

Research Article

In-vitro Cytotoxic Activity of *Enicostemma axillare* Extract against HeLa Cell Line

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ABSTRACT:

This study was aimed for the evaluation of the anticancer activity of the whole plant of *Enicostemma axillare* on the *HeLa* cell line. The whole plant of *Enicostemma axillare* extract was tested for its inhibitory effect on *HeLa* cell line. The percentage viability of the cell line was carried out by using Tryphan blue dye exclusion method. The cytotoxicity of *Enicostemma axillare* on *HeLa* cell line in concentration range between 31.25 to 1000 µg/ml by using MTT assay. IC₅₀ values of *Enicostemma axillare* on *HeLa* cell line were 340 and 320 µg/ml respectively by MTT assay and Tryphan blue dye exclusion technique. From the performed assay, methanolic extract of *Enicostemma axillare* shows greater activity on *HeLa* cell line and that mean *Enicostemma axillare* can be used in anticancer activity.

Key words: Cytotoxicity, MTT assay, Tryphan blue dye exclusion technique, *Enicostemma axillare*, *HeLa* cell line.

INTRODUCTION:

Cancer is a general term applied to a series of malignant diseases which may affect many different parts of the body. If the process is not arrested, it may progress until it causes the death of the organism^[1]. Cancer is one of the major causes of death in developed countries, together with cardiac and cerebrovascular diseases^[2].

According to the American Cancer Society, on an average, 559,312 people die of the disease each year^[3] despite tremendous efforts to find methods of control and cure. Thus, not surprisingly, every fourth citizen of a developed country will be stricken sometime during his/her life and approximately 400 new incidents emerge per 100,000 people annually^[4, 5]. The statistics released by WHO in 2008 and GLOBOCAN indicate that there is a high likelihood of developing countries approaching the same incident rates of cancer as developed ones, because of life style changes, average age of the population, tobacco usage, etc^[6, 7]. In a scenario, where conventional medicine has failed to develop techniques that could reduce the incidence of death due to cancer, complementary and alternative medicine (CAM) is slowly emerging as an option. A variety of ingredients of traditional medicines and herbs are being widely investigated in several parts of the world to analyse their potential as therapeutic agents^[8, 9, 10]. According to the National Institute of Health's National Centre for Alternative and Complementary medicine, around 36% of people in the US use alternative medicine in some form or the other. Several studies indicate that a majority of cancer patients use CAM extensively as a mode of treatment or as a means to reduce the side effects of conventional treatment methods^[11, 12]. Cervical cancer forms an important part of cancer subtypes among women and is more common in Hispanic and

African-American women than in Caucasians. It is the fifth most common type of cancer among women with 471,000 new cases each year with 11,070 cases in US in 2008 and 3,870 deaths according to the data released by the National Cancer Institute^[14]. A *HeLa* cell is an immortal cell line used in medical research. The cell line was derived from cervical cancer cells taken from Henriette Lacks, who died from her cancer in 1951. Initially, the cell line was said to be named after a "Helen Lane" in order to preserve Lacks's anonymity^[14].

Drug discovery from medicinal plants has played an important role in the treatment of cancer and, indeed, most new clinical applications of plant secondary metabolites and their derivatives over the last half century have been applied toward combating cancer^[15, 16]. In this study, the methanolic extract of the plant *Enicostemma axillare* was prepared and their antiproliferative activity was screened against *HeLa* cell lines and compared with the standard. *Enicostemma axillare* is used in folk medicine for the treatment of various diseases such as antidiabetic, anti-inflammatory, stimulant, astringent, diuretic and useful in skin diseases. The present study was carried out to analyse the invitro cytotoxic activity of *Enicostemma axillare* through standard techniques such as MTT assay, Tryphan Blue dye Exclusion Technique.

MATERIALS AND METHODS:

Plant Material:

The whole plants of *Enicostemma axillare* were collected in fresh from Coimbatore region of Western Ghats, Tamil Nadu. Further identified by Botanical Survey of India (Southern Circle), Coimbatore. The plant was dried under shade then ground into a uniform powder using a blender and stored in polythene bags at room temperature.

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Table 1: Percentage of Cytotoxicity of the test sample against *HeLa* cell line by MTT assay.

