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CYTOTOXIC EFFECT OF BITTER GOURD ON BREAST, COLON, LUNG AND PROSTATE CANCER CELLS

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ABSTRACT

The present investigation was carried out on *Momordica charantia* (bitter gourd), to evaluate its *in vitro* anticancer efficiency in which systematic bioassays were performed on seven human cancer cell lines from six different tissues such as MCF-7 and T-47D (breast), HCT-116, (colon), A-549 (lung), MDA-MB-435 (melanoma), PC-3 (prostate), A-498 (renal). The results demonstrated that bitter gourd suppressed the proliferation of all the seven tested human cancer cell lines in the range of 74-86%. The extract was evaluated at lower concentrations and again it showed activity against breast (MCF-7), colon (HCT-116), lung (A-549) and prostate (PC-3) cancer cell lines at 50µg/ml. Further, fractionation of this active extract was carried out and it was observed that chloroform, ethyl acetate and acetone soluble fractions showed significant results at 100 µg/ml against breast (MCF-7), colon (HCT-116), lung (A-549) and prostate (PC-3) cancer cell lines. When evaluated at lower concentrations, the acetone soluble fraction showed 99% and 97% growth inhibition of lung cancer cell line (A-549) at 50 and 10 µg/ml respectively. However, the same fraction also exhibited 75% growth inhibition of breast (MCF-7) and 93% growth inhibition of colon (HCT-116) and prostate (PC-3) cancer cell lines at 50µg/ml. Further, IC_{50} values were calculated and it was observed that acetone fraction showed $IC_{50} < 10$ in case of all the tested four human cancer cell lines namely breast, colon, lung and prostate with least IC_{50}=1.36 µgm/ml in case of lung cancer cells.

Keywords **:** Bitter gourd, Cancer cells, *In vitro* cytotoxicity, Extract, Fraction.

Introduction

Momordica charantia, also known as balsam pear /bitter gourd/ karela and belonging to family cucurbitaceae is commonly consumed as vegetable, widely cultivated in Asia, Africa, South America and extensively used in folk medicines as a remedy for diabetes, specifically in India, China, Central America (Grover *et al.,* 2002). In India, various medicinal properties are claimed for *M. charantia* that include antidiabetic, abortifacient, anthelmintic, contraceptive, antimalarial, laxative and is used for treatment of dysmenorrhea, eczema, emmenagogue, galactagogue, gout, jaundice, kidney stone, leprosy, leucorrhea, piles, pneumonia, psoriasis, rheumatism, scabies (Anila and Vijayalakshmi, 2000). Multiple types of extracts from bitter gourd had *in vivo* and *in vitro* anticancer activity (Chiampanichayakul *et al.,* 2001; Nagasawa *et al.,* 2002; Kohno *et al.,* 2004; Yasui *et al.,* 2005).

Eleostearic acid (α-ESA), which is a conjugated linolenic acid that makes up 60% of bitter gourd seed oil, can block breast cancer proliferation and induce apoptosis through a mechanism that may be oxidation dependent (Grossmann *et al.,* 2009). *In vitro* studies using pure α-ESA have reported anticancer activity as α-ESA significantly reduced viability of transformed NIH-3T3 mouse fibroblast (SV-T2) and monocytic leukaemia (U-937) cells (Suzuki *et al.,* 2001) . In additional reports, DLD-1 colorectal adenocarcinoma cells treated with α-ESA *in vitro* were growth inhibited and underwent DNA laddering indicative of apoptosis

(Tsuzuki *et al.,* 2004). Both Caco-2 and HT-29 colon cancer cells had decreased viability and increased DNA fragmentation when treated with α-ESA (Yasui *et al.,* 2006).

