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Research Article

IN-VITRO CYTOTOXICITY ACTIVITY OF SOLANUM NIGRUM EXTRACT AGAINST *HELA* CELL LINE AND *VERO* CELL LINE.

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ABSTRACT

The study was aimed to evaluation of the anticancer activity of the fruits of Solanum Nigrum on the *HeLa* cell line. The fruits of Solanum Nigrum methanolic extract were tested for its inhibitory effect on *HeLa* Cell Line. The percentage viability of the cell line was carried out by using Trypan blue dye exclusion method. The cytotoxicity of Solanum Nigrum on *HeLa* cell was evaluated by the SRB assay and MTT assay. Solanum Nigrum methanolic extract has significant cytotoxicity effect on *HeLa* Cell Line in concentration range between 10 mg/ml to 0.0196 mg/ml by using SRB assay and study also showed that inhibitory action on *HeLa* cell line in concentration range between 10 mg/ml to 0.0196 mg/ml by using MTT assay. IC_{50} value and R^2 value of Solanum Nigrum on *HeLa* cell and *Vero* cell were 847.8 and 0.8724, 9088 and 0.1017 respectively by SRB assay. IC_{50} value and R^2 value of Solanum Nigrum on *HeLa* cell was 265.0 and 0.9496 respectively by MTT assay. IC_{50} value of Solanum Nigrum on *Vero* cell was 6.862 by MTT assay. R^2 value of Solanum Nigrum was not found by MTT assay. From the performed assay, methanolic extract of these drug shows greater activity on *HeLa* cell line and little activity on *Vero* cell line and that mean Solanum Nigrum can be used as anticancer activity.

Keywords: Cytotoxicity Activity, SRB Assay, MTT Assay, *Solanum nigrum*, *HeLa* Cell Line, *Vero* Cell Line

INTRODUCTION

Since last many years, plants have beneficial activity in different type of diseases producing in human beings. As per WHO calculate that about 80% of the world's inhabitants problem should treated by medicinal herbal drug for their primary health care[1-2]. Plants have long history used in the treatment of cancer. Active constitutes of Catharanthus roseus, Angelica Gigas, Podophyllum peltatum, Taxus brevifolia, Podophyllum emodii, Ocrosia elliptica, and Campototheca acuminata have been used in the treatment of advanced stages of various malignancies[3]. There are various medicinal plants reported to have anti-cancer as well as anti-inflammatory activity in the Ayurvedic system of medicine. Solanum Nigrum is one of them with proven anti-cancer as well as anti-inflammatory activity[4-7].

Solanum Nigrum belongs to family solanaceae[8]. Commonly it is known as black night shade, makoy, deadly nightshade. It possesses medicinal properties like anti-microbial, anti-oxidant, cytotoxic properties, antiulcerogenic, and hepatoprotective activity[9-11]. Solanum Nigrum is a potential herbal alternative as anti-cancer agent and one of the active principles reported to be responsible for this action is Diosgenin[12-14].

A *HeLa* cell is an immortal cell line used in medical research. The cell line was derived from cervical cancer cells taken from Henrietta 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[15].

MATERIAL AND METHOD

Materials

Plant material collection

The fruits of Solanum Nigrum were collected from Hakeem Chichi Sons, Hakeem Chichi Street, Rani Talao, Surat, and Gujarat, India. All parts of plant were identified at Department of Biological Sciences; Veer Narmad South Gujarat University, Surat by Dr. Minoobhai Parabia, Dr. Ritesh Vaidh.

Cytotoxicity Screening

Cell line used:

African green monkey kidney Normal cell line (*Vero)*, Cervical cancer cell line *(HeLa*).

Table 1: Details of cell lines[16].

Cell line	Morph-ology	Origin	Species	Ploidy	Charecteristics	Supplier
HeLa	Epithelial	Cervix	Human	Aneuploid	G6PD type A	NCCS, Pune
Vero	Epithelial	Kidney	Monkey	Aneuploid	Viral subtract and assay	NCCS, Pune

Reagents

Trypan blue (Hyclone, Lot no: JRH27098), Sodium bicarbonate (MP Biomedicals, Lot No: 2048J), EDTA (MP Biomedicals, Lot No: 6941H), DPBS (Dulbecoo's phosphate buffer saline) (MP Biomedicals, Lot No: C1290), Trypsin (Invitrogen, Lot No: 1376596), SRB Dye, MTT Salt

Cell proliferation kit

MTT (Roche applied sciences, Cat. No. 11 465 007 001)

