ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 12, Issue, 07, pp.12392-12396, July, 2020

DOI: https://doi.org/10.24941/ijcr.39118.07.2020

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

MORINGA OLEIFERA LEAVES EXTRACT ACTS AS ANTI-CANCER AGAINST BREAST CANCER CELL LINES

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ARTICLE INFO

Article History:
Received 21st April, 2020
Received in revised form
30h May, 2020
Accepted 07th June, 2020
Published online 28th July, 2020

Key Words:
Moringa Oleifera Leaves Extract,
Cell Lines, Cytotoxicity, Anticancer.

ABSTRACT

Medicinal plants are most important indigenous source of herbal medicine for developing country. There are few of medicinal plants were used for treatment of breast cancer have been documented. The present study aimed to assess the cytotoxic effect of Moringa oleifera plant leaf extracts on three different cell lines (MCF-7, MDA-MB-468 and MDA-MB 231). Different doses of plant extract and standard were taken and introduced into cancer cells lines were recorded at 72 hrs respectively it clearly showed an effective cell growth inhibition of human breast cancer cell lines MCF-7, MDA-MB-468 and MDA-MB-231 in a dose and specific time duration for the inhibition of cells. The present study demonstrates that M. oleifera leaf extract have anti-proliferative and pro-apoptotic effects on breast cancer cell lines in a concentration and time-dependent manner, which suggests their potential to be used as a new therapeutic strategy for breast cancers. These results highlight the novelty of this finding because earlier no report has yet been cited on the effectiveness of Moringa leave extracts found in the indigenous environment as an anti-cancer agent against breast. Our study is the first of its kind to evaluate the anti-malignant properties of Moringa leaves. These findings suggest the leaf extracts of Moringa collected from the widely distributed in different Indian region possess anti-cancer activity that can be used to develop new drugs for treatment of breast cancers.

Citation: ***Ramanuj K Gupta, Dharmshila Kumari Aniket K. and Ajit K Saxena*. 2020.** "*Moringa oleifera* leaves extract acts as anti-cancer against breast cancer cell lines", *International Journal of Current Research*, 12, (07), 12392-12396.

INTRODUCTION

Moringa oleifera (MO) belongs to family Moringaceae is angiosperm plants [Olson, 2002]. This plant mostly found in the Himalayan region that is widely cultivated throughout tropical and sub-tropical countries of the world including India. [Mbikay, 2012; Alaklabi, 2014]. The plant has numerous medicinal applications and is used as indigenous medicine for the treatment of various diseases like skin, hypertension, diabetes, anemia, cancer etc [Patel *et al*., 2010; Tiloke *et al*., 2013; Emmanuel *et al*., 2014; Jung, 2014;]. Additionally, the pharmacological importance of the leaves extract containing bio-active compounds were earlier reported Leone *et al* (2015) about 74% of the known anti-cancer medicines are derived from various plant species. Indeed, there are many household dietary products exhibiting anti-cancer potential with minimal side effect that are currently under clinical trials for cancer treatment [Mann, 2002; Garg *et al*., 2005]. The present study based on analysis MO plant extract therapeutic mechanisms to identify the DNA copy number variation of TNF α, GST and SOD of treatment as well as control.

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TNF antagonists may offer therapeutic potential in solid tumors, but patients who have high serum levels of TNF-α fail to respond to infliximab, suggesting consumption of the circulating antibody and loss of transmembrane TNF-α on tumors by ectodomain shedding (Yu M, *et al.,* 2013). Glutathione S-transferase (GST), a Phase II detoxification enzyme, has recently been implicated in protection against apoptosis. Expression of GST protein, an established apoptosis marker in cases of infiltrative ductal breast carcinomas (Huang J, *et al.,* 2003). Superoxide dismutase (SOD), one of the antioxidant enzymes, plays an important role in defense against reactive oxygen species (ROS). Many previous studies reported the association between SOD polymorphism and the cancer susceptibility (Kang, 2015). Hence, *MO* leaf water extract had no activity on MCF-7 cells while the ethanol extract inhibit the MCF-7, MDA-MB231 and MDA-MB453 human breast cancer cell lines in a time- and dose-dependent manner (Zhang *et al*., 2015). This compound detoxifies carcinogens, serves as an intracellular antioxidant (protecting cell membranes and intracellular components from damage by free radicals) and assists in the regulation of DNA synthesis. Stimulation of glutathione (GSH) not only functions as a co substrate for the family of GST, enzymes necessary for conjugating GSH to electrophiles and thus blocking DNA-adduct formation, but also serves as a reductant for glutathione