MCP_{30}, a protein isolated from bitter gourd seeds selectively induces prostate cancer apoptosis (Xiong *et al.,* 2009). Fruit and leaf extracts (50% methanol) from *M. charantia* possess chemopreventive potential on dimethyl benz(a)nthracene (DMBA) induced skin tumorigenesis, melanoma tumor and cytogenicity (Agrawal and Beohar, 2010). Methanolic extract as well as momordin of bitter gourd showed cell toxicity against human cancer cell lines (Lee *et al.,* 1998). Chronic treatment with hot water extract of karela inhibited uterine adenomyosis and mammary tumor growth in mice (Nagasawa *et al.,* 2002). It was demonstrated that maximal anticarcinogenic activity is found in the peel of *M. charantia* (Singh *et al.,* 1998)). The crude aqueous extract from bitter gourd showed *in vivo* antitumor activity (Jilka *et al.,* 1983). Alcoholic extract from the leaves of bitter gourd have an anti-metastatic effect against rat prostate cancer progression both *in vitro* and *in vivo* (Pitchakarn *et al.,* 2010). 9, 11, 13-octadecatrienoic acid (α-eleostearic acid), a major linolenic acid in bitter gourd seeds strongly inhibited the growth of some cancer and fibroblast cell lines including those of HL-60 leukemia and HT-29 colon carcinoma (Kobori *et al.,* 2008). In the present investigation, *in vitro* cytotoxic effect of karela has been evaluated against seven human cancer cell lines from six different tissues.

Materials and Methods

Authentication, collection, chopping and drying of plant material

Momordica charantia was authenticated at site by Dr. RK Samnotra Professor, Division of Vegetable Science, SKUAST-Jammu and enough quantity of fresh fruits were collected from Assar village of Doda District, Jammu, J&K. The freshly collected vegetable was chopped, shade-dried and ground into powdered form.

Extraction method

Standard protocol (Kandil *et al.,* 1994) was followed for the extraction of bitter gourd. Powdered dried fruit material (100 g) was placed in a percolator of appropriate size. The material was then submerged in 99% (v/v) methanol depending on the need. After standing for about 16 h (overnight), the percolate was collected and filtered if required. The process was repeated four times, which was generally sufficient for exhaustive extraction of the plant material. The methanolic extract (collected in four attempts) was evaporated to dryness under reduced pressure at 60 ^{0}C using rotavapor and round bottom flask (RBF). The final drying was done in a vacuum desiccator. The dried extract was scrapped off from the RBF and transferred to a tared wide mouth glass container of appropriate size. The container was weighed to calculate the quantity of the extract obtained. This formed the "stock extract" of the plant. Generally, 8 to 10 g crude extract was obtained from 100 g of the dried plant material. The plant extract obtained, was stored at –20 ^{0}C under desiccation in deep freezer for further testing.

Fractionation with different solvents

The methanolic extract of bitter gourd was fractionated in different solvents namely n-hexane, chloroform, ethyl acetate, acetone and methanol in the order of increasing polarity. Different fractions were obtained and evaporated using rotary vacuum evaporator followed by lyophilisation. Yield of the different fractions thus obtained was noted and stored at 4 ^{0}C until use.

Preparation of reagents

- Prepared RPMI–1640 with 2 mM L-glutamine (10.4 g), 2 g of sodium bicarbonate (2 g/L) and 100 g of streptomycin (100 µg/ml) were dissolved in 1L of double distilled water and mixed thoroughly. The pH of the medium was adjusted 7.2 and the medium was sterilized by filtration (0.22 µ filter) and stored in a refrigerator (2-8 ^{0}C).
- Complete growth medium contained FBS (10%) and penicillin (1%) in growth medium
- Medium for cryopreservation (freezing medium) contained FBS (20%, v/v) and DMSO (10%, v/v) in growth medium
- Gentamycin medium contained gentamycin (1%, w/v) in complete growth medium
- 9.6 g of PBS was dissolved in 1L of DW, mixed gently, autoclaved and stored in a refrigerator (2-8 ^{0}C)
- 0.05% trypsin and 0.02% EDTA were dissolved in PBS and filtered.
- 6.25 mg/ml pencillin in PBS and filtered
- 5 mg/ml gentamycin in PBS and filtered
- 0.4% (w/v) SRB was dissolved in 1% (v/v) acetic acid.
- 10 M (pH 10.5) 1.210 g tris base in 1L double distilled water
- 500 g of TCA in 1L of double distilled water

Source of human cancer cell lines

National Centre for Cell Science, Ganeshkhind, Pune–411007 (India) and National Cancer Institute, DTCD, Frederick Cancer Research and Development

Center, Fairview Center, Suite 205, 1003 West – 7th Street, Frederick, MD 21701 – 8527 (U.S.A).

