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Evaluation of *in vitro* anticancer activity of stem of *Tinospora cordifolia* against human breast cancer and Vero cell lines

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Abstract
Tinospora cordifolia, also known as Giloy, Guduchi or Amrita, is used in the treatment of various diseases in the traditional medicinal system in India and is also an immune system modulator. In the current study, *in vitro* cytotoxic activity of 50% methanolic extract of stem of *Tinospora cordifolia* was evaluated against human breast cancer cell line MDA-MB-231 and normal Vero epithelial cell line. Methanolic extract of *Tinospora cordifolia* showed significant anticancer activity against MDA-MB-231 human breast cancer cell line. IC_{50} values of TC methanolic extract with respect to MDA-MB-231 were found to be 59 ± 4.05 µg/ml in 0.25% DMSO and 50± 2.01 µg/ml in 0.5% DMSO. Methanolic extract of *Tinospora cordifolia* was shown to possess cytotoxic activity against human breast cancer cells. The methanolic extract would be studied further for isolation and characterization of active components for lead optimization studies.

Keywords: Anticancer, cytotoxicity, *in-vitro*, MDA-MB-231, breast cancer, *Tinospora cordifolia*

1. Introduction
Plants are being used as indigenous cure in folklore or traditional system of medicine for treatment of various kinds of illness including cancer [1]. Recently, a greater emphasis has been given towards research on complementary and alternative medicine that deals with cancer management [2]. In traditional medicine, plants are being used for healing purposes and are effective as they contain biologically active components which are non toxic [3]. With an understanding of cell biology, mechanism based bioassays have become increasingly important and bio-activity guided phytochemical investigation has resulted in the isolation and characterization of several new molecules possessing interesting medicinal properties [4]. Ayurveda, a traditional sect of Indian system of medicine mainly based on plant drugs has been successful since very early times for preventing or suppressing ailments [5]. Plants represent the principal therapy in traditional medicine since time immemorial [6]. Early documentation about the use of medicinal plants has been mentioned in Discorides and Ayurveda [7]. Epidemiological studies suggest that consumption of diets containing fruits and vegetables which are the major sources of phytochemicals and micronutrients reduce the risk of developing cancer [8]. Certain products from plants are known to induce apoptosis in neoplastic cells but not in normal cells [9].
Tinospora cordifolia (TC) (Menispermaceae) also known as Giloy or Guduchi, is an important medicinal plant cultivated throughout the Indian subcontinent. Through centuries, it has been extensively use in various Ayurvedic preparations for the treatment of various ailments [10, 11]. There are several herbal preparations used in the indigenous system of medicines which can enhance the body's immune status. TC, used in several indigenous drug preparations for general health and other disease conditions, has been shown to possess immunomodulatory antiallergic [12] antidiabetic [13], antihepatotoxic [14] and antipyretic [15] properties.
TC has also been evaluated for its role as an anti-neoplastic agent [16]. It has also been found that TC can kill HeLa cells very effectively *in vitro* [17]. Anticarcinogenic and antimutagenic effect of TC has been evaluated in C57 Bl mice and Swiss albino mice [16]. Application of TC extract has been shown to significantly prevent micronucleus formation in bone marrow of mice, in a dose dependent manner and significant reduction of tumor as compared to control. The anticancer activity of TC has also been studied in Ehrlich's ascites carcinoma [18].
The current study involved a methodical evaluation of cytotoxicity and cell proliferation effects of TC in human cancer cells. The study revealed that methanolic extract of TC harbors

dose-dependent cytotoxic activity. As per National Cancer Institute (NCI, USA) for anti-cancer screening on a panel of human cell lines, an IC_{50} value of less than 100 µg/ml for medicinal plant extracts would be considered as potential anticancer agents and recommended for further isolation and characterization of bio-active molecules. The data suggest that TC methanolic extract exhibited potent cytotoxic activity with an IC_{50} value less than 100 µg/ml. Therefore, methanolic extract of TC may be considered for further isolation and characterization of bio-active molecules.

