

In Vitro Cytotoxicity Activity of *Semecarpus anacardium* Extract Against Hep 2 Cell Line and Vero Cell Line

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Abstract : Cancer is the major public difficulty and one of the top causes of death in the prosperous countries. Conventional plants are precious source of novel cytotoxic agents and are still in performance better role in health concern. The study was intended to estimation of the anticancer activity of Methanolic Extract of *Semecarpus anacardium* nut on the human epidermoid larynx carcinoma cell line (Hep 2). The Nuts of *Semecarpus anacardium* methanolic Extract was tested for its inhibitory effect in 96 micro plate formats against Hep 2 Cell Line. The percentage viability of the cell line was carried out by using Trypan blue dye exclusion method. The cytotoxicity of *Semecarpus anacardium* on Hep 2 cell was evaluated by the SRB assay. Cyclophosphamide was used as positive control. Dose response curves constructed between the ranges between 10 mg/ml to 0.0196 mg/ml for *Semecarpus anacardium*. IC₅₀ value and R² value of *Semecarpus anacardium* on Hep 2 cell was 468 µg/ml and 0.688 respectively by SRB assay. IC₅₀ value of *Semecarpus anacardium* on Vero cell was not found and R² value 0.008 by SRB assay.

Key words: Cytotoxicity activities, SRB assay, *Semecarpus anacardium*, Hep 2 cell line, Vero cell line.

Introduction

Cancer is the major public difficulty and one of the top causes of death in the prosperous countries. Most of the larynx carcinoma cancers are develops from the squamous cells, which are majority of the laryngeal epithelium. Cancer can develop in any part of the larynx, but location of the tumor affect cure rate. As per anatomical regions, the larynx is divided into three major parts: (a) Glottis, (b) Supraglottis and (c) Subglottis [1-5]. Most of the laryngeal cancers originate in the glottis part. Supraglottic cancers are less seen, and subglottic tumours are least frequent seen [1-7].

Conventional plants are valuable source of novel cytotoxic agents and are still in performance better role in health concern. Throughout history and crosswise the world, the plant kingdom has provided a diversity of medicines for cancer treatment. In current period, plants have been a source of Analgesics, Anti-inflammatories, Antiasthmatics, Antiarrhythmic agents, Antihypertensives Antimicrobial agents known to be frequent.

Semecarpus anacardium belongs to family *Anacardiaceae* and commonly well known as Bhilawa in Hindi. The Parts of these plants are nearly everyone

normally used in Ayurvedic system of medicine for various ailments, mainly alimentary tract and certain dermatologic conditions. It has potential action reported on heart, blood pressure, respiration, cancer and neurological disorders [8-10]. Chemically, it contains active principal of Bhilwanol, Jeediflavone, Semecarpuflavone, Gulluflavone, Anacardoside, Biflavanone and Anacardic acid.

Material Method

Plant material collection

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

Cell lines

African green monkey kidney Normal cell line (Vero), Human Epidermoid Larynx Carcinoma cell line (Hep 2).

Reagents

Trypan blue, Sodium bicarbonate, EDTA, DPBS (Dulbecco's phosphate buffer saline), Trypsin, SRB Dye, DMEM (Dulbecco's Modified Eagles medium, high glucose), DMEM (Dulbecco's Modified Eagles medium, low glucose), FBS (Fetal Bovine Serum)

Preparation of plant extracts

The nuts were shed dried for about 20 days and then subsequent to reduce coarse drug particle into fine powder using pestle and mortar. The extraction carried out by Soxhlet extraction techniques. Solvent was used consecutively with gradient polarity. The extract was evaporated to complete dryness by using vacuum distillation and kept in refrigerator for further use ^[12].

Subculture of adherent cell lines (Hep 2)

Cultures were viewed using an inverted microscope to assess the degree of confluency and the absence of bacterial and fungal contaminants was confirmed. Cell monolayer was washed with PBS without $\text{Ca}^{2+}/\text{Mg}^{2+}$ using a volume equivalent to half the volume of culture medium. Trypsin/EDTA was added on to the washed cell monolayer using 1ml per 25 cm^2 of surface area. Flask was rotated to cover monolayer with trypsin. Flask was returned to the CO_2 incubator and left for 2- 10 mins. The cells were examined using an inverted microscope to ensure that all the cells were detached and floated. The cells were resuspended in a small volume of fresh serum containing Hep-2 medium. 100-200 μl was removed to perform a cell count. The required number of cells were transferred to a new labeled flask containing pre-warmed Hep-2 medium and incubated as appropriate for the cell line.

