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Screening of *Moringa Oleifera* Leaf Extract on Various Human Cancerious Cell Line Using Microtiter Plate Based Assay

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Abstract

The Moringa plant (*Moringa oleifera*) has been the object of much research due to its multiple uses and well-known medicinal properties. It has been reported that leaf extract of *Moringa oleifera* has anti-proliferative effect on the human cervical cancer cell line, KB cells and alveolar cancer cell line A549 cells. The object of the present work is to investigate the anti-cancer effect of *Moringa oleifera* leaf extract on various human cancerious cells like DU-145, MCF-7, HEP-3B, K-562 and HCT-15. Cold extraction of powdered leaves was carried out using hydro-alcoholic mixture (water: methanol; 30:70 v/v). Cell viability was measured by MTT assay and IC_{50} value was calculated using Graph Pad Prism Ver. 6.01 and Microsoft word excel 2008 at one third dilutions with ten different points which was compared with standard anticancer drug Colchicin. The effect of the extract on VERO (normal) cell was evaluated as well. The leaf extract showed a dose-dependent inhibition on cell proliferation K-562 (IC_{50} value 32.43 µg/ml, R^2=0.9253), DU-145 (IC_{50} value 42.74 µg/ml, R^2=0.9862), HCT-15 (IC_{50} value 5.213 µg/ml, R^2=0.9647), MCF-7 (IC_{50} value 24.76 µg/ml, R^2=0.9701), HEP-3B (IC_{50} value 29.37 µg/ml, R^2=0.9772). It showed low cytotoxicity in the normal cells. Results of DNA fragmentation study showed significant fragmentation pattern in case of K-562, DU-145 and HCT-15 cells when treated by IC50 concentration. The present study suggests that the hydro-alcoholic leaf extract of *M. Oleifera* induces anticancer effect on K-562, DU-145 and HCT-15 cancer cells.

Keywords

Moringaoleifera, Leaves, Hydro-alcoholic Extract, Cell Lines, MTT Assay, DNAFragmentation

1. Introduction

During the last two decades, the use of microbial and plant products, has gained an increased interest in cancer therapy [1, 2]. Several compounds isolated from herbaceous medicinal plants were found to possess anticancer activities [3]. Furthermore, medicinal plants and their extracts represented a good source for anticancer bioactive compounds currently applied in clinical trials for cancer treatment. Paclitaxel, which is isolated from *Taxusbrevifolia* Nut, is used against ovarian cancer and advanced breast cancer [4]. Podophyllotoxin, extracted from *Podophyllumemodi*is used with its derivatives (etoposide and teniposide) for the treatment of lymphomas as well as bronchial and testicular cancers [5]. Vincristine extracted from *Catharanthusroseus* G. Don. is mainly used with other cancer chemotherapeutics for treating different cancer types, *i.e.* leukemia, breast and lung cancer [6]. Despite the recent advancements in chemotherapeutics, chemotherapy is still associated with severe adverse effects such as nephrotoxicity, nausea, hair loss, skin irritation, anemia, and infertility [7, 8]. Therefore, naturally occurring anticancer compounds from natural plants, especially those with low toxicity and high potency, have important implications for chemotherapy and chemoprevention. Natural plants have drawn much attention