Preparation of test material

A stock solution of 20 mg/ml was prepared in DMSO. For 99% (v/v) methanolic extract, DMSO was used. Stock solutions were prepared atleast one day in advance. Stock solutions were not filtered / sterilized, but microbial contamination was controlled by the addition of gentamycin in complete growth medium used for dilution of stock solutions to prepare working test solutions.

Working test solution (200 µg/ml) was prepared on the day of assay, an aliquot of frozen stock solution was thawed at room temperature. Working test solution was prepared by dilution of stock solution with gentamycin medium. (10 µl of stock solution +990 µl of gentamycin medium = 1000 µl).

Positive controls

Positive controls were prepared in DW (5–flurouracil, mitomycin-C, tamoxifen) / DMSO (paclitaxel). They were further prepared in gentamycin medium to obtain working test solutions.

Chemicals

RPMI-1640 medium, dimethyl sulfoxide (DMSO), EDTA, fetal bovine serum (FBS), sulphorhodamine blue (SRB) dye, phosphate buffer saline (PBS), trypsin, gentamycin, penicillin and 5-flurouracil were purchased from Sigma Chemical Co., USA. All other chemicals were of high purity and obtained locally with the brand Sigma-Aldrich Chemicals Pvt. Ltd. and S.D. Fine Chemicals Pvt. Ltd. from Ramesh Traders, Panjthirthi-Jammu, J&K.

***In vitro* assay**

Cytotoxicity of the test samples was performed against various human cancer cell lines originated from different tissues (Monks *et al.,* 1991). Number of 96-well flat bottom tissue culture plates were dependent upon the number of test samples along with appropriate positive controls (standard drugs for cancer). There were four types of wells in the tissue culture plates, control blank (CB, without cells, complete growth medium only) and control growth (GC, with cells alone in the absence of test material) to determine 100% growth. The growth in the presence of test material was determined from the difference of test growth (GT, cells with test material) and test control (CT, test material without cells).

Preparation of cell suspension for assay

The desired human cancer cell lines were grown in multiple tissue culture flasks at 37 ^{0}C in an atmosphere of 5% in CO_2 and 90% relative humidity in complete growth medium to obtain enough number of cells as per requirement depending upon number of test samples. The flask with cells at sub-confluent stage was selected. The cells were harvested by the treatment of Trypsin – EDTA and the complete growth medium was added to stop the action of trypsin. Cells were separated to single cell suspension by gently pipetting action. Viable cells were counted in haemocytometer and viable cell density was adjusted to 5,000 – 40,000 cells/ 100 µl depending upon the cell line. At this stage, cell suspension was ready for addition to tissue culture plates.

Addition of cell suspension in tissue culture plates

Cell suspension 100 µl / well was added into GC and GT wells. Complete growth medium 100 µl/well was added into CB and CT wells. The plates were incubated at 37 ^{0}C for 24 h in an atmosphere of 5% CO_2 and 90% relative humidity in a CO_2 incubator. After 24 h test material and positive controls were added.

Addition of test material

Working solutions of the test material or positive controls (100 µl) were added to respective CT and GT wells and equivalent complete growth medium was added to CB and GC wells into tissue culture plates prepared 24 h in advance, containing either cells or complete growth medium (100 µl).The plates were incubated at 37 ^{0}C for 48 h in an atmosphere of 5% CO_2 and 90% relative humidity in a CO_2 incubator. The cell growth was determined after 48 h by SRB assay (Skehan *et al.,* 1990).

