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Evaluation Of Haemolytic Potential, Cytotoxic Effect And Anti-Proliferative Property Of *Boerhavia Diffusa* (Linn) Root Extract Using Hela Cell Line By Clonogenic Assay

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Abstract

Plant origin natural compounds believed to be safe for human have received much attention all over the world in the recent years. In ayurveda and unani medicine, different parts of the renowned herbal medicinal plant Boerhavia diffusa have been used for treating several human disorders. But very little is known about the antiproliferative activity of B. diffusa root extract. Hence investigation was carried out on the growth inhibitory property of B. diffusa on HeLa cell line. Results showed maximum of 28.95 % of hemolysis at 320 µg/ml test concentration and good cytotoxiic effect with an IC_{50} value of 54.06 µg/ml inhibition on HeLa cell line. The present research work using the ethanolic extract of B. diffusa root has demonstrated the antiproliferative property on HeLa cell line.

Keywords: *Haemolytic, Cytotoxic, Antiproliferative, B. diffusa, HeLa, Clonogenic.*

1. Introduction

Cancer is one of the most dangerous diseases all over the world. In order to overcome the side effects of synthetic drugs using for cancer treatment, as an alternative natural herbal product are important and safe source of pharmacological active compounds. In traditional ayurvedic system of medicine many plants have been used to treat wide range of ailments without side effects. The ethno-medicinal property of these plants needs to be exploited properly for therapeutic purposes. Hence screening the chemical constituents is essential for drug discovery from natural products[1]. Several plants have been evaluated for their medicinal property, including antiproliferative activity [2].

Boerhavia diffusa commonly called as Punarnava belongs to family nyctaginaceae is a perennial creeping herb found all over India[3, 4]. It is widely used in traditional medicine to treat various health related problems[5,6]. Pharmacological study of this plant exhibited wide variety of medicinal properties like diuretic, antibacterial, immunomodulatory property etc[3]. This medicinal plant has received much attention, as it was used to cure several ailments in human including abdominal tumor [7].

As the plant has the long history of medicinal values, analysis of potential anticancer activity of this plant is required. Hence in the present study considering the ethonomedicinal potential, antiproliferative property of ethanolic extract of popular medicinal plant *B. diffusa* root has been assessed in vitro using HeLa cells.

2. Materials and methods

The plant sample used in the present study was root of *Boerhaavia diffusa*. Required quantity of the dried plant root sample was collected from the Amruth kesari depot in Avenue road, Bengaluru. Identification of the plant root sample was confirmed by the experts in the department of Botany in Nrupathunga University. The crude extraction of *B. diffusa* root sample was prepared by using ethanol as a solvent.

2.1. Haemolytic potential

Five ml of blood was collected from healthy volunteers in the tubes containing 5.4 mg of EDTA to prevent coagulation and centrifuged at 1000 rpm for 10 min at 4ºC. Plasma and the white buffy layer was completely removed by aspiration with a pipette with utmost care. The erythrocytes were then washed for additional three times with 1X PBS, pH 7.4, stored at 4ºC and used within 6 h for the haemolysis assay.
50 µl of 10 dilution (100µl erythrocytes suspension: 900 µl 1X PBS) erythrocytes suspension was taken in 2 ml Eppendorf tube and 100 µl of test sample plant root extract was added. 100 µl of 1X PBS was negative control and 100 µl of 1% 1% SDS was maintained as positive controls. This reaction mixture having erythrocyte suspension with test material was incubated for 1 hour during rotation at 37ºC. Adjust the volume of reaction mixture to 1 ml by adding 850 µl of 1X PBS. After incubation, samples were centrifuged at 3000 rpm for 3 min to obtain supernatant, containing free haemoglobin. Finally the resulting haemoglobin in the supernatant was measured at 540 nm by spectrophotometer to determine the concentration of haemoglobin. The following formula was used to calculate the percentage of haemolysis.

$$\% \text{ Haemolysis} = \frac{(\text{Control OD} - \text{Sample OD})}{\text{Control OD}} \text{x } 100$$

2.2. Cytotoxic effect of plant root sample on HeLa cell lines by MTT assay

The MTT system is a means to measure the activity of living cells via mitochondrial dehydrogenases. The key component MTT or (3-[4, 5- dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide) is a water soluble tetrazolium salt yielding a yellowish solution when prepared in media or salt solutions lacking phenol red. Dissolved MTT is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by mitochondrial dehydrogenase enzymes of viable cells. This water insoluble formazan can be solubilized using DMSO, acidified isopropanol or other solvents (Pure propanol or ethanol). The resulting purple solution is spectrophotometrically measured. An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of effects caused by the test material.
The important materials used for this experiment are, MTT Powder, DMSO, CO_2 incubator and Tecan plate reader.

