ejpmr, 2020,7(4), 505-510

SJIF Impact Factor 6.222

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

INVITRO CYTOTOXIC EFFECT AND APOPTOSIS INDUCTION OF HYDROALCOHOLIC EXTRACT OF *BOERHAAVIA DIFFUSA* LINN AGAINST HUMAN CANCER CELL LINES

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Article Received on 06/02/2020 **Article Revised on 26/02/2020** **Article Accepted on 16/03/2020**

ABSTRACT

The aim of the present study is to evaluate the effect of *in-vitro* anticancer activity of the Hydroalcoholic extract of *Boerhaavia diffusa* linn against MCF-7 breast cancer & HCT-116 colon cancer cell lines. The viability of cells were evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method. The percentage of cell viability was found to be 97% at 6.25µg/ml on HCT-116 which decrease to 28% with increase in concentration of extract at 100µg/ml. Similarly for MCF-7 cell line, cell viability shows 70% at a concentration of 6.25µg/ml that reduced to 40% at 100µg/ml. The effect of hydro-alcoholic extracts of *Boerhaavia diffusa* linn on cell morphology of MCF-7 and HCT-116 cells was examined using phase-contrast microscope. Treatment with plant extract showed typical morphological changes on MCF-7 & HCT-116 exhibited condensed chromatin, apoptotic bodies with membrane blebs and cell shrinkage. Apoptosis changes and chromatin condensation were also determined by fluorescent microscope. The results indicate that *Boerhaavia diffusa* is a considerable source of natural bioactive substances with antiproliferative activity on HCT-116 & MCF-7 cells & significant morphological effects.

KEYWORDS: Antiproliferative, MTT assay, apoptosis, chromatin condensation, *Boerhaavia diffusa* linn.

INTRODUCTION

Cancer is a major life-threatening health problem throughout the world and is characterized by uncontrolled proliferation of cells. Among various types of cancer, the breast cancer is a major type of cancer next to lung cancer, (Canfeza. et.al, 2002) and the mortality is 30% in females and a small but increasing number is from men (Parkin, 2001) and it is a major public health problem in the developing countries like India. Breast cancer is both genetically and histopathologically heterogeneous, and the mechanism(s) underlying breast cancer development remains unclear(Hedenfalk. et.al, 2002) but the involvement of several genes i.e. either activated or inactivated are there to promote malignancy. (Ingrasson. et.al, 2001) Colorectal cancer metastases result in a significant number of cancer related deaths and colon cancer is commonly ascribed to the transformation of normal colon epithelium to adenomatous polyps and ultimately invasive cancer.

In vitro tumor models have provided important tools for cancer research display a powerful tool to interrogate the efficiency of chemotherapeutic drugs for growth inhibition and tumor cell kill. The increasing costs of conventional treatments (chemotherapy and radiation) and the lack of effective drugs to cure solid tumors encouraged people to depend on folk medicine which is rooted in medicinal plants use. There are excellent sources of bioactive components from medicinal plants which make them targets for potential anticancer treatments. The different parts of the plant *Boerhaavia diffusa* has been used in different system of traditional medication for the treatment of diseases and ailments of human beings. The preliminary review of literature indicates that plants are rich in antioxidant phytochemicals. Therefore the present studies aim to evaluate the plants for anticancer activity by *in vitro* studies**.**

Preparation of Extract

Aerial parts of *Boerhaavia diffusa* Linn was collected & extracted using Soxhlet apparatus by hot continuous percolation method. The powdered material were soaked in the extractor, macerated for 30h with petroleum ether, refluxed with petroleum ether to de-fat the material. The marc was dried and then extracted with a mixture of alcohol and water by continuous hot percolation method using Soxhlet apparatus for 40h separately. The hydro-alcoholic extract was filtered with Whatmann filter paper No. 40 and concentrated under vacuum using rotary flask evaporator under reduced pressure.