SRB assay

After 48 h incubation of cells with test material, the plates were taken out and 50 µl of chilled 50% TCA was gently layered on top of the medium in all the wells to produce a final concentration of 10%. After that tissue culture plates were incubated at 4 ^{0}C in a refrigerator to fix the cells attached to the bottom of the wells. After 1 h the plates were taken out from refrigerator and all the contents of all the wells were pipetted out and supernatent 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 the liquid in the wells was discarded by sharply flicking plate over a sink. Plates were air dried and can be stored until use. SRB solution (100 µl) was added to each well of the plates and the plates were incubated for 30 minutes at room temperature. The unbound SRB

was removed quickly (to avoid desorption of protein bound dye) by washing the wells of the plates five times with 1% (v/v) acetic acid. Plates were then air dried. After that Tris buffer (100 µl/well) was added in the plates. The plates were gently stirred for 5 minutes on a mechanical shaker and the optical density was recorded on ELISA reader at 540 nm.

The SRB staining method offer several advantages for very large scale screening. The SRB assay is simpler, faster and more sensitive. It provides better linearity with cell number. It was less sensitive to environmental fluctuations and independent of intermediary metabolism. It provides a fixed end point that does not require a time sensitive measurement of initial velocity (Alley *et al.,* 1988; Scudiero *et al.,* 1988; Rubinstein *et al.,* 1990).

Calculations

The cell growth was determined by subtracting average absorbance value of respective blank from the average absorbance value of experimental set. Percent growth in presence of test material was calculated as under:

- OD Change in Presence of Control = Mean OD of Control – Mean OD of Blank
- OD Change in Presence of Test Sample = Mean OD of Test sample – Mean OD of Blank
- % Growth in Presence of Control = 100/OD change in presence of control
- % Growth in Presence of Test Sample = (% growth in presence of control) × OD change in presence of test sample
- % Inhibition by Test Sample = 100 – % growth in presence of test sample

Criteria for activity

The growth inhibition of 70% or above was considered active while evaluating extracts and fractions at different concentrations.

Results and Discussion

The methanolic extract derived from the fruit part of bitter gourd showed striking observations as the extract suppressed the proliferation of all the seven tested human cancer cell lines in the range of 74-86%. Maximum growth inhibition *i.e.,* 86% was observed against MCF-7, a cancer cell line from breast origin. The extract showed 83% growth inhibition against colon cancer cell line (HCT-116). 82% growth inhibition was observed against prostate cancer cell line (PC-3) and 79% growth inhibition was observed against lung cancer cell line (A-549). The extract suppressed 75% proliferation of breast (T-47D) and melanoma (MDA-MB-435) cancer cells. Moreover, a growth inhibition of 74% against A-498, a cancer cell line from renal origin, was also displayed by this methanolic extract of *M. charantia*). The methanolic extract of bitter gourd was evaluated at lower concentrations and the extract showed activity against breast, colon, lung and prostate cancer cell lines at 50µg/ml. Growth inhibition of 71% was observed against MCF-7, a cancer cell line from breast origin. The extract showed 72% growth inhibition against colon cancer cell line (HCT-116). 71% growth inhibition was observed against lung cancer cell line (A-549) and 70% growth inhibition was observed against prostate cancer cell line (PC-3). However, the extract showed negligible activity, when evaluated at 10 and 1µg/ml (Table 1).

The observations produced by the fractions of the methanolic extract from *Momordica charantia* are summarised in Table 2. Most significant results, that is strong antiproliferative effect on a range of human cancer cell lines was displayed by chloroform, ethyl acetate and acetone soluble fractions. These three fractions showed significant results against all the tested four human cancer cell lines, *viz.*, MCF-7, HCT-116, A-549, PC-3 originated from breast, colon, lung and prostate respectively. The chloroform fraction exhibited growth inhibition in the range of 72-98% as it suppressed 98% proliferation of colon, 88% of breast, 77% of lung and 72% of prostate cancer cell line. Similarly, ethyl acetate fraction showed growth inhibition in the range of 75-100% as it suppressed 100% proliferation of colon, 95% of breast, 79% of lung and 75% of prostate cancer cell line. Surprisingly, acetone fraction of bitter gourd produced very striking results showing growth inhibition in the range of 84-100%. This particular fraction displayed 100% growth inhibition of colon, lung and prostate cancer cells whereas 84% growth inhibition was observed in case of breast cancer cells. However, n-hexane and methanolic soluble fractions of the same plant showed negligible activity against all the tested four human cancer cell lines.