Preparation of test solutions

For cytotoxicity study, 32 mg/ml stocks were prepared using DMSO. Serial two fold dilutions were prepared from 320 µg/ml to 10 µg/ml using DMEM plain media for treatment.

Cell lines and culture medium

All the cell lines were procured from ATCC, stock cells was cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) in a humidified atmosphere of 5% CO_2 at 37ºC until confluent. The cell was dissociated with cell dissociating solution (0.2 % trypsin, 0.02 % EDTA, 0.05 % glucose in PBS). The viability of the cells are checked and centrifuged. Further, 50,000 cells /well was seeded in a 96 well plate and incubated for 24 hrs at 37ºC, 5 % CO_2 incubator. Reagents like DMEM, FBS, Pen Strep and Trypsin used for the experiment were procured from Invitrogen.

Procedure

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10^5 cells/ml using respective media containing 10% FBS. To each well of the 96 well microtiter plate, 100 µl of the diluted cell suspension (50,000 cells/well) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of test drugs were added on to the partial monolayer in microtiter plates. The plates were then incubated at 37ºC for 24 hrs in 5% CO_2 atmosphere. After incubation the test solutions in the wells were discarded and 100 µl of MTT (5 mg/10 ml of MTT in PBS) was added to each well. The plates were incubated for 4 h at 37ºC in 5% CO_2 atmosphere. The supernatant was removed, 100 µl of DMSO was added and the plates were gently

shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 590 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% (IC_{50}) values is generated from the dose-response curves for each cell line.

$$\% \text{ Inhibition} = \frac{(\text{Control OD} - \text{Sample OD})}{\text{Control OD}} \text{x } 100$$

IC_{50} values can be calculated for a given antagonist by determining the concentration needed to inhibit half of the maximum biological response of the agonist. IC_{50} values for cytotoxicity tests were derived from a nonlinear regression analysis (curve fit) based on sigmoid dose response curve (variable) and computed using graph pad prism 6 (Graph pad, San Diego, CA, USA).

Nonlinear regression

In statistics, nonlinear regression is a form of regression analysis in which observational data are modeled by a function which is a nonlinear combination of the model parameters and depends on one or more independent variables. The data are fitted by a method of successive approximations.

2.3. Clonogenic Assay

A typical clonogenic survival experiment using adherent cell lines involves three distinct components. Preparation of single cell suspensions and plating an appropriate number of cells in dishes, treatment of the cell monolayer in tissue culture flasks and fixing and staining colonies following a relevant incubation period. This could range from 1-3 weeks, depending on the cell line.

Protocol

Harvest cells and plate 1 x 10^3 numbers of cells per 35mm dish. (The number of cells for seeding should be determined by the aggressiveness of the treatment). Incubate cells for 24 hours in a CO_2 incubator at 37 °C. Treat the cells as necessary with samples at desired concentrations and control is maintained without treatment. After 18-24 h of treatment, the number of cells in each sample were counted carefully using a hemocytometer and diluted such that appropriate cell numbers were seeded into petri dishes (5000 cells/dish). Incubate the cells in a 5 % CO_2 incubator at 37°C for 1 week, change medium after 1 week and further incubate until cells in control plates have formed colonies that are of a substantially good size (50 cells per colony is the minimum for scoring). Colonies of greater than 50 cells were counted to determine the surviving fraction.

Colony Counting

Colonies containing more than 50 individual cells were counted using ImageJ. Digital images after staining of the colonies were obtained using a camera or scanning device.

Fixing and staining colonies

Gently remove the media from each of the plates by aspiration. Wash each plate with 1 ml 1X PBS. Fix the colonies with 1 ml 3.7% PFA solution for 15-30 minutes. Stain with 1 ml 0.05% crystal violet in distilled H_2O/PBS for 30-60 minutes. Wash excess crystal violet with distilled H_2O and allow dishes to dry. Colonies were counted using ImageJ software.

Calculation:

$$\text{Plating efficiency (PE)} = \frac{\text{Number of colonies counted}}{\text{Number of cells plated}} \times 100$$

Where: Number of cells plated = 5000/well.

$$\text{Surviving fraction (SF)} = \frac{\text{PE of treated sample}}{\text{PE of control}} \times 100$$

3. Results

3.1. Haemolytic potential of plant root extract

Results of the haemolytic study using *B. diffusa* root extract did not show significant haemolytic activity. The obtained data has negligible haemolysis and the sample screened can be considered for pharmaceutical studies. Sample has shown maximum of 28.95 % of hemolysis at its highest (320 µg/ml) test concentration. Details of evaluation of haemolytic potential of plant root extract for positive control, negative control and the samples are given in table 1 and figure 1.