2. Materials and Methods

2.1 Reagents

0.4% Trypan blue, PBS (pH=7.2, 1X), 0.25% Trypsin-EDTA (1X), DMEM/F-12 (1X) (Dulbecco's Minimum Essential Medium) and Antibiotic (100X) were obtained from Gibco, Life Technologies; whereas FBS and MTT were from Himedia. Doxorubicin hydrochloride solution was purchased Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl Sulfoxide (DMSO) was purchased from Calbiochem. All other chemicals used in the study were of reagent grade.

2.2 Collection of plant material

Fresh stems of TC were collected and dried under the shade and then blended into fine powder with a grinder.

2.3 Sample Preparation

For experimental purpose, 25g of powder was extracted with 50% methanol (1:8). After 24 h, the upper layer of solvent was collected in a beaker and the procedure was repeated thrice in the interval of 24h continuously till the color of solvent disappeared. All extracts were pooled together and filtered using Whatman No.1 filter paper (125 mm). The filtered extracts were concentrated at 100°C in water bath. The semi-solid paste formed was transferred to a Petri plate and kept in hot air oven till it attained a powdered form. The total weight of powder was measured and stored in air tight container for further use. For biological studies, 20 mg of methanolic extract was dissolved in 10% and 50% DMSO respectively at a concentration of 20 mg/ml. The extracts were passed through 0.22 µm sterile Millipore syringe filter units (Fisher Scientific) prior to being used in cell culture studies.

2.4 Biological evaluation

2.4.1 Cell line

MDA-MB-231 (human breast carcinoma, ER^-, tumorigenic and invasive) and Vero (ATCC- CCL-81) normal kidney epithelial cell lines were obtained from the National Centre for Cell Science (NCCS), Pune, India, and as such, were maintained by sub-culturing and passaging as monolayers in 25 and 75 cm^2 cell culture flasks (Nest, Tarsons) at 37 °C in Tissue and Cell Culture Lab, Era's Medical College, Lucknow, in an incubator gassed with an atmosphere of 5% CO_2 at 95% humidity, in advanced Dulbecco's Minimum Essential Medium (DMEM) containing phenol red as a pH indicator and supplemented with 5% FBS. The medium, prior to being used in cell culture experiments was vacuum filtered using a Corning filtration system. The medium requires an atmosphere of 5% CO_2 to produce HCO_3 buffering capacity to maintain pH at 7.4 for normal cell growth.

2.4.2 Cell Culture

For experiments, cells were trypsinized and cultured in 6-well (0.5×10^5 cells/well) initially for 24 h, so as to allow the cells to attach. After 24h of incubation, the cells were exposed to 50 and 100 µg/ml of methanolic extracts of TC (in 50% DMSO) for the next 48h. Suitable untreated controls (containing 50% DMSO as vehicle) were also concomitantly employed. Each dose was tested in at least 3 replicate wells. Results were interpreted as cell viability *versus* time period graph.

2.4.3 Morphological Study

For morphological analysis, cells in 6-well plate were observed under phase contrast microscope & photographed (Nikon Eclipse Ti, Japan).

2.5 Cytotoxicity assays

2.5.1. Trypan blue dye exclusion assay

A cell suspension was made at a suitable dilution (1.0×10^5 cells/ml) in PBS. 50 µl of cell suspension was taken and mixed with an equal volume of 0.4% trypan blue. The solution was mixed thoroughly and allowed to stand for 5 min at room temperature. 50 µl of the solution was transferred to a hemocytometer and viable cells were counted as clear cells and dead cells as blue ones. The number of live cells per ml was calculated using the following formula: % viability = (live cell count/total cell count)*100.

