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Anticancer evaluation of Tinospora *cordifolia* extracts

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Abstract
Cancer becomes a global disease and there is no proper treatment of cancer. Natural Products plays important role in the drug discovery of cancer. The plants Tinospora *cordifolia* (common name guloy) have been investigated by various *in vitro*. However, detailed investigations on anticancer properties of the selected medicinal plants are unknown. In this direction, we have studied the detailed anticancer activities of Tinospora *cordifolia* in cancer cells (A349, PC3, Cervical Cancer) using a cell proliferation assay and the ethanolic extract of plant showed potent inhibition against cancer cells (A549, Hela and PC3) compare to aqueous extract of Tinospora *cordifolia.*

Keywords: Tinospora *cordifolia*, cancer cell lines, A549, PC3, cervical cancer, Hela

Introduction
Chemotherapy aims at treatment of cancer by destroying the rapidly proliferating cancer cells. It is also called a systemic treatment as the drug enters through the bloodstream, travels throughout the body and kills cancer cells at their sites (Pluen *et al*., 2001) [12]. However, malignant tumors are often resistant to chemotherapy and even develop acquired chemoresistance or show multi-drug resistance as a consequence of the previous treatment (Gottesman, 2002) [4]. Among the cellular mechanisms proposed to mediate multidrug resistance, over expression of a family of plasma membrane efflux transporters, ATP-binding cassette (ABC) transporters, has received extensive investigation. It is observed that the over expression of these ABC transporters, predominantly by ABCB1 (MDR1), ABCC1 (MRP1) and ABCG2 (BCRP) (Shukla *et al*., 2008) [16] in cancer cells limits the intracellular accumulation of anticancer drugs for efficient activity through the active extrusion of the cytotoxic drugs. Classical multidrug resistance to plant based hydrophobic compounds has been shown to be due to the elevated expression of cell-membrane transporters, which result in an increased efflux of the cytotoxic drugs from the cancer cells, thus lowering their intracellular concentrations (Nobili *et al*., 2011) [8].
Plants are being used as indigenous cure in folklore or traditional system of medicine for treatment of various kinds of illness including cancer (Pandey *et al*., 2011) [11]. Recently, a greater emphasis has been given towards the research on complementary and alternative medicine that deals with cancer management (Sawadogo *et al*., 2012) [13]. In traditional medicine, plants are being used for healing purposes and are effective as they contain biologically active principles which are non-toxic (Duffy *et al*., 2012) [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 (Pan *et al*., 2012) [10]. Ayurveda, a traditional sect of Indian system of medicine mainly based on plant drugs had been successful since very early times for the prevention or suppressing ailments (Liu, 2011) [7]. Plants represent the principal therapy in traditional medicine since time immemorial (Ogilvie, 2003) [9]. Early documentation about the use of medicinal plants has been mentioned in Discorides and Ayurveda (Spitzer, 2011) [17]. 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 (Davis and Milner, 2010) [2]. The current study investigated, one indigenous medicinal plants, *Tinospora cordifolia* of the family Menispermaceae. The selection of plants were based on valuable information obtained from Ayurveda on anticancer properties, detailed ethno-botanical review, results obtained from the preliminary anticancer screening and contain potential anticancer agents like sesquiterpene lactones. The pharmacological activities of the selected plants, *Tinospora cordifolia* have been investigated by various *in vitro*. However, detailed investigations on anticancer properties of the selected medicinal plants are unknown. In this direction, we have studied the detailed anticancer activities of *Tinospora cordifolia* in cancer cells (A549, PC3, Cervical Cancer) using a cell proliferation assay.
Specific activity of *Tinospora cordifolia* in comparison with human normal epithelial cells.

Materials and Methods
Identification, collection and preparation of crude extracts of selected medicinal plant
The stem part of *Tinospora cordifolia* (Wild.) Hook. F. & Thomas of the family Menispermaceae and were collected from the Uttarakhand (India) in the month of January 2009 and were authenticated by Forest Research Institute Dehradun. The authenticated material Shade dried and coarsely powdered plant (2 kg) was extracted in a soxhlet apparatus for 72 hours using absolute ethanol as solvent. The crude ethanolic extracts were

concentrated in a rotary evaporator under reduced pressure and stored in a desiccator and dried completely. Similarly, for the preparation of aqueous extracts of selected medicinal plants, shade dried and coarsely powdered plant materials were extracted with double distilled water in a glass beaker by repeated warming at 50-60°C for 72 hours. Each extract was then filtered through whattman filter paper. Crude aqueous extracts were concentrated in a rotary evaporator under reduced pressure and stored in a desiccator and dried completely. The crude ethanolic and aqueous extracts were further dried by lyophilization. For the biological studies, crude ethanolic extracts were dissolved in DMSO (Sigma) at 20 mg/ml and aqueous extracts were dissolved in sterile MilliQ water at 10 mg/ml.

