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Malabar spinach potentiates cytotoxic activity through apoptosis in human breast cancer cell lines

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Abstract

Breast cancer, a highly diverse and invasive disease ranking second in cancer-related fatalities originates in the breast cells. It is the most common cancer among women worldwide and can also occur in men, albeit rarely. Hence, it is essential to conduct comprehensive research on cancer and explore nature-derived therapeutic interventions. The significance of plant-based medications lies in their natural compounds that offer diverse therapeutic benefits with potentially fewer side effects. *Basella alba* (Malabar Spinach), is a green leafy vegetable with documented properties of gastro protective, ulcer-healing, anti-inflammatory and wound-healing activities. Consequently, we have selected this plant for an in-depth study to investigate its potential anticancer activity against MDA-MB-231 and MCF-7cell lines. In the present *in vitro* anti-cancer study, the IC_{50} values for methanol extract *B. alba* (MBA) were 102.43 ± 9.29 µg/mL for MDA-MB-231 cells and 113.26 ± 5.46 µg/mL for MCF-7 cells. Cytological changes, including nuclear fragmentation, membrane blebbing, apoptotic bodies and chromatin condensation, were observed through acridine orange/ethidium bromide dual (AO/EB) staining. Additionally, Hoechst 33258 staining revealed bright blue fluorescent cells having apoptotic features such as nuclear fragmentation, marginalisation and condensed chromatin in extract -treated cells. Furthermore, MBA treatment induced loss of mitochondrial membrane potential, resulting in fluorescent green cells in both cell lines. The extract notably reduced Bcl-2 gene expression, with a more significant impact on MCF-7 cells. Western blotting confirmed a substantial down regulation in Bcl-2 levels for MBA-treated MDA-MB-231 and MCF-7 cells, underscoring the anticancer potential of MBA, as observed in this study.

Keywords

Basella alba; MDA-MB-231; MCF-7; breast cancer; apoptosis

Introduction

Cancer is one of the leading causes of mortality worldwide, affecting the socio-economic development of the populations owing to the quick hike in the prevalence and death rates (1, 2). Breast cancer is ranked top amongst the most diagnosed subtypes, with around 2.3 million cases newly diagnosed around the globe (3). More than three-quarters of breast cancers are hormone-mediated and the rest are non-hormone-mediated subtypes (4). Major risk factors attributing to breast cancer include female gender, age, genetics, early puberty, waist-hip ratio (WHR), late pregnancy, late

menopausal age, low physical activity, high triglyceride levels and radiation exposure (5). Managing adverse effects, increased resistance to treatment and symptom recurrence post therapy has become a major global challenge in cancer treatment (6).

Natural products from herbs are beneficial in developing anticancer drugs as a safer alternative. These natural products work by modulating the activity of AMP-activated protein kinase and suppress breast cancer cells. Through a variety of metabolic signaling pathways, including the inhibition of the expression of antiapoptotic Bcl-2 gene by relying on the HIF-1α-induced Cav-1 expression pathway in Cav-1-free RT4 bladder tumour cells and the hypoxia-induced inhibitory effect on the anti-apoptotic pathway due to Cav-1-dependent AMPK activity, Adenosine 5′-monophosphate (AMP)-Activated Protein Kinase (AMPK) can control metabolic reprogramming and counteract the “Warburg effect” in breast cancer (7-9). The anti-apoptotic protein Bcl-2, which is produced by the Bcl-2 gene, is essential for controlling the ratio of cell death to growth in normal cells. But in aberrant situations, like cancer, amplification of the Bcl-2 gene can accelerate the growth of cancerous cells by preventing apoptosis and encouraging tumor expansion. Research has demonstrated a correlation between elevated levels of Bcl -2 and a number of malignancies, including lung, hepatocellular, prostate, gastric and breast cancers. Unusual overproduction of pro-survival anti-cancer therapies find the Bcl-2 family proteins to be appealing targets since they have the potential to promote the growth of cancer and increase treatment resistance (10-12). Research on how these proteins control apoptosis, cancer growth and treatment resistance has focused on targeting BCL-2 family proteins as a possible anticancer therapeutic approach (10, 12). The Bcl-2 family of proteins, which are mostly found in the endoplasmic reticulum and mitochondria, is involved in controlling apoptosis. Pro-apoptotic proteins can cause apoptosis directly by interacting with mitochondria, whereas anti-apoptotic proteins function in concert with other proteins to prevent apoptosis and increase cell viability (10). Current research in cancer subtypes suggests that medicinal plants and their derivatives are reliable as primary and adjuvant therapeutic agents against them. Therefore, continuous and adequate research is required to develop novel efficient compounds (13-19). *Basella alba* is an edible plant with various applications in human medicine. It belongs to the family Basellaceae and is widely known as Indian Spinach, Malabar Spinach or Vine Spinach. It grows profusely in tropical Asia and Africa and is commonly consumed as a leafy vegetable. It is a fast-growing, soft-stemmed vine that may grow 10 m long. Local tribes and researchers have recently documented its anti-inflammatory, antibacterial, cytotoxic, anticonvulsant, antioxidant and other therapeutic effects (19, 20). The anticancer qualities of *B. alba* have been investigated, yet there is no concrete proof or study connecting it to the Bcl-2 gene or its function in controlling apoptosis. Nonetheless, studies do show that Malabar Spinach exhibits antiproliferative action against the cancer cell line Ehrlich's Ascites Carcinoma (EAC) (21). Hence, the current work thus sought to examine potential for *B. alba* for anticancer activity using *in vitro* techniques.