Trypan blue dye exclusion technique

Trypan Blue is a blue acid dye that has two azo chromophores group. Trypan Blue is an essential dye, use in estimating the number of viable cells present in a population ^[13]. Wash hemacytometer and coverslip with the help of 70% Isopropanol and blot dry with kimwipe. Observe hemacytometer and coverslip under microscope to check that are clean or not. Mix culture sample to resuspend cells. Take 20 μl of cell culture sample and fill into sterile microfuge tube. Add 20 μl of 0.4% Trypan Blue Solution into the same tube. Mix well above solution by gently aspirating and dispensing the solution with the help of micropipette ^[14]. Fix the coverslip on the centre top of the hemacytometer. The metal notches should be partially exposed. Take 10 μl mixture of the cell culture/Trypan Blue mixture from microfuge tube with the help of micropipette. Hold the micropipette straight up and dispense the 10 μl of the cell/Trypan Blue solution into a notch of the hemacytometer. Put hemacytometer assembly on microscope stage for examines. Focus light on the hemacytometer grid using 100 X magnification, observed live cells as clear, and dead cells as blue in colour. These is the most important part in these technique, there are four main in haemocytometer and that are subdivided into 16 squares.

Beginning with quadrant 1 and moving through to quadrant 4, count the cells in a serpentine fashion: work left to right across the top row of the quadrant. Move down to the second row and count the cells in each square moving right to left. Change to opposite direction each time a row is completed. Record the number of live and dead cells. Formula to determine live cell count:

$$C = (N/V) \times D$$

Where,

C=live cell count in cells per milliliter

N = total number of live cells counted in the four main quadrants

V = volume of counting area. The total volume of the four quadrants is 0.0004mL. (Each quadrant is 0.0001mL.)

D = dilution factor.

Calculate percent viability by using formula:

$$\% \text{ viability} = (\text{live cell count}/\text{total cell count}) \times 100$$

Sulphorodamine B assay

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 ^[15-16]. The monolayer cell culture was trypsinized and the cell count was adjusted to 0.5-1.0 x 10⁵ 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°C for 72 hours in 5% CO_2 incubator and 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 a overall concentrations 10%. The plates were incubated at 4°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 100 μl 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 solubilize the dye. The plates were shaken vigorously for 5 minutes. The absorbance was measured using microplate reader at a wavelength of 540nm ^[17].

The percentage growth inhibition was calculated using following formula,

$$\% \text{ cell inhibition} = 100 - \left\{ \frac{(At - Ab)}{(Ac - Ab)} \right\} \times 100 \dots \dots \dots [1]$$

Where,

At= Absorbance value of test compound

Ab= Absorbance value of blank

Ac=Absorbance value of control

Results and Discussion

In vitro Confirmation of *Semecarpus anacardium* toxicity on Hep 2 and Vero cell lines. Percentage of viable cell can be obtained by performing Trypan blue dye exclusion technique. The cytotoxicity activity was carried out by using SRB assay. The effect of Methanol extract of *Semecarpus anacardium* nut (test) and cyclophosphamide (control) on the growth of Hep 2 cell line was examined by SRB assay. Dose response curves constructed between the range 10 mg/ml and 0.0196 mg/ml for *Semecarpus anacardium*, express decreasing number of viable cells with increasing concentration of extract. Calculation of IC₅₀ and R² value was done using graphs generated from Microsoft excel 2003 edition. (Figure 1 and Figure 2). The susceptibility of cells to the extract exposure was characterized by IC₅₀ values (Table 3). Results indicate that the antiproliferative effect strengthens with increase in the concentration of extract.

Viability and Characterization of Cell Lines

Cell lines obtained from NCCS, Pune were free from any kind of bacterial and fungal contamination. Percentage cell viability of cell lines was carried out by using Trypan

blue dye exclusion technique. From the table 2, it showed that the % viability of Hep 2 cell line & Vero cell line are 87.5% & 81.13% respectively, which are most suitable to perform cytotoxicity studies.

Cytotoxicity activity

The cytotoxicity study was carried out for plant extract of *Semecarpus anacardium* nut. *Semecarpus anacardium* extract was screened for its cytotoxicity against Hep 2 and Vero cell lines at different concentrations to determine the IC₅₀ (50% growth inhibition) by SRB assay.

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

Results are tabulated in table 3 and graphically represented in Figure 1 and Figure 2. The percentage growth inhibition was found to be increasing with increasing concentration of test compounds, and that show in Figure 1 *Semecarpus anacardium* effect on Hep 2 cell line upto 0.0196 mg/ml (Table 3 and Figure 1, 2) and that IC₅₀ value on Hep 2 cell line was 468, while *Semecarpus anacardium* has no significant action on Vero cell line, so difficulty occur in find out IC₅₀. That means *Semecarpus anacardium* has no effect on normal healthy body cell So *Semecarpus anacardium* second-hand as antitumour action to diminish the side effect. If drug has effect on Vero cell line that denote it cause on normal healthy body cell and turn out side effect. While in casing of *Semecarpus anacardium* superior result on Hep 2 cell but not effect on Vero cell. So its give anticancer activity.

