Cell morphology

The morphology of the anthocyanin treated cells has been observed initially under an inverted microscope as presented in (Fig. 4; a-d). Cell detachment, irregular shape, vacuolation and agglutination of cells were seen in the affected onco cell lines as compared to control cells. The cell morphology was also evaluated using AO/PI staining showed induced morphological abnormalities in treated cells, such as cell shrinkage, cell membrane blebbing, rounding off of cells and reduced cell volume. However, no visible changes were seen in the case of normal cells (Fig. 5 a-d). The apoptotic cells were shrunken condensed with fragmented nuclei after exposure of purified anthocyanin extract from the *Osbeckia* species for 48 h (Fig. 6a-d). Meanwhile the control non-apoptotic cells, revealed a low fluorescence, smooth, flattened nuclear shape and normal nuclei, as well as homogenously dispersed chromatin components. In fact, DNA fragmentation into oligo nucleosomal ladders is the marker of apoptosis, recent evidence refers that all cells never undergo such extensive DNA fragmentation. [8] Further, the DNA fragmentation into bp-size seems to be an early marker of apoptosis before ends into the complete digestion of DNA to multiples of nucleosomal sized DNA fragments. [9] The positive control experiment showed necrotic cells with shrinked kidney shaped nuclei under fluorescent microscope. It was found that anthocyanin extract of *O. reticulata* showed significant cytotoxic ativity against He La cell lines (Fig. 7; a-d) followed by HT29 (Fig. 8;a-d) colon cancer and MG63 (Fig. 9; a-d) bone cancer cell lines.

Overall, the present study reveals that purified anthocyanin extract of *Osbeckia* species displayed potential cytotoxic activity on Hela cells. This study also provides basic data of DNA laddering and apoptosis. Further studies are warranted for isolation and identification of biologically active lead molecules from these extracts.

Herbal extracts to be useful in therapeutic application displays potential toxicity against the targeted cells or interfere directly with specific metabolic pathway without any side effects on the normal cell or interfere with the routine functional pathways. In categorization of drugs safety, IC_{50} value of 20µg/ml and below were considered to be significantly cytotoxic in *in vitro* assay according to US National Cancer Institute (NCI) plant screening program following incubation for more than 48 h. [10] But, the application of natural plant based product is safe and cost effective even though the IC_{50} value is greater than 20µg/ml as compared to synthetic drugs.

The cellular toxicity potentials of anthocyanin extracts were analysed by MTT-formazan viability assay. Cellular viability and proliferation are considered as functional features of normal healthy cells. Increased cell viability reveals cell proliferation, and its decrease reflects cell death as a result of either toxic effect of the drug or sub optimal culture conditions. The cell viability noticed for the negative control (DMSO) at the highest concentration of 1000µg/ml under the similar experimental condition suggests the optimal culture conditions. Therefore, the anthocyanin extracts of *Osbeckia* species tested may be used for treating diseases. Interestingly, the anthocyanin extracts showed cytotoxicity range against the screened onco cell lines but not with the mouse fibroblast cells (Normal, data not shown). Therefore, the use of anthocyanin extracts in the traditional drug can be validated. It is also important to record that no toxicity has been documented for the ethnic usage of anthocyanin extracts of *Osbeckia* species by the local people. Similarly, the cellular toxicity does not necessarily reflect toxicity at the organism level due to its possible interactions in the gut and bioavailability issues.

Several cytotoxic and anti-tumour fractions of stilbenoids like Combretastin A and Combretastatin 85 (IC_{50} value < 20µg/ml) have been isolated from *Combretum* species. Toxicity is generally recorded due to irrational usage leading to the accumulation of potentially toxic components or interactions between the crude drug and conventional treatments. The general toxic clinical symptoms noticed with synthetic drugs were diarrhoea, weight loss, agitation, hispid hair, convulsions, tremors, dyspnoea among other and mortality. [11] Similarly, some medicinal plant secondary metabolites can also leads to irritative reactions like the release of mucus from goblet cells, hypersecretion of crypt cells, and mal adsorption causing diarrhoea and emesis. Treatments with high dose of phytochemicals may cause impacts like necrosis, haemorrhage, and ulceration. Herbal drugs often have acute/chronic additional toxicity or more directly life-threatening impacts on other organ system.

Nemati *et al*., evaluated the cytotoxic properties of some medicinal plant extracts from Mazandaran, Iran. [12] Tantengco and Jacinto compared cytotoxicity of crude extracts from *Premna odorata*, *Artocarpus camansi* and *Gliricidia sepium* against selected human cancer cell lines. [13] Siegel *et al*., reviewed the alarmingly increased number of cancer patients across the world. [14] Zulkipli *et al*., documented that herbals were potential source of phytochemicals for regulating cell division. [15] Canoy *et al*., analyzed chemotherapeutic potential of endemic indigenous plants of Kanawan, Morong, Bataan Province of Philippines. [16] Olarte *et al*., proved the *in vitro* antitumor properties of leaf extracts of *Cassia alata*. [17] The obtained results of *Osbeckia* species were comparable with that of *Kalanchoe tubiflora*. [18] Tantiado attempted a field survey on ethnopharmacology of hebals in Iloilo, Philippines especially related with antitumour potentials. [19] Lirio *et al*., analyzed the antitubercular constituents from *Premna odorata*. [20] Abe and Ohtani made an ethnobotanical analyzes of medicinal plants and traditional therapies on Batan Island, the Philippines. [21]

The present study on purified anthocyanin from *in vitro* callus vultures of *Osbeckia aspera* and *O. reticulata* were important because they show selective toxicity against selected cancer cell lines effectively. Thus, an appropriate usage of the molecules as therapeutics for curing ailments is recommendable. *In vivo* acute toxicity analysis, a pre-requisite to confirm the safety level of the drugs but, the *in vitro* assay results always not necessarily translate into *in vivo* activity. Mutagenicity and genotoxicity analysis are warranted for establishing the long term impact of the use of anthocyanin. *In vivo* animal models are costly and require much work to establish the optimal concentrations or histological evaluation of toxicity. It is also plausible to carry *ex vivo* analysis using major organs of mammals.

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