Materials and Methods

Collection of plant material and authentication

Basella alba, a whole plant was collected from the Vashi market region of Mumbai City, Maharashtra, India. Southern Regional Centre (The Botanical Survey of India), T.N.A.U. Campus, Coimbatore, Tamil Nadu, India taxonomically recognized and authenticated the plant material, as collection no. BIS/SRC/5/23/2019/Tech./2932. A voucher specimen was deposited at the Department of Veterinary Pharmacology, CVAS, Mannuthy, Thrissur, Kerala, India.

Extraction using methanol

The entire plant of *B. alba* was air-dried at room temperature, finely pulverized and extracted with methanol at 55 °C using the Soxhlet equipment. It was then concentrated using a rotating vacuum evaporator at low pressure and temperature (55 °C). After completely evaporating the solvent, the extract was refrigerated in an airtight container.

Sample preparation

Basella alba methanol extract was diluted in dimethyl sulphoxide (DMSO) to produce a stock solution with 1 mg/mL concentration. This stock solution was then diluted to the appropriate concentrations using phosphate-buffered saline (PBS). The final concentration of DMSO in the wells was kept to less than 1 % w/v.

Cell culture

The study utilized authenticated cell lines, MDA-MB-231 and MCF-7, obtained from NCCS, Pune, India. RPMI-1640 media with 1 % antibiotic antimycotic solution containing amphotericin B and penicillin-streptomycin and 10 % foetal bovine serum was used for sub culturing the cells. The cells were maintained in a laboratory CO_2 incubator (5 %) at 37 °C. The cells were sub cultured by enzymatic digestion trypsin (0.25 %) and ethylene diamine tetra acetic acid solution (1 mM) after reaching 70 % confluency. The trypsinised cells were used for 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay.

In vitro cytotoxic analysis of MBA

Cytotoxicity of methanol extract of *B. alba* (MBA) was assessed using MTT (22). The absorbance was assessed at 570 nm using an ELISA plate reader. Graph pad prism version 9.1.1 was used to calculate the extract's half-maximal inhibitory concentration (IC_{50}) by graphing the concentration versus % cell viability.

Microscopic studies

Trypsinised cells were plated into a six-well plate at a concentration of $1x10^5$ cells and left to develop for 24 h. The concentrations of the plant extract were chosen for subsequent studies based on the MTT assay. Acridine orange ethidium bromide (AO/EB) (23), Hoechst 33258 (24), and JC-1 (25) staining methods were used.

Doxorubicin was selected as the positive control at a 0.58 µg/mL concentration. For analysis, trinocular research fluorescence microscope (DM 2000 LED, Leica) was used.

B cell Lymphoma -2 (Bcl-2) gene expression study

RT-qPCR was used to evaluate the Bcl-2 gene expression in cell culture samples. The corresponding IC_{50} concentrations of the extract were added to the cells for 24 h. RT-qPCR was performed using Maxima SYBR green qPCR master mix following manufacturer's instructions using human Bcl-2 primer sets (Sigma). Human GAPDH served as a positive control. qRT-PCR was done on a real-time PCR cycler (Applied Biosystems, USA). The level of Bcl-2 gene expression was measured using the fold change formula $2^{-\Delta\Delta C_T}$. Expression fold change in gene and protein was assessed using the one-sample t-test (26).