Cell lines

HCT- 116 (Human Colorectal Cancer) and MCF-7 (Human Breast Adenocarcinoma) cells was initially procured from National Centre for Cell Sciences (NCCS), Pune, India and maintained Dulbecco's modified Eagles medium, DMEM (Sigma aldrich, USA). The cell line was cultured in 25 cm^2 tissue culture flask with DMEM supplemented with 10% FBS, L-glutamine, sodium bicarbonate (Merck, Germany) and antibiotic solution containing: Penicillin (100U/ml), Streptomycin (100µg/ml), and Amphotericin B (2.5µg/ml). Cultured cell lines were kept at 37ºC in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany).

Cells seeding in 96 well plate

Two days old confluent monolayer of cells were trypsinized and the cells were suspended in 10% growth medium, 100µl cell suspension ($5x10^4$ cells/well) was seeded in 96 well tissue culture plate and incubated at 37ºC in a humidified 5% CO_2 incubator.

Preparation of compound stock

1mg of **HAEBD** extract was weighed and dissolved in 1mL DMEM using a cyclomixer. The sample solution was filtered through 0.22 µm Millipore syringe filter to ensure the sterility.

Experimental design

After 24 hours the growth medium was removed, freshly prepared each compounds in 5% DMEM were five times serially diluted by two fold dilution (100µg, 50µg, 25µg, 12.5µg, 6.25µg in 500µl of 5% DMEM) and each concentration of 100µl were added in triplicates to the respective wells and incubated at 37ºC in a humidified 5% CO_2 incubator. Non treated control cells were also maintained. The viability of cells were evaluated by direct observation of cells by Inverted phase contrast microscope and followed by MTT assay method.

Cell Morphology(Gini. et.al, 2014)

Entire plate was observed after 24 hours of treatment in an inverted phase contrast tissue culture microscope (Olympus CKX41 with Optika Pro5 CCD camera) and microscopic observation were recorded as images. Any detectable changes in the morphology of the cells, such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells were considered as indicators of cytotoxicity.

Cytotoxicity studies by MTT Assay(Liotta. et.al, 1983)

Fifteen mg of MTT (Sigma, M-5655) was reconstituted in 3 ml PBS until completely dissolved and sterilized by filter sterilization. After 24 hours of incubation period, 30µl of reconstituted MTT solution was added to all test and cell control wells, the plate was gently shaken well, then incubated at 37ºC in a humidified 5% CO_2 incubator for 4 hours. After the incubation period, the supernatant was removed and 100µl of MTT Solubilization Solution (Dimethyl sulphoxide, DMSO, Sigma Aldrich, USA) was added and the wells were mixed gently by pipetting up and down in order to solubilize the formazan crystals. The absorbance values were measured by using microplate reader at a wavelength of 540 nm (Laura B. Talarico et al., 2004).

The percentage of growth inhibition was calculated using the formula:

$$\% \text{ of viability} = \frac{\text{Mean OD Samples x } 100}{\text{Mean OD of control group}}$$

Chromatin condensation & Apoptosis studies (Zhang.et, 1998)

`DNA-binding dyes Acridine Orange (AO) and Ethidium Bromide (EtBr)(Baskic. et.al, 2006) (Sigma, USA) were used for the morphological detection of apoptotic and necrotic cells (Zhang et al, 1998). After treatment with sample of LC 50 concentration (HCT-116 –59.03µg/mL and MCF-7 - 56.2235µg/mL) for 24 hours, the cells were washed by cold PBS and then stained with a mixture of Acridine orange (100 µg/ml) and Ethidium Bromide EtBr (100 µg/ml) at room temperature for 10min. The stained cells were washed twice with 1X PBS and observed by a fluorescence microscope in blue filter of fluorescent microscope (Olympus CKX41 with Optika Pro5 camera). The cells were divided into four categories as follows: living cells (normal green nucleus), early apoptotic (bright green nucleus with condensed or fragmented chromatin), late apoptotic (orange-stained nuclei with chromatin condensation or fragmentation) and necrotic cells (uniformly orange-stained cell nuclei).

