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Annihilating efficacy of *Euphorbia hirta* L. extracts on *Artemia salina* Nauplii and human breast adenocarcinoma cells (MCF-7 & MDA-MB-231)

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ABSTRACT

Cancer is a leading cause of death both in developed and developing countries. Recently the secondary metabolites produced by plants are being investigated due to their promising anticancer activities. Accordingly, in the current study the anti-cancer potentials of *Euphorbia hirta* L., a renowned medicinal plant was explored for its anticancer activity. The methanol and aqueous extracts of *Euphorbia hirta* L. (EHA and EHM) were tested for their toxicity against *Artemia salina* nauplii, and for cytotoxic potentials in MDA-MB-231 and MCF-7 human breast adenocarcinoma cell lines. Both the extracts EHA and EHM showed maximum toxicity towards *Artemia salina* among which the methanol extract was able to annihilate all the nauplii in its highest concentration. Excitingly, *Euphorbia hirta* L. extracts exhibited potent cytotoxicity selectively against human breast cancer cells (MDA-MB-231 and MCF-7) than in normal cells (VERO). In conclusion, the results suggest that EHM extract of the selected plant might possess beneficial ingredients to treat human breast cancers especially against ER-positive breast cancer.

KEYWORDS: *Euphorbia hirta* L., *Artemia salina*, selective-cytotoxicity, human breast cancer, MCF-7, MDA-MB-231

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INTRODUCTION

Cancer is well-known as a multifactorial disease due to abnormal proliferation of cells and is one of the major causes of death worldwide. Various genetic and epigenetic events create an accumulative phenomenon that lead to cancer development and progression (Wang *et al.*, 2001). Treatment of cancer is particularly challenging and it inflicts a severe burden on the public health system. Conventional chemotherapy is associated with anticancer drugs with low therapeutic index and results in severe side effects (Khandare *et al.*, 2006). Recent evidences suggest that natural products are good sources for the treatments of human diseases. Medicinal plants have been used to treat human diseases for centuries (Sharma and Moin, 2020). According to the World Health Organisation (WHO) few nations still rely on plant-based treatment as their main source of medicine (Parasuraman *et al.*, 2014). The history dates back to several years in using plants for their medicinal benefits, especially for cancer (Conforti *et al.*, 2008). Chemotherapy

seems to be promising where 65% of the drugs used are of natural origin (Cragg & Newman, 2005). Due to the above-said reasons, medicinal plants might prove potential to aid in the development of alternative therapy against cancer.

Euphorbia hirta L. is a very popular herb amongst practitioners of traditional herbal medicine and belongs to the Euphorbiaceae family. Traditionally, aqueous extracts of various *E. hirta* L. parts were noted to be used to treat various syndromes that include hay asthma, bowel disease, cough, bronchial disease, worm infestation, kidney stones, to decrease lactation, etc. They were also recorded for their benefits as a sedative, anxiolytic, analgesic, antipyretic, and anti-inflammatory agent. Furthermore, studies revealed that *E. hirta* L. possesses galactogenic, anti-anaphylactic, antimicrobial, antioxidant, anticancer, anti-feedant, antiplatelet aggregation, anti-inflammatory, aflatoxin inhibition, antifertility, anthelmintic, anti-plasmodial, anti-amoebic, antimalarial and larvicidal activities (Rajeh *et al.*, 2010). Based on the afore-mentioned

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literature, the aqueous and methanol extracts of *E. hirta* L. were investigated to identify potential anticancer properties.

MATERIALS AND METHODS

Plant Materials

The fresh plants of *E. hirta* L. was collected from its natural habitat from Indur village of Dharmapuri district (Tamil Nadu, India). Collected specimens were recorded by maintaining a voucher specimen (GACDPIEH1) in the laboratory for future references. Whole plants were subjected to shade drying and powdered coarsely in a mechanical mixer-grinder.

Preparation of Extracts

Hot extraction was performed in a soxhlet apparatus for the powdered plant material using methanol and water as solvents [sample (g): methanolic/aqueous] = 1:6 ratio]. Obtained extracts were then concentrated in rotavapor R-215 (BUCHI Labortechnik AG, Switzerland) under reduced pressure (72 mbar) at 40 °C to yield dry extracts. Concentrated extracts were then stored in a vacuum desiccator at room temperature until further use. The extracts were named as EHA (*Euphorbia hirta* L. aqueous) and EHM (*Euphorbia hirta* L. methanol).

