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Cytotoxic Effect of Moringa Oleifera Leaf Extracts on Human Multiple Myeloma Cell Lines

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Abstract: In the present study different organic solvent extracts of dried leaves of country variety of *Moringa oleifera* (L) were evaluated for their cytotoxic potentiality on human Multiple myeloma cell lines. Different extracts of aqueous methanol, ethanol, ethyl acetate and chloroform from *Moringa oleifera* and *Vinca rosea* leaves were analysed for their cytotoxic effect by performing neutral red dye uptake assay to check for the viability of myeloma cells after treatment. *Moringa* leaf extracts exhibited pronounced cytotoxic effect (<15% viability at 0.2 mg mL^{-1}) compared to *Vinca* extracts. Of the organic extracts, methanolic extracts of *Moringa* leaves showed least viability at highest dose (2%) and comparatively less viability at lowest dose (12%) with a sigmoidal dose responsive viability profile on cells representing an ID_{50} value of 0.32 μg mL^{-1}. Overall, methanol was expected to be suitable organic solvent for the extraction of anti cancer i.e., antimyelomic compounds from *Moringa oleifera*.

Key words: *Moringa oleifera*, *Vinca rosea*, multiple myeloma, cytotoxic effect

INTRODUCTION

Multiple Myeloma (MM) is a malignancy of B-lymphocytes characterized by monoclonal proliferation of malignant plasma cells within bone marrow (Barlogie *et al*., 1989) and presence of malignant plasmacytomas secreting monoclonal immunoglobulins in the serum and urine with a concomitant decrease in normal immunoglobulin levels and subsequent formation of lytic bone lesions. (Hideshima *et al*., 2003; Iida *et al*., 2003). It remains incurable despite recent advances in high dose chemotherapy followed by autologous stem cell transplantation (Attal *et al*., 1996; Kumar *et al*., 2003) that improved overall survival to 49-62 months respectively (Barlogie *et al*., 1997). As patients relapse with refractory diseases and succumb to this malignancy, new biologically based therapies are needed which make use of plant products.

Most of the researches performed so far focus on development of drugs to treat cancer. Several drugs currently used in chemotherapy were isolated from plant species or derived from natural phenotypes like vincristine and vinblastin from *Vinca rosea* (Cragg *et al*., 1993). Over 50% of drugs in clinical trials for anti cancer activity were isolated from natural plant sources (Cragg and Newman, 2000).

Moringa oleifera (L) (Horse radish tree, Drumstick tree) belongs to Moringaceae family with 14 species of deciduous tree (Jahn, 1988). It is a multipurpose tree widely distributed in India, Burma, Srilanka, Thailand, Pakistan, etc., (Okuda *et al*., 2001) and widely used in Indian traditional medicine. Various parts of *Moringa* have been studied for many pharmacological actions. Juice of its leaves showed anti bacterial activity (Bhawasar *et al*., 1965). Fifty percent Ethanolic extracts of the plant excluding roots showed anti cancer activity in mice (Dhawan *et al*., 1980). According to Hartwell (1967-71) leaves were used in folk remedies for tumors and widely recognized as food source for

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human (Makkar and Beekar, 1997). *Moringa* plant extracts were already reported for antioxidant property (Siddhuraju and Becker, 2003), fungicidal activity, hypocholesterolemic activity, antidiabetic activity, anti tumor activity (Richa *et al*., 2005).

The objective of this study was to determine the *in vitro* cytotoxic potential of *Moringa oleifera* extracts, on human Multiple myeloma cell lines using neutral red dye uptake cell viability assay. This data will be used further to isolate, purify and charaterize anti cancer i.e., anti myelomic compounds from its leaves which would aid herbal medicinal therapy.

MATERIALS AND METHODS

Plant Material

Plant samples of *Moringa oleifera* and *Vinca rosea* were collected in Tambaram, Chennai (India). The leaves of these plants were dried under shade and used for further experimentation.