Statistical analysis

Statistical comparisons between control and treatment mean values of two parameters were analyzed using the Student's *t*-test. Multiple comparisons were done using ANOVA. The differences were statistically significantat $P < 0.01$; $P < 0.05$.

RESULTS

Table 1 & 2 showed that **HAEBD** at the dose of 25, 50, 100 µg/ml reduced the percentage of cell viability from the MTT assay in a concentration dependent manner on MCF-7 & HCT-116 cell lines. The effect of **HAEBD** on the cell morphological studies (**Fig:2**) indicate that, untreated MCF-7 cells exhibited typical growth patterns and a smooth, flattened morphology with central nuclei, whereas treated cells exhibited condensed chromatin, apoptotic morphological changes with protrusion of plasma cell membrane and detachment from the surface. In addition, it showed apoptotic bodies with cytoplasmic condensation indicating apoptosis like changes. Similar results were observed with HCT-116 cells on treatment with HAEBD. The effects of **HAEBD** on chromatin condensation and apoptosis of MCf-7 & HCT-116 cells (**Fig:3 & 4**) were examined by using Acridine orange & Ethidium bromide. AO is taken up by both viable and non-viable cells and emits green fluorescence intercalated into double stranded nucleic acid (DNA). EtBr is taken up only by non-viable cells and emits red fluorescence by intercalation into DNA. These

observations provide evidence that an apoptotic pathway was triggered with the **HAEBD** treatment in both cancer cell line.

Effect of Hydro Alcoholic Extract of *Boerhaavia diffusa* linn on cell viability by MTT Assay on MCF-7 cell line.

Sample Concentration (µg/mL)	OD value I	OD value II	OD value III	Average OD	Percentage Viability
Control	1.0062	0.9832	1.1635	1.0510	100.00
6.25	0.7267	0.7391	0.7419	0.7359	70.02
12.5	0.7115	0.7043	0.7118	0.7092	67.48
25	0.5851	0.5916	0.5846	0.5871	55.86
50	0.4387	0.4802	0.4467	0.4552	43.31
100	0.4216	0.4273	0.4379	0.4289	40.81

Effect of Hydro Alcoholic Extract of *Boerhaavia diffusa* linn on cell viability by MTT Assay on HCT-16 cell line.

Sample Concentration (µg/mL)	OD value I	OD value II	OD value III	Average OD	Percentage Viability
Control	1.4721	1.4625	1.4700	1.4682	100
6.25	1.4401	1.4336	1.4162	1.4300	97.40
12.5	1.2156	1.2015	1.2209	1.2127	82.60
25	1.0381	1.1503	0.9822	1.0569	71.98
50	0.5614	0.6271	0.6017	0.5967	40.64
100	0.4221	0.4174	0.4322	0.4239	28.87

