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Original Article

Moringa oleifera Methanolic Extract Induces Apoptosis in Human Breast Cancer Cells

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ABSTRACT

Background: *Moringa oleifera* Lam. (Family: *Moringaceae*), commonly known as 'drumstick tree' is beneficial to obtain sustenance as a food source and applied as a traditional medicine. The therapeutic potential of *Moringa oleifera* emphasizes on the inhibition of cancer proliferation and its versatile applications in Pharmacology which provide novel by-products and nutritive dietary supplements for human consumption. The objective of the present study is to investigate the efficacy of *M. oleifera* leaves methanolic extract (MOME) against the invasive breast cancer cell lines such as T-47D and MDA-MB-231. **Materials and Methods:** The different concentrations of MOME were cultured individually with breast cancer cells, T-47D and MDA-MB-231. The cell viability was determined after 24 hrs of treatment using MTT Assay (-(4, 5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) to analyse the anti-proliferative effect of MOME on cell lines, T-47D and MDA-MB-231. The cell cycle assay and apoptosis were evaluated by Annexin V- FITC Apoptosis Detection Kit, followed by the analysis through Attune flow cytometer. **Results:** The IC_{50} values of MOME in T-47 D and MDA-MB231 were found 45.33 ± 5.2 µg/mL and 24.44 ± 3 µg/mL, respectively. The G2M phase population exhibited an increase of 60.4% in MOME treated T-47D cells and 11.6% in MDA-MB231 cells. The population of early apoptosis cells increased to 37.1% in MOME treated cells. The time and dose dependent treatment with MOME inhibited the growth and induced apoptosis in T-47D and MDA-MB-231 cells. The externalization of phosphatidylserine on membrane of T-47D and MDA-MB-231 cells confirmed the apoptosis. **Conclusion:** These findings suggest that MOME actively contributes to cell cycle arrest and inhibits the proliferation of breast cancer cells in a dose-dependent manner; thus, evaluated as the first report of apoptosis induced in triple negative breast cancer cells by MOME. The cell cycle arrest at G2/M phase depicted G2/M enrichment to emphasize on the efficacy of MOME, thus representing as an ideal anti-cancer agent against human breast cancer cells. The dose-dependent methanolic extract of leaves of *M. oleifera* was found as a promising anti-proliferating agent against the invasive breast cancer cells.

Key words: Apoptosis, Cell cycle arrest, Cell lines, MDA-MB-231, *Moringa oleifera*, T-47D.

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INTRODUCTION

Moringa oleifera L. (Family: Moringaceae) is utilized as an important food commodity, which has an enormous attention as the 'natural nutrition of the tropics.[1,2] The value for products extracted from plant sources is increasing due to its potential applications in medicinal field, livestock feed, cosmetics and industries. Clinical studies have suggested that all parts of *M. oleifera* depicted pharmacological significance and therapeutic properties, therefore known as the 'miracle tree'. The root bark, leaves, stem bark, pods, and/or seeds of M. oleifera are used traditionally in courses of nutritional therapy within many countries over the world owing to their high nutritive value. In India, it is believed that the diet containing *M. oleifera* has been shown to prevent more than 300 diseases, as per Ayurveda.[3] The *M. oleifera* plant preparations have been reported to detoxify blood and liver, strengthening the heart by reducing the risk of heart disease, increase fat metabolism to promote weight loss, and act as an anti-worm.[4,5] The other medicinal activities reported are, anti-pyretic, anti-bacterial, anti-tumour, anti-diabetic, anti-epileptic, anti-inflammatory, diuretic, cholesterol lowering, anti-ulcer, antispasmodic, antioxidant, hepatoprotective and antihypertensive.[6] *M. oleifera* is an economically valuable plant, which provides an impact on social, environmental and health-related perspectives.