Extraction Procedure

Five grams of both *Moringa* and *Vinca* leaf powders were dissolved in 50 mL of absolute methanol, ethanol, ethyl acetate and chloroform respectively. The solutions were kept in shaking incubator (Sciegenics, India) at 20°C, 500 rpm for 48 h. The solutions were filtered with filter papers and allowed to evaporate for 3 days at room temperature. The residues left were obtained dry of 0.1 g for both plants.

Preparation of Extracts

Dry extracts of both plant leaves were dissolved in 5 mL of respective solvents to give a desired stock solution of extracts (20 mg mL^{-1}). All the extracts were stored at 4°C till experimentation. During the experiment, stock solutions were diluted to obtain an original concentration of 0.2 mg mL^{-1} (10 μL of stock solution made up to 1 mL with RPMI 1640).

Cell Culture Conditions

U266B1(Human B-lymphocyte Plasmacytoma), Myeloma cell culture was obtained from National Center for Cell Science, Pune, India and used in this study. The cell lines were grown at 37°C at humidified 5% CO_2 in RPMI 1640 medium supplemented with 2 mM L-Glutamine, 1.5 g L^{-1} Sodium bicarbonate, 4.5 g L^{-1} glucose, 10 mM Hepes, 1 mM Na-pyruvate, 15% Fetal calf serum and conserved in a log growth phase after sub culturing with 83% viability.

Cytotoxicity Studies

The effect of administration of leaf extracts of *Moringa* and *Vinca* at serial dilutions from 200 to 0.5 μg mL^{-1} for a period of 60 min was studied to assess the cytotoxicity on myeloma cells. The apoptotic response of the cells was recorded with the use of neutral dye uptake method of viability measurements.

Experimental Set up

Cells were seeded into two 96 well microtitre culture plates at 2×10^4 cells $well^{-1}$ (150 μL) and incubated for 24 h at 37°C, in a humidified atmosphere. Each plate is divided into four sets, each set with two rows, to maintain duplicates

Set 1: Treated with methanol extracts
Set 2: Treated with ethanol extracts
Set 3: Treated with ethyl acetate extracts
Set 4: Treated with chloroform extracts

In the first column of all sets cell culture alone was maintained at a volume of 150 µL. The second column received cell culture along with 1% of respective solvent in respective set which was maintained as control.

To the first plate, 30 µL of the respective *Moringa* leaf extract was loaded into the third column (total volume of 190 µL) representing the highest dose 200 µg mL^{-1}. From this 30 µL of the well mixed medium was serial diluted to the adjacent columns till the least dilution of 0.512 µg mL^{-1} obtained. (Column-11). These eight concentration ranges were maintained for all the four solvent extracts. Similar method is followed to treat the cells in the second plate with *Vinca* leaf extracts. This was followed by the incubation of plates at 37°C, for 60 min in flat bed shaker.

Neutral Red Dye Uptake Assay

Cytotoxicity testing using viability assays gives a dose responsive curve over a concentration range. This assay reproduces better with neutral red dye uptake property of the treated cells (Borenfreund *et al*., 1985). The extract treated wells were replaced with 150 µL of neutral red medium (2 mg mL^{-1} stock in RPMI 1640 medium) and incubated for 60 min at 37°C. Cells were washed with warm PBS (37°C) to remove excess stain and replaced by 150 µL of destainer (glacial CH_3COOH : CH_3OH : H_2O in 1:50:49) to fix the cells and elute neutral red in the cells. Absorbance of the cells in each well was read at 540 um against blank well with medium alone. The ID_{50} concentration of plant extract was analysed from Fig. 1 and Table 1 and 2.

