

Evaluation of Antiproliferative Activity of *Trichosanthes dioica* Root Against Ehrlich Ascites Carcinoma Cells

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Abstract: *Trichosanthes dioica* Roxb. (Cucurbitaceae), called pointed gourd in English is a dioeciously climber plant grown in India and used traditionally for various medicinal purposes. Present study evaluated antiproliferative effect of hydroalcoholic extract from *T. dioica* root (TDA) on Ehrlich ascites carcinoma (EAC) cells *in vitro*. The cytotoxic activity of TDA (1 to 10 µg/ml) against EAC cells was assessed *in vitro* by trypan blue cell viability assay and MTT cell proliferation assay. TDA at all test concentrations exhibited significant ($p < 0.001$) increment in non-viable cells in trypan blue cell viability assay as compared to vehicle control; the percentage of non-viability increased up to a concentration of 4 µg/ml of TDA (48.45%), followed by decrease at higher concentrations. Similarly, in MTT cell proliferation assay, the percent cytotoxicity increased up to a concentration of 2 µg/ml of TDA (34.58%) followed by gradual decrease on increasing TDA concentrations. From the present study it can be concluded that the hydroalcoholic extract of *T. dioica* root demonstrated significant antiproliferative effect at lower concentrations against Ehrlich ascites carcinoma cells *in vitro*, thus suggesting the feasibility of its possible promise as natural anticancer agent.

Key words: *Trichosanthes dioica* • MTT • Cytotoxic • Viability • Root

INTRODUCTION

Cancer can be defined as a rapid, abnormal, uncoordinated proliferation of aberrant cells in any tissue or organ of the body which may mass together to form a growth or tumor, or proliferate throughout the body indicating abnormal growth at other sites. If the process is not arrested, it may progress until it causes the death of the organism [1]. Cancer is considered as one of the most fearsome causes of morbidity and mortality in all over the world. Although the disease has often been regarded principally as a problem of the developed world, more than half of all cancers occur in the developing countries [2]. Unfortunately, currently available cancer chemotherapeutic agents insidiously affect the host cells especially bone marrow, epithelial tissues, reticulo-endothelial system and gonads [3]. Most of the antineoplastic agents produce serious chronic or delayed toxicities that may be irreversible, particularly in heart, lungs and kidneys, thereby increasing morbidity [4].

Plants have a long history of use in the treatment of cancer. The approach for minimizing unwanted toxicity is to employ newer natural products that may act by different and distinct mechanism(s) and/or precipitate less serious adverse effects. A number of plant or other natural product extracts have been studied for anticancer activity leading to the development of several clinically useful anticancer agents [5]. Hence, the natural products now have been contemplated of exceptional value in the development of effective anticancer drugs with minimum host cell toxicity.

Trichosanthes dioica Roxb. (Cucurbitaceae), called pointed gourd in English, *Potol* in Bengali and *Patola* in Sanskrit, is a dioecious climber found wild throughout the plains of North and North-East India from Punjab to Assam and Tripura states of India. It is also commercially cultivated in India for its consumable fruits, a common culinary vegetable in India. In India, all parts of this plant have been traditionally used for various medicinal purposes. According to Ayurveda, the traditional system

of Indian medicine, its root is a strong purgative. The root has been traditionally used in India as purgative and as tonic, febrifuge, in treatment of jaundice, anasarca and ascites [6-9]. In our earlier studies, we have reported anthelmintic effects of leaf and root, antibacterial and antimitotic activities of the root of *T. dioica* [10-13]. The present study evaluated the influence of treatment of hydroalcoholic extract of *T. dioica* root on proliferation of Ehrlich ascites carcinoma (EAC) cells *in vitro*.

MATERIALS AND METHODS

Plant Material: The mature tuberous roots of *T. dioica* were collected during December 2008 from Majdia, Nadia district, West Bengal, India. The species was identified by Dr. M. S. Mondal, at the Central National Herbarium, Botanical Survey of India, Howrah, West Bengal, India and a voucher specimen (CNH/I-I/57/2009/Tech.II/493) was deposited at Pharmacognosy Research Laboratory, Bengal School of Technology, Delhi Road, Hooghly 712102, India. Just after collection, the plant material was washed thoroughly with running tap water and shade dried at room temperature (24-26°C) and ground mechanically into a coarse powder.

Drugs and Chemicals: Trypan blue and 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma-Aldrich Chemical Corporation, USA. All the other reagents used were of analytical reagent grade obtained commercially. Doubled distilled water from all-glass still was employed throughout the study.

Preparation of Extract (TDA): The powdered plant material (644 g) was macerated at room temperature (24-26°C) with 20% ethanol water (950 mL) for 4 days with occasional shaking, followed by re-maceration with the same solvent for 3 days. The macerates were combined, filtered and evaporated to dryness *in vacuo* (at 35°C and 0.8 MPa) in a Buchi evaporator, R-114. The dry extract (TDA, yield: 12.15%) was kept in a vacuum desiccator until use.