Brine Shrimp Lethality Assay (BSLA)

Toxicity of the plant extracts were evaluated primarily on brine shrimp, *Artemia salina* nauplii. The lethality assay was performed following the method of Meyer *et al.* (1982). Briefly, eggs were obtained from STORI Salt Lake Artemia Cysts, Fish Cave Seller, Howrah, WB, India and stored at 4°C in a refrigerator until further use. 1g of the cysts was sprinkled into the glass container (1L capacity) containing sterile artificial sea water (prepared by dissolving 38g of sea salt in 1 litre of sterile water) for hatching. This setup was incubated at room temperature (25-29°C) for 24hrs with strong aeration and continuous light regime. Following which, newly hatched pink-coloured free-swimming nauplii were harvested from the bottom of the container. Different dilutions of the plant extracts for this assay were prepared by adopting the procedure of McLaughlin *et al.* (1998). 20mg of the aqueous and methanol plant extracts were separately dissolved in 2ml of suitable solvents (stock solution). These were further diluted to prepare a series of concentrations 1000, 100, 10, and 1ppm (µg/ml).

The assay system was prepared with 4.5ml of sterile seawater containing 0.5ml of the chosen concentration of the plant extracts in watch glass and transferred with 10 nauplii. The setup was allowed to remain for 24hrs, under constant illumination of florescent lamp and the number of survived and dead nauplii were counted and recorded with a hand lens. Larvae were considered dead if they did not exhibit any internal or external movement during several seconds of observation. Experiments were performed in triplicates including solvent and a negative control. Percentage mortality of the extracts was calculated using the formula: % Mortality= No. of dead nauplii/Total

No. of nauplii × 100. LC₅₀ values were estimated using a probit regression analysis (Finney, 1971) at 95% confidence intervals.

Cell Cultures and Maintenance

MDA-MB-231 (human breast carcinoma), MCF-7 (human breast carcinoma) and VERO (African Monkey kidney epithelial) cell lines were procured from National Centre for Cell Science (Pune, India). MDA-MB-231 cells were maintained in L-15 (Leibovitz's) culture medium, and MCF-7 and VERO were maintained in Minimum essential medium (MEM) (Eagle) with Non-essential amino acids, all with 10% fetal bovine serum in a humidified atmosphere at 37°C (with 5% CO₂ for MCF-7 and VERO only). The cell lines were maintained in their growing phase at 70% confluency with regular passaging.

Cytotoxicity Assay

MTT assay was performed as described by Mosmann (1983) with slight modifications. Briefly, a 96-well plate was seeded with a cell concentration of 10,000 cells from column 2-11 and each well were made up to 200µl using respective fresh medium. 200µl of fresh culture medium alone was loaded in control columns 1 and 12. All sides of the plate was sealed and incubated at 37°C in a humidified atmosphere for 24hrs. After 24hrs, existing medium was aspirated and wells were replenished with 200µl of media containing varying concentrations (12.5, 25, 50, 100, 200µg/ml) of plant extracts and incubated for 24hrs. After incubation, the extract medium was aspirated and fresh medium was added along with 10µl MTT reagent (final concentration of 0.45µg/ml). The plate was then covered with aluminium foil and placed for incubation in a humidified atmosphere for 4 hours at 37°C. After which, the medium with MTT was removed and 100µl Solubilization solution (DMSO) was added to each well to dissolve formazan crystals. Absorbance was recorded at 570 nm using a Dynex Opsys MR™ Microplate Reader (Dynex Technologies, VA, USA) with a 630nm reference filter. Wells containing cells without extract treatments served as the control. Percentage cytotoxicity was calculated by using the formula:

$$\% \text{ Cytotoxicity} = \frac{A_c - A}{A_c} \times 100$$

Where, A_c is the Absorbance of control cells and A is the absorbance of the extract treated cells

The IC₅₀ value was determined by using linear regression equation i.e. Y = Mx+C.

Here, Y = 50, M and C values were derived from the viability graph.

Statistical Analysis

All analyses were carried out in triplicates. For each assay, data were presented as mean ± SD from three independent experiments (n = 3). Statistical analyses were performed by

one-way ANOVA and significant differences between groups were determined at $P < 0.05$. To evaluate relationships between experimental parameters, results were analyzed for correlation and tested for significance by Student's t-test ($P < 0.05$). MATLAB ver. 7.0 (Natick, MA, USA), GraphPad Prism 5.0 (San Diego, CA, USA) and Microsoft Excel 2007 (Roselle, IL, USA) were used for the statistical and graphical evaluations.