for their pharmacological effects in the treatment and prevention of various diseases due to their high biocompatibility, low toxicity, and potential biological activity [9]. Among them, edible M. oleifera is known to be a rich source of various nutrients and has therefore been regarded as an important crop [10]. Additionally, the plant has been considered a multipurpose plant that could be used as a medicinal plant; vegetable; animal fodder; and a source of vegetable oil, which is used in condiments and the manufacture of perfumes, cosmetics, and hair care products [11, 12]. Among the various parts of M. oleifera, the roots, pods, seeds, and gum are used to treat rheumatism and to relieve edema and arthritis [13, 14] the leaves have been reported to have hypocholesterolemic [15], hepatoprotective [16, 17], antimicrobial [18], anti-gastric ulcer [19], antiviral [20], and hypotensive [21] effects and have been used in the prevention of cardiovascular diseases and as antioxidant [22]. However, because of the importance and versatility of the plant, most of the published reports focused on compositional analysis and on its use as a dietary supplement. M. oleifera is also used as a health food and cosmetic in many countries, but its medicinal effects have not yet been well established. However, only a few studies have reported the anticancer activity of M. oleifera seed oil, and much of them have focused on the evaluation of their efficacy with respect to tumor suppressive activity in breast cancer, but not on the human colon carcinoma, mammary gland carcinoma, liver carcinoma, leukemia and prostate cancer cell inhibition activity. Additionally, most studies have been conducted using solvent extracts of leaves and but not their hydro-alcoholic soluble extracts [23-27]. Solvent extraction is the most frequently used technique for the isolation of bioactive compounds from plants. Therefore, the recovery of bioactive compounds from M. oleifera has been typically accomplished using various solvents, such as methanol and ethanol, as well as hot water and buffers. Nevertheless, the majority of the studies focused on solvent extracts because the efficacy of solvent extraction is higher than simple water extraction. In fact, the buffer extract of M. oleifera leaves was much less effective than the hydro-alcoholic extracts for hepatic carcinoma cells [28]. Moreover, hydro-alcoholic system can dissolve the many useful organic molecules found in plants, such as phenolic compounds. Based on above discussion, our aim for the present investigation was to prepared a hydro-alcoholic extract of the leaves of MO plant and investigated the possibility as anticancer drugs in different types of human cancer cell lines. Finally, the medical value of a hydro-alcoholic extract of the leaves of MO will be discussed base on intensity of DNA fragmentation efficacy on various cancerious cell line.

2. Material and Methods

2.1. Cell lines and Media

All the cancer cells and African green monkey kidney (VERO) cells used in this study were purchased from NCCS, Pune, India. The cells were grown in DMEM (i.e., HCT-15, MCF-7, HEP-3B, K-562 and DU-145 and VERO) (Hi-Media Lab, Mumbai, India) supplemented with 10% fetal bovine serum (FBS; Hi-Media) and 1% penicillin-streptomycin. Cells were inoculated at a density of 100,000 cells in a 96-well plate and were maintained at 37°C in a humidified atmosphere containing 95% air and 5% CO_2. Thioglycollate medium (TGM) and Tryptone soya broth (TSB) were purchased from Hi-Media Laboratory Pvt., Ltd, Mumbai, India.

2.2. Preparation Plant Leaves Extract

Dried leaves of *M. oleifera* Lam. were obtained from botanical garden of ICAR-DMAPR, Anand, Gujarat. The leaves were ground into powder (20#) using a mortar and pestle. 100g of the powdered leaves were extracted in 500ml conical flasks using 30 ml of deionized distilled water and 70% ethanol. The conical flasks were plugged with rubber corks, then shaken at 120 rpm for 30 min and allowed to stand at room temperature for 5 days with occasional manually agitation of the flask using a sterile glass rod at every 24 hour. The extracts were separately filtered using sterile Whatman no. 1 filter paper. The resulting filtrates were then concentrated in a rotary evaporator and subsequently lyophilized to dryness.