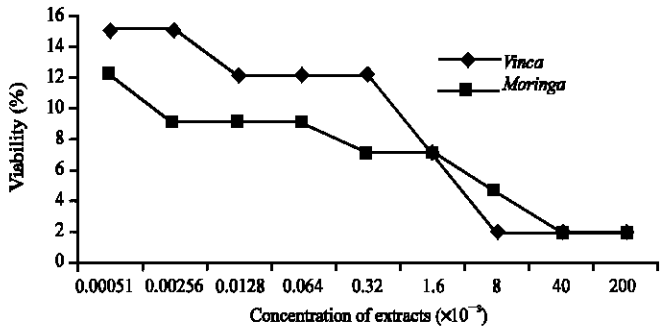

Fig. 1: Cytotoxic effect of methanolic extracts

Table 1: The absorbance at 540 nm by the cells after treatment with various extracts of *Moringa* and *Vinca* for 60 min

		Absorbance at 540 nm							
		Row A,B (methanolic)		Row C,D (ethanolic)		Row E,F (ethanolic)		Row G,H (chloroform)	
Column	Concentration of extracts (mg mL^{-1})	V	M	V	M	V	M	V	M
1	Control medinm	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
2	Extract control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
3	200×10^{-3}	0.01	0.01	0.01	0.02	0.01	0.04	0.01	0.03
4	40×10^{-3}	0.01	0.01	0.01	0.02	0.01	0.04	0.01	0.03
5	8×10^{-3}	0.01	0.02	0.01	0.02	0.01	0.03	0.01	0.03
6	1.6×10^{-3}	0.03	0.03	0.01	0.02	0.01	0.01	0.01	0.04
7	0.32×10^{-3}	0.06	0.03	0.01	0.00	0.01	0.01	0.01	0.04
8	0.064×10^{-3}	0.06	0.04	0.01	0.03	0.01	0.07	0.01	0.04
9	0.0128×10^{-3}	0.06	0.04	0.01	0.03	0.01	0.07	0.01	0.04
10	0.0256×10^{-3}	0.07	0.04	0.01	0.07	0.01	0.08	0.01	0.04
11	0.000152×10^{-3}	0.07	0.06	0.03	0.11	0.01	0.08	0.01	0.05

Table 2: Comparison of viability in *Moringa* and *Vinca* extract

	Absorbance		Viability (%)	
Type of extracts and concentration (mg mL^{-1})	V	M	V	M
Methanol				
20010^{-3}	0.01	0.01	2	2.0
40×10^{-3}	0.01	0.01	2	2.0
8×10^{-3}	0.01	0.02	2	4.5
1.6×10^{-3}	0.03	0.03	7	7.0
0.32×10^{-3}	0.06	0.03	12	7.0
0.064×10^{-3}	0.06	0.04	12	9.0
0.0128×10^{-3}	0.06	0.04	12	9.0
0.00256×10^{-3}	0.07	0.04	15	9.0
0.000512×10^{-3}	0.07	0.06	15	12.0
Ethanol				
200×10^{-3}	0.01	0.02	2	4.5
40×10^{-3}	0.01	0.02	2	4.5
8×10^{-3}	0.01	0.02	2	4.5
1.6×10^{-3}	0.01	0.02	2	4.5
0.32×10^{-3}	0.01	0.00	2	0.0
0.064×10^{-3}	0.01	0.03	2	7.0
0.0128×10^{-3}	0.01	0.03	2	7.0
0.00256×10^{-3}	0.01	0.07	2	15.0
0.000512×10^{-3}	0.01	0.11	2	22.0
Ethyl acetate				
200×10^{-3}	0.01	0.04	2	9.0
40×10^{-3}	0.01	0.04	2	9.0
8×10^{-3}	0.01	0.03	2	7.0
1.6×10^{-3}	0.01	0.01	2	2.0
0.32×10^{-3}	0.01	0.01	2	2.0
0.064×10^{-3}	0.01	0.07	2	15.0
$0.0128\times^{-}10^{-3}$	0.01	0.07	2	15.0
0.00256×10^{-3}	0.01	0.08	2	17.0
0.000512×10^{-3}	0.01	0.08	2	17.0
Chloroform				
200×10^{-3}	0.01	0.03	2	7.0
40×10^{-3}	0.01	0.03	2	7.0
8×10^{-3}	0.01	0.03	2	7.0
1.6×10^{-3}	0.01	0.04	2	9.0
0.32×10^{-3}	0.01	0.04	2	9.0
0.064×10^{-3}	0.01	0.04	2	9.0
0.0128×10^{-3}	0.01	0.04	2	9.0
0.00256×10^{-3}	0.01	0.04	2	9.0
0.000512×10^{-3}	0.01	0.05	2	11.0