Drugs and Chemicals

Doxorubicin hydrochloride solution, Dulbecco's modified eagle medium (DMEM), Trypsin-EDTA, Hank's Balanced Salt Solution (HBSS), Thiazolyl Blue Tetrazolium Bromide (MTT), Acridine orange, Ethidium bromide, Hoechst 33342 (Bisbenzimide Trihydrochloride), Propidium iodide, RNase A, Agarose, HEPES buffer, Proteinase K, Nonidet P40, Tris-HCl, were purchased Sigma Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was purchased from Gibco, Invitrogen, USA. Dimethyl Sulfoxide (DMSO) was purchased from Sigma Aldrich, India. All other fine chemicals were obtained from Qualigens fine chemicals (Mumbai, India).

Cell culture

A349 (Lung Cancer carcinoma, tumorigenic and invasive), HeLa (Human cervical carcinoma, tumorigenic and invasive) and PC3 (Prostate Cancer cells) Cell line human were cultured in DMEM with high glucose supplemented with 10% FBS, 100 units/ml penicillin and 100 µg/ml streptomycin, in a humidified 5% CO_2 incubator at 37°C. Cells were cultured in healthy condition and exponentially growing cells were taken for the experiments.

Cytotoxicity assay (Methyl tetrazolium-MTT assay)

Determination of optimal cell number for assay

In order to determine optimal cell number required for the assay, serial dilutions of the A349, Hela, PC3, (2,000, 4,000, 6,000, 8,000, 12,000, 14,000, 16,000 and 18,000 cells/100 µl) were made in cell culture media and seeded in 96 well microtiter tissue culture plates. Cells were cultured, in a humidified 5% CO_2 incubator at 37°C for 24 hours. At the end of the incubation period, 20 µl of MTT solution (Stock concentration, 5.00 mg/ml in HBSS) was added to each well and incubated for 4 hours under the same conditions. Thereafter, medium containing MTT was gently replaced by 200 µl DMSO to dissolve formazan crystals and the absorbance values were measured by a microtiter plate reader (Biotek ELx800 - MS) at 540 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 densities of cell lines corresponding to absorbance values of 0.9 to 1.0 in the assay were selected for each of the cell lines such as A349, HeLa, PC3, facilitate measurement of both stimulation and inhibition of cell proliferation within the linear range.

Evaluation of Cytotoxicity

Briefly, specified cell types were trypsinized and resuspended in the culture medium to get a defined cell number for A349, PC3, HeLa in a 96-well microtiter tissue culture plate and cultured in a humidified 5% CO_2 incubator at 37°C for 24 hours. Defined concentrations of the extracts in culture media were freshly prepared by serial dilution to get final concentration of 12.5, 25, 50, 100 and 200 µg/ml (for *Tinospora cordifolia* ethanolic extract); 25, 50, 100 and 200 µg/ml (for *Tinospora cordifolia)* aqueous extracts. 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 0.50% (v/v). After 24 hours of incubation, cells were treated with above mentioned concentrations of *Tinospora cordifolia* extracts in triplicates for 48 hours. 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 was made in HBSS at 5.00 mg/ml) reagent was added to each well and incubated for further 4 hours. 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 630nm. Percentage cell viability (Y-axis) was calculated from O.D values and plotted against concentration in µg/ml (X-axis). The absorbance values of both test and the control (untreated cells) were used for calculating the percentage cell viability (% Cell viability = O.D of Test/O.D of Control x 100). Cell viability in untreated control was normalized to 100%. The dose response curve was plotted with concentration of the drug in the ordinate and percentage cell viability in the abscissa. IC_{50} values of the extracts were obtained from the graph as the concentration which decreases percentage cell viability to 50. IC_{50} values for *Tinospora cordifolia* ethanolic and aqueous extracts; doxorubicin hydrochloride were determined by nonlinear regression (curve fit) analysis by plotting log (inhibitors) vs. normalized response using software, Graph pad prism 8.