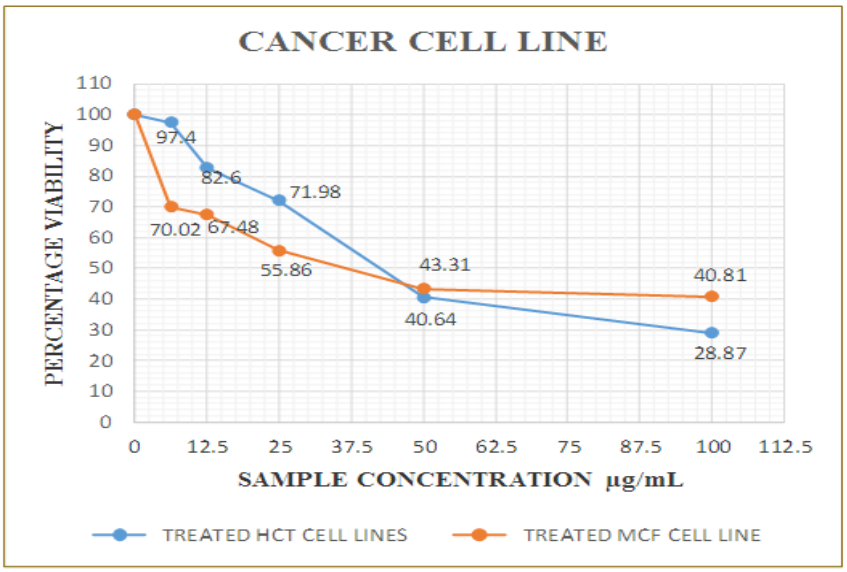


Fig. 1: Effect of HAEBD on cell viability by MCF-7 & HCT-116.

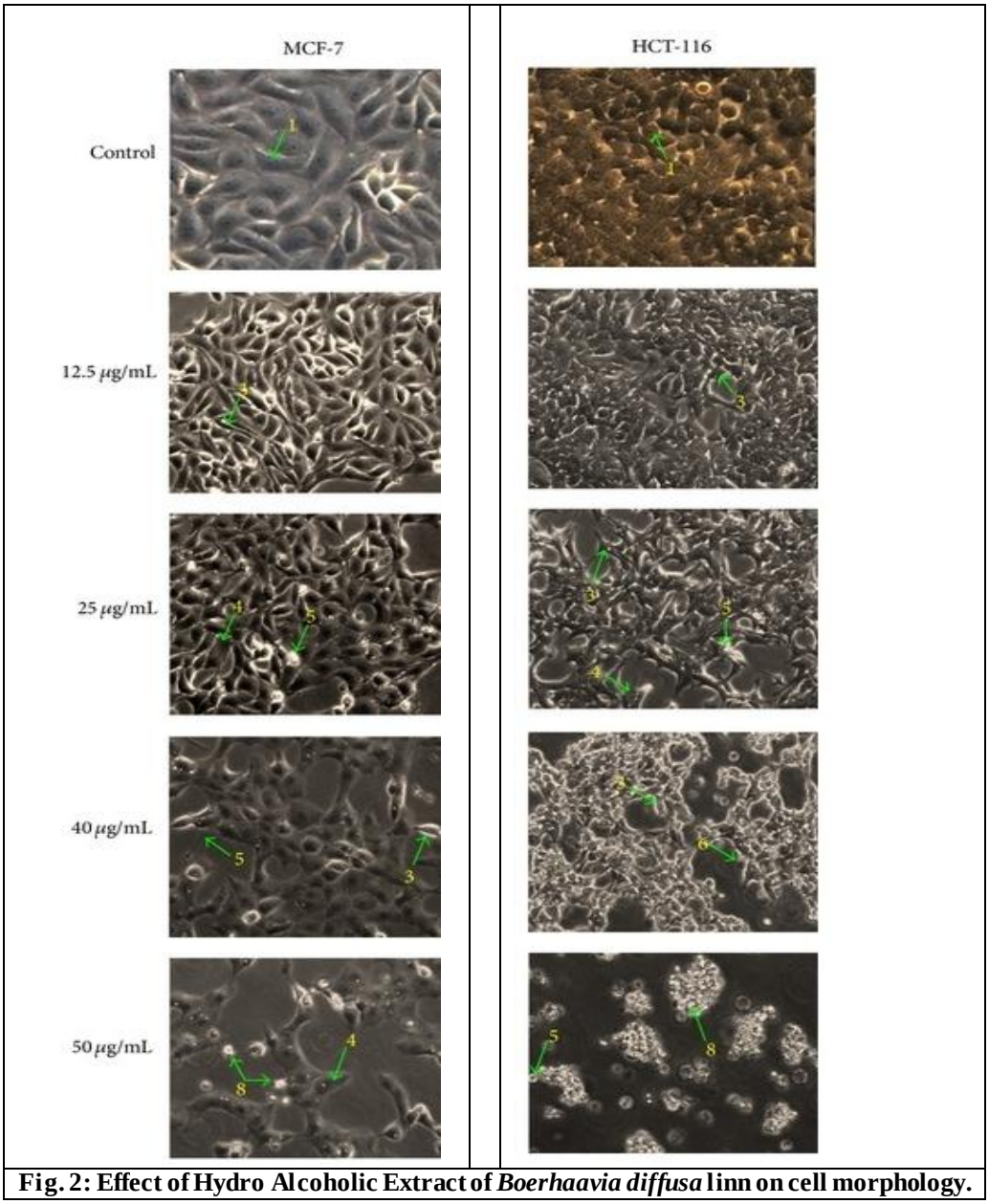
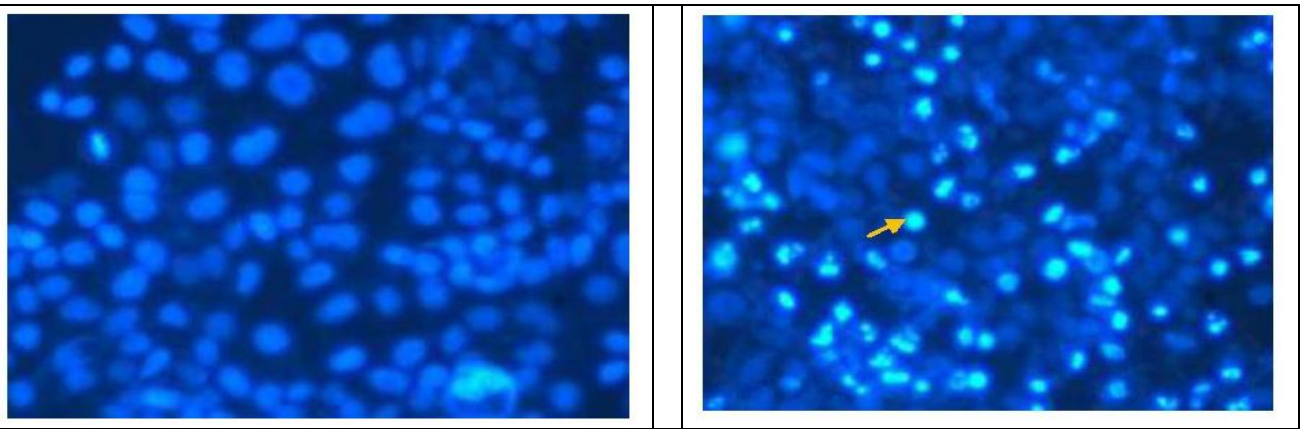