Media

DMEM (Dulbecoo's Modified Eagels medium, high glucose), DMEM (Dulbecco's Modified Eagels medium, low glucose), FBS (Fetal Bovine Serum) (Bioclot, Lot No: 07310)

Glasswares and plastic wares

96-well micro titer plate, Tissue culture flasks, Falcon tubes, Reagent bottles

Equipments

Fluorescence inverted microscope (Leica DM IL), Biosafety cabinet classII (Esco), cytotoxic safety cabinet (Esco), CO_2 incubator (RS Biotech, mini galaxy A), Deep freezer, ELISA plate reader (Thermo), Micropipettes (Eppendorff), RO water system (Millipore)

Methods

Preparation of plant extracts

Accurately weighed 5 gms of Solanum Nigrum powder was extracted with 25 ml methanol by stirring at 50^0 C for 1 hr. The filtered extract was concentrated under reduced pressure to remove the solvent. The extract was obtained by drying the concentrated pooled extract under vacuum[17].

Cytotoxicity Assay

Trypan blue dye exclusion technique

Principle

Trypan Blue is a blue acid dye that has two azo chromophores group. Trypan blue will not enter into the cell wall of plant cells grown in culture. Trypan Blue is an essential dye, use in estimating the number of viable cells present in a population[18].

Procedure

Make a cell suspension in a fixed volume of cells (e.g. 1ml). Although an aseptic

technique is not essential in all stages of this procedure, it is good laboratory practice to maintain sterility throughout the procedure. Take 50uL of cell suspension and mix it with an equal volume of trypan blue. Mix solution well using a pipette. Transfer to a hemocytometer and count the live cell as clear form and dead cell as blue cells. After staining with trypan blue solution counting should commence <5minutes as after that time the cells will begin to take up the dye. Using a pipette place some of the cell suspension: trypan blue mix into the hemocytometer and overlay with a coverslip. The cell suspension will pass under the coverslip by capillary action unless there is an air bubble. Make sure the wells are no overfilled and that the coverslip is not moved once it is place on the grid and the cell solution is added. Place the hemocytometer on the stage of an inverted microscope. Adjust focus and power until a single counting square fills the field. Calculate the number of cells per ml, and the total number of cells[19], using the following formula

Calculate percent viability by using formula:

% viability = (live cell count/total cell count)*100

Sulphorodamine B assay

Principle

Sulphorodamine B (SRB) is a bright pink Aminoxanthine dye with two sulfonic groups. Under mild acidic conditions, SRB binds dye to basic amino acid residues in TCA (Trichloro acetic acid) fixed cells to provide a sensitive index of cellular protein content that is linear over a cell density range of visible at least two order of magnitude[20-21].

Procedure

The monolayer cell culture was trypsinized and the cell count was adjusted to 0.5-1.0 x 10^5 cells/ml using medium containing 10% new born sheep serum. To each well of the 96 well microtitre plate, 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 hours, when a partial monolayer was formed, the supernatant was flicked off, washed once and 100 µl of different test compound concentrations were added to the cells in microtitre plates. The plates were then incubated at 37^{o}C for 72 hours in 5% CO_2 incubator, microscopic examination was carried out, and observations recorded every 24 hours. After 72 hours, 25 µl of 50% trichloroacetic acid was added to the wells gently such that it forms a thin layer over the test compounds to form overall concentration 10%. The plates were incubated at 4^{o}C for one hour. The plates were flicked and washed five times with tap water to remove traces of medium, sample and serum, and were then air-dried. The air-dried plates were stained with 100µl SRB and kept for 30 minutes at room temperature. The unbound dye was removed by rapidly washing four times with 1% acetic acid. The plates were then air-dried. 100 µl of 10mM Tris base was then added to the wells to solubilise the dye. The plates were shaken vigorously for 5 minutes. The absorbance was measured using microplate reader at a wavelength of 540nm[22]. The percentage growth inhibition was calculated using following formula,

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%cell inhibition= 100-{(At-Ab)/ (Ac-Ab)} x100

Where,

At= Absorbance value of test compound

Ab= Absorbance value of blank

Ac=Absorbance value of control

Microculture tetrazolium (MTT) assay

Principle

This Colorimetric assay is based on the capacity of Mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into an insoluble, colored formazan product which is measured spectrophotometrically[23-24]. Since reduction of MTT can only occur in metabolically active cells, the level of activity is a measure of the viability of the cells.

