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In vitro cytotoxic effect of leaves and stem bark of *Azadirachta indica* on human colon, liver, neurablastoma and prostate cancer cell lines

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Cancer still remains the major cause of mortality and morbidity all over the world. In the search of potential anticancer agents from the medicinal plants, the present research work was carried out to examine the anticancer properties of *Azadirachta indica* leaves and stem bark against human cancer cell lines via., ethanolic, hydro-ethanolic and aqueous extracts using sulphorhodamine B (SRB) dye. All the three extracts from leaf part and stem- bark showed *in vitro* cytotoxicity against all the human cancer cell lines at 100µg/ml. At lower doses (10 and 30 µg/ml) aqueous extract from leaf part was found to be more active than ethanolic and hydro-ethanolic extracts in dose dependent manner. Results showed the potent anticancer effect of *Azadirachta indica* (leaves and stem bark) on human cancer cell lines of colon, liver, neurablastoma and prostate origin and the plant can be explored for probable anticancer lead molecules for the drug development.

Key words : *Azadirachta indica*, Human cancer cell lines, *In vitro* cytotoxicity, Anticancer.

INTRODUCTION

Azadirachta indica A. Juss., commonly known as neem and belongs to the Meliaceae family is well known in India and its neighbouring countries for more than 2000 years as one of the most versatile medicinal plants. It possesses a wide spectrum of biological activity and every part of this tree has been used as traditional medicine for house hold remedy against various human ailments (Chopra *et al.*, 1956; Chatterjee and Parkash, 1995). This plant shares high reputation in tradition where as its goodness is extensively documented in Ayurveda, Unani and Homeopathic medicine and has become a cynosure of modern medicine (Schmutterer, 1995). Several pharmacological activities and medicinal utilities have been described, especially for leaf and stem bark. The leaf of this plant and its constituents have been demonstrated to exhibit immunomodulatry, anti-inflammatory, antihyperglycemic, antiulcer, antimalarial, antifungal, antibacterial, antiviral, antioxidant, antimutagenic and anticarcinogenic properties (Subapriya and Nagini, 2005). In addition, leaves of *Azadiractita indica* has been reported to possess hepatoprotective (Chattopadhyay, 2003), antifertility (Khillare and Shrivastav, 2003), antiulcer (Chattopadhyay *et al.*, 2004), antimalarial (Udeinya *et al.*, 2008) and anti-inflammatory (Okpanyi and Ezeukwv, 1981) activities. Compound nimbolide, (a limonoid) isolated from leaves and flowers showed cytotoxic effects on human choriocarcinoma (BeWo) cells (Kumar *et al.*, 2008) and flavanones which are present in the flowers contains antimutagenic constituents against heterocyclic amines (Nakahara *et al.*, 2003) Oil from the leaves and bark possess a wide spectrum of antibacterial action against gram-negative and gram positive microorganisms (Satyavati *et al.*, 1976). Bark extract has also shown insecticidal (Oigiangbe *et al.*, 2007) and antimalarial (Aliero, 2003) activity. Current investigation was carried out to determine the *in vitro* cytotoxic potential of the plant against human cancer cell lines of colon, liver, neurblastoma and prostate origin, for developing the potent anticancer agents from the plant.

MATERIALS AND METHODS

The plant was collected from Nagrota region of Jammu, J&K, India, in the month of April and authentication was done by Dr Yashpal Sharma, at the herbarium of the Botany Department, University of Jammu, Jammu. The collected plant material (leaves, stem and bark) was chopped, shade dried and ground into powder. Powdered dried plant material was then extracted with different solvents at room temperature.

Preparation of plant extracts:

For the ethanolic extract, dried and powdered plant material (100g) was percolated with 95 % ethanol (500ml), and evaporated to dryness under reduced pressure. Hydro-ethanolic extract was prepared by percolating

another lot of dried powdered plant material (l00g) with 50 % ethanol with water (500ml) and then concentrating it to dryness under reduced pressure. The hot water extract was obtained by boiling powdered plant material (l00g) for 30 min in distilled water (300ml). Stock solutions 20μg/ml were obtained by dissolving ethanolic extract in Dimethyl sulfoxide (DMSO), the hydro-ethanolic extract in 50% DMSO and the hot water extract in sterile water. The microbial contamination was controlled by the addition of 1% gentamycin in complete growth medium *i.e.*, used for dilution of stock solution to prepare working test solutions 200 μg/ml.

Preparation of positive controls:

Positive controls were prepared in distilled water (Adrimimycin, 5-flurouracil, Mitomycin-C) and then diluted in gentamycin medium to obtain desired concentrations of $2x10^{-4}$ M and $2x10^{-5}$ M.