RESULTS AND DISCUSSION

Cancer is the most common and deadly diseases in the modern era and in addition, adverse effects of synthetic anticancer drugs are the major limitations in treating cancer. In Asian cultures, medicinal plants are used traditionally to prevent and treat cancers. Medicinal plants have been reported as a good source for the discovery of new chemotherapeutic drugs with high efficiency and fewer side effects (Harvey & Cree, 2010).

Yield of extracts

Extraction is an important step for the detection of bioactive components from plant materials. With the aid of a solvent, desirable constituents are removed from those that are not required from the plant material (Dhanani *et al.*, 2017). In the present study, fifty grams of *E. hirta* L. whole plant powder upon soxhlet extraction resulted in a yield of 9.2g (percentage extract yield: 18.4% of dry weight) of aqueous (EHA) and 7.3g (percentage extract yield: 14.6% of dry weight) of methanol (EHM) crude extracts. The sample was further stored in a vacuum desiccator until further use.

Brine shrimp lethality bioassay

The brine shrimp lethality bioassay is widely used in the evaluation of toxicity of heavy metals, pesticides, plant extracts, etc. It's a preliminary toxicity screen for further experiments on mammalian animal models (Price *et al.*, 1974; Sorgeloos *et al.*, 1978). The brine shrimp assay has advantages of being rapid (24hrs), inexpensive, and simple. It easily utilizes a large number of organisms for statistical validation and requires no special equipment (McLaughlin *et al.*, 1998). Both methanol and aqueous extracts of *E. hirta* L. exhibited significant mortality against *Artemia* nauplii. The percentage mortality for *E. hirta* L. whole plant aqueous and methanol extracts were depicted in (Table 1 and Table 2). Accordingly, EHA exhibited percentage mortality ranging from 27% to 73% whereas, EHM exhibited 50% to 100% mortality at 1 μ g/ml to 1000 μ g/ml concentrations. EHM found to exhibit better mortality in *A. nauplii* when

compared to EHA with an LC_{50} of $1 \pm 5.4 \mu\text{g/ml}$ than the later $43.25 \pm 9.6 \mu\text{g/ml}$. As mentioned by Meyer (1982), both the plant extracts exhibited an LC_{50} value of less than 1000 $\mu\text{g/ml}$ which is considered toxic. The methanol extract (EHM) in its highest concentration (1000 $\mu\text{g/ml}$) had killed all the nauplii treated.

Selective-cytotoxicity of EHA and EHM towards breast cancer cells

The efficacy of *E. hirta* L. extracts to inhibit the proliferation of cancer cells were further evaluated using two cancer cells (MDA-MB-231 and MCF-7) and a normal cell (VERO). Both the extracts have exhibited significant cytotoxicity in cancer cells when compared to normal cell (Figure 1 and Figure 2). Similar results were also made by Weisburg *et al.* (2004), wherein selective destruction of cancer cells was demonstrated. Among the study extracts, EHM had lower IC_{50} values of 82.81 and 24.31 $\mu\text{g/ml}$ than EHA (92.86 and 53.08 $\mu\text{g/ml}$) in MDA-MB-231 and MCF-7 cells respectively. The extracts EHA and EHM were more effective in controlling the proliferation of MCF-7 cells rather than MDA-MB-231 cells. According to the American National Cancer Institute (NCI), the crude plant extract with an IC_{50} value $< 30 \mu\text{g/ml}$ indicates the promising plant material for further cytotoxic agent purification (Suffness and Pezzuto, 1990). Accordingly, the extracts, EHA and EHM had lower IC_{50} values in MCF-7 cells, close and within the range of reported values by NCI when compared to the values of these extracts in MDA-MB-231 cells. Moreover, VERO cells had recorded the maximum

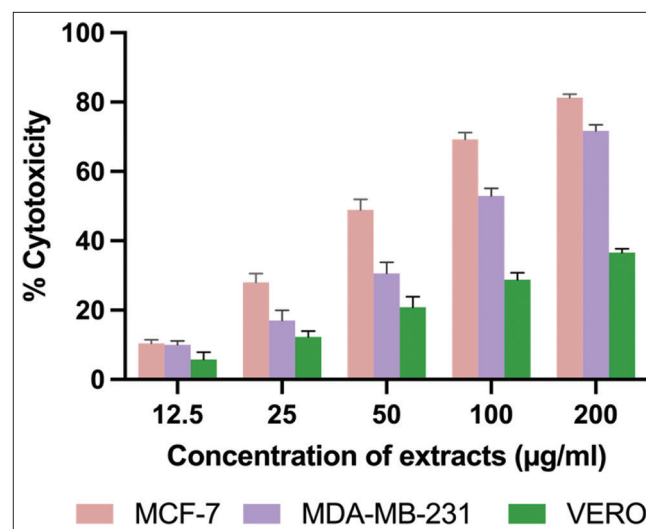


Figure 1: Cytotoxicity of EHA on MDA-MB-231 and MCF-7 breast adenocarcinoma cells, and VERO cell lines

Table 1: Percentage mortality and LC_{50} value of *A. salina* nauplii after 24hrs post exposure to the *E. hirta* L. whole plant aqueous extract (EHA).

