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Cytotoxic Activity and Colony Survival Analysis of Ethanolic Leaf Extracts of *Boerhavia diffusa* L. against Achn Cell Line

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Abstract

Plants and plant-based medicaments are the basis of many of the modern pharmaceuticals we use today for our various ailments. In plants, as a result of metabolic processes, many different kinds and types of organic compounds or metabolites are produced. In the present study reported that the cytotoxicity and clonogenic survival of ethanolic leaf extracts of *Boerhavia diffusa* against ACHN cell line. In the present study showed the minimum cell viability (34.81±2.5%) and maximum cell inhibition (65.19±5.7%) was noted in 500 µg/ml concentrations of ethanolic extracts of *B. diffusa* in ACHN cell line. This tumour cells are also treated with various concentrations (125, 250 and 500 µg/ml) of ethanol extracts of *B. diffusa* leaf. The plate showed the significant inhibition of colony forming capability when compared to control. Of these, highest inhibition of colony survival (8.4±2.1%) was noted in the 500 µg/ml of ethanol of extracts against ACHN cell line. The colony survival was inhibited by the depending upon the concentrations. Overall, the present study concluded that the ethanolic extracts of *B. diffusa* has inhibited the growth of ACHN cancer cells by preventing the formation of colony and there by inducing apoptosis.

***Key words*:** *Boerhavia diffusa,* Cytotoxicity, Clonogenic assay, Ethanol extracts, ACHN cell line, MTT assay

Human beings have depended on nature for their simple requirements as being the sources for medicines, shelters, food stuffs, fragrances, clothing, flavours, fertilizers and means of transportation throughout the ages. For the large proportions of world's population medicinal plants continue to show a dominant role in the healthcare system and this is mainly true in developing countries, where herbal medicine has continuous history of long use. The development and recognition of medicinal and financial aids of these plants are on rise in both industrialized and developing nations [1]. Cancer constitutes serious public health problems in both developed and developing countries. The limited success of clinical therapies including radiation, chemotherapy, immune modulation and surgery in treatment of cancer indicates that there is an imperative need of alternative strategies in cancer management [2]. Chemoprevention, which consists of the use of synthetic or natural agents (alone or in combination) to block the development of cancer in human beings, is an extremely promising strategy for cancer prevention. The control of cell proliferation is fundamental in maintaining cellular homeostasis and loss of this mechanism is a principle hallmark of cancer cells. Synthetic chemotherapeutic agents are used to stop the growth and eliminate cancer cells even at distant sites from the origin of primary tumour.

Plants are the effective source of anticancer agents and over 60% anticancer agents are derived from plants [3]. Medicinal plants have thus become a focal point to improve the present and future health needs against cancer. This is because secondary metabolites present in medicinal plants could maintain the health and cure various diseases including cancer with less harmful effects. *Boerhaavia diffusa* Linn. (Nyctaginaceae) is a well-known traditional medicinal plant in Indian medicine system as well as other parts of world, such as Southern American and African continent. Various parts of this plant, especially roots, have been used for gastrointestinal, hepatoprotective, and gynecological indications [4]. *B. diffusa* is a traditional herb of which has been proposed for the treatment of cancer, tumors, jaundice and liver disorders. The root is generally used as an infusion to treat internal inflammation. It is also used for the treatment of diabetes [5] and to treat seminal weakness and high blood pressure [6]. Previous studies also reported that *B. diffusa* shown potential anticancer activity by effectively reducing the metastases formation by B16F10 cells, a highly metastatic melanoma cell line [7]. Based on the previous pharmacological reports of *B. diffusa*, there were no scientific reports on anticancer activity against human kidney cancer ACHN cell. Hence, the present investigation has been carried out to evaluate the in vitro cytotoxic effects of ethanolic leaf extracts of *B. diffusa* using ACHN cell lines.

MATERIALS AND METHODS

24-well plates, Millipore double distilled water, ACHN line, RPMI 1640 media, L-glutamine, 10% fetal bovine serum, streptomycin, penicillin, PBS (phosphate buffer saline), MEM

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(Minimum Essential Media), TPVG (Trypsin Phosphate Versene Glucose), FCS (Foetal Calf Serum), MTT (3- 4, 5-dimethyl-2-thiazolyl) -2, 5-diphenyl - tetrazolium bromide), DMSO (Dimethyl sulphoxide), 0.5% crystal violet, methanol and CO_2 incubator were used for these experiments.