When evaluated at lower concentrations, the ethyl acetate fraction showed 83, 91, 70 and 70% growth inhibition of breast (MCF-7), colon (HCT-116), lung (A-549) and prostate (PC-3) cancer cell lines respectively at 50 µg/ml. However, at 10 and 1µg/ml, the same fraction did not exhibit any significant effect against any of the above mentioned human cancer cell lines. The acetone soluble fraction showed 75, 93, 99 and 93% growth inhibition of breast (MCF-7), colon (HCT-116), lung (A-549) and prostate (PC-3) cancer cell lines respectively at 50 µg/ml. The same fraction also exhibited growth inhibition of 97% of lung (A-

549) cancer cell line at 10 µg/ml. However, at 1µg/ml, the fraction showed negligible *in vitro* cytotoxic effect against all the tested four human cancer cell lines. Further, IC_{50} values were calculated and it was observed that ethyl acetate fraction showed IC_{50}<10 in case of colon cancer cell line - HCT-116 (Figure 1) whereas acetone fraction showed IC_{50}<10 in case of all the tested four human cancer cell lines *viz*., MCF-7, HCT-116, A-549 and PC-3 (Figure 2).

Cancer is a deadly disease, becoming a big load on families and economies. The cancer cases are on rise in Jammu and Kashmir with lung cancer becoming most prominent due to smoking. There is an increase in the number of cancer patients in the State from last five years. The number of individuals living with cancer is continuing to expand, but most of the drugs used in cancer chemotherapy exhibit cell toxicity and can induce genotoxic, carcinogenic and teratogenic effects in non tumor cells. Therefore, the research for alternative drugs of natural origin, which are less toxic, endowed with fewer side effects and more potent in their mechanism of action, is an important research line. There is strong, consistent evidence that high intake of vegetables protect against various cancers. These protective effects of high vegetable consumption are attributed to the active micronutrients (vitamins and minerals) and non-nutritive components (phytochemicals) that exhibit a potential for modulating human metabolism in a manner favorable for the prevention of cancer.

In the present investigation, *in vitro* assay for cytotoxic activity was conducted by using SRB dye with appropriate positive controls and the prime finding is the remarkable *in vitro* anticancer efficiency from fruit part of *Momordica charantia* (bitter gourd) as mentioned above. The data was compared with literature values and it was found that the data was in good agreement with the published data. Human breast cancer cells (MCF-7 and MDA-MB-231) were used to assess the efficacy of bitter gourd extract as an anticancer agent and it was found that the extract inhibits breast cancer cell proliferation by modulating cell cycle regulatory genes and promotes apoptosis (Ray *et al.,* 2010) . *M. charantia* was found effective on highly metastatic PC-3 prostate cancer cell line (Rao *et al.,* 2004). Moreover, the fruit part of karela from Baisht village of Jammu Division was found active against lung (NCI-H322), leukemia (THP-1) and glioblastoma (U-87MG) cancer cell lines (Hussain *et al.,* 2013).

The results from the investigation forms a good basis for the selection of this vegetable of Doda district of Jammu division for further phytochemical and pharmacological analysis to offer new drugs from natural sources which would be less toxic and more potent for the efficient management of cancer. To conclude, isolation and characterization of active ingredients is required from the most active fraction of the bitter gourd to provide a great promise and service to patients especially with breast, colon, lung and prostate carcinoma.

