



IN VITRO CYTOTOXIC ACTIVITY OF INDIAN MEDICINAL PLANTS USED TRADITIONALLY TO TREAT CANCER

SHANTHY SUNDARAM, SATISH KUMAR VERMA, PRIYANKA DWIVEDI
Centre for Biotechnology, Nehru Science Centre, University of Allahabad, Allahabad-211002
Uttar Pradesh, India E mail: res_biotech@rediffmail.com

ABSTRACT

The SRB assay was used to test *in vitro* cytotoxicity against four human cancer cell lines of six Indian medicinal plant species which are being used by traditional people in tribal regions for the treatment of ulcers and other diseases of patients. The ethanolic and aqueous extracts were tested against human cancer cell lines such as human neuroblastoma cell line (IMR-32) and colon cell lines (HT-15 & HT-29) and lung cancer cell lines A-549. The results showed that plants *Calotropis procera*, *Ocimum sanctum* and *Cannabis sativa*, exhibited a very high degree of *in vitro* cytotoxic activity. The results showed a certain degree of selectivity against the different cell types with different extracts.

Key words: Human cancer cell lines; *In vitro* Cytotoxicity test; SRB assay; Indian medicinal plants.

INTRODUCTION

Herbal drugs have been used since ancient times as medicines for the treatment of a range of diseases. Medicinal plants have played a key role in world health. An increasing number of research papers and reviews clearly indicate that medicinal plants exhibit a variety of therapeutic properties (Ahmad et al. 1998; Datta et al. 1998; Abo et al. 2000; Graf, 2000; Ankli et al. 2002; Neto et al. 2002) and provide health security to rural people in primary health care. *In vitro* cytotoxicity screening models provide important preliminary data to help select plant extracts with potential antineoplastic properties for future work (Cardellina et al. 1999). Bilal et al. 2003 showed the immunomodulatory effects of fenugreek (*Trigonella foenum graecum* L.) extract in mice. In Indian medicine both aqueous and ethanolic extracts are used for most of these species. Hence, two different extracts of each plant were made, one with hot water and other with ethanol, except in case *Solanum nigrum* where only alcoholic extract were used. Most of the negative results were neglected.

METHODS

Plant material:

The parts of each species, reported to be used against cancer in Indian folk medicines were collected from February to March 2004. The places of collection were from Chitrakoot region and Allahabad of Uttar Pradesh. Leaf of *Calotropis procera*, seed and inflorescence of *Ocimum sanctum* and leaf of *Solanum nigrum*, leaves and inflorescence of *Canabis sativa*, seeds of *Trigonella foenum graecum* and leaves and stem of *Chenopodium rubrum* were used. Authentication of plant materials were carried out at the herbarium of the Centre for Biotechnology, University of Allahabad, Allahabad. A duplicate set has been deposited in herbarium of the Centre.

Preparation of plant extracts:

Plant materials were dried at 37°C, powdered and extracted in different solvent. The aqueous SWW extract was obtained by boiling dried ground plant material (100 g) for 30 minutes in distilled water (300 ml). All extracts were fine-filtered and freeze dried. For the ethanolic extracts, dried ground plant material (100 g) was percolated with 95% ethanol and concentrated to dryness under reduced pressure. The aqueous extracts were dissolved in sterile water and the ethanolic extracts in Dimethylsulphoxide (DMSO) to form stock solutions 20mg/ml which were filter sterilized (0.2 µm) before testing on cell lines.

Human cell lines:

HT-15 and HT-29 human cancer cell lines of colon grown in RPMI medium were obtained from National Centre for Cell Sciences, Ganeshkhind, Pune, India. IMR-32 neuroblastoma cell line was grown in Minimal Essential Media (MEM) obtained from Centre for

Cellular and Molecular Biology, Hyderabad, India and A-549 which was grown in RPMI was obtained from National Cancer Institute, DTCD, Frederick Cancer Research & Development Center, Madison, USA.

Preparation of test material:

Stock solution: Stock solution of 20 mg/ml were prepared. DMSO was used for 95% reconstitution of MeOH/EtOH extract, and distilled water for hot aqueous extract. Stock solutions were prepared one day in advance. Multiple aliquots of each sample were stored for initial tests and retests, if necessary. Stock solutions were filtered sterilised and microbial contamination was controlled by addition of gentamycin to the complete growth medium.

Working test solution: On the day of assay, thaw an aliquot of frozen stock solution at room temperature. Prepared 100 µg/ml concentration of the extract by serial dilution of stock solution using the complete growth medium containing 50 mg/ml of gentamycin.