peroxidise (Lubos *et al*, 2011). A solvent of *MO* (ethanol) in different concentrations were tested against human breast cancer cell lines and checked for cell viability percentage. The percentage of cell viability decreased with increased concentration of ethanol solvent (Kumari et.al 2017). However, the cell lines (CLs) are frequently used in many laboratory research work such as in-vitro models in cancer research. CL have their own advantages like easy to handle and represent an unrestricted self-replicating source of cells that can be grown in desired quantities. [Osborne *et al*., 1987]. CLs can used as an initial screen for source that might regulate drug resistance activities. To establish more appropriate prototypes of drug resistance and explore the differences that occur in the various drug resistant by different treatments methods [Kashyap, *et al*., 2011]. In this study we used MO leaves extract to observe its efficacy as an anti-cancer agent on breast cell lines. To interpret the effectiveness of leaves extracts, we analyzed cell motility and survival condition to measure the phenotypic changes in MCF-7, MDA MB231 and MDA-MB-453 breast cancer cell lines. The rationale behind this study and selection of this cell lines are; easy to access and availability also more importantly, prevalence of this cancer in a major population of the Indian.

MATERIALS AND METHODS

Cell lines and culture medium: Human breast cancer cell line MCF-7, MDA-MB-231, and MDA-MB-453 was obtained from National Center for Cell Sciences (NCCS), Pune and grown in Eagles minimum essential medium (EMEM) containing 10% fetal bovine serum (FBS) at 37°C, 6.5% humidified CO_2 incubator, 95% air and 100% relative humidity. The MCF-10A cell line was cultured and supplemented with 10% FBS. Maintenance of cultures was changed twice a week.

Extract preparation: Preparation of Extract: MO leaves powder were purchased from Vokin Biotech Pvt. Ltd., Haryana (India), fssai no. 13316002000336). About 50g of dried leaves powder specimens was taken in round bottom flask and 500 ml of ethanol was added. The extraction was continued for 6–8 hrs until all the soluble constituents dissolved in the solvent. The methanol extracts were concentrated by evaporating at a reduced pressure using rotary evaporator. The concentrated extracts were further dried at 37^0 C for 3 to 4 days in order to facilitate complete evaporation of the solvents. Extracts thus obtained, were collected and stored at 4°C until further use.

In Vitro Assay for Cytotoxic Activity: For this study, the stock solution 50mg of extracts were dissolved in 1.0 ml of ethanol and filtered through a 0.22µM filter and cell culture medium to a final stock concentration of 10mg/ml and then diluted with complete culture medium to reach the desired concentrations. For doing the test, at first, detached cells were counted with a neobar chamber. Then, cells were seeded into 96-well plates having 400 cells/well, a density that allowed the untreated control to grow exponentially for 72 hrs. Further, after seeding of 24 hrs, cells were treated with different concentrations of plant extract including 0, 25, 50, 100, 250, 500 µg/ml for 48 and 72 hrs inculcated 37°C, 5% CO_2. For MTT test, control samples were incubated with equivalent amount of DMSO as a solvent of plant extracts. There were three replicates for each concentration of plants extracts.

MTT assay: The effect of ethanol extract of the plants on the viability of cells was determined by using MTT assay. For MTT assay, at the end of incubation period (48 and 72 hrs), 20 µL of (3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (Atocel) (5 mg/ml) was added into each well. After 3 hrs. of incubation at 37 °C, media was removed and 100 µL of DMSO was added to each well. Further, optical densities (ODs) were carried out by an enzyme-linked immunosorbent assay (ELISA) reader (BioTek-ELx800, USA) at 490 nm. ODs were used to calculate the viability of cells, percentage of viable cells and dividing the mean absorbance of treated cells with mean absorbance of its control cells.