Procedure

The monolayer cell culture was trypsinized and the cell count was adjusted to 3-lakh-cells/ml using medium containing 10% newborn calf serum. To each well of 96 well microtitre plates, 0.1ml of diluted cell suspension was added. After 24 hours, when the monolayer formed the supernatant was flicked off and 100 µl of different test compounds were added to the cells in microtitre plates and kept for incubation at 37ºC in 5 % CO_2 incubator for 72 hour and cells were periodically checked for granularity, shrinkage, swelling. After 72 hour, the sample solution in wells was flicked off and 50µl of MTT dye was added to each well. The plates were gently shaken and incubated for 4 hours at 37^{o}C in 5% CO_2 incubator. The supernatant was removed, 50 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 490 nm[25]. The percentage growth inhibition was calculated using the formula below:

The percentage growth inhibition was calculated using following formula,

%cell inhibition= 100-{(At-Ab)/(Ac-Ab)}x100

Where,

At= Absorbance value of test compound

Ab= Absorbance value of blank

Ac=Absorbance value of control

Data interpretation

Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely, a higher absorbance rate indicates an increase in cell proliferation. Rarely, an increase in proliferation may be offset by cell death; evidence of cell death may be inferred from morphological changes.

%cell survival= {(At-Ab)/ (Ac-Ab)} x100

Where,

At= Absorbance value of test compound

Ab= Absorbance value of blank

Ac=Absorbance value of control

% cell inhibition= 100-cell survival

RESULTS AND DISCUSSION

In-vitro confirmation of their toxicity on *HeLa* and *Vero* cell lines. Percentage of viable cell can be obtained by performing trypan blue dye exclusion technique. The cytotoxicity activity is carried out by using SRB assay and MTT assay.

Viability and characterization of cell lines

Cell lines derived from NCCS, Pune were free from any kind of bacterial and fungal contamination.

Table 2: Percentage cell viability and characterization of cell line.

Cell line	% Viability	Live cell count	Total cell count	pH
VERO	81.13%	1.72*105	2.12*105	7.5
HeLa	70-72%	$1.728*10^5$	$2.40*10^5$	6.9

Percentage cell viability of cell lines were carried out by using Trypan blue dye exclusion technique. From the Table 2, it showed that the % viability of *HeLa* cell line & *Vero* cell line are 70-72% & 81.13% respectively, which are most suitable to perform cytoxicity studies.

Cytotoxicity activity:

The cytotoxicity study was carried out for plant extract of Solanum Nigrum fruits. These extract was screened for its cytotoxicity against *HeLa* and *Vero* cell lines at different concentrations to determine the IC_{50} (50% growth inhibition) by SRB assay and MTT assay.

Determination of Total Cell protein content by Sulphorhodamine B (SRB) assay

Table 3: Determination of cytotoxicity by SRB assay.

Plant Extract	Conc. mg/ml	*Hela*		IC_{50}	R^2	*Vero*		IC_{50}	R^2
		Absor-bance	% Inhibition			Absor-bance	% inhi-bition		
						0.113	49.80		
						0.148	91.14		
						0.104	39.17		
						0.118	55.70		
						0.172	120.07		
						0.204	157.28		
						0.250	212.20		
						0.128	66.929		
						0.160	105.31		
						0.228	186.22		

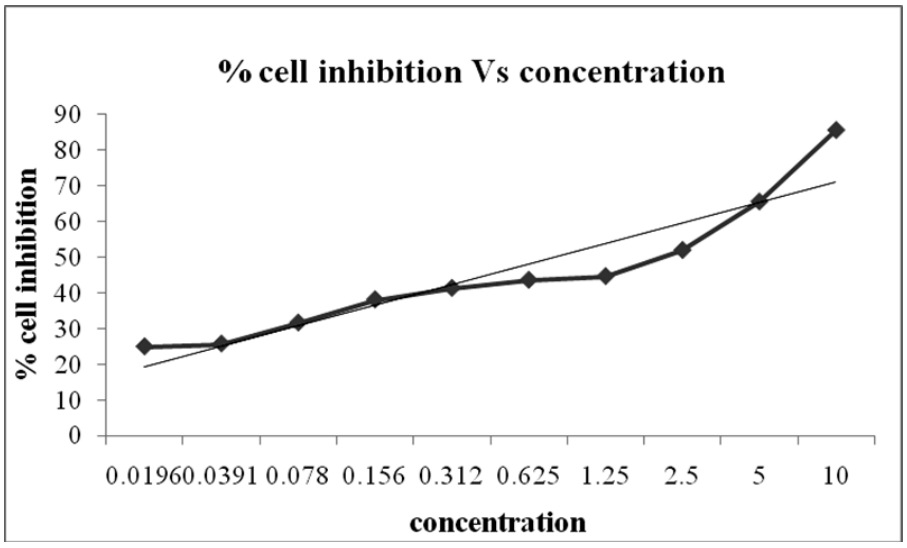

Fig. 1: DRC of methanolic extract of Solanum Nigrum for *HeLa* cell line by SRB assay.