2.5.2 (Methyl tetrazolium-MTT assay)

a. Determination of optimal cell number for assay

In order to determine optimal cell number required for the assay, serial dilutions of MDA-MB-231 (2,000, 4,000, 6,000, 8,000, 12,000, 14,000, 16,000 and 18,000 cells/100 l) were made in cell culture media and seeded in 96 well microtiter tissue culture plates (Linbro, MP Biomedicals). Cells were cultured, in a humidified 5% CO_2 incubator at 37°C for 24 h. At the end of the incubation period, 20 µl of MTT solution (Stock concentration, 5.0 mg/ml in PBS) was added to each well and incubated for 4h under the same conditions. Thereafter, medium containing MTT was gently replaced by 200 µl DMSO to dissolve formazan crystals and the absorbance values were read in an ELISA plate reader at (Biorad PW41) at 550 nm with a reference wavelength of 630 nm. A graph was plotted with the number of cells in X-axis and absorbance at 570/630 nm in Y-axis. Optimal cell density of cell line corresponding to absorbance values of 0.9 to 1.0 in the assay was selected for MDA MB 231 to facilitate measurement of both stimulation and inhibition of cell proliferation within the linear range.

2.6. Evaluation of cytotoxicity and cell viability

Briefly, MDA-MB-231 cells were trypsinized and resuspended in the culture medium to get a defined cell number for MDA-MB-231 (16,000/100 µl) in a 96-well microtiter tissue culture plate and cultured in a humidified 5% CO_2 incubator at 37°C for 24 h. Defined concentrations of the extracts in 10% and 50% DMSO were freshly prepared in culture media by serial dilution to get final concentration of 20, 40, 60 and 100 µg/ml (for TC methanolic extract). Serial dilution was carried out in cell culture media in such a way that the final concentration of DMSO in the well did not exceed 0.5% (v/v). Three control wells containing medium alone to serve as blanks were also included. After 24 h of incubation, cells were treated with the above-mentioned concentrations of TC extract in triplicates for 48h. Doxorubicin hydrochloride, an anticancer drug was used as a positive control. Equal volume of DMSO was used as a vehicle control. At the end of treatment, 20 µl of MTT (stock made in PSS at 5.0 mg/ml) reagent was added to each well and incubated for further 4 h. Thereafter, the culture medium was removed and formazan crystals were dissolved in 200µl of DMSO. The plates were read in a 96 well microplate reader (Biotek-ELx-800) at a wavelength of 570 nm with a reference

wavelength of 630 nm. Percentage cell viability (Y-axis) was calculated from absorbance and plotted against concentration in µg/ml (X-axis).

% Cell survival was calculated as = $\{(A_T-A_B)/(A_C-A_B)\}$ x100 where,

A_T= Absorbance of treatment well

A_B= Absorbance of blank

A_c=Absorbance of control well

% cell inhibition= 100-Cell Survival

IC_{50} values of the extracts were obtained from the graph as the concentration which decreased cell by viability 50%.

2.7 Comparison of Cytotoxic Activity of Extracts

The question whether TC extract-mediated cytotoxicity was selective to cancer cells, and not to normal cells was addressed by evaluating the effects of the methanolic extract of TC on normal epithelial cells (Vero) at the concentration that was cytotoxic to human breast cancer cells. Briefly, Vero cells were seeded (14,000/100 µl) in 96-well microtiter tissue culture well plates initially for 24 h and then treated with TC methanolic extract (50 µg/ml), and doxorubicin (0.50 µM) for the next 48 h. At the end of the treatment, cells were subjected to MTT assay. Percentage cell viability (Y-axis) was calculated from absorbance and plotted against concentration in µg/ml (X-axis).

2.8 Data interpretation and Statistical Analysis

Absorbance values that were lower than the control wells indicated a reduction in the rate of cell proliferation. Conversely, a higher absorbance value indicated an increase in cell proliferation. Rarely, an increase in proliferation might be offset by cell death; evidence of cell death was inferred from morphological analysis.

Results were expressed as mean ± SD of experiments done in triplicates.