Moringa leaves are a good source of natural antioxidants like β-carotene, ascorbic acid, calcium, flavonoids, phenolic, carotenoids and potassium.[7,8] As reported by researchers, about 74% of the known anti-cancer medicines are derived from various plant species.[9] The pharmacological importance of the leaves extract containing bio-active compounds were earlier reported and about 74% of the known anti-cancer medicines are derived from various plant species.[10] The investigations from researchers portrays the exploration of *M. oleifera* through determination of its efficacy and functional ability to represent as a model system against anti-tumour agents.

According to the World Health Organization (WHO) report, 2.3 million women were diagnosed with breast cancer and 685,000 deaths worldwide in 2020. At the end of 2020, there were 7.8 million women alive those who had been diagnosed with breast cancer in the last 5 years, making it the most common cancer in the world. In the present study, methanol extract of *M. oleifera* leaf (MOME) was used to test its efficacy against invasive breast cancer cell lines T47D and MDAMB231. Cell viability was analysed using the MTT assay to determine MOME IC_{50} values in breast cancer cells. The study focuses on the dose-dependent effect of the methanolic extract of *M. oleifera* leaves on the cell cycle of breast cancer cell lines, T47D and MDAMB231 and the determination of apoptosis to

evaluate a significant inhibitory effect on the lines. Cells of breast cancer and, therefore, its antiproliferative effect on the invasive cells of triple negative breast cancer.

MATERIALS AND METHODS

Collection of *Moringa oleifera* Leaves

The leaves of *Moringa oleifera* were collected from agricultural farms of Marathwada region, India and authenticated by Botanical Survey of India (Western regional office, Pune letter no.2020/9, Dated, 28/10/2020). They were then subjected to shade drying for 15 days in a cool and dry place.

Preparation of the Extracts

The dried leaves of *Moringa oleifera* were used for the preparation of methanolic extract described in Roham *et al.*[10] with minor modifications. Briefly, 500 gm of leaves were mixed with 10 volumes of methanol and extracted at 60°C by using a Soxhlet apparatus. The obtained solvent extract was cooled down at room temperature, then stored at -20°C and were analysed for further experimentation. This leaf extract was labelled as MOME (*Moringa oleifera* methanolic extract).

Cell Lines

Two Human breast cancer cells – T-47D (invasive ductal carcinoma cell line; highly invasive, ER+, PR+/- and HER2–) and MDA-MB-231 (highly invasive, ER^-, PR^- and $HER2^-$) were procured from the National Centre for Cell Sciences (NCCS) Pune, India. The T-47D cells were maintained in complete growth medium RPMI-1640 (Thermo Inc, USA), which was supplemented with 10% heat-inactivated fetal bovine serum (Himedia, India), 0.2 units/ml bovine insulin, 1% penicillin and streptomycin. L-15 medium (Himedia, India), supplemented with 10% heat-inactivated fatal bovine serum (Himedia, India) was used for MDA-MB-231. The cells were maintained as described earlier.[11]

Analysis of Cell Viability by MTT Assay and IC_{50}

MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay (Himedia, INDIA) was used for the analysis of anti-proliferative activity of MOME on two human breast cell lines, T-47D and MDA-MB-231 as described previously with minor modifications to the manufacturer's instructions.[12] Briefly, ~ 1×10^3 cells were seeded in 100 µL cell culture (RPMI-1640 or L-15) and incubated in CO_2 incubator using 96 well plates. After 12 hr of incubation; 0, 0.25, 5, 10, 15, 20, 40, 80, 100 µg/mL (w/v) MOME was added in respective wells making triplicates for each of the above concentrations. After incubation in a CO_2 incubator at 37° C, the cell viability was determined at 12 hr, 24 hr and 48 hr respectively, by recording the absorbance at 490 nm using a 96 well Multiscan Ascent (Thermo Inc. USA). Each concentration of MOME was repeated as three replicates for each cell line. The inhibitory effect of MOME on cell growth was assessed as percent cell viability, where the cells without treatment were considered as 100% viable. The experiment was repeated three times for each concentration.