Determination of IC50: Inhibition concentration 50 (IC50) were calculated by using the concentration of compound required to inhibit 50 % cell growth by plotting a graph of Log (concentration of Extract) in compare with % cell inhibition. A line drawn from the 50 % value on the Y axis meets the curve and incorporate to the X axis. The X axis value gives the Log (concentration of the compound). The antilog of that value gives the IC50 value. Percentage inhibition of novel compounds against all cell lines was calculated using the following formula:

$$\% \text{ cell survival} = \frac{(\text{At} - \text{Ab})}{(\text{Ac} - \text{Ab})} \times 100$$

Where, At = Absorbance of Test, Ab= Absorbance of Blank Media, Ac= Absorbance of control cells and % cell inhibition = 100 − % cell survival

DNA Isolation & Real Time PCR: DNA isolation kit (ReliaPrep Blood gDNA Miniprep System) was acquired from Promega (Promega Corporation, USA) and SYBR Green PCR Master Mix (Bio-Rad USA). Primers were obtained from Eurofines listed in table 1. To determine the DNA copy number variation of TNFα for cell signaling, SOD & GST treated as oxidative stress marker and GAPDH was used as housekeeping gene. The PCR assay will be carried out by using specific forward and reverse primers of gene of interest in total volume of 20 µl which contain 50 ng of DNA, 10 picomole of each primer, sybergreen green master mix (2.5mM $MgCl_2$ 2.5mM dNTP and 5 Unit of Taq DNA polymerase).

Statistical Analysis: Statistical analysis was done by using Student's t-test to find experimental differences. Experimental data were represented as the mean ± SD. A p-value of less than 0.05 was considered to be statistically significant value.

RESULTS

Proliferative effects of MO on Cell Lines: The effect of anticancer property of MO on MCF 7, MDA MB 231 and MDA MB 453 was evaluated through MTT assay. The different concentration of ethanolic extract from MO were use and effective doses were calculated from dose response curve. Results of the cytotoxicity evaluation against MCF 7 cell lines of the MO extract are shown in figure 1 (A & B), MDA MB 231 in figure 2 (A & B) and MDA MB 453 shown in figure 3 (A & B).

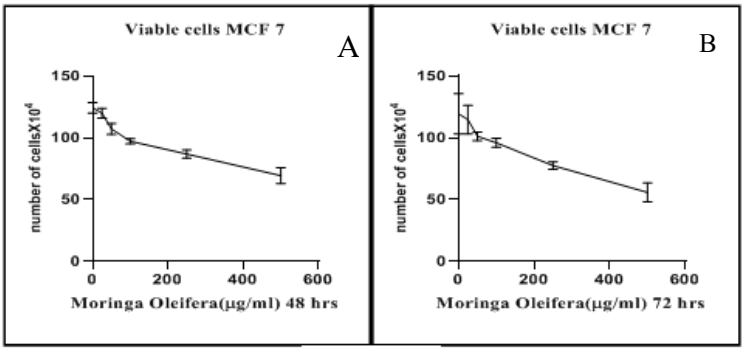


Figure 1 A & B: Showing dose response curves of MCF 7 cell line of M.O. extract during 48 and 74 hrs treatment.

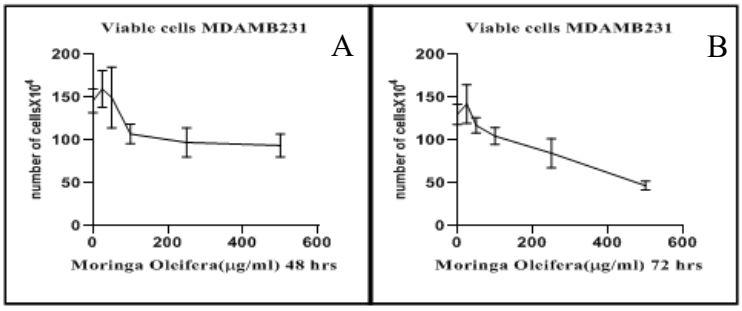


Figure 2 A & B: Showing dose response curves of MDA MB 231cell line of M.O. extract during 48 and 74 hrs treatment.