3. Results

3.1 Methanolic extract of TC shows cytotoxicity against human breast cancers cells with less effect on normal cells

Cytotoxic effect on human cancer cell line MDA-MB-231 was studied by MTT assay (Fig. 1). Optimal cell densities corresponding to absorbance values of 0.9 to 1.0 in MTT assay were selected to facilitate measurement of inhibitions of cell proliferation within the linear range. The optimal cell number to be seeded for a cytotoxicity assay for MDA-MB-231, was determined from the plot was found to be 16,000 cells/100l respectively. In order to evaluate the cytotoxic effects of the methanolic extract of TC, MDA cells were treated with specified concentrations of the extract for 48 h. Doxorubicin was used as a positive control. The methanolic extract of TC showed cytotoxic and dose dependent inhibitory effects on human breast cancer cells MDA-MB-231. IC_{50} values of TC methanolic extract with respect to MDA-MB-231 were found to be 59 ± 4.05 µg/ml in 0.25% DMSO and 50± 2.05 µg/ml in 0.5% DMSO.

Figures 2 and 3 respectively depict the morphological analysis of untreated versus treated cells with respect to TC methanolic extract (50µg/ml). It is evident from the figures that TC showed cytotoxic effect on human breast cancer cells MDA-MB-231. The positive control, doxorubicin imparted cytotoxic and dose dependent inhibition of cell proliferation and the IC_{50} value of doxorubicin on MDA-MB-231 cells was found to be 0.50 ± 0.03 µM (Fig.4). The next question was to determine whether TC extract-mediated cytotoxicity was selective to cancer cells, and not to normal cells. This would be a highly desirable trait for a potential therapeutic anti-cancer agent. This question was addressed by determining the cytotoxic effect of bio-active TC methanolic extract on Vero normal kidney epithelial cell line. Results indicated that methanolic extract of TC possesses cytotoxic activity with an IC_{50} value of >100± µg/ml (Fig. 1c). However, the results revealed that the methanolic extract of TC failed to induce cytotoxicity in normal cells at the concentration that was cytotoxic to human breast cancer cells (Fig. 5).

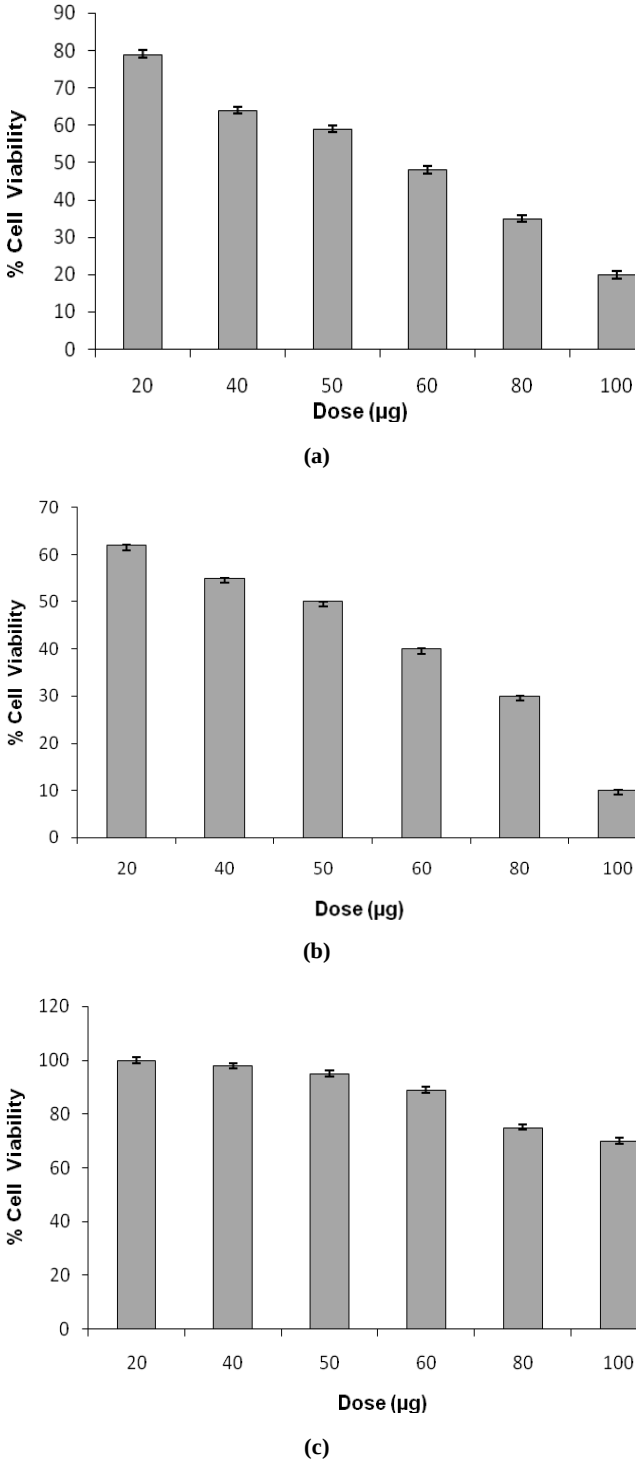
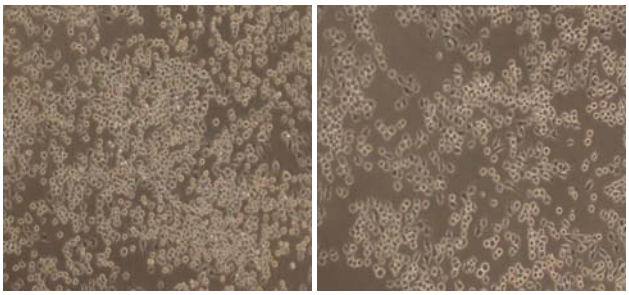
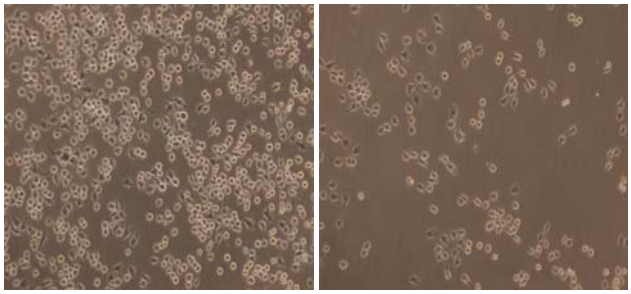