Collection of plant sample

Boerhaavia diffusa leaf was collected from in and around region of Thanjavur, Tamil Nadu, India. The plant parts were identified taxonomically and authenticated according to various literatures, Flora of Madras Presidency and Wealth of India including other pertinent taxonomic literature. The collected plant leaves were rinsed with distilled water left at room temperature for 7 days in the dark and then oven dried for 24 h at 50°C. Then the plant material made to coarse powder using mixer grinder. The powdered sample was stored in refrigerator for further use.

Extract preparation

The Soxhlet apparatus was used for successive solvent extraction of the collected leaf powder of *Boerhavia diffusa*. 35g of plant powder was extracted with ethanol for 12 hrs. After complete extraction, the contents of extraction were concentrated by distillation. The concentrated extract was evaporated to dryness and stored separately at 4 ºC in air tight containers for further experimental studies.

ACHN cell culture

The human kidney cancer cell line, ACHN cell lines were procured from American type culture collection (ATCC CRL – 1611) and maintained with RPMI 1640 with L-glutamine supplemented with 10% fetal bovine serum, 100 µg/ml streptomycin, and 100 U/ml penicillin (Invitrogen), maintained at 37°C in a 5% CO2 incubator.

Maintenance of cell line

The cell lines were obtained from National Centre for Cell Sciences (NCCS), Pune. The tissue culture bottles that showed confluent monolayer were selected by observing them under an inverted microscope. Growth medium was removed from the bottle, washed with PBS/MEM without FCS and 5 mL of TPVG was added dispersing evenly on the monolayer and left in contact with the cells for 2-3 minutes until there is a cloudy appearance on the monolayer. TPVG was removed and the cells were resuspended in 5 ml of growth medium (MEM containing 10%FCS). The suspension was aspirated few times to break the cell clumps. The cell suspension was then transferred to a 24 well plate. 1ml (1 lakh cells/ml) of the cell suspension was added to each well. The plate was then incubated in a CO_2 incubator maintained with 5% CO_2.

Cell viability by MTT assay

Cells (1×10^5/well) were plated in 24-well plates and incubated in 37°C with 5% CO_2. After the cell reaches the confluence, the various concentrations of the ethanol leaves extracts of *B. diffusa* were added and incubated for 24 h. After incubation, the samples were removed from the well and washed with phosphate-buffered saline (pH 7.4) or MEM without serum. Then 100µl of 0.5% of MTT (3- 4, 5-dimethyl-2-thiazolyl) -2, 5-diphenyl - tetrazolium bromide) was added individually to each well and incubated for 4h. After incubation, 1ml of DMSO was added in all the wells. The absorbance at 570nm was measured with UV-Spectrophotometer using DMSO as the blank. Measurements were performed and the concentration required for 50% inhibition (IC_{50}) was determined graphically. The % cell viability was calculated using the following formula:

Cell viability (%) = A570 of treated cells / A570 of control cells × 100

Graphs are plotted using the % of Cell Viability at Y-axis and concentration of the sample in X-axis. Cell control and sample control is included in each assay to compare the full cell viability assessments [8].

Clonogenic assay

The cytotoxic effect of potent plant extracts was also further confirmed by clonogenic assay [9], where the cells were incubated separately into 25 cm^2 Petri dishes and allowed to attach for 16 h. Thereafter, cells were treated with various concentrations (125, 250 and 500 µg/ml) of *B. diffusa* leaf extracts and left undisturbed for 10 days for the formation of cell clones. The cultures were then removed and stained using gentian violet. The total number of colonies was counted.

Fixation and staining of colonies

Medium was removed in both dishes and plates; cells were rinsed with PBS. Fixation and staining of clones were done with a mixture of 0.5% crystal violet in 50/50 methanol/water for 30 min. Dishes were rinsed with water and left for drying at room temperature. Counting of clones was done on the following day.