Table 1: Heamolytic potential of plant root extract

Sample	Conc. in µg/ml	Abs at 540 nm	% Hemolysis
Control	PBS	0.532	0.00
Positive Control (SDS)	1%	0.04	92.48
Boerhaavia diffusa Root Extract	10	0.512	3.76
	20	0.485	8.83
	40	0.465	12.59
	80	0.435	18.23
	160	0.401	24.62
	320	0.378	28.95

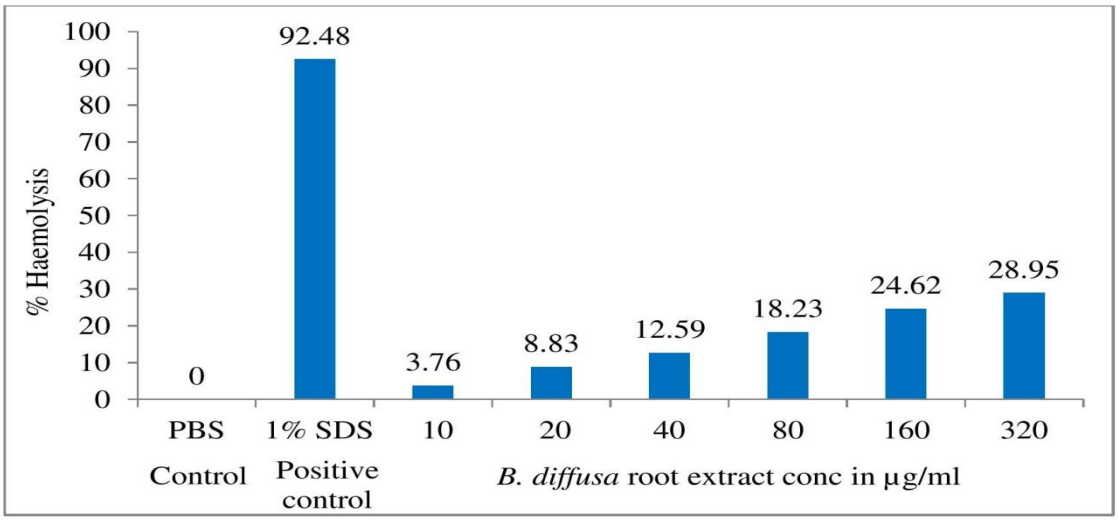

Figure 1: Graphical representation of haemolysis assay

3.2. Cytotoxic effects of medicinal plant extracts on HeLa cell lines by MTT assay

In the cytotoxic study the standard colchicine showed an IC_{50} value of 16.91 µM on HeLa cells. (Table 2 and figure 2). Sample *B. diffusa* root extract has showed an IC_{50} value of 54.06 µg/ml inhibition on HeLa cell line. The details of percentage of inhibition at different concentrations of *B. diffusa* root extract are given in table 3 and figure 3. From the data it is clear that the percentage of inhibition is directly proportional to the concentration of the sample and the IC 50 value is found to be effective.

Table 2: Cytotoxic effect with colchicine

Compound Name	Conc. µM	OD at 590nm	% Inhibition	IC_{50} µM
Control	0	0.553	0.00	16.91
Colchicine	3.13	0.491	11.33	
	6.25	0.409	25.99	
	12.5	0.330	40.29	
	25	0.256	53.67	
	50	0.149	73.16	
	100	0.099	82.18	