Fig 1: Dose dependent effect of TC methanolic extract (a) 0.25 % (b) and 0.5 % DMSO on viability of MDA cells (c) Dose dependent effect of TC methanolic extract in 0.5 % DMSO on viability of Vero cells in *vitro.*



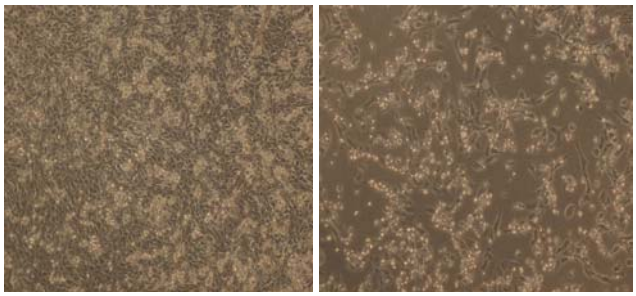
(a) **(b)**

Fig 2: (a). Control showing untreated MDA human breast cancer cells in presence of 0.25% DMSO and (b) Cytotoxic activity of TC methanolic extract (50 µg/ml) in 0.25% DMSO after 48 h (Magnification 10X).



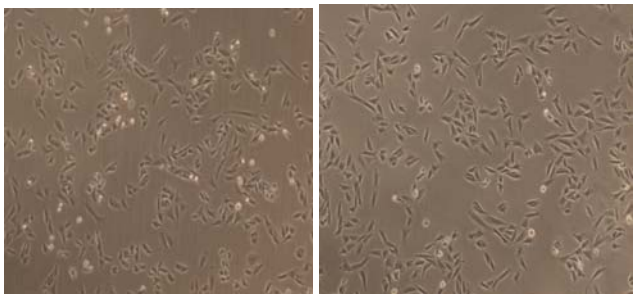
(a) **(b)**

Fig 3: (a). Control showing untreated MDA human breast cancer cells in presence of 0.5% DMSO and (b) Cytotoxic activity of TC methanolic extract at (50 µg/ml) in 0.5% DMSO after 48 h (Magnification 10X).