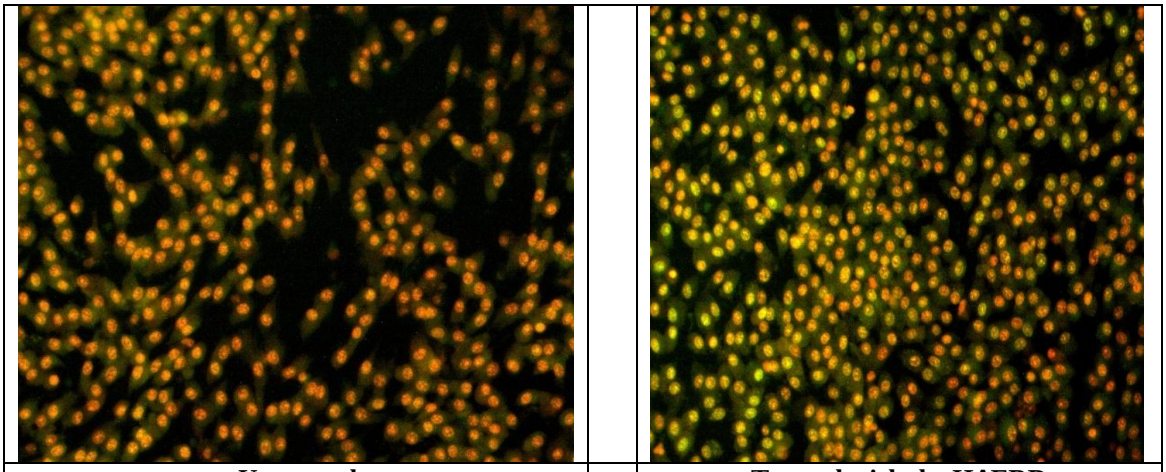
Fig. 2: Effect of Hydro Alcoholic Extract of *Boerhaavia diffusa* linn on cell morphology.