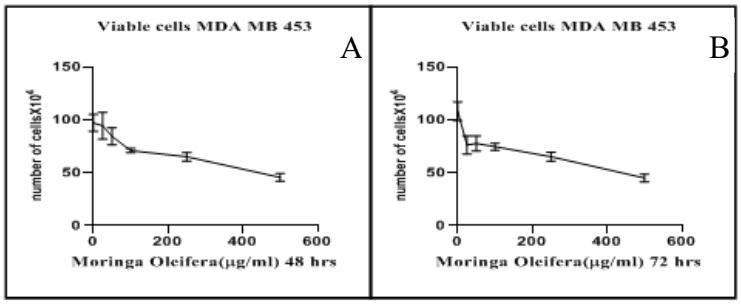
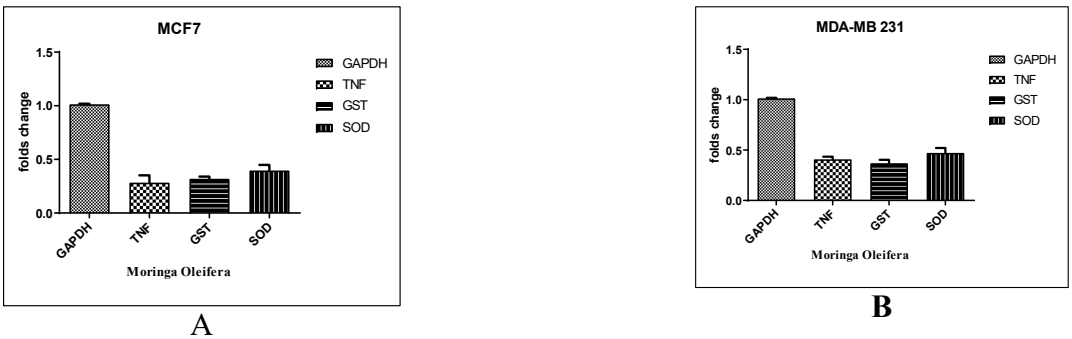


Figure 3 A & B: Showing dose response curves of MDA MB 435 cell line of M.O. extract during 48 and 74 hrs treatment



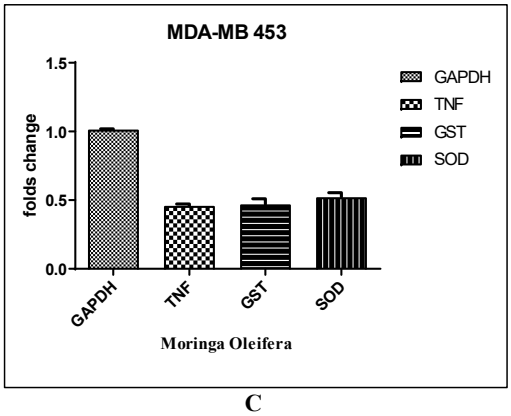


Figure 4 A, B & C: Showing dose response curves of MDA MB 435 cell line of M.O. extract during 48 and 74 hrs treatment

Table 1. Primer for Real time PCR designed by primer blast software (NCBI), standardized and analysis done on following PCR condition

Sl.No.	Gene	Forward primer	Reverse primer	PCR Condition X 40 cycle
1	TNF α	3'CTCTTCTGCCTGCTGCACTTTG 5'	3'ATGGGCTACAGGCTTGTCACTC 5'	94˚C -4 min 94˚C -30sec 58˚C -30sec
2	GST	3,TGGACATGGTGAATGACGGCGT 5'	3'GGTCTCAAAAGGCTTCAGTTGCC 5'	94˚C -4 min 94˚C -30sec 58˚C -30sec
3	SOD	3'CTCACTCTCAGGAGACCATTGC 5'	3'CCACAAGCCAAACGACTTCCAG 5'	94˚C -4 min 94˚C -30sec 57˚C -30sec
4	GAPDH	3'GCACAGTCAAGGCCGAGAAT 5'	3'GCCTTCTCCATGGTGGTGAA 5'	94˚C -4 min 94˚C -30sec 57˚C -30sec

Table 2. Statistical analysis of different leaf extract on cell lines.