Standardization of TDA: TDA was subjected to preliminary phytochemical and planar chromatographic studies. Qualitative phytochemical analysis revealed the presence of reducing sugars, amino acids, triterpenoids and steroids in TDA [14]. Presence of cucurbitacin type triterpenoid aglycones in TDA was ascertained by planar chromatography on silica gel pre-coated high performance

thin layer chromatography (HPTLC) plates (Silica gel 60 F₂₅₄ Merck, Germany) detected with vanillin-phosphoric acid reagent [15]. TDA was dispersed in phosphate buffered saline as per required concentrations and sonicated for 10 min immediately prior to use in the bioassays.

Animals: Adult male Swiss albino mice of about 2 months of age weighing 20 ± 2 g were obtained from Laboratory Animal Centre, Department of Pharmaceutical Technology, Jadavpur University, Kolkata, India. The mice were grouped and housed in polyacrylic cages (38×23×10 cm) with not more than four animals per cage and maintained under standard laboratory conditions (temperature 25 ± 2°C, relative humidity 48%, with dark/light cycle 12/12 h). They were allowed free access to standard dry pellet diet (Hindustan Lever, Kolkata, India) and water *ad libitum*. The mice were acclimatized to laboratory conditions for 10 days before commencement of the experiment. All procedures described were thoroughly reviewed and approved by the University Animal Ethical Committee, Jadavpur University (Reg. no. 367001/C/CPCSEA).

Preparation of Tumor Cells: The transplantable murine tumor cell line namely Ehrlich ascites carcinoma (EAC) cells were obtained from the Chittaranjan National Cancer Institute (CNCI), Kolkata, India. The EAC cells were maintained in the ascitic form *in vivo* in Swiss albino mice by means of sequential intraperitoneal transplantation of 2×10⁶ cells/mouse after every 10 days. Ascitic fluid was drawn out from EAC bearing mouse 8 days after transplantation from the peritoneal cavity by aspirating the ascitic fluid into a sterile isotonic saline solution. The freshly drawn fluid was diluted with ice-cold sterile phosphate buffered saline (PBS, 0.2 M, pH 7.4) and the tumor cell count was adjusted to 2 × 10⁷ cells/mL by sterile PBS immediately before the studies.

In vitro Antiproliferative Evaluation

Cell Viability Assay: The viability of tumor cells was judged by trypan blue dye exclusion assay. Briefly, 0.1 ml of freshly prepared EAC cell suspension was mixed with various concentrations of TDA (1 to 10 µg/ml) in PBS resulting in a final volume of 1 ml and incubated at 37°C for 3 hours. PBS without the extract served as control. After the incubation the resultant dispersion was placed on the Neubauer counting chamber and the cells were then stained with trypan blue (0.4 % in PBS) dye. The cells that did not take up the dye were viable and those

took the dye were non-viable. These viable and non-viable cells in the 64 small squares were counted under the light microscope. The study was performed in triplicate and the results averaged [16].

Cell count = (No. of cells \times dilution factor) / (area \times thickness of liquid film).

% viable cells = $[1.00 - (\text{Number of blue cells} \div \text{Number of total cells})] \times 100$

Cell Proliferation Assay: The proliferation of tumor cells was judged by MTT proliferation assay. Briefly, 0.1 ml of freshly prepared EAC cell suspension was seeded in each well of 96-well microtiter plates. Cells were incubated with different concentrations of TDA (1 to 10 $\mu\text{g/ml}$) for 48 hrs at 37°C, 5% CO₂ with 98% relative humidity. The medium was replaced with fresh medium containing 100 $\mu\text{g/mL}$ of 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 4 hrs. The supernatant was aspirated and MTT-formazan crystals were dissolved in 100 μL of dimethyl sulfoxide (DMSO). Absorbance was measured at 570 nm by using an ELISA plate reader. Cell growth was calculated by comparing the absorbance of treated and untreated cells. The study was performed in triplicate and the results averaged [17].

Statistical Analysis: The data are presented as the mean \pm standard error of mean (SEM). The data of trypan blue cell viability assay were analyzed for statistical significance by Student's 't' test. *P* values less than 0.001 ($p \leq 0.001$) were considered as statistically significant.

RESULTS

The results of the present study revealed that TDA at all test concentrations exhibited significant ($p < 0.001$) increment in non-viable cells in trypan blue cell viability assay as compared to vehicle control but the percentage of non-viable cells were found to be increased up to a concentration of 4 $\mu\text{g/ml}$ of TDA, followed by a decrease i.e, on further increasing the TDA concentration, the non-viable cells were found to be decreased thereby demonstrating the maximum cytotoxic effect at the concentration of 4 $\mu\text{g/ml}$. The results are summarized in Table 1.