Determination of Apoptosis in MOME Treated Cells

An evaluation on the quantitative assessment of apoptosis was performed by using Annexin V-FITC Apoptosis Detection Kit (Thermo Inc. USA) as per the manufacturer's instructions. The protocol has been described in our previous report.[11] Cells were cultured at a density of ~ 1×10^5 cells in 500 µL complete cell culture medium (RPMI-1640 or L-15) with 10 % v/v FBS in 6-well plates and incubated for 24 hr at 37°C in a CO_2 incubator. After achieving confluent cell growth, the cells were treated with 20 µg/mL and 30 µg/mL concentrations of MOME to respective wells. After 24 hr, cells were harvested, washed with cold PBS and re-suspended in 200 µL of Annexin binding buffer [50mM HEPES, 700 mM NaCl, 2.5mM $CaCl_2$, pH 7.4]. Annexin V-FITC 5 µL and 1 µL PI (100 µg/mL) were then added to 100 µL cells suspensions, after 15 min of incubation at room temperature, the cells were collected, washed twice with cold PBS, and re-suspended in binding buffer prior to analysis by Attune flow cytometer (Thermo Inc. USA) to assess the growth inhibition of cell lines, T-47D and MDA-MB-231 by MOME. The apoptotic induction was calculated by Attune cytometric software v2.1 (Thermo Inc. USA).

Cell Cycle Arrest in MOME Treated Cells

The cell cycle assay was assessed to detect the effect of *M. oleifera* leaf methanolic extract (MOME) on cell cycle arrest. Approximately, 1×10^5 cells of each cell were plated 500 µL of (RPMI-1640 or L-15) with 10 % v/v FBS in six-well plates and allowed to adhere overnight. After 24 hrs of serum-starvation, 40 ug/mL MOME was added. The spent media were collected and spun at 1000 × *g* for 5 min to collect detached cells or floaters, which were combined with adherent cells for cell cycle distribution analyses by flow cytometry. The cells were washed twice with the PBS and fixed in 70% (v/v) ice-cold ethanol at 4°C for 24 hr. 50 µL of RNase A solution (100 µg/mL in PBS) was added to the cells. The fixed cells were stained with propidium iodide (Thermo Inc. USA). The samples were then analysed in an Attune flow cytometer (Thermo Inc. USA).

Statistical Analysis

The data generated from all the experiments were evaluated by statistical analysis using Student's two tailed t-tests, further followed by Analysis of variance (ANOVA) test. The difference was considered statistically significant value if $P<0.05$. The Graph pad Prism software had been applied for the interpretation of statistical data.

RESULTS

The discovery of the action of *Moringa oleifera* leaves methanolic extract (MOME) on invasive breast cancer cells, T-47D and MDA-MB-231 cells suggests that the MOME in human breast cancer cells (T-47D and MDA-MB-231) induces apoptosis and also arrests the cell cycle at G2/M phase. This is the first report of apoptosis induced in triple negative breast cancer cells by MOME.

Cell Viability in MOME Treated Invasive Breast Cancer Cells

Human breast cancer cells, T-47 D, MDA-MB-231 were treated with MOME, and the antiproliferative effect was evaluated by MTT assay. As shown in (Figure 1a-b), a dose dependent decrease in viability of cancer cells was observed with increasing concentration of the MOME. After 24 hr of exposure of cells to MOME, IC_{50} value of MOME in T-47 D was found low i.e., 45.33 ± 5.2 µg/mL (Figure 1a). Since the lower the IC_{50} value the higher the cytotoxic effect of the sample, the IC_{50} was calculated 24.44 ± 3 µg/mL in MDA-MB-231 cells (Figure 1b). The cell viability was decreased after 24 h in both the cell lines (Figure 1a and b). After 48 hr exposure to MOME, IC_{50} was 12.39± 1.2 µg/mL and 16.55± 5.2 µg/mL, for T-47D and MDA-MB-231 cells, respectively. These findings suggested that time and dose dependent treatment with MOME inhibited the growth and induced apoptosis in T-47D and MDA-MB-231 cells. Significantly decreased cell viability was found in MOME treated T-47D and MDA-MB-231 cells ($P<0.0001$ compared to untreated control). In previous reports, *M. oleifera* extracts were found cytotoxic against colon cancer (Colo-320 DM), breast cancer (MCF-7), ovary cancer (PA-1), and oral cancer (KB-403) cell lines with IC90 value of 3.98, 17.60,12.86, and 8.40 µg/mL, respectively.[13] Aqueous leaf extract of *M. oleifera* was reported to reduce HeLa cell viability with IC_{50} of 70 µg/mL.[14] The present study has investigated on the efficacy of MOME