Results and Discussion

Tinospora *cordifolia* extracts inhibits cancer cell proliferation

Cytotoxic and growth inhibitory effects of the selected plant extracts on human cancer cells were studied by MTT assay. Optimal cell densities corresponding to absorbance values of 0.9 to 1.0 in MTT assay was selected for each of the cell lines to facilitate measurement of both stimulation and inhibition of cell proliferation within the linear range. The optimal cell number to be seeded for a cytotoxicity assay in, HeLa, determined from the plot and was found to be 14,000, 16,000, 6,000 and 12,000 cells/100 µl respectively. In order to evaluate the cytotoxic effects of the ethanolic and aqueous extracts of *Tinospora cordifolia* and in human lung cancer cells (A549), human cervical cancer cells (HeLa) and Human Prostate Cancer cell line). HeLa cells were treated with specified concentrations of the extracts for 48 hours. Doxorubicin was used as a positive control. The ethanolic extracts of *Tinospora cordifolia* showed cytotoxic and dose dependent inhibitory effects on cell proliferation of human lung cancer cells (A549) and cervical cancer cells (HeLa). IC_{50} values of *Tinospora cordifolia* ethanolic extract in HeLa cells were found to be 155.30 ± 6.48, Human Prostate Cancer cell line (PC3) 84.40 ± 2.68 µg/ml, Lung Cancer cell line 66.39 ± 3.08 µg/ml. Aqueous extracts of *Tinospora cordifolia* did not show any concentration dependent cytotoxicity or growth inhibitory activity in any of the cell lines (IC_{50} >300.0 µg/ml. Thus, among

the crude extracts screened for cytotoxicity and inhibition of cancer cell proliferation, *Tinospora cordifolia* ethanolic extracts demonstrated dose dependent anticancer activity against human lung cancer (A549) PC3 (Prostate cancer cell line) and cervical cancer cells (HeLa). The positive control, doxorubicin imparted cytotoxic and dose dependent inhibition of cell proliferation and the IC_{50} values of doxorubicin was found to be 1.25 ± 0.05 µM for A549 (human lung cancer cells), 0.70 ± 0.03 µM for Hela (Cervical Cancer cells) and 2.12 ± 0.14 µM for PC3 (Prostate cancer cells), respectively

Table 1: IC_{50} values of *Tinospora cordifolia* extracts IC_{50} µg/ml

Groups	A549	Hela	PC3
Tinospora cordifolia Etanolic Extacts	66.39±3.08	155.30±6.48	84.40±2.68
Tinospora cordifolia Aqueous Extracts	430.90±13.44	589.80±24.56	830.70±38.54
Doxorubicin	1.25±0.05	0.70±0.03	2.12±0.14

IC_{50} (Concentration of the drug required to reduce the percentage cell viability to 50 percentage) were obtained from the graph by non-linear regression analysis as best curve- fit values. IC_{50} values of Tinospora *cordifolia* extracts and standard anticancer drug, doxorubicin were compared with human Lung cancer cells (A549); human cervical cancer cells (HeLa) and human prostate cancer cells.

*****The IC_{50} of doxorubicin is represented as µM. Numerical data are means ± S.D of three independent experiments (n=3). Statistically significant difference from IC_{50} values for the control cells exposed to vehicle control (blank) ($p < 0.001$).

The next question was whether Tinospora *cordifolia* extract-mediated suppression of cell viability and growth was selectively to cancer cells, and not to normal cells. This would be a highly desirable trait for a potential therapeutic anti-cancer agent. Results indicated that ethanolic extract of *Tinospora cordifolia* possess cytotoxic and cell growth inhibitory effects with A549 66.39±3.08 (good Inhibitory effect) whereas Hela (human cervical cancer cells) 155.30±6.48 and PC3 (Prostate cancer cells), 84.40±2.68 were showed less inhibitory effect comparison to A549 (human lung cancer cells). However, cell viability was not significantly affected by *Tinospora cordifolia* ethanolic extract treatment at the concentrations that were cytotoxic to in human lung cancer cells (A549), human cervical cancer cells (HeLa) and Human Prostate Cancer cell line (PC3) cells. Moreover, an increased IC_{50} value for *Tinospora cordifolia* ethanolic extracts against Hela cells as compared to in human lung cancer cells (A549), Human Prostate Cancer cell line) and HeLa cells indicate less cytotoxic effects. Table.1 shows a comparison profile of IC_{50} values for *Tinospora cordifolia* extracts and the standard, doxorubicin in human lung cancer cells (A549), human cervical cancer cells (HeLa) and Human Prostate Cancer cell line). These data suggested *Tinospora cordifolia* ethanolic extracts do impart cancer cell-specific cytotoxic effects on lung cancer cells & prostate cancer cells. The data represent ± SD of three independent experiments (n=3).

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Conflict of Interest: Author shows no conflict of interest

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