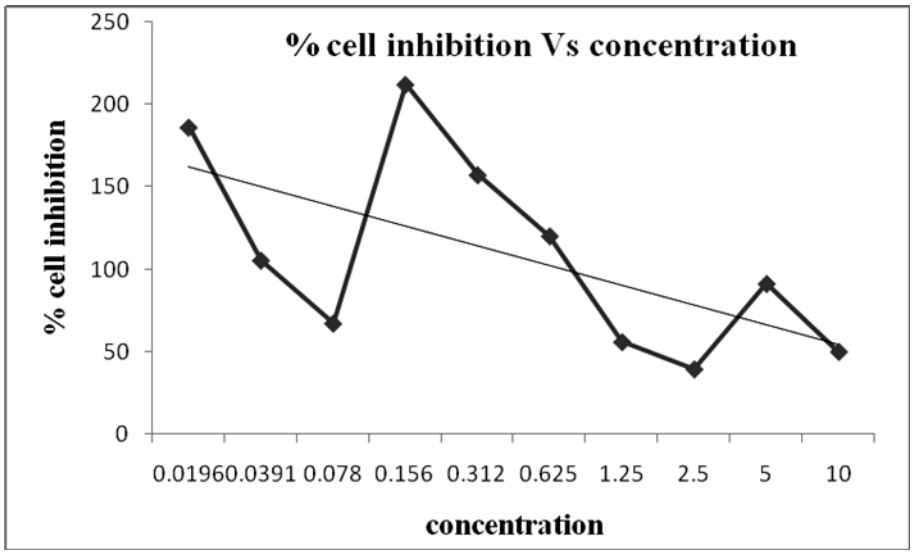

Fig. 2 : DRC of methanolic extract of Solanum Nigrum for *Vero* cell line by SRB assay.

Results are tabulated in Table 3 and graphically represented in Fig. 1 and Fig. 2. The percentage growth inhibition was found to be increasing with increasing concentration of test compounds, and that show in Fig. 1. Solanum Nigrum effect on *HeLa* cell line up to 0.0196 mg/ml (Table 3 and Fig. 1) and that IC_{50} value on *HeLa* cell line was 847.8 and R^2 value was 0.8724 while IC_{50} value on *Vero* cell line was 9088 and R^2 value was 0.1017 on *Vero* cell line.

Determination of Cytotoxicity by MTT assay

Table 4: Determination of cytotoxicity by MTT assay.

Plant extract	Conc. mg/ml	*Hela*		IC_{50}	R^2	*Vero*		IC_{50}	R^2
		Absor-bance	% inhi-bition			Absor-bance	% Inhi-bition		
	10	1.519	62.61			0.332	313.70		
	5	1.560	60.56			0.719	-99.46		
	2.5	1.62	57.54			1.080	-484.85		
	1.25	1.63	57.04			1.392	-817.97		
Solanum	0.625	1.658	55.62			1.935	-1397.68		
Nigrum	0.312	1.735	51.79	265.0	0.949	2.284	-1770.28	6.862	-
	0.156	1.745	51.29			2.271	-1756.40		
	0.078	1.918	42.66			2.205	-1685.40		
	0.0391	1.93	42.03			2.307	-1794.84		
	0.0196	2.005	38.28			2.259	-1743.59		