2.3. Cell Proliferation Assay (MTT Assay)

The cytoxicity study of the methanolic and ether extracts of leaves and root of plant was studied on cultured cells using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (29). Cells were seeded in 96-well plates in DMEM supplemented with 10% FBS. After 24 h of incubation in a humidified 5% CO_2/air environment at 37°C, when cells became 70-80% confluent, then the cells were grown in 96-well plates for establishment of monolayer at a density of 1×10^5 cells per well according to the instruction given in kit manual (EZcount MTT Cell Assay Kit, PC:CCK003, Hi-Media). After achieving desired cell density, cells were treated with one third dilution series of various extract concentrations (0.05, 0.15, 0.46, 1.37, 4.12, 12.35, 37.04, 111.11, 333.33 and 1000 µg/ml) and incubated further for 24 hrs. Then after next day, 25 µl of the MTT solution (5 mg/ml) was added to each well, and the plate was re-incubated for 4 hrs. Finally, 100 µl of DMSO:IPA (60:40) solubilizing mixture was added to dissolve formazan crystals. Then absorbance of the plate was measure at 570 nm by using a 96 well micro plate reader (Multiskan Reader CF3, Thermoscientific). Percentage cell growth inhibition or percentage cytotoxicity was calculated by following formula:

$$\% \text{ Viability} = (A_T - A_B) / (A_C - A_B) \times 100$$

Where, A_T = Absorbance of treated cells (drug), A_B = Absorbance of blank (only media) and A_C = Absorbance of control (untreated).

2.4. Statistical Analysis of Data

The cytotoxicity data was reported in form of IC_{50} value which was calculated using GraphPad Prism software Ver. 6.01. Linear regression analysis with 95% confidence limit and R^2 were used to define dose-response curves and to compute the concentration of chemical agents needed to reduce absorbance of the formazan by 50% (IC_{50}).

2.5. DNA Fragmentation Study

The DNA fragmentation analysis was carried out to distinguish the apoptosis from necrosis, and is among the most reliable methods for detection of apoptotic cells. The DNA fragmentation study was performed by selecting the IC_{50} dose of the various extracts of *Withania Somnifera* against cancer cell lines K-562, DU-145. Standard kit manual protocol (Apoptotic DNA Ladder Kit, KH01021, Thermofisher, USA) was followed to perform DNA fragmentation study [30].

2.6. Microscopy

To monitor cell morphology, cells were visualized by light microscopy (Leica Microsystems, DMIL, Germany). Images were captured with a Power Shot S45 Canon Digital Camera system.

3. Results and Discussions

In the present study, hydro-alcoholic leaves extract of *Moringa oleifera* was evaluated for in- vitro cytotoxicity assay against five different cell lines; DU 145 (Prostate cancer), MCF 7 (Breast cancer), K 562 (Leukemia), HCT 15 (colon cancer) and VERO (African green monkey's kidney normal cell line). In preliminary experiment, I tested several tetrazolium salts by incubation with cells for several hours. The most promising reagent was MTT, a pale yellow substrate that produced a dark blue formazon product when incubated with living cells. The MTT formazon reaction product was only partially soluble in the medium and so the mixture of IPA with Hexane (60:40%v/v) was used to dissolve the formazon and produce a homogeneous solution suitable for measurement of optical density. The cleavage of MTT has several desirable properties for assaying cell survival and proliferation. MTT is cleaved by all living, metabolically active cells that we have tested, but not by dead cells. In the present work, the extract was evaluated against selected cell lines named K-562, DU-145, MCF-7, HCT-15, HEP-3B and VERO cell line (Normal cell). For each tested compound, Dose Response Curve (DRC) against all cell lines was plotted with 10 analysis points i.e. with 10 different drug concentrations