After counting clones, the plating efficiency (PE) and surviving fraction (SF) were calculated as mean X. by the following formula:

PE = No. of colonies formed / No. of cells seeded × 100

SF = (No. of colonies formed after treatment / No. of cells seeded × PE) × 100

Statistical analysis

All the results were expressed as the mean ± standard error mean of triplicate analysis (n = 3). The statistical analysis was carried out by one-way analysis of variance followed by Dunnett's test and anticancer activity of ethanolic leaf extract of *B. diffusa* evaluated as IC50 values (defined as the concentration of the compound required to inhibit cell proliferation by 50%) using SPSS software version 2.0. *P ≤ 0.05, **P ≤ 0.01, and ***P ≤ 0.001 represent a significant difference compared with the control group.

RESULTS AND DISCUSSION

Cytotoxic effect

The present study revealed that the anticancer activity of ethanol leaves extracts of *B. diffusa* was investigated using MTT assay on ACHN cell line. The cell morphology was viewed through the microscope, which clearly shows the cell degradation and granulation at higher when compared to control cell. The minimum cell viability (34.81±2.5%) and maximum cell inhibition (65.19±5.7%) was noted in 500 µg/ml concentration of ethanolic extracts of *B. diffusa* in ACHN cell line. The IC_{50}value (82.25 µg/ml) was calculated for anticancer activity of ethanolic extract of *B. diffusa* against ACHN cell line. The lowest cell inhibition (13.77±1.3%) was recorded in the lowest concentration and highest cell inhibition (65.19±5.7%) was noted in the higher concentration of ethanolic extract of *B. diffusa*. The percentage of cell inhibition was noted in the different concentrations of leaf extract ranges from 7.8 to 500 µg/ml. Standard anticancer drug 5-Fluorouracil (100 µg/ ml) was shown 80.95±8.2% cytotoxicity on ACHN cell line (Table 1). The MTT assay is a method to measure the effectiveness of the sample in inhibiting the biological or

biochemical function. The MTT assay is based on the formation of dark colored formazan dye by reduction of the tetrazolium salt MTT by metabolically active cells [10]. The ethanolic extract of *Pleurotus ostreatus* inhibited the cell proliferation in a dose-dependent manner in HL-60 leukemia cells [11]. In previous study reported that the methanolic extract of *Piper sarmentosum* possessed anticarcinogenic properties in HepG2 cells [12].

Table 1 *In vitro* anticancer activity of ethanol extracts of *B. diffusa leaves* against ACHN cell line

Sample concentrations (µg / ml)	Percent of cell viability (Triplicate values)				Percent of cytotoxicity	IC50 (µg/ ml)
	1	2	3	Average		
Control	100	100	100	100	0	
7.8	86.23 ± 5.2	87.14 ± 5.4	85.32 ± 5.4	86.23 ± 5.6	13.77 ± 1.3	
15.6	76.15 ± 5.1	80.64 ± 5.0	78.43 ± 5.2	78.43 ± 5.1	21.57 ± 1.7	
31.2	60.25 ± 4.3	64.38 ± 5.1	62.25 ± 4.8	62.25 ± 4.8	37.75 ± 2.3	82.25
62.5	49.12 ± 3.7	53.42 ± 4.1	51.27 ± 4.6	51.27 ± 4.5	48.73 ± 2.8	
125	44.13 ± 4.1	47.64 ± 4.5	45.29 ± 3.7	45.68 ± 3.8	54.31 ± 3.9	
250	38.20 ± 2.6	41.24 ± 3.1	40.58 ± 2.8	40.00 ± 2.9	60.00 ± 5.2	
500	33.41 ± 2.2	36.82 ± 2.8	34.20 ± 2.5	34.81 ± 2.5	65.19 ± 5.7	
Standard (100)	17.25 ± 1.5	20.38 ± 2.4	19.53 ± 1.7	19.05 ± 1.8	80.95 ± 8.2	42.35