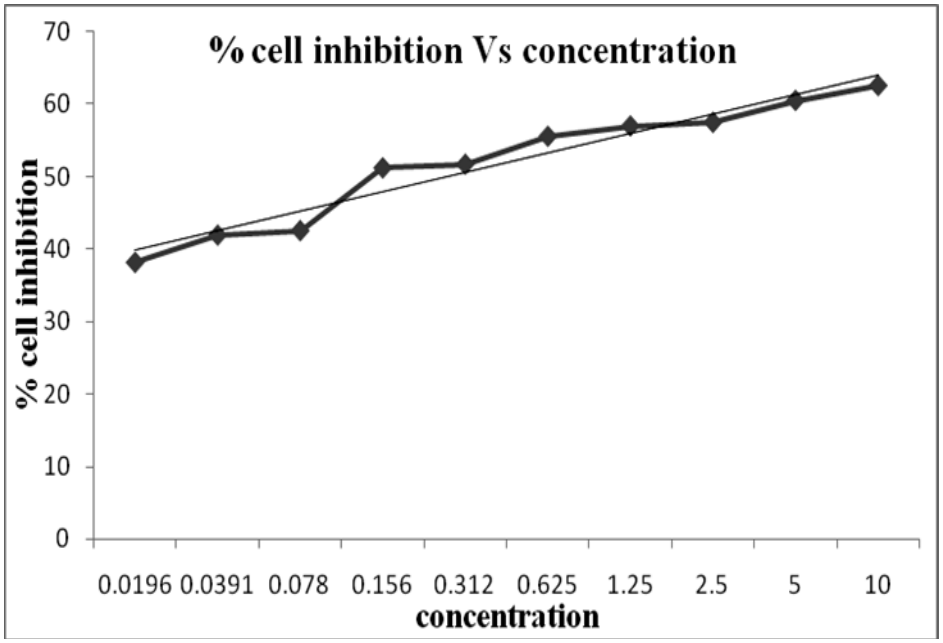

Fig. 3: DRC of methanolic extract of Solanum Nigrum for *HeLa* cell line by MTT assay.

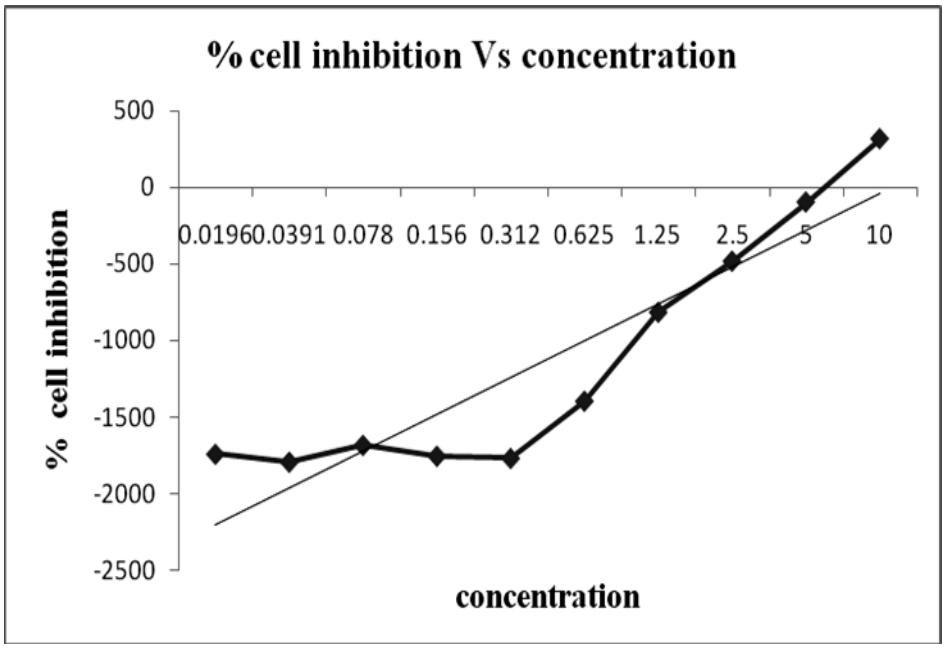

Fig. 4: DRC of methanolic extract of Solanum Nigrum for *Vero* cell line by MTT assay.

As per SRB assay *Solanum nigrum* shows considerable activity on *HeLa* cell and little beat effect on *Vero* cell, and these activity was checked by using second cytotoxicity assay, MTT assy. MTT assay also shows significant effect on *HeLa* cell and had little beat significant value on *Vero* cell.

The results are tabulated in Table 4 and

graphically represented in Fig. 3 and Fig. 4. It was found that the % growth inhibition increasing with increasing concentration steadily up to 0.0196 mg/ml on *HeLa* cell line and IC_{50} value of this assay was 265.0 and R^2 value was 0.9496. while in case of *Vero* cell, more fluctuation occur and so that IC_{50} value was 6.862e+008, and more difficulty was produce for find out R^2 value of these assay. Now overall study evaluate that Solanum Nigrum has potential activity on *HeLa* cell and less effect on *Vero* cell so these drug has considerable anticancer activity on cervical cancer.

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