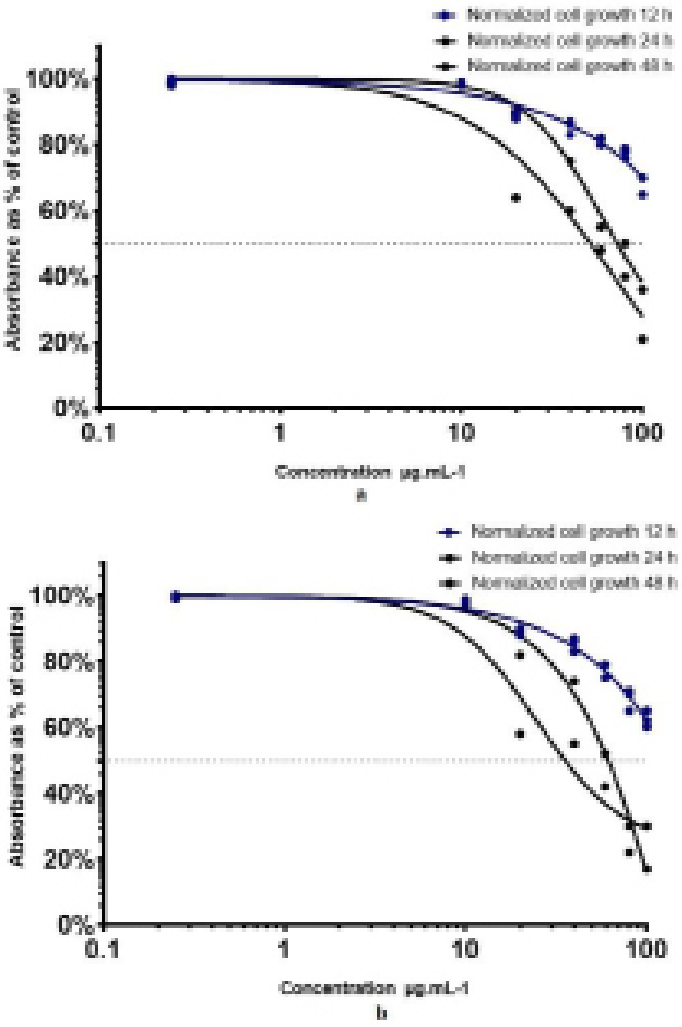

Figure 1: Evaluation of viability of MOME treated Human breast carcinoma cells –T-47D and MDA-MB-231 cells.

T-47D and MDA MB 231 cells were treated with 0, 0.25, 5, 10, 15, 20, 40, 80, 100 µg.mL^{-1} MOME. Where, a) T-47D cells treated with MOME for 24 h, b) MDA-MB-231 cells treated with MOME for 24 h.

Cell viability was monitored by MTT assay. The percentage of viability was calculated as the following formula: (viable cells) %=(OD of drug-treated sample/OD of untreated sample)×100. Measurements were made in triplicate, and standard bars represent the standard deviation. The Student's two-tailed t-test was used to determine statistical significance of the differences between untreated cells and cells treated with the various concentrations of MOME.

on viability of invasive breast cancer cells- T-47D and MDA-MB-231 cells.