Human cancer cell lines:

The human cancer cell lines were obtained from National center for cell science, Pune, India. The cell lines namely colon (COLO 205, HT-29, SW-620) were grown and maintained in RPMI-1640 medium, pH 7.4, whereas DMEM was used for liver (HEP-2), neuroblastoma (IMR-32), prostate (DU-145) The media were supplemented with FCS (10%), penicillin (100 units/ml), streptomycin (100 μg/ml) and glutamine (2mM).

In vitro cytotoxic assay against human cancer cell lines:

Test material (extracts) was subjected to *in vitro* anticancer activity against various human cancer cell lines (Monks *et al.*, 1991). For the assay (in brief) the cells were grown in tissue culture flask in growth medium at 37^0C in an atmosphere of 5% CO_2 and 90 % relative humidity, in a CO_2 incubator. The cells at subconfluent stage were harvested from the flask by treatment with trypsin (0.05% trypsin in Phosphate Buffer Saline containing 0.02 % Ethylene diamine tetra acetic acid) and suspended in growth medium. Cells with more than 97% viability (Trypan blue exclusion) were used for determination of cytotoxicity. An aliquot of 100μl of cells (10^5cells/ml) was transferred to a well 96 well tissue culture plate. The cells were allowed to grow for 24 h. Test material was then added to the wells and cells were further allowed to grow for another 48 h.

Sulphorhodamine B (SRB) assay:

The antiproliferative SRB assay was performed to assess growth inhibition which estimates cell number indirectly by staining total cellular protein with the dye SRB (Skehan *et al.*, 1990). In brief, the cell growth was stopped by gently layering 50μl of 50% (ice cold) tri-chloro acetic acid (TCA) on the top of growth medium in all the wells. The plates were incubated at 4^oC for an hour to fix the cells attached to the bottom of the wells. Liquid of all the wells were then gently pipetted out and discarded. The plates were washed five times with distilled water and air dried. SRB 100μl (0.4% in 1% acetic acid) was added to each well and the plates were incubated at room temperature for 30 min. Excess dye was removed by washing with 1% acetic acid and the bound dye was dissolved in tris buffer (100 μl, 0.01 M, pH 10.4). Plates were gently stirred on a mechanical shaker for 5 min and the optical density was recorded at 540nm. Suitable blanks and positive controls were also included. Each test was done in triplicate and the values reported herein are mean values of three experiments.

Calculations:

The cell growth was determined by subtracting average Absorbance (OD) value of respective blank from the average Absorbance (OD) value of experimental set.

Per cent growth in the presence of test material was calculated as under:-

$$\frac{\text{Growth in the presence of test material}}{\text{Growth in the absence of test material}} \text{x100}$$

Per cent growth inhibition in the presence of test material was calculated as under:-

100 – per cent growth in the presence of test material.

Criteria for activity:

The growth inhibition of 70% or above was considered active.

Results and Discussion

In the first phase of experiment, three extracts (ethanolic, hydro-ethanolic and aqueous) of the leaf part of the plant were investigated each at the concentration of 10, 30 and 100μg/ml against six human cancer cell lines from colon, liver, neuroblastoma and prostate origin. Out of the three extracts evaluated, aqueous extract had shown highest growth inhibition against all the human cancer cell lines at the concentration of 10, 30 and 100μg/ml (Table 1). The per cent growth inhibition was observed in the range of 73-96% for aqueous extract. Hydro-ethanolic and ethanolic extracts at 100μg/ml showed significant cytotoxic effect on five human cancer cell lines *viz.*,

Table 1 : *In vitro* cytotoxicity of leaves of *Azadirachta indica* against human cancer cell lines

Plant's generic name	Part used	Extract	Conc.	Human cancer cell lines from particular tissues					
				Colon			Liver	Neuroblastoma	Prostate
				COLO -205	HT-29	SW-620	HEP-2	IMR-32	DU-145
				Growth inhibition (%)					
		Ethanolic	10 (µg/ml)	07	04	33	31	24	09
Azadirachta			30 (µg/ml)	09	72	90	70	83	13
indica			100 (µg/ml)	70	99	89	92	97	30
		Hydro-	10 (µg/ml)	30	42	37	38	54	27
	Leaves	ethanolic	30 (µg/ml)	42	77	91	72	87	38
			100 (µg/ml)	81	99	92	96	95	51
		Aqueous	10 (µg/ml)	78	87	82	81	73	77
			30 (µg/ml)	87	90	89	88	85	87
			100 (µg/ml)	90	95	91	96	92	95
Positive controls									
Adriamycin			$1x10^{-5}$M	--	--	--	--	70	--
Mitomycin-C			$1x10^{-5}$M	--	--	--	77	--	73
5-Flurouracil			$1x10^{-5}$M	40	36	42	--	--	--

OD was recorded on ELISA reader at 540nm
Growth inhibition of 70% or above has been indicated in bold members.
The mark (-) means that the particular human cancer cell line was not treated with that specific positive control.