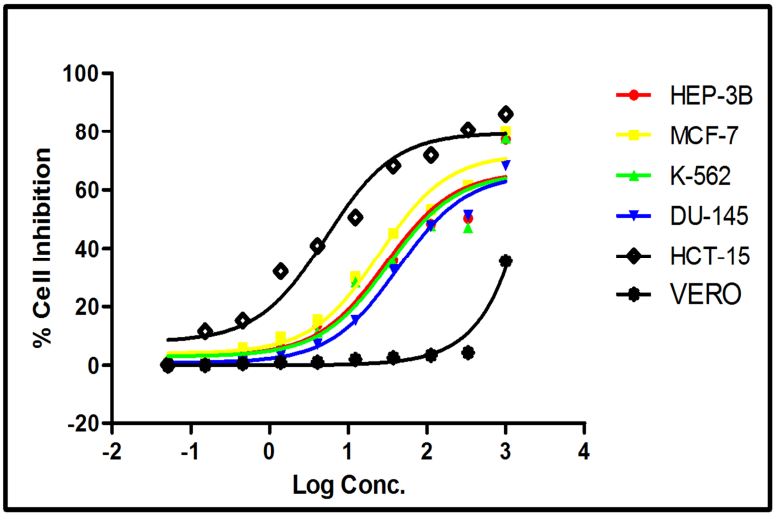

Figure 1. *Dose Response Curve of hydro-ethanolic extract of Moringa oleifera leaves on various cell lines.*

This assay was also performed for Colchicine as standard compound against same cell lines. Result for each cell lines were reported as the IC_{50} value. The concentration causing 50% cell growth inhibition (IC_{50}) was determined from DRC using *Graph Pad Prism* software (Ver. 6.01) (Graph Pad Software, Inc., USA) and Micorsoft Excel 2007 (Microsoft Corporation, USA) application

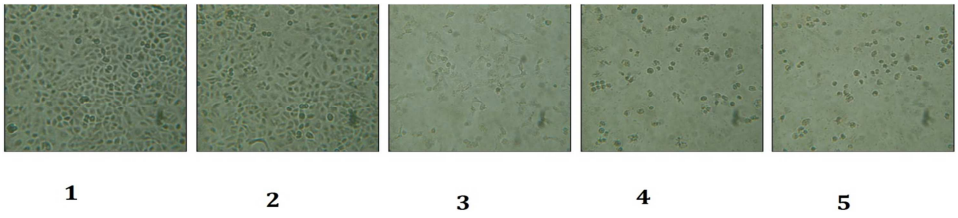

Figure 2. *Inhibitory effect of IC_{50} concentration on various cell proliferations.*

(1) MCF-7 cell (2) HEP-3B cell (3) K-562 (4) HCT-15 cell (5) DU-145 cell under Microscopic view (×1000)

Table 1. *Percentage cell inhibition by Hydro-Ethanolic leaves extracts of Moringaoleifera.*

Con. µg/ml	Log Con.	% Cell Inhibition on different cell lines					
		HEP-3B	MCF-7	K-562	DU-145	HCT-15	VERO
0.05	-1.29	1.033	0.72	1.232	0.3487	0.17	-0.240
0.15	-0.82	1.598	2.65	1.427	0.4089	11.65	0.000
0.46	-0.34	2.517	6.018	2.614	1.3481	15.22	0.560
1.37	0.14	6.051	9.587	5.982	3.2682	32.07	0.980
4.12	0.61	13.88	15.46	11.37	7.2642	40.88	1.060
12.35	1.09	29.15	30.16	28.36	15.331	50.67	2.030
37.04	1.57	35.87	45.26	34.89	32.562	68.29	2.560
111.11	2.05	48.35	53.26	47.82	47.592	71.88	3.450
333.33	2.52	50.32	61.53	47.25	51.342	80.62	4.350
1000	3.00	77.31	80.25	77.65	68.25	86.07	35.620
IC_{50} (µg/ml)		29.37	24.76	32.43	42.74	5.213	>1000
R^2		0.9372	0.9707	0.9253	0.9862	0.9647	0.9467
ColchicineIC_{50} (µM/ml)		8.779	1.455	35.11	40.1	8.779	1.455