Externalization of *Phosphatidylserine on Membrane of MOME Treated Cells*

The key features of apoptotic cell are DNA condensation and phosphatidylserine (PS) externalization.[15] Annexin-V staining method was utilized for the analysis of the morphological changes during induction of apoptosis. Morphological changes clearly indicated the induction of apoptosis in the breast cancer cells–T-47D and MDA-MB-231. Annexin-V binds to externalized phosphatidylserine (PS) of apoptotic cells membranes. Subsequent research studies demonstrated that PS exposure preceded other features of apoptosis, such as membrane permeabilization and nuclear condensation, confirming that annexin V-mediated detection of PS is a viable technique for the early detection of apoptotic cells.[16] The occurrence of early and late apoptosis is validated by the increase of Annexin-V positive cells in dose dependent experiment (Figure 2 a-d). Annexin V, Alexa Fluor® 568 conjugate /PI flow cytometric assay was performed to confirm this finding and the results are summarized in (Figure 2 a-d). The majority of the untreated control cells were found to be viable (93%). In MOME treated T-47D cells, the viability of the cells was found to be decreased from 90 % (Untreated) to 51.7% (10 ug/mL of MOME) (Figure 2a), 38.6% (20 ug/mL of MOME) (Figure 2b) and 36.6% (30 ug/mL of MOME) (Figure 2c). The population of early apoptosis cells was increased to 37.1% in MOME treated cells (Figure 2a). The dose-dependent increase in early apoptotic cells population's i.e.; 48 % (Figure 2b) and 55.38% (Figure 2c) was observed in 20 µg/mL and 30 µg/mL MOME cells, respectively. A remarkable increase in population of late apoptosis 8.8 % and 11% was found in 10 µg/mL and 20 µg/mL of MOME treated T-47D cells, respectively (Figure 2 a-c). Apoptosis induction was also detected in MDA-MB-231 cells treated with MOME. The viability of the cells was decreased from 93 % to 90.4 % (Figure 2d), 87.2% (Figure 2e) and 78.5% (Figure 2f) in 10 µg/mL, 20 µg/mL and 30 µg/mL MOME treated cells, respectively. The early apoptosis was observed in 8.9% cells containing 10 µg/mL MOME (Figure 2d). A dose dependent increase in early apoptotic cells 12.1% (Figure 2e) and 20.9% (Figure 2f) was observed in 20 and 30 µg /mL MOME treated cells, respectively. The growth inhibition led to apoptosis in MOME treated MDA-MB-231 and T-47D cells. The potency of *Moringa oleifera* inhibited cell growth and induced apoptosis in breast cancer cells, thus confirmed presence of potent anti-cancer properties.

Cell cycle arrest in MOME treated T-47D and MDA-MB-231 cells

The effects of MOME on the cell cycle of T-47D and MDA-MB-231 cells were evaluated by using MOME treated and untreated cells. The Flow Cytometry was used to determine whether MOME induced apoptosis was related to arrest of cell cycle progression in breast cancer cells and

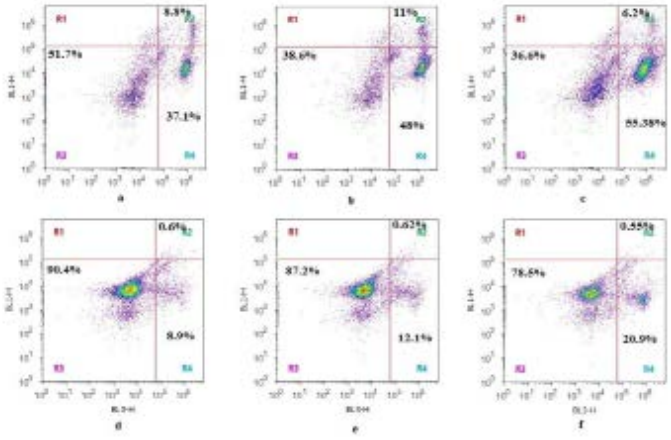

Figure 2: Detection of membrane proteins on MOME treated cells.