RESULTS

In vitro Cytotoxicity

Cytotoxicity of different leaf extracts of *Moringa oleifera* showed drastic effect on Multiple myeloma cell lines compared to *Vinca rosea* extracts with least viability profiles as shown in Fig. 1-4. Methanolic extracts of *Moringa* leaf extracts showed basic sigmoidal dose responsive curve with much more cytotoxic effect than the respective extract from *Vinca rosea*. Table 1 represents the absorbance at 540 nm by the cells after treatment with various extracts for 60 min. Fig. 5a and b shows the apoptosis of cells before and after use of extracts under bright field microscope (400X).

Cell Viability Profiles

Table 2 shows the percentage viability profiles of the extracts of *Moringa* and *Vinca* on U266B1 human Multiple myeloma cell lines. Methanolic extracts of *Moringa* and *Vinca* showed perfect cytotoxic profile with reduced viability of cells *in vitro* aiding to the apoptotic induction. In case of methanol extracts, *Moringa oleifera* leaves exhibited even less viability compared to *Vinca* leaves with

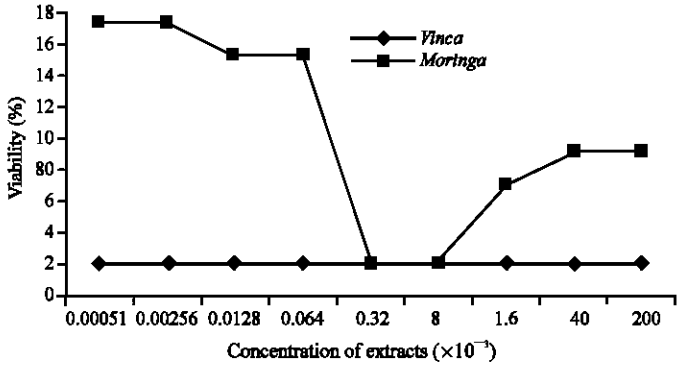

Fig. 2: Cytotoxic effect of acetate extracts

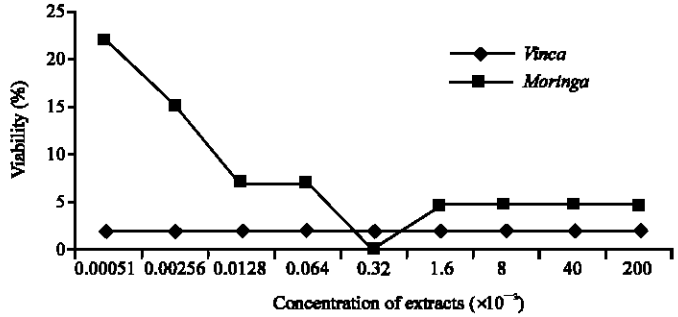

Fig. 3: Cytotoxic effect of ethanolic extracts

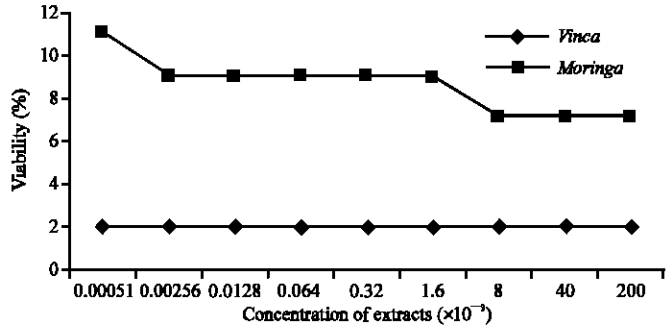

Fig. 4: Cytotoxic effect of choloroform extracts

least viability at highest dose (2%) and less viability at lowest dose of 0.5 μg mL^{-1} (12%). Viability of cells during the assay is represented in Fig. 1-4. Typical sigmoidal behaviour of viability of cells is observed in case of methanol extracts representing the aggressive action of *Moringa* leaves, (Fig. 1) with an ID_{50} value of 0.32 μg mL^{-1}.

DISCUSSION

The search for anti cancer agents from natural sources has been successful world wide. *Vinca rosea* has been reported to be utilized in treating patients with cancer (Myeloma) in USA (Kufe *et al*., 2003). Vinorelbine, a new *Vinca* alkaloid has been used to treat advanced malignant human myeloma cell lines (Naoya *et al*., 2002). Some traditional Thai rhizomes like *Langus galanga* with