In case of MTT cell proliferation assay, the percent cytotoxicity increased up to a concentration of 2 $\mu\text{g/ml}$ of TDA, followed by a decrease i.e. further increase in TDA concentration led to gradual decrease in cytotoxicity

Table 1: Influence of TDA on EAC cells by trypan blue cell viability assay

Treatment	Concentration ($\mu\text{g/ml}$)	% Non-viable cells
Control	-	3.74 \pm 1.23
TDA	1	39.23 \pm 0.56*
TDA	2	46.32 \pm 0.78*
TDA	4	48.45 \pm 1.12*
TDA	6	35.12 \pm 1.07*
TDA	8	22.23 \pm 0.68*
TDA	10	14.18 \pm 1.25*

Data are mean \pm SEM ($n = 3$). * $p < 0.001$ compared to control

Table 2: Influence of TDA on EAC cells by MTT cell proliferation assay

Treatment	Concentration ($\mu\text{g/ml}$)	% Cytotoxicity
Control	-	-
TDA	1	26.65 \pm 0.66
TDA	2	34.58 \pm 1.20
TDA	4	29.13 \pm 0.84
TDA	6	20.45 \pm 0.71
TDA	8	17.23 \pm 1.16
TDA	10	16.81 \pm 0.95

Data are mean \pm SEM ($n = 3$)

thereby demonstrating the maximum antiproliferative effect at the concentration of 2 $\mu\text{g/ml}$. The results are presented in Table 2.

In both of the present bioassays, the effective concentration for 50% cytotoxicity (EC_{50} value) could not be determined as 50% effect was not observed in any case.

DISCUSSION

The present study attempted to assess the possible antiproliferative property of *T. dioica* root extract (TDA) against EAC cells *in vitro*. In the present study, the *in vitro* cytotoxicity of TDA against EAC cells was assessed by trypan blue cell viability assay and MTT cell proliferation assay.

Conventionally, the determination of cell proliferation or death is determined by counting viable cells after staining with a vital dye. It is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue, eosin or propidium, whereas dead cells do not. Trypan blue, a diazo dye, is a vital stain used to selectively colour dead tissues or cells blue. Live cells or tissues with intact cell membranes are not coloured. Since cells are very selective in the compounds that pass through the membrane, in a viable cell, trypan blue is not absorbed; however, it traverses the membrane in a dead cell. Hence, dead cells are shown as a distinctive blue colour under a light microscope.

Since live cells are excluded from this staining, hence, this staining method is also described as a dye exclusion method [16, 18].

Trypan blue staining is a simple and rapid way to evaluate cell membrane integrity (and thus assume cell proliferation or death) but the method is not sensitive and cannot be adapted for high throughput screening. Measuring the uptake of radioactive substances, usually tritium-labeled thymidine, is accurate but it is also time-consuming and involves handling of radioactive substances. Yellow MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole compound) is reduced to purple formazan in the mitochondria of living cells. The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 and 600 nm) by using a spectrophotometer. The absorption maximum is dependent on the solvent employed. This reduction takes place only when mitochondrial reductase enzymes are active and therefore conversion can be directly related to the number of viable (living) cells. When the amount of purple formazan produced by cells treated with an agent is compared with the amount of formazan produced by untreated control cells, the antiproliferative effectiveness of the agent in causing death of cells can be calculated [17, 19].

From the present study it is evident that the TDA is a moderately active cytotoxic agent *in vitro*, although failed to exert more than 50% death or cytotoxicity of EAC cells *in vitro* but definitely have significant effect in lower concentrations (1-4 µg/ml). The trypan blue dye exclusion assay showed higher cytotoxicity (maximum 48.45%) whereas the MTT proliferation assay exhibited comparatively lower cytotoxicity (maximum 34.58%). In higher concentrations however, inverse relationships were observed. The effects (increasing or decreasing) were found to be roughly concentration dependent. In both of the bioassays, at the concentrations below 1 µg/ml of TDA no appreciable results were observed. Significant activity was observed in lower test concentrations but after a certain concentration the expected increase in activity was not observed. The decrease in activity on increasing extract concentration requires further definitive studies.

The presence of triterpenoids and cucurbitacin aglycones was affirmed in TDA by qualitative phytochemical analysis and planar chromatographic studies (HPTLC). Cucurbitacins are known to possess several important biological activities including anticancer property [20]. The presence of putative cucurbitacin

aglycones could provide the chemical basis of its cytotoxic effect against EAC cells. Recently, the authors have reported antimitotic potential of *T. dioica* root by Allium test [13]. This notable property could be responsible for its antiproliferative activity against murine tumor cells *in vitro*.

From the present preliminary investigation, it can be concluded that the hydroalcoholic extract of *T. dioica* root demonstrated significant antiproliferative effect at low concentrations against Ehrlich ascites carcinoma cells *in vitro*, thus suggesting the feasibility of its possible use as natural anticancer agent. Further antitumor studies on *T. dioica* root using animal cancer cell lines *in vivo* are presently underway.

ACKNOWLEDGEMENT

The authors are thankful to the authority of Jadavpur University, Kolkata 700032, India, for providing necessary facilities for the present study.

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