Clonogenic assays

Clonogenic assays are commonly used to investigate the capacity of cells to produce progeny. A Clonogenic assay was performed in petri plates, with clone produced by ACHN tumour cells. This tumour cells are treated with various concentrations (125, 250 and 500 µg/ml) of ethanol extracts of *B. diffusa* leaf. The plate showed the significant inhibition of colony forming capability when compared to control. Of these, highest inhibition of colony survival (8.4±2.1%) was noted in the 500 µg/ml of ethanol of extracts of *B. diffusa* leaf against ACHN cell line when it compared to other concentration of extracts. Standard drug 5-Fluorouracil (100 µg/ ml) was shown the 5.8±1.5% of survival fraction. After treatment many cells are still divided 2 to 3 times and small colonies of less than 50 cells are formed but not scored for survival. The colony survival was inhibited by the depending upon the concentrations (Fig 1). In the control showed the 100% survival fraction ACHN cells. Overall, these results reported that the ethanolic extracts of *B. diffusa* has inhibited the growth of ACHN cancer cells by preventing the formation of colony and there by inducing apoptosis (Fig 2). In the literature, several studies on comparability of MTT and clonogenic assay can be found.

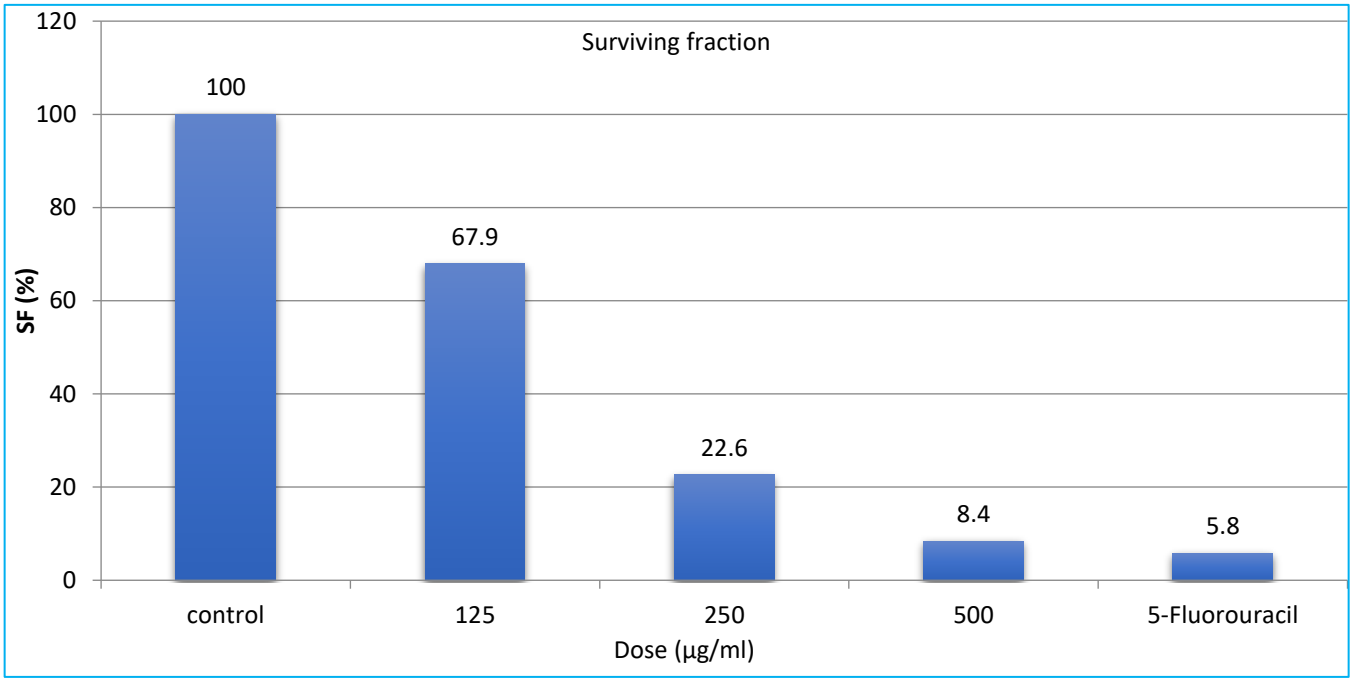

Fig 1 Effect of different concentration of ethanol extract of *B. diffusa* leaves on ACHN cell lines for inhibition of colony survival