Test Drug	Test Concn. (µg/ml)	% Cytotoxicity	CTC ₅₀ (µg/ml)
Methanolic Extract	1000	80.33±0.10	340
	500	79.64±4.12	
	250	33.09±2.92	
	125	17.91±0.96	
	62.5	14.50±3.30	
	31.25	14.08±1.19	

CTC₅₀ = Concentration of test drug needed to inhibit cell growth by 50%

Preparation of Extracts: A 15g of air dried plant powder was soaked in 150 ml of organic solvent, viz., Methanol for 24 hours in a round bottomed flask at room temperature. Extracts were filtered through the Whatman filter paper No.1. The filtrate was allowed to dry at room temperature and methanol extract was obtained. Condensed extract was weighed and stored in air-tight containers at 4°C till further investigation.

$$\% \text{ Growth Inhibition} = 100 - \left(\frac{\text{Mean OD of individual}}{\text{Mean OD of control group}} \right) \times 100$$

Anti-cancer assays:

Chemicals: Trypan blue, Fetal Bovine serum (FBS), Phosphate Buffered Saline (PBS), Dulbecco's Modified Eagle's Medium (DMEM) and Trypsin were obtained from Sigma Aldrich Co, St Louis, USA. EDTA, Glucose and antibiotics from Hi-Media Laboratories Ltd., Mumbai. Dimethyl Sulfoxide (DMSO) and Propanol from E.Merck Ltd., Mumbai, India.

Cell lines and Culture medium: *HeLa* cell lines, was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells of *HeLa* was cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO₂ at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm² culture flasks (Tarsons India Pvt. Ltd., Kolkata, India).

Preparation of Test Solutions: For cytotoxicity studies, each weighed test drugs were separately dissolved in distilled DMSO and volume was made up with DMEM supplemented with 2% inactivated FBS to obtain a stock solution of 1 mg/ml concentration and sterilized by filtration. Serial two fold dilutions were prepared from this for carrying out cytotoxic studies.

Determination of cell viability by MTT Assay: The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10⁵ cells/ml using DMEM containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) 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 microtitre plates. The plates were then incubated at 37°C for 3 days in 5% CO₂ atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37°C in 5% CO₂ atmosphere. The supernatant was removed and 100 µl of propanol 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 540 nm. The percentage growth inhibition and concentration of test drug needed to inhibit cell growth by 50% (CTC₅₀) values is generated from the dose-response curves for each cell line.

Determination of cell viability by trypan blue dye exclusion technique

The monolayer cell culture was trypsinized and the cell count was adjusted to 1.0 x 10⁵ cells/ml using DMEM medium containing 10% FBS. To each of 40mm petri dish, 1 ml of the diluted cell suspension (approximately 100,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was removed, washed the monolayer once with medium and 1 ml of different test concentrations of test drug was added on to the partial monolayer in culture dishes. The dishes were then incubated at 37°C for 3 days in 5% CO₂ atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 h, the drug solutions in the wells were removed and cells were trypsinized. The cells were suspended in PBS and centrifuged to separate cell pellet, resuspended in 1 ml of fresh medium and performed dye exclusion test i.e. equal quantity of the drug treated cells and trypan blue (0.4%) were mixed and left for a minute. It was then loaded in a haemocytometer and viable and non-viable count was recorded within two minutes. The percentage growth inhibition was calculated and CTC₅₀ value is generated from the dose-response curves for each cell line.

$$\% \text{ Growth Inhibition} = 100 - \left(\frac{\text{Total Cells} - \text{Dead Cells}}{\text{Total Cells}} \right) \times 100$$

RESULTS AND DISCUSSION

Cytotoxicity assays: See table

MTT assay: See table

Trypan blue dye exclusion assay

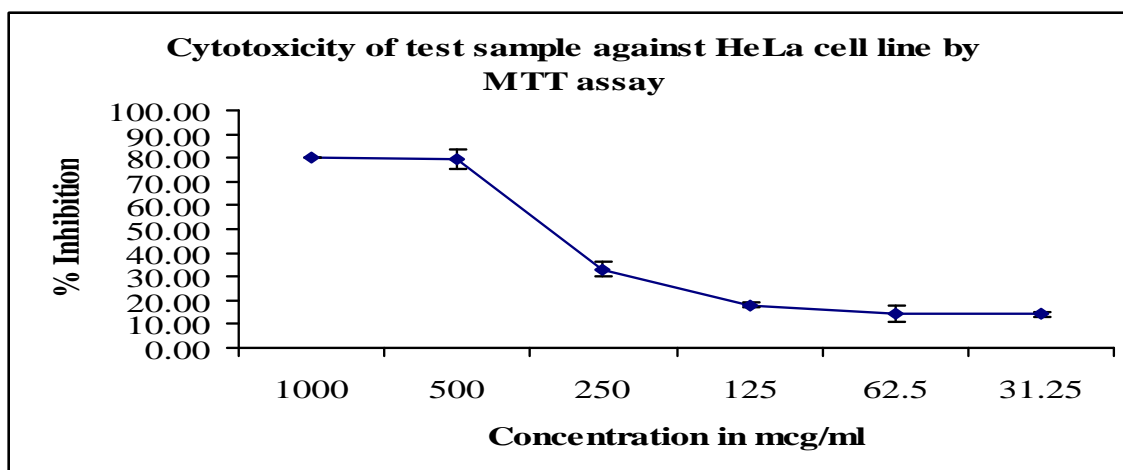


Fig. 1: Graphical representation of Cytotoxicity results. Cytotoxicity of test sample against HeLa cell line by MTT assay. CTC₅₀ value of the methanolic extract=340µg/ml