Western Blot Analysis

Lysates of control and extract treated (IC_{50} concentration) cells were prepared by homogenizing the cells on ice for 1h after washing twice in 1XPBS using radioimmunoprecipitation assay buffer, followed by centrifugation at 18728 g, 4 °C for 15 min. Total protein concentration was determined by taking an aliquot of the lysate using the Lowry method (Genei kit protocol). Using 12 % SDS-PAGE, proteins were separated and transferred to the PVDF membrane (Hoefer semidry transfer apparatus). β- actin was used as an internal control to ensure uniform protein loading. Primary antibodies of Bcl-2 (1:1000, Sigma-Aldrich) and β-actin (1:2000, Sigma-Aldrich) were used to incubate the membranes. The binding of antibodies was visualized by incubating the blots with HRP-conjugated secondary antibody (Cell Signaling Technology) followed by a colour reaction with DAB substrate buffer. The western blotting band strength was determined by the Image J density measurement program (http://imagej.en.softonic.com). Expression fold change in protein expression was assessed using the one-sample t-test (27).

Results

In vitro anticancer study

The results of the MTT assay after 48 h treatment with MBA in MCF-7 and MDA-MB-231 cancer cell lines are shown in Table 1. The viability of both treated cells showed significant ($p < 0.05$) reduction at 80 µg/mL concentration. The IC_{50} for MBA was found to be 102.43 ± 9.29 µg/mL for MDA-MB-231 cells and 113.26 ± 5.46 µg/mL for MCF-7 cells.

Acridine orange or ethidium bromide dual (AO/EB) staining.

Most of the MDA-MB-231 cells were in the early apoptotic stage with yellowish-green fluorescence and MCF-7 cells were primarily late apoptotic emitting orange to red fluorescence after treatment with IC_{50} concentration of MBA (Fig. 1 and Fig. 2). Cytological alterations like nuclear fragmentation, membrane blebbing, apoptotic bodies and chromatin condensation were also noted. Most of the

Table 1. The cell viability of MDA-MB-231 and MCF-7 cells after 48 h treatment with MBA

% Cell Viability	Conc. (µg/mL)							IC_{50} (µg/mL)
	10	20	40	80	160	320	500	
MDA-MB-231	98.16^{a} ± 5.27	100.51^{a}± 11.71	94.52^{a} ±4.23	89.96^{a} ±2.96	39.13^{b} ±1.28	36.14^{a} ±4.10	38.54^{a} ±0.99	102.43 ±9.29
MCF-7	94.30^{a} ± 5.82	90.76^{a} ± 4.56	91.07^{a} ±5.12	76.61^{a} ±0.10	42.26^{a} ±6.80	30.55^{a} ±5.92	25.11^{a} ±1.99	113.26 ±5.46

Note. Values are expressed as Mean ± SE (n = 3). Means bearing the different superscript (a-c in rows) vary significantly at $p < 0.05$.