Apoptosis in T-47D and MDA-MB-231 cells was assessed after 24 h of treatment with MOME by Annexin V, Alexa Fluor® 568 conjugate /PI binding and measured by flow cytometry. Where, (a) Untreated T-47D cells, (b) T-47D cells with 20 ug/ml MOME, (c) T-47D cells with 30 ug/ml MOME, (d)Untreated MDA-MB-231 cells, (d) MDA-MB-231 cells with 20 ug/ml MOME.,(e) MDA-MB-231 cells with 40 ug/ml MOME.

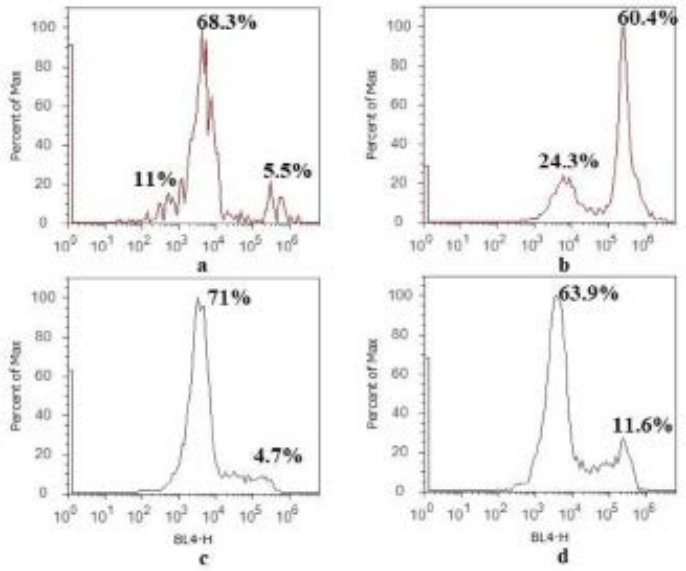

Figure 3: Cell cycle analysis in MOME treated T-47D and MDA-MB-231 cells. Cell cycle in T-47D and MDA-MB-231 cells was assessed after 24 h of treatment with MOME by PI staining and measured by flow cytometry analysis. Where, (a) T-47D cells without drug, (b) T-47D cells with MOME (40µg/mL) ,(c) MDA-MB-231 cells without drug, (d) MDA-MB-231 cells with MOME (40µg/mL). Numbers indicate the percentage of cells in each gate /phase.

quantitate the cell cycle distribution under treatment with MOME at a concentration of 40 µg/ml. The number of cells in G2/M phase were increased, while that in the G0/G1 phase were decreased in MOME treated cells (Figure 3 a-d). After the confirmation of induction of apoptosis in MOME treated T-47D cells and MDA-MB231 cells, the cell cycle was analysed. In untreated T-47D cells, 68.3 % of the cell populations were found in G1 phase (Figure 3a). The G2/M phase population was recorded as 5.5 % in untreated cells (Figure 3a), and had increased to 60.4 % in MOME treated T-47D cells (Figure 3b). The cell population of the G1 phase showed a decrease to 24.3 % in MOME treated T-47D cells (Figure 3b). The cell cycle arrest in the G2/M phase was also observed in MDA-MB231 cells. The population of G2/M phase was increased from 4.7 % to 11.6 % in MOME treated cells (Figure 3 c-d). These experimental finding implies that MOME induced apoptosis in breast cancer cells via G2/M phase arrest. The dose-dependent cell cycle halt was observed in MOME treated T-47D and MDA-MB-231 cells. The G2/M arrest is associated with the intrinsic pathways of apoptosis induction in MOME treated breast cancer cells. Similarly, cell cycle arrest in G1, S, and G2/M was reported in hepatocellular carcinoma cells treated with *M. oleifera* leaf extract.[17] In the current study, the cell cycle arrest in G2/M phase which confirmed apoptotic induction in invasive breast cancer cells, T-47D and MDA-MB-231 cells.