COLO-205, HT-29, SW-620 (colon), HEP-2 (liver) and IMR-32 (neuroblastoma), except DU-145 (prostate). Further on lower doses at 30µg/ml both the extracts showed significant activity of 70-91% only against four human cancer cell lines (HT-29, SW-620, HEP-2 and IMR-32) but at 10µg/ml such type of significant activity was not observed.

In the second phase of experiment, the extracts (ethanolic, hydro-ethanolic, aqueous) of the stem bark of the plant were investigated at the concentrations of 10, 30 and 100 µg/ml against same human cancer cell lines as mentioned in the first phase (Table 2). Remarkable growth inhibition in the range of 90- 95% was observed by the aqueous extract at the 100 µg/ml, as it suppressed the growth of all the six different human cancer cell lines from four different tissues. But at the lower concentrations

Table 2 : *In vitro* cytotoxicity of stem-bark of *Azadirachta indica* against human cancer cell lines

Plant's generic name	Part used	Extract	Conc.	Human cancer cell lines from particular tissues					
				COLON			Liver	Neuroblastoma	Prostate
				COLO-205	HT-29	SW-620	HEP-2	IMR-32	DU-145
				Growth inhibition (%)					
		Ethanolic	10 (µg/ml)	09	04	03	07	02	05
Azadirachta			30 (µg/ml)	11	23	09	20	10	07
indica			100 (µg/ml)	72	70	71	70	74	73
		Hydro-	10 (µg/ml)	23	13	04	17	09	10
	Stem-	ethanolic	30 (µg/ml)	49	47	19	38	17	21
	bark		100 (µg/ml)	80	81	75	79	84	86
		Aqueous	10 (µg/ml)	38	29	06	39	16	05
			30 (µg/ml)	49	43	21	49	23	33
			100 (µg/ml)	92	95	90	89	91	94
Positive controls									
Adriamycin			$1x10^{-5}$M	--	--	--	--	74	--
Mitomycin-C			$1x10^{-5}$M	--	--	--	76	--	69
5-Fluorouracil			$1x10^{-5}$M	42	37	45	--	--	--

OD was recorded on ELISA reader at 540nm
Growth inhibition of 70% or above has been indicated in bold members.
The mark (-) means that the particular human cancer cell line was not treated with that specific positive control.

of 10 and 30 µg/ml, growth inhibition was below 50 per cent. Other two extracts (ethanolic and hydro-ethanolic) demonstrated 70-86% growth inhibition against all the human cancer cell lines at the concentration of 100µg/ml but at lower doses 10 and 30µg/ml, the extracts did not exhibit any significant cytotoxic effect.

Azadirachta indica i.e., Neem, is the source of a wide variety of products including adhesive, beauty aids, fertilizers, pesticides, numerous pharmaceuticals etc. and these products are variously derived mainly from the stem bark and leaves (Biswas *et al.*, 2002). Extracts of Neem, often called "Nature's drugstore", have been used in medicine for over 2500 years. Each part of the neem tree possesses medicinal property and more than 140 compounds have been isolated from different parts of neem. Therefore, in view of the above facts, the research work was carried out for the exploitation of this most useful traditional medicinal plant in the management of cancer. Accordingly, *in vitro* cytotoxic assay of the leaves and stem bark of the plant was conducted with appropriate positive controls against six human cancer cell lines from four different origins *viz.*, colon, liver, nuroblastoma and prostate. Results yielded very interesting and significant consensus from medicinal view point. All the extracts (ethanolic, hydro-ethanolic and aqueous) from the leaf part of the plant showed significant cytotoxic effect against all the human cancer cell lines at the concentration of 100µg/ml (70-96 % growth inhibition was observed in case of aqueous extract) except DU-145 on which ethanolic and hydro-ethanolic extracts showed lower than 50% growth inhibition. The extracts (ethanolic, hydro-ethanolic, aqueous) from the stem-bark of the plant also possess remarkable anticancer property as they also inhibited the growth of all the human cancer cell lines at the concentration of 100 µg/ml (90-95 % growth inhibition was observed in case of aqueous extract).

To conclude, the aqueous extract from the both parts (leave and stem-bark) of the plant showed a very curious and significant observation which can be explored further for its possible use as anticancer drug. The active ingredient(s) from this particular extract of the leaves and stem-bark can act as lead molecule(s) for the development of drugs for liver, neuroblastoma, prostate and colon carcinomas to provide a great service to cancer patients.

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