Table 1: Details of Cell Lines ^[11]

Cell line	Morphology	Origin	Species	Ploidy	Charechteristics	Supplier
Hep 2	Epithelial	Larynx	Human	polyploid	Plating efficiency, 44% Hela like	NCCS, Pune
Vero	Epithelial	Kidney	Monkey	Aneuploid	Viral substract and assay	NCCS, Pune

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*10 ⁵	2.12*10 ⁵	7.5
Hep 2	87.5%	1.75*10 ⁶	2.0x10 ⁶	7.5

Table 3: Determination of Cytotoxicity by SRB assay

Plant Extract	Conc. mg/ml	Hep 2		Ic ₅₀	R ²	Vero		Ic ₅₀	R ²
		Absor-bance	% Inhibition			Absor-bance	% Inhi-bition		
<i>Semecarpus anacardium</i>	10	0.032	130.56	468	0.688	0.22	126.54	-	0.008
	5	0.069	110.53			0.07	10.38		
	2.5	0.0703	99.26			0.09	23.08		
	1.25	0.0852	88.35			0.12	46.92		
	0.625	0.0934	66.34			0.26	153.08		
	0.312	0.10	43.65			0.14	61.54		
	0.156	0.103	36.45			0.23	131.15		
	0.078	0.125	23.12			0.15	67.31		
	0.0391	0.132	12.56			0.18	93.46		
	0.0196	0.134	10.23			0.25	145.77		

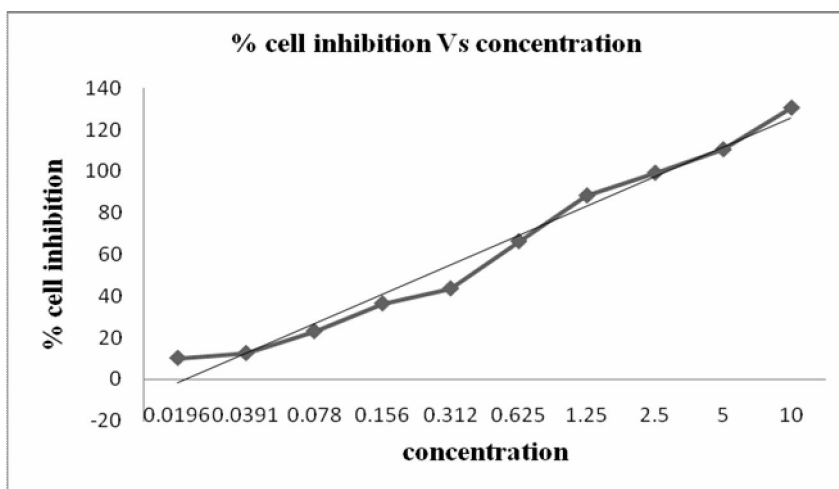


Figure 1: DRC (Drug response curve) of Methanolic extract of *Semecarpus anacardium* for Hep 2 cell line by SRB assay.

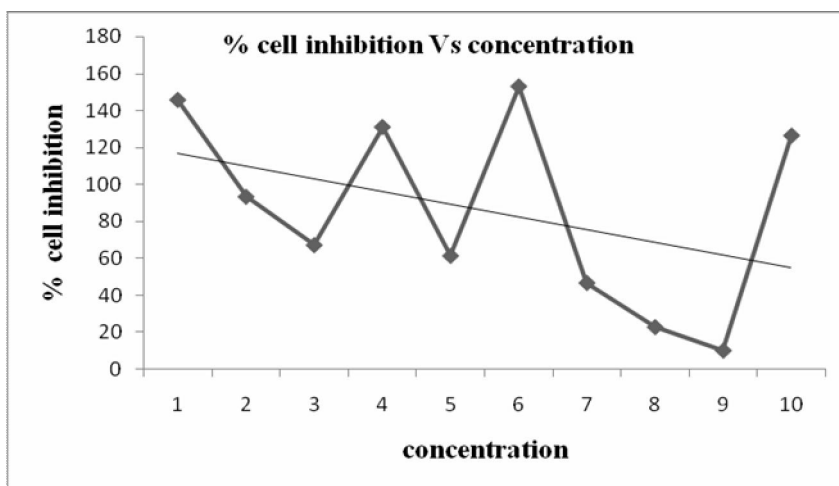


Figure 2: DRC of methanolic extract of *Semecarpus anacardium* for Vero cell line by SRB assay.

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