Sl. No.	Conc. of Plant extract (ppm or $\mu\text{g/ml}$)	Log C Conc.	Number of Surviving Nauplii after 24hrs				Mean \pm SD	% Mortality	Probit % Mortality	LC_{50} ($\mu\text{g/ml}$)
			T1	T2	T3	Total				
1	1	0	8	7	7	22	7.33 ± 5.77	27	4.39	43.25 ± 9.56
2	10	1	6	5	3	14	4.67 ± 15.20	53	5.08	
3	100	2	4	3	4	11	3.67 ± 5.77	63	5.33	
4	1000	3	4	2	2	8	2.67 ± 11.50	73	5.61	

Note: *T=Trials (No. of nauplii taken in each trial = 10); There is no probit value for 0% and 100%

Table 2: Percentage mortality and LC50 value of *A. salina* nauplii after 24hrs post exposure to the *E. hirta* L. whole plant methanol extract (EHM).

Sl. No.	Conc. of Plant extract (ppm or µg/mL)	Log C Conc.	Number of Surviving Nauplii after 24 hrs				Mean ± SD	% Mortality	Probit % Mortality	LC50 (µg/ml)
			T1	T2	T3	Total				
1	1	0	6	4	5	15	5.00±10.00	50	5.00	1 ± 5.39
2	10	1	4	3	3	10	3.33±5.77	67	5.44	
3	100	2	2	2	3	7	2.33±5.77	77	5.74	
4	1000	3	0	0	0	0	0.00±0.00	100	0	

Note: *T=Trials (No. of nauplii taken in each trial = 10); There is no probit value for 0% and 100

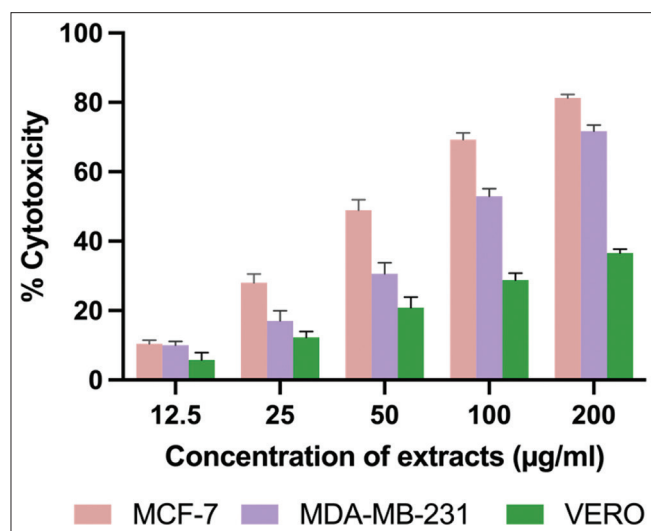


Figure 2: Cytotoxicity of EHM on MDA-MB-231 and MCF-7 breast adenocarcinoma cells, and VERO cell lines

IC₅₀ values, 689.32 and 226.69µg/ml for the extracts EHA and EHM, respectively which indicates significantly ($P < 0.05$) lower cytotoxicity in these cells. Hence, the results reveal that the *E. hirta* L. extracts were selectively targeting cancer cells and more specifically ER-positive breast cancer cells (MCF-7).

CONCLUSION

The study has revealed that the aqueous and methanol extracts of *E. hirta* L. plant had higher toxicity against the Brine shrimp, *A. salina* nauplii, where the methanol extract could annihilate all of the nauplii in its highest concentration. Both the extracts exhibited higher cytotoxicity in cancer cells (MCF-7 and MDA-MB-231) when compared to normal VERO cells. The results clearly indicate that both the extracts might possess beneficial therapeutic components specifically against ER-positive breast cancer with lesser damage towards normal cells. Hence, these extracts may further be exploited to isolate novel and potential sources of anticancer agents.

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