Table 1 showed results of in-vitro cytotoxicity study of K-562, DU-145, HCT-15, MCF-7, HEP-3B and VERO cell lines for hydro-alcoholic extract. It showed order of cytotoxicity activity in comparision to standard Colchicindrug. Potency of the extract for cell inhibition obtained was K-562 (IC_{50} value 32.43 µg/ml, R^2=0.9253) > DU-145 (IC_{50} value 42.74 µg/ml, R^2=0.9862) > HCT-15 (IC_{50} value 5.213 µg/ml, R^2=0.9647)) > MCF-7 (IC_{50} value 24.76 µg/ml, R^2=0.9701)) > HEP-3B (IC_{50} value 29.37 µg/ml, R^2=0.9377). *Moringa oleifera* leaves extract showed good cytotoxicity activity against K-562 cell line while, same induced less activity in MCF-7 and HEP-3B. Moreover, *Moringa oleifera* leaves extract was also studied for normal cell cytotoxicity effect using VERO cell line. Study results of normal cell are summarized in Table 1 which showed >1000 µg/ml indicated they are safe for normal body cell when it will use for therapeutic purposes. The cytotoxic effect of extract against various cell lines was also examined using microscopy. For the further confirmation of anticancer activity of all four methanolic extracts, DNA fragmentation study was carried out.

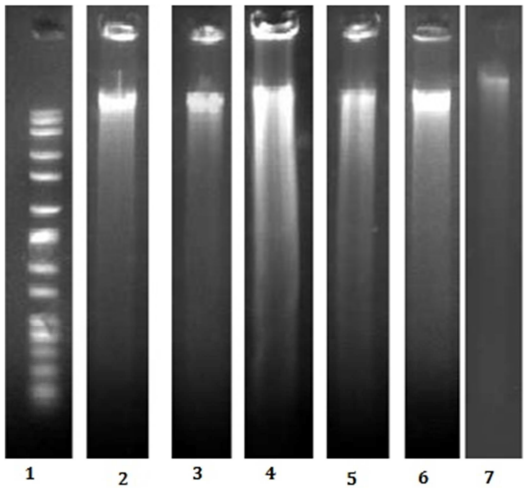

Figure 3. *DNA fragmentation study on different cancer cell lines.*

Lane 1: 100 BP DNA ladder Lane 2: HEP-3B cell line Lane 3: MCF-7 cell line Lane 4: K-562 cell line Lane 5: DU-145 Lane 6: HCT-15 cell line Lane 7: VERO cell line

From the results of DNA fragementation, it was found that the extract was exhibited excellent DNA fragmentation pattern in K-562, DU-145 and HCT-15 comparision with standard DNA ladder, which confirms the apoptosis mechanism rather necrosis properties. In addition, less DNA fragmentation pattern was observed in MCF-7 and HEP-3B indicated less cytotoxicity effect. The possible reason behind cytotoxicity activity is because of the leaves contains most of nitrile glycoside derivatives. But further study is required to confirm the same for its molecular mechanisms by studying various cancer pathways.

4. Conclusion

In conclusion, the obtained results clearly confirm that the leaves extract obtained from *Moringa oleifera* has a significant inhibitory effect on different cell lines investigated. The current investigation can be considered as a primary investigation paving the way for future work investigating the possible mechanisms and modes of action on various human cancerious cell lines. Results of anticancer activity study showed that extract of leaves induced severe cell cytotoxicity in K-562 and DU-145 cancer cells; however it was not the case anymore in normal cells. Further confirmation for anticancer activity of extract using DNA fragmentation assay demonstrated good DNA fragmentation properties in leukemic and prostate cancer cells, which indicates good apoptosis effect. Overall, these data suggest that the hydro-alcoholic leaves extract of *Moringa oleifera* may become a good candidate for anticancer therapy in leukemia and prostate cancer. Further studies are required in this regard.

Conflict of Interest

The authors have no any conflict of interest.

Abbreviations

MTT: [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

KB cell: Keratin forming tumor cell
A-549: adenocarcinomic human alveolar basal epithelial cells
DU-145 (human prostate cancer cell)
HCT-15:Human Colorectal adenocarcinoma
K-562: human immortalized myelogenousleukemia
MCF-7:breast cancer cell line
HEP-3B: human hepatomacell line

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