Sl. No.	Plant Extract	S.E	95% C.I	P Value
1	*Moringa Oleifera MCF 7*	0.2336	0.1537 to 0.05679	0.002
2	*Moringa Oleifera* MDA-MB-231	0.0345	0.2304 to 0.01203	0.002
3	*Moringa Oleifera* MDA-MB-453	0.4336	0.1537 to 0.05679	0.002

Interestingly, we observed significant decrease in cell survival in these cancer cell lines when treated with the extracts of leaves. A graphical presentation was constructed summarizing the effect of MO extracts on growth of these three cancer cell lines mentioned in figures 1-3.

IC50 value determination of plants extract: In this study, the cytotoxicity of ethanol extracts of MO were determined using MTT assay cell lines 0-500 µg/ml of extracts at two incubation period of 48 and 72 and IC50 values were determined. On the treatment with MO extract, the MCF-7, MDA MB 231 and MDA MB 453 cell lines showed an increased rate of cell death at a higher concentration of the plant extract.

Quantification of DNA copy number variation (CNV): Cells treated with leaf extract showed significant decreases in TNF-α, GST and SOD levels with respect to housekeeping gene (GAPDH) shown in figure 4 A-C. The results of the whole study indicate that MO extract is a good antioxidant scavenger, as it decreases cell viability. However, MO leaf extract alone is cytotoxic to MCF 7, MDA MB 231 and MDA MB 453 cancerous cell lines and reduces the CNVs, suggesting the very significant correlation with cell viability. In addition, the appearance of TNF-α, GST and SOD gene were significantly reduces the efficiency of cell lines. Whereas statistical analysis was calculated using comparative Ct value analyses considered as statistically significant shown in Table 2.

DISCUSSION

In our study, we use MO, a common medicinal plant belong to family Moringaceae, is distributed worldwide and has been known by a number of different names, including the horseradish tree (English), Soanjna (Hindi), Shobhanjana (Sanskrit) (Sahoo *et al*., 2014) This plant extract containing numerous anti-oxidant and anti-cancer properties [Abdull *et al*., 2014]. The plant exhibits anti-cancer potential by interfering with the signal transduction mechanism that promotes cancer cell proliferation and progression [Tiloke *et al*., 2013].

These antioxidant compounds possess anti-inflammatory, anti-tumor, anti-mutagenic, anti-carcinogenic in nature (Mitscher *et al*., 1996; Owen *et al*., 2000; Sala *et al*., 2002). In addition, Cai and co-worker reported that total phenolic content of Chinese medicinal plants showed a positive significant linear relationship with antioxidant activity. A plant extract that will act successfully as an anti-cancer drug should kill cancer cells without causing excessive damage to normal cells. Furthermore, MCF-7 cells are estrogen-receptor (ER) positive and classified as low-grade and luminal type. MDA-MB-231 and MDA-MB-453 cells are ER negative and classified as high-grade and basal type (Kenny *et al*., 2007). In this study, we evaluated the anti-cancer properties of MO leaves against MCF 7, MDA MB 231 and MDA MB 453 breast cancer cell lines and we found very significant result showing anti cancerous activity. However, further experiments are needed to evaluate the specific molecules in the apoptotic pathway and cytotoxic effect and induced DNA fragmentation on a breast cancer cell line mechanism that seemed to use the apoptosis pathway (Carvalho *et al*., 2012; Sriwiriyajan *et al*., 2014; Silva *et al*., 2014). Further, the hypothesized, the effectiveness of leave extract treatment for breast cancer using locally and widely grown plants may be a good herbal treatment of breast cancer.

Conclusion

These results suggest, MO leave extract exhibited anti cancerous and cytotoxic activity against breast cancer cells and lower toxicity. The cytotoxic effect of this fraction inhibited cell growth and appears to have induced apoptosis in MCF-7, MDA-MB-231 and MDA-MB-453 cells. Further molecular level study required to obtain a potentially active and pure compound will be considered as future herbal medicine to cure breast cancer.

Acknowledgments

I thankfully acknowledge my Ph.D. supervisor, my co-guide for providing infrastructural facilities for the completion of this research work and support.

Conflicts of Interest: The authors declare no conflicts of interest.

Funding: There is no any funding to carry out this research work. Infrastructure and support provided by AIIMS, Patna.

Author Contributions

Experimental work and Analysis has been perform by RKG, Manuscript preparation and Discussion done by AK, and technical support given by DK and AKS.

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