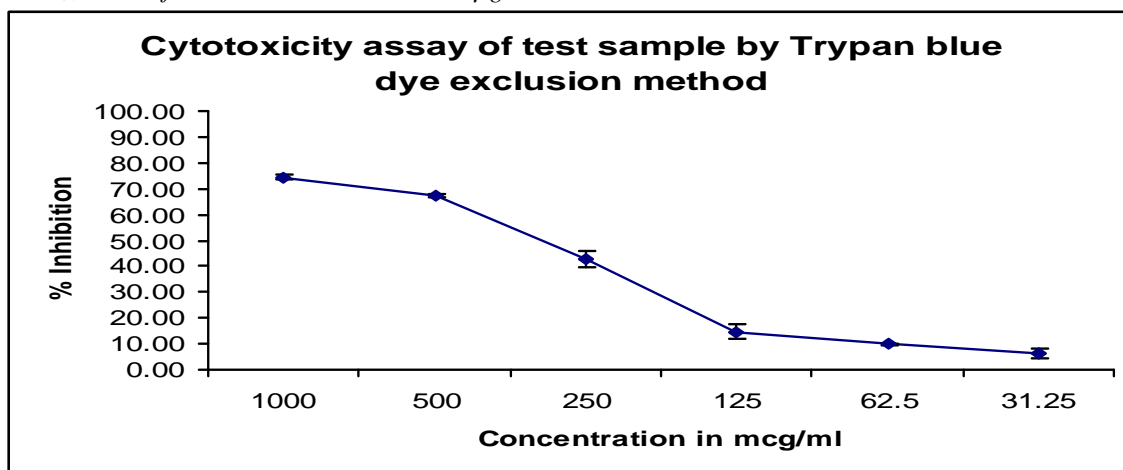


Fig. 2: Graphical representation of Cytotoxicity results. Cytotoxicity of test sample against HeLa cell line by trypan blue dye exclusion method. CTC₅₀ value of the methanolic extract=320µg/ml

Table 2: Percentage of Cytotoxicity of the test sample against HeLa cell line by Trypan blue dye exclusion Technique.

Test drug	Test Conc. In µg/ml	Viable cell Number		% Cytotoxicity		Mean% Cytotoxicity	CTC ₅₀ in µg/ml
		n1	n2	n1	n2		
Methanolic Extract	1000	10	11	75.00	73.81	74.41±0.84	320.00
	500	13	14	67.50	66.67	67.09±0.59	
	250	22	25	45.00	40.48	42.74±3.20	
	125	35	35	12.50	16.67	14.59±2.95	
	62.5	36	38	10.00	9.52	9.76±0.34	
	31.25	37	40	7.50	4.76	6.13±1.94	
	Control	40	42	0.00	0.00		

Complementary and alternative medicine (CAM) reports on multiple holistic approaches, including herbal medicines [17]. Recently, CAM has directed its interest towards therapies focused on important diseases throughout the world [18]. Drug discovery from natural sources is an area pertinent to CAM [19] and natural sources such as plants, animals and microorganisms provide a basis for the isolation of unique and potentially potent bioactive compounds [20]. Ethnopharmacologists can

therefore provide CAM practitioners with relevant new information on therapies from natural sources [20]. This information helps to establish modern CAM treatment modalities, which may offer efficacious treatment to large populations affected with different diseases including cancer [21].

The cytotoxicity study was carried out for plant extract of *Enicostemma axillare*. These extract was screened for its cytotoxicity against *HeLa* cell lines at different

concentrations were determined using the MTT assay and Tryphan Blue Dye Exclusion method. The MTT assays and tryphan blue dye exclusive method was presented respectively in figures 1 and 2 and the corresponding IC₅₀ are summarized in table 1 and 2.

In MTT assay, methanolic extract of *E.axillare* showed significant growth inhibitory effect. It was found that the % growth inhibition increases with the increasing concentration and IC₅₀ value of this assay was 340µg/ml. From Tab.1 it was showed that E.axillare has potential activity on *HeLa* cell and so this indicates that E.axillare may possess relative selective cytotoxicity to cervical cancer cells.

Percentage growth inhibition of *HeLa* cell line was carried out by using Tryphan blue dye exclusion technique. From Tab.2 it was shown that the IC₅₀ value of percentage growth inhibition was 320µg/ml. Fig 2 represent that when the concentration of the plant extract increases, there is a decrease in the number of cells. Tryphan blue dye exclusion method also shows significant effect on *HeLa* cell line.

CONCLUSION:

To conclude that *Enicostemma axillare* exhibits strong anticancer activity on *HeLa* cell line. So this drug has considerable anticancer activity on *HeLa* cell line.

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