Table 1 : Growth inhibitory effect of *Momordica charantia* extract along with positive controls against human cancer cell lines at different concentrations

English name of the vegetable	Extract E Extract	Conc. (µg/ml)	Human cancer cell lines from six different tissues						
			Breast	Breast	Colon	Lung	Melanoma	Prostate	Renal
			MCF-7	T-47D	HCT-116	A-549	MDA-MB-435	PC-3	A-498
			Growth Inhibition (%)						
Bitter gourd	**Methanolic**	**100**	**86**	**75**	**83**	**79**	**75**	**82**	**74**
		50	**71**	**-**	**72**	**71**	**-**	**70**	**-**
		10	32	**-**	26	42	**-**	35	**-**
		1	05	-	13	24	-	08	**-**
Positive control (Standard drugs)		**Conc. (µM)**							
5-Fluorouracil		**20**	**-**	**-**	65	-	-	-	-
Mitomycin-C		**1**	-	-	-	-	-	63	70
Paclitaxel		**1**	77	72	-	71	-	-	-
Tamoxifen		**1**	-	-	-	-	75	-	-

Growth inhibition of 70% or more in case of extracts has been indicated in bold numbers

Mark (-) indicates that particular human cancer cell line was not treated with that particular extrac/positive control

Table 2 : *In vitro* anticancer potential of fractions of *Momordica charantia* at different concentrations

English name of the vegetable	Fractions	Conc. (µg/ml)	Human cancer cell lines from four different tissues			
			Breast	Colon	Lung	Prostate
			MCF-7	HCT-116	A-549	PC-3
			Growth Inhibition (%)			
Bitter gourd	n-hexane	100	43	21	39	32
	Chloroform	100	**88**	**98**	**77**	**72**
	Ethyl acetate	100	**95**	**100**	**79**	**75**
		50	**83**	**91**	**70**	**70**
		10	29	51	13	47
		1	12	31	00	27
		IC_{50}	19.73	**8.61**	32.43	12.04
	Acetone	100	**84**	**100**	**100**	**100**
		50	**75**	**93**	**99**	**93**
		10	58	57	**97**	65
		1	0	14	31	15
		IC_{50}	7.17	7.6	**1.36**	5.64
	Methanol	**100**	50	32	42	44
Positive controls (Standard drugs)		**Conc.(µM)**				
Paclitaxel		**1**	77	-	71	-
5-Fluorouracil		**20**	-	65	-	-
Mitomycin-C		**1**	-	-	-	63

Growth inhibition of 70% or more in case of fractions and least IC_{50} has been indicated in bold numbers
Mark (-) indicates that particular human cancer cell line was not treated with that particular positive control

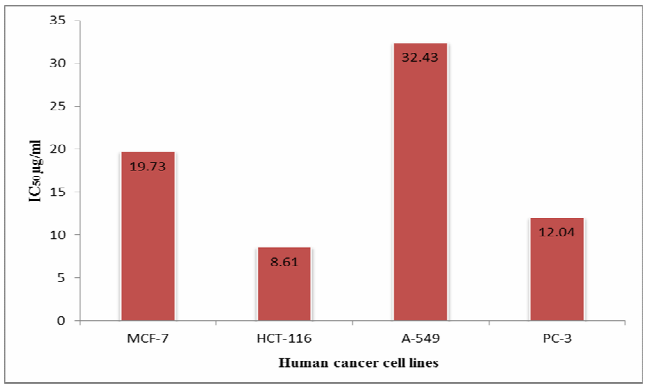

Fig. 1: IC_{50} values of ethyl acetate fraction of *Momordica charantia*

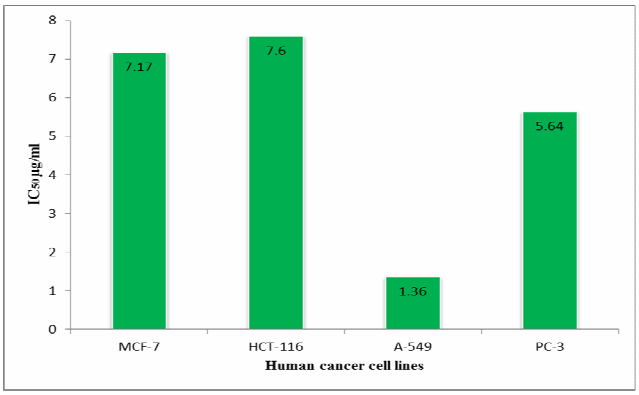

Fig. 2: IC_{50} values of acetone fraction of *Momordica charantia*

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