DISCUSSION

The risk of breast cancer has become a challenging perspective with the discovery of novel therapies and potent medicinal drugs in indigenous medical system. The evaluation of patients with breast cancer through treatments of surgical oncology, chemotherapy, radiation therapy, hormone therapy and targeted therapy have been practised in the medical field, which can imply adverse side-effects. The remedy to avoid these side-effects, the synthesis of potential anti-cancer agents from plant sources needs validation and thus, can develop the molecular mechanisms with effective therapeutic properties. Research on the adverse side effects with doses achievable by oral ingestion should still go on, since currently there are no scientifically confirmed clear toxic and harmful effects of *M. oleifera* extracts and products on both human and animal models.[18]

In a recent comparative study, the diethyl extract of leaves of *Moringa peregrina* (P/DEE) induces cell arrest at S phase, along with ethyl acetate extracts of leaves of *Moringa peregrina* (P/EA), diethyl extract of leaves of *Moringa oleifera (*O/DEE), and ethyl acetate extracts of leaves of *Moringa oleifera (*O/EA) that induce cell cycle arrest at G2/M phase.[19] In accordance with certain research work, *Moringa peregrina* leaf extracts have shown to induce apoptosis and cell cycle arrest at G2/M phase of hepatocellular carcinoma.[19] The comparative analysis of the components of different parts and extracts of *M. oleifera* can further elucidate its high efficacy when treated against cancer cells. The study of Sreelatha *et al.*[20] showed that aqueous extract of *Moringa oleifera* leaves containing flavonoids, such as quercetin and kaempferol triggered apoptosis. The anti-carcinogenic potential of aqueous extract of *M. oleifera* (AEMO) *in vitro* and *in vivo* was studied and revealed that AEMO treatment did not induce significant alterations of liver and kidney function and haematological parameters.[21] The research by Luetragoon *et al.*[22] found that the extract of *M. oleifera* leaf can up-regulate cysteine, downregulate the anti-apoptotic factors, and induce apoptosis of cancer cells. The treatment using the methanolic extracts of *Moringa oleifera* leaves (MOME) induced apoptosis with G2/M enrichment in breast cancer cells, thus enhance the immunological potential of *M. oleifera*. The treatment of Methanolic *Moringa oleifera* leaves Extract (MMLE) in cervical cancer cells showed decreased cell growth in a dose and time dependent manner.[23] The detailed study of the composition, bioactive compounds of *M. oleifera* in correlation with the applications in the field of pharmaceuticals and environmental sciences, which would implement innovative therapeutic strategies. In anti-cancer studies, *M. oleifera* were found to slowdown the cancerous process through targeting chemoprevention, inhibiting carcinogen activation and inducing carcinogen detoxification, anti-inflammation, anti-proliferation of tumour cells, and inducing apoptosis of cancer cells.[24] The study on the proliferative effectiveness of the methanolic extract of *M. oleifera* leaves exhibited cytotoxic activity against human breast cell lines, T-47D and MDA-MB-231 and thus, beneficial as a potentially active anti-cancer agent.

CONCLUSION

The invasive triple negative Human breast cancer cells T-47D and MDA-MB-231 cells were treated with the methanolic leaves extract of *Moringa oleifera*. The reduced cell viability in cancer cells stimulated the antiproliferative effect of MOME on T-47D and MDA-MB-231. The phosphatidylserine exposure on the membrane of the cells confirmed the mitochondria mediated intrinsic apoptosis induction in the MOME treated cells. The cell cycle arrest at G2/M phase emphasized on G2/M enrichment, which was used to confirm the effect of MOME on the cell health and the irreversible nature of the apoptosis in presence of MOME. The dose-dependent methanolic extract of leaves of *M. oleifera* was found as a promising anti-proliferating agent against the invasive breast cancer cells. The phytochemicals of *M. oleifera* could be developed as an innovative therapeutic strategy for the prevention of invasive growth of breast cancer tumours. The exemplary applications of *M. oleifera* in the medicinal field, which was focused upon traditional remedies are now

being proven optimistically and explored with scientific postulates and evidences.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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