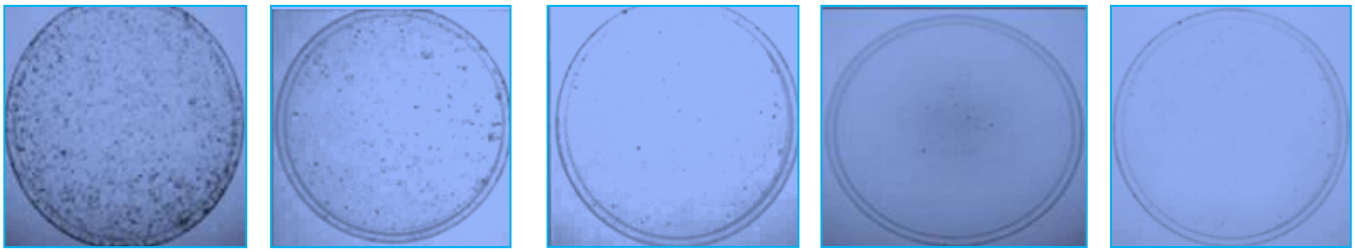

Fig 2 Effect of ethanol extract of *B. diffusa* leaves *on* ACHN cell line for inhibition of colony formation

CONCLUSION

Plants and plant-based medicaments are the basis of many of the modern pharmaceuticals we use today for our various ailments. The present study indicates the efficacy of ethanolic leaf extract of *B. diffusa* as a significant cytotoxic agent against ACHN cancer cell lines. Our results also suggest that the clonogenic assay in order to determine survival of irradiated tumor cells. Further, the study has been focused on the isolation of bioactive anticancer compounds present in the ethanolic leaf extract of *B. diffusa*. In the present concluded that the ethanolic leaf extract of *B. diffusa* showed a vital role of cytotoxic activity against human kidney cancer (ACHN) cell line.

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Conflicts of interest

The authors declare no conflict of interest.

LITERATURE CITED

1. WHO. 1998. Regulatory situation of herbal medicines. A worldwide review. Geneva, Switzerland. pp 1-5.
2. Dai J, Mumper RJ. 2010, Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules* 15(10): 7313-7352.
3. Cragg GM, Newman DJ. 2005, Plant as source of anticancer agents, *Journal of Ethnopharmacology* 100: 72-79.
4. Mishra M, Mishra PK, Kumar U, Prakash V. 2014. NaCl phytotoxicity induces oxidative stress and response of antioxidant systems in *Cicer arietinum* L. cv. Abrodhi. *Botany Research International* 2(2): 74-82.
5. Pari L, Amarnath SM. 2004. Antidiabetic activity of *Boerhaavia diffusa* L. effect on hepatic key enzymes in experimental diabetes. *Jr. Ethnopharmacology* 91(1): 109-113.
6. Gaitonde BB, Kulkarni HJ, Nabar SD. 1974. Diuretic activity of Punarnava (*Boerhaavia diffusa*). 2nd Edition. Bombay, India. pp 24.
7. Leyon PV, Lini CC, Kuttan G. 2005. Inhibitory effect of *Boerhaavia diffusa* on experimental metastasis by B16F10 melanoma in C57BL/6 mice. *Life Science* 76: 1339-1349.
8. Mosmann T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, *Journal of Immunological Methods* 65: 55-63.
9. Puck TT, Markus PI. 1956. Action of X-rays on mammalian cells. *Jr. Exp. Med*. 103: 653-666.
10. Hansen J, Bross P: 2010. A cellular viability assay to monitor drug toxicity. *Methods Mol Biology* 648: 303-311.
11. Venkatakrishnan V, Shenbhagaraman R, Kaviyarasan V, Gunasundari D, Radhika K, Dandapani R, Jagadish LK. 2010. Antioxidant and antiproliferative effect of *Pleurotus ostreatus*. *Jr. Phyto*. 2: 22-28.
12. Ariffin SHZ, Omar WHHW, Ariffin ZZ, Safian MF, Senafi S, Wahab RMA. 2009. Intrinsic anticarcinogenic effects of *Piper sarmentosum* ethanolic extract on a human hepatoma cell line, *Cancer Cell Int*. 9: doi:10.1186/1475- 2867-9-6.