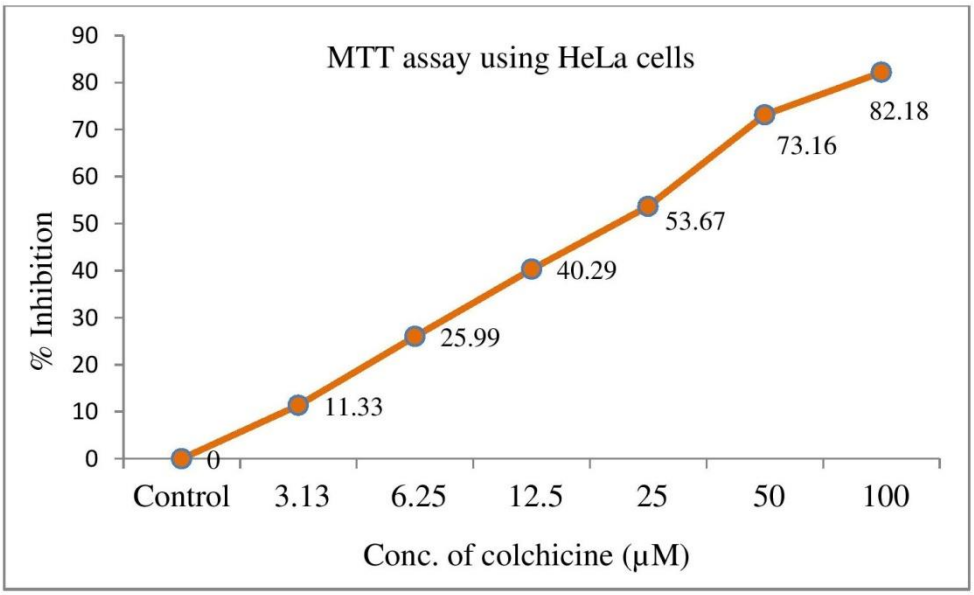

Figure 2: MTT assay using HeLa cell line with colchicine

Table 3: Cytotoxic effects with *B. diffusa* plant root extract

Compound Name	Conc. µg/ml	OD at 590nm	% Inhibition	IC50 µg/ml
Control	0	0.793	0.00	54.06
Root extract	10	0.682	13.96	
	20	0.541	31.82	
	40	0.438	44.80	
	80	0.329	58.50	
	160	0.254	67.99	
	320	0.093	88.34	

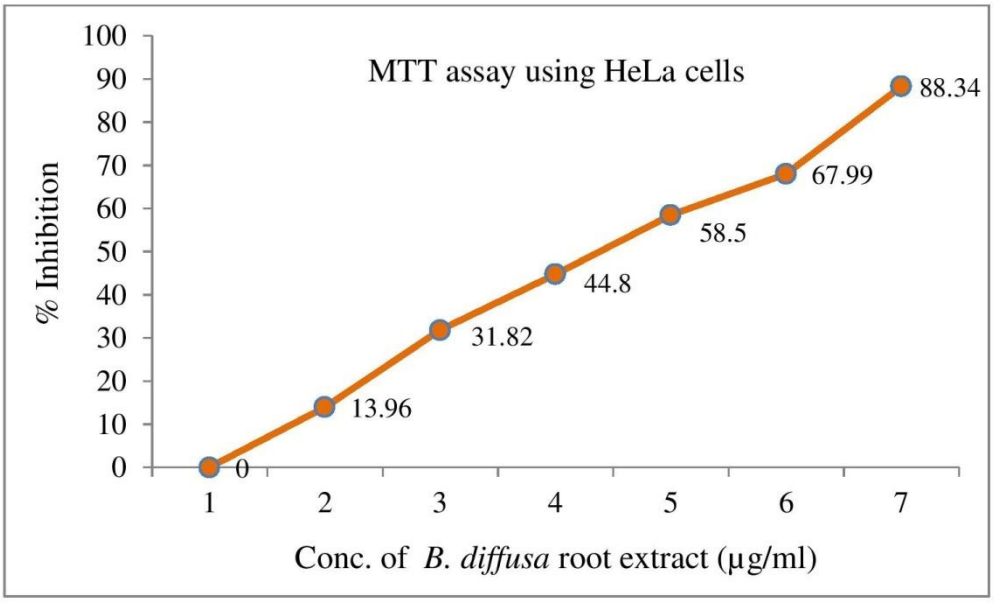

Figure 3: MTT assay using HeLa cells with *B. diffusa* root extract

3.3. Anti-proliferative property of *B.diffusa* root extract on HeLa cell line by clonogenic assay.

HeLa cells showed 100% surviving fraction for control, 75.74% and 37.87% for 40µg/ml and 80µg/ml of sample *B. diffusa* root extract treatment respectively. Standard colchicine showed 11.49% surviving fraction. The details of surviving fraction of HeLa cells in control, treated groups and colchicine are summarized in table 4. The post crystal violet stained colonies of HeLa cell lines is given in figure 4. The result shows very

low percentage and effective surviving fraction in the 80 µg/ml concentration of *B. diffusa* root extract treated group.