(a) **(b)**

Fig 4: (a). Control showing untreated MDA human breast cancer cells in presence of 0.5% DMSO and (b) Cytotoxic activity of doxorubicin chloride at 0.5µM in 0.5% DMSO after 48 h (Magnification 10X)



(a) **(b)**

Fig 5: (a). Control showing untreated normal epithelial cells (Vero) in presence of 0.5% DMSO and (b) in presence of TC methanolic extract at 50 µg/ml in 0.5% DMSO after 48 h (Magnification 10X).

4. Discussion

A few studies have reported anticancer activity of TC against some cell lines, but the cytotoxic activity of the methanolic extract is being reported for the first time [19]. Thus, this study revealed the enormous medicinal potential of stem of this plant. It would be our endeavor in future to determine the *in vitro* effect of TC methanolic, ethanolic and aqueous extract at higher doses on a panel of cancer and normal cell lines. This would be a highly desirable trait for a potential therapeutic anti-cancer agent.

The mechanism of action of ethanolic extract of TC on cancer cell lines has been reported previously [19]. Ethanolic extract of TC has been shown to induce apoptosis in breast cancer cells. Acridine orange-ethidium bromide and Hoechst 33342 assays by fluorescent microscopy have revealed that the ethanolic extracts of TC induce apoptosis, but not necrosis, in breast cancer cells. DNA fragmentation is a hall mark property of apoptosis [20] and DNA fragmentation assay has corroborated the fact that the ethanolic extracts of TC induce apoptosis in breast cancer cells [19].

Several anti-cancer drugs are known to cause their effects by blocking cell cycle [21]. Cancer cell cycle specific drugs have drawn considerable attention as they act on specific cancer cell cycle checkpoints (G_o/G_1 phase, S phase, G_2/M phase) and inhibit cancer cell proliferation (G_o/G_1 phase arrest), or DNA replication (diminished S phase) or mitosis (G_2/M phase arrest). Additionally, Sub-G_o DNA content in cells indicates apoptosis [22]. Cell cycle specific pharmacological effects of TC ethanolic extracts against human breast cancer cells have been investigated by propidium iodide based cell cycle analysis using flow cytometry. The data has revealed that TC ethanolic extracts cause significantly increased sub-G_0 phase indicating induction of apoptosis but without altering the cell cycle.

Anti-cancer drugs with minimal side effects on normal cells are highly desirable for therapeutic purposes [23]. Hence, the current study also addressed the question whether TC methanolic extract-mediated suppression of cell viability was selective to cancer cells. Cytotoxicity study against normal epithelial cell line (Vero) revealed that the methanolic extract of TC possesses less cytotoxic activity against non-cancerous cells. Agents that are capable of inducing selective apoptosis of cancer cells, without causing much harm to normal cells, have received considerable interest in the development of novel cancer chemotherapeutic drugs [24]. We found that concentrations of methanolic extract of TC that were cytotoxic to human breast cancer cells failed to induce apoptosis in Vero cells. In contrast, doxorubicin, a conventionally used anticancer drug, was found to have cytotoxic and apoptotic effects in Vero cells as well as in cancer cells at the same concentrations.

These results suggest that methanolic extract of TC possesses anti-cancer activity in human breast cancer cells with less cytotoxic effects against normal cells. Thus, this extract can be evaluated further for potential anticancer properties and isolation of bio-active phytochemicals.

5. Conclusion

This study showed that 50% methanolic extract of stem of TC is cytotoxic to the human breast cancer cell line MDA-MB-231.

6. Competing interests

The authors declare that they have no competing interests.

7. Authors' contributions

RA carried out experiments, analyzed and interpreted data and drafted the manuscript. ANS and MAK conceived experiments, study design and coordination. All authors read and approved the final manuscript.

8. Acknowledgements

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