Acetoxychavicol (ACA) a natural compound has been reported to inhibit cellular growth of multiple myeloma cells *in vivo* and *in vitro* through apoptosis (Keisuke Ito *et al*., 2005). Further compounds like β-lapachone from *Tabebuia avellanedae* showed anti tumor potential in multiple myeloma cell lines which are drug resistant. (Deepak *et al*., 2002) and mushrooms like *Ganoderma lucidum* reported apoptosis in multiple myeloma cells (Claudia *et al*., 2006).

The present study assayed different leaf extracts of *Moringa* and *Vinca rosea* representing different cytotoxic and antiproliferative effects against human Multiple myeloma (U266B1) cells through *in vitro* screening (Fig. 5a and b).

Methanolic extracts of *Moringa* compared to *Vinca* leaves had greater activity with ID_{50} value of 0.32 μg mL^{-1}. All the organic extracts of *Moringa* showed their kind of antimyelomic activity which was reliable in case of *Vinca* extracts. Nevertheless, methanol extracts of *Moringa* are most active in accord to neutral red dye uptake assay (Borenfreund and Puerner, 1985) where the optical density and consequent cell percentage viability suggested the effect of compounds present in the extracts on cell proliferation (Table 2). The assay period of 60 min incubation showing drastic antiproliferation of cells with less dye uptake refers to the active participation of methanol extracted compounds present in *Moringa* leaves that aid for apoptosis in Myeloma cells.

Finally the random study on cytotoxic effect of *Moringa* leaf extracts favours further experimentation to detect the antimyelomic compounds present in *Moringa*, their purification, characterization and site of action. It is expected that flavonoid or alkaloid group of compounds similar to vincristine and vinblastin might be present in *Moringa* leaves that could be used in the herbal treatment of Myeloma patients.

Fig. 5a: Microscopic examination of live cell

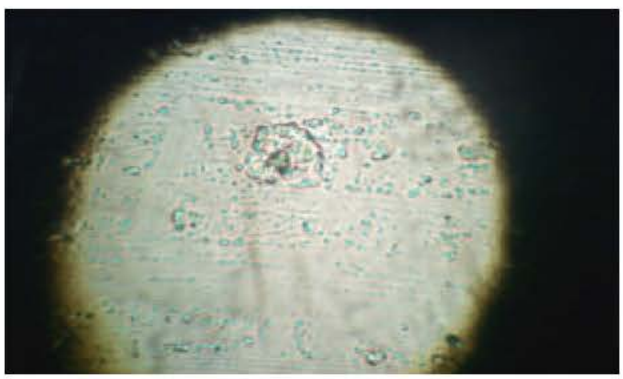

Fig. 5b: Microscopic examination of dead cell

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