Table 4: HeLa Colony count in control and different concentrations of tested sample

Sample	Conc. µg/ml	Colony count	Plating efficiency %	Surviving fraction %
Control	0	235	4.7	100
Root	40	178	3.56	75.74
	80	89	1.78	37.87
Colchicine	25 µM	27	0.54	11.49

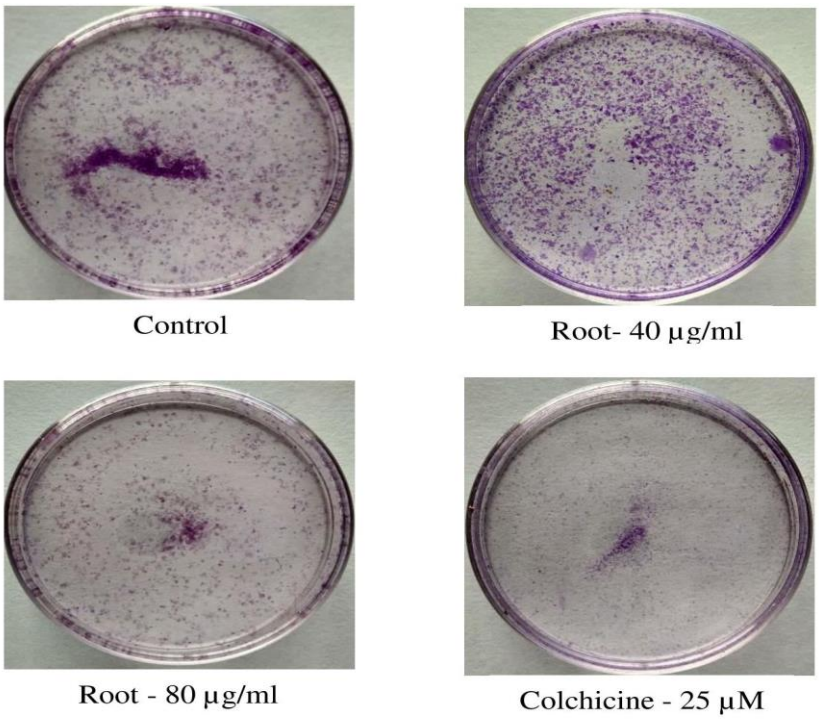

Figure 4: Post crystal violet stained colonies of HeLa cell line

4. Discussion

Various plant derived products have been used for treating tumors in traditional medicine. *B. diffusa* was extensively used for various purposes in ayurveda clearly indicates the medicinal property of this plant. Presence of alkylating agents in phytochemical analysis of *B. diffusa* is an indication of antiproliferating property of this plant. Sreekumar and Sreeharshan [7] and Apu et al.,[8] have reported the cytotoxic and antiproliferative property of this plant respectively. In the present study the sample did not show significant haemolytic activity. Results of the study reported IC_{50} value 54.06 µg/ml for the sample, which clearly indicates cytotoxic effect of *B. diffusa* root extract on HeLa cells. Rakhi Srivastava[3] reported that the root crude extract of the *B. diffusa* show varied cytotoxic effect on cell lines having different histological origin, due to the presence of different types of compounds in the extract.

Study conducted by Mehrotra, et al.,[2] using *B. diffusa* reported the inhibition of different cell lines in mouse and human. Bharali et al.,[9] reported the inhibition of growth of leukemia cell lines and prevented DMBA induced skin carcinogenesis in mice treated with *B. diffusa* root extract. Study carried out by Rakhi Srivastava, et al.,[3] using methanolic crude extract of *B. diffusa* root for anti-proliferative effect showed positive result on cancerous cell lines. They have reported the significant reduction in the growth and morphological changes in HeLa cells after 48 hours of exposure to the concentration of 200 µg mL-1. Antiproliferation in HeLa cells could be due to obstruction of DNA synthesis during cell cycle.

Salman Khan et al.,[10] reported that flavonoids present in this plant are responsible for its medicinal properties. The results of the study could be due to the effect of combination of the compounds. Even though the study showed low surviving fraction in the 80 µg/ml concentration treated group, further studies are required to find out the particular active molecules responsible for the anti-proliferative property of *B. diffusa* root.

5. Conclusion

Cytotoxic study results clearly indicate that the root extract can be an effective source to control the growth and multiplication of cells. The results of clonogenic assay showed that *B. diffusa* root extract has a promising active compound to inhibit the growth of HeLa cells. This can be a good source for further studies to identify the bioactive compounds for developing anticancer drug.

Conflict of interest
The authors declare no competing financial interest.

Author contributions
Mohan Kumar H. M., Sanjay K. L. and Sharada Devi J. N. conceived the study, performed the experiment and wrote the article.

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