Positive controls

The positive controls used was Mitomycin-C.

In vitro Assay for cytotoxic activity

The anticancer activity is determined by the cytotoxic potential of the test material using human cancer cell lines which were allowed to grow on tissue culture plates in the presence of test material. The cell growth was measured on ELISA reader after staining with Sulforhodamine B dye (SRB) which binds to basic amino acid residues in the trichloroacetic acid (TCA) fixed cells.

Preparation of Cell suspension for assay:

The desired human cancer cell line were grown in multiple TCFs at 37°C in an atmosphere of 5% in CO₂ and 90% relative humidity in complete growth medium to obtain enough number of cells as per requirement depending upon number of test samples. The flasks with cells at sub-confluent stage were selected. Cells were harvested by treatment with Trypsin- EDTA and added to complete growth medium to stop the action of trypsin. Cells were separated to single cell suspension by gentle pipetting action and the viable cells were counted in a hemocytometer using trypan blue. Cell viability at this stage should be >97%. Viable cell density was adjusted to 5,000 - 40,000 cells/100µl depending upon the cell line (Monks et al., 1991). Cell suspension is ready for addition to tissue culture plates. 100µl of cell suspension together with 100µl of complete growth medium was added into each well. The plates were incubated at 37°C for 24 hours in an atmosphere of 5% CO₂ and 90% relative humidity in a CO₂ incubator. After 24 hours, the test material, DMSO (vehicle control) and positive controls were added.

Table 1: Percent growth inhibition of different cell lines having different plant extracts with respect to the control. The concentration of the extracts is 100 µg/ml against different cell lines.

Name of plant (1-6) and Control (7)	Extracts in solvent	Concentration		A-549	HT-15	IMR-32	HT-29
1. <i>Calotropis procera</i>	Hot water	100		80	74	56	72
2. <i>Canabis sativa</i>	Ethanol	100	90	93	97	90	
3. <i>Trigonella foenum graecum</i>	Methanol	100		77	89	96	99
4. <i>Solanum nigrum</i>	Ethanol	100	60	57	15	65	
5. <i>Chenopodium rubrum</i>	Ethanol	100	38	70	43	72	
6. <i>Ocimum sanctum</i>	Methanol	100	81	78	92	75	
7. Mitomycin -C	Water	1X10 ⁻⁴		85	73	71	84

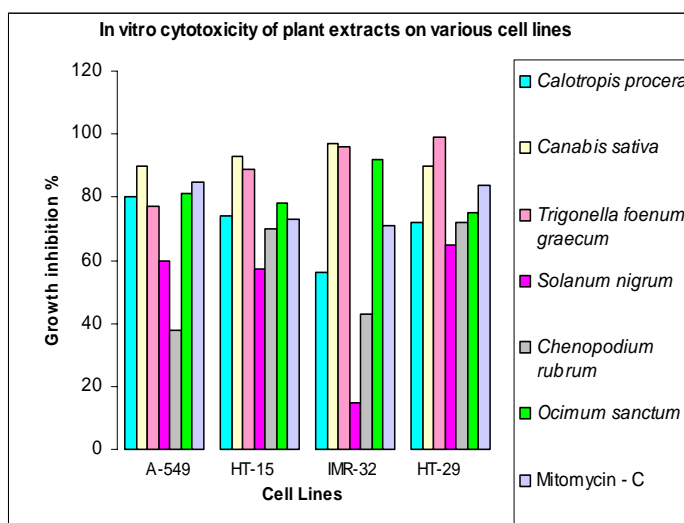


Figure 1: Invitro cytotoxicity of plant extracts on various cell lines

Addition of test materials

Added 100µl of working solutions of the test materials and positives controls along with equivalent complete growth medium into these wells in the tissue culture plate. It was prepared 24 hours in advance containing either cells or complete growth medium in a final volume of 100 µl. The plates were incubated at 37°C for 48 hours in an atmosphere of 5% CO₂ and 90% relative humidity. The cell growth was determined after 48 hours by SRB assay.

Sulforhodamine B (SRB) assay

Assay was carried out as described by Skehan et al (1990) using SRB dye. The microtiter plates were taken out after 48 hours incubation of cells with test materials and gently layered with 50µl of chilled 50% TCA on top of the medium in all the wells to produce a final concentration of 10%. Tissue culture plates were incubated at 4°C for one hour to fix the cells attached to the bottom of the wells.