Hydro Alcoholic Extract of *Boerhaavia diffusa* linn on chromatin condensation & apoptosis effect on MCF-7 & HCT-cell line



Untreated	Treated with the HAEBD

Fig 3: Apoptosis effect of HAEBD on Treated and Untreated MCF-7 Cell lines.



Untreated		Treated with the HAEBD

Fig 4: Apoptosis effect of HAEBD on Treated and Untreated HCT-116 Cell lines

DISCUSSION

The hydro-alcoholic extract of *Boerhaavia diffusa* linn was subjected to ***in vitro* cytotoxic activity** on cell lines MCF-7 and HCT-116 by MTT assay, cell morphological studies, studies on chromatin condensation, apoptosis measurement. Measurement of cell viability and proliferation forms the basis for numerous *in vitro* assays of a cell population's response to external factors. MTT assay is used to measure the mitochondrial activity in viable cells based on the activity of mitochondrial dehydrogenase enzyme that reduces the yellow tetrazolium MTT into purple formazan crystal. The amount of the purple formazan formed indicates the number of metabolically active viable cells. (Twentyman. et, 1987) The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability were observed.

In cell morphological studies, untreated MCF-7 retained its normal angular or polygonal shape and has intact and large vesicular nuclei with prominent nucleoli. Most of the cells lost these features after treatment with increasing concentrations of the extract **(Fig.2).** Cell shrinkage with lesser cytoplasm mass and even apoptotic bodies were observed. These morphological changes of the cells observed after treatment with the extract has provided some insight on the effect of the extract against the HCT116.

In cancer therapy, one approach to suppress tumour growth is by activating the apoptotic machinery in the cell, (Fan. et.al, 1998) i.e. by the activation of an intrinsic cellular suicide programme when the cells are no longer needed or when they are seriously damaged.(Hannun, 1997) On **HAEBD** treatment MCF-7 cells exhibited condensed chromatin, apoptotic morphological changes with protrusion of plasma cell membrane In addition, it showed apoptotic bodies with cytoplasmic condensation indicating apoptosis like changes **(Fig.3 & 4)** Although significant results were demonstrated indicating that the **HAEBD** exhibited cytotoxic effect on colon cancer cell line, it should be noted that using MTT assay is not possible to differentiate between cell growth inhibition and an increase in cell death. A successful anticancer drug should kill or incapacitate cancer cells without causing excessive damages to normal cells and this is achievable by inducing apoptosis in cancer cells**.** (Lowe, 2000) These observations provide evidence that an apoptotic pathway was triggered with the **HAEBD** treatment in both cancer cell line MCF-7 & HCT-116. The loss of chromatin integrity may be due to activation of caspases.

CONCLUSION

This study concluded that hydro alcoholic extract of *Boerhaavia diffusa* linn exhibited marked cytotoxicity effect and significant morphological changes on MCF-7 and HCT-116 cell lines. The studies also indicated that the **HAEBD** primarily possessed apoptosis activity had shown promising *in vitro* anti cancer activity against MCF-7 and HCT-16 cell lines.

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