All the contents of all the wells were pipetted out gently and the supernatant was discarded. The plates were washed five times with distilled water to remove TCA, growth medium, low molecular weight metabolites, serum proteins etc. For washing, the wells of tissue culture plates were filled with distilled water and then discarded the excess liquid in the wells by sharply flicking plate over a sink. Plates were air dried and stored until use. 100µl of SRB solution was added to each well of the plates and incubated at room temperature for 30 minutes. The unbound SRB was removed quickly (to avoid desorption of protein bound dye) by washing the wells five times with 1 % acetic acid and then the plates were air dried.

100µl of Tris buffer (0.01 M, pH =10.4) was added and shaken gently for 5 minutes on a mechanical shaker. Optical density was recorded on ELISA reader at 515 nm and then the data was recorded.

Calculations

Cell viability and growth in presence of test material was calculated as follows:

Percent growth in presence of test material

$$= \frac{\text{Growth in presence of test material}}{\text{Growth in absence of test material}} \times 100$$

Percent growth inhibition in presence of test material was calculated as under:

100- Percent growth in presence of test material

Criteria for Determination of Activity: The test sample showing growth inhibition of >70% at 100 µg/ml is considered to be active. Following table describes the results of *in vitro* cytotoxicity studies carried out against human cancer cell lines in the present investigations.

RESULTS AND DISCUSSION

The foregoing experiment shows various levels of *in vitro* cytotoxic activities of the alcoholic extracts, hot water extracts of different plants / their parts. The observations from the table 1, showed very few of them are active against cancer cell lines. This implies that the active extracts have specific cytotoxic activities against specific cell lines and that they are not generally cytotoxic. The activity was done using 100µg/ml.

The *in-vitro* cytotoxicity was performed on six Indian medicinal plants against four cell lines namely of lung (A-549), colon (HT-15, HT-29) and neuroblastoma (IMR-32).

The plant *Ocimum sanctum*, *Calotropis procera*, *Canabis sativa*, *Trigonella foenum graecum* shows more than 70% of growth inhibition and hence it has anticancer activity, *Solanum nigrum*, showed less than 70% of growth inhibition and therefore, these are showing no activity against any cell line. *Chenopodium rubrum* showed the activity against colon cell lines HT-29, HT-15 but had no activity against the cell lines, neuroblastoma IMR-32 and lung cancer cell line A-549.

Hence, the different extracts of plant in different solvents have cytotoxic activity showing a certain degree of selectivity against the different cell types. Alcoholic extract showed more degree of inhibition against the cell lines while aqueous extracts showed lesser degree of inhibition. It might be because the metabolites which are active against the cell line are best extracted in non polar solvents while some in polar solvents when it is hot extracted.

This piece of work shows that the different plant extracts used in our study respond very differently to the different cancer cell lines possibly since their specificity for these cell lines are different.

REFERENCES

1. Abo KA, Adeyemi AA, Adeite DA (2000) Ethnobotanical survey of plants used in the treatment of infertility and sexually transmitted diseases in southwest Nigeria. *Afr J Med Med Sci* 29, 325–327
2. Ahmad I, Mehmood Z, Mohammad F (1998) Screening of some Indian medicinal plants for their antimicrobial properties *J Ethnopharmacol* 62, 183–193
3. Ankli A et al. (2002) Yucatec Mayan medicinal plants: evaluation based on indigenous uses *J Ethnopharmacol* 79, 43–52
4. Bilal Bin-Hafeez et al. (2003) Immunomodulatory effects of fenugreek (*Trigonella foenum graecum* L.) extract in mice *International Immunopharmacology* 3, 257-265
5. Cardellina et al. (1999) Evolving strategies for the selection dereplication and prioritization of antitumor and HIV-inhibitory natural products extracts. In: Bohlin, L., Bruhn, J.G. (Eds.), *Bioassay Methods in Natural Product Research and Development*. Kluwer Academic Publishers Dordrecht, pp. 25–36
6. Datta BK, Rahman I, Das TK (1998) Antifungal activity of Indian plant extracts *Mycoses* 41, 535–536
7. Graf J (2000) Herbal anti-inflammatory agents for skin disease. *Skin Therapy Lett* 5, 3–5
8. Monks et al. (1991) Feasibility of a high-flux anticancer drug screen using a diverse panel of cultured human tumor cell lines *J Natl. Cancer Inst.* 83, 757-766
9. Neto C C et al. (2002) Antibacterial activity of some Peruvian medicinal plants from the Callejon de Huaylas. *J Ethnopharmacol* 79, 133–138.
10. Skehan, P et al. (1990) New colorimetric cytotoxicity assay for anticancer drug screening *Journal of National Cancer Institute* 82, 1107–1112.