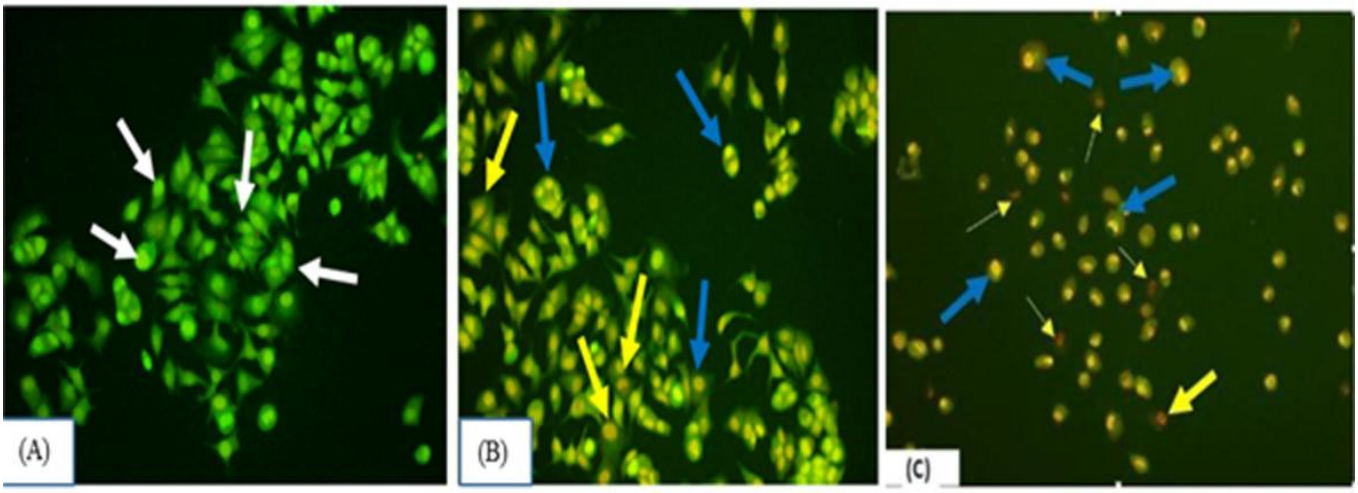

Fig. 1. Morphological changes of MDA-MB-231 cells by acridine orange/ethidium bromide staining, 40X. A- Control cells; B- Cells treated with doxorubicin 0.58 µg/mL; C- Cells treated with MBA at IC_{50} concentration. White arrow- normal cells, Blue arrow - early apoptotic cells, Yellow arrow-late apoptotic cells, Red arrow - nuclear fragmentation.

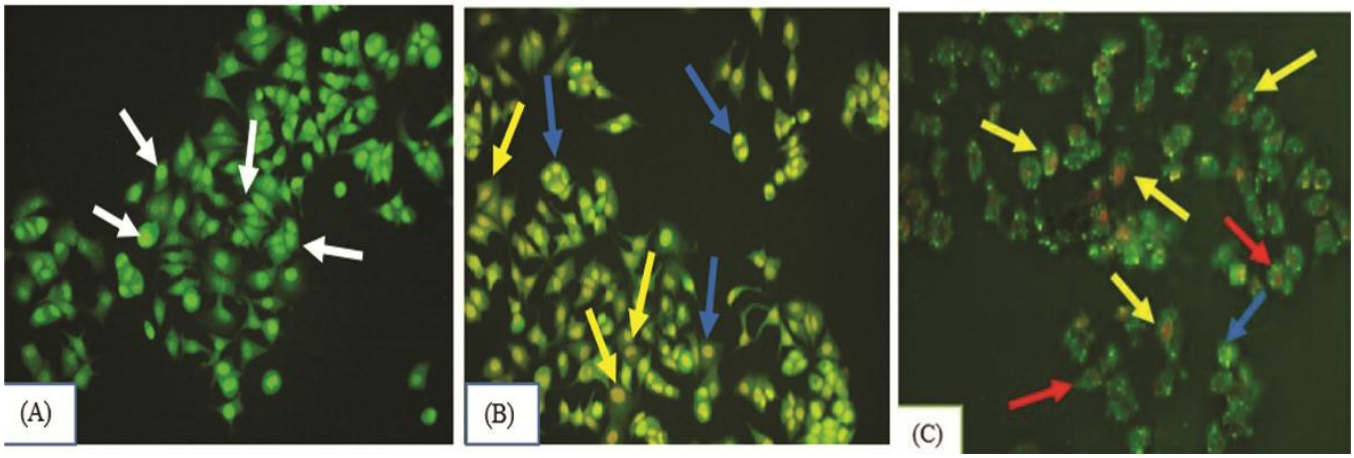

Fig. 2. Morphological changes of MCF-7 cells by acridine orange/ethidium bromide staining, 40X. A- Control cells; B- Cells treated with doxorubicin 0.58 µg/mL; C - cells treated with MBA at IC_{50} concentration. White arrow- normal cells, Blue arrow - early apoptotic cells, Yellow arrow-late apoptotic cells, Red arrow - nuclear fragmentation.

doxorubicin-treated cells were in the early apoptotic stage for both cell lines.

Analysis of morphological changes in the nucleus

Hoechst 33258 staining in MDA- MB-231 and MCF-7 cells treated with IC_{50} concentration of MBA (Fig. 3 and Fig. 4) presented live control cells with uniform blue fluorescence. Bright blue fluorescent cells characterized by apoptotic variations like nuclear fragmentation, marginalization and condensed chromatin were seen in positive control and extract-treated cells.

Analysis of mitochondrial transmembrane potential (MMP)

JC-1 aggregates with red/ orange fluorescence were observed in both control cells suggesting a higher mitochondrial membrane potential. Fluorescent green cells were obtained in both the cell lines treated with MBA indicating loss of mitochondrial membrane potential. The findings demonstrated that MCF-7 cells expressed more mitochondria-dependent intrinsic apoptotic pathway than MDA-MB-231 cells (Fig. 5 and Fig. 6).

Bcl-2 gene expression study

The relative *Bcl-2* gene expression in the cells on treatment with MBA is shown in Table 2. Compared with control cells, a significant ($p < 0.01$) drop in Bcl-2 gene expression level was obtained for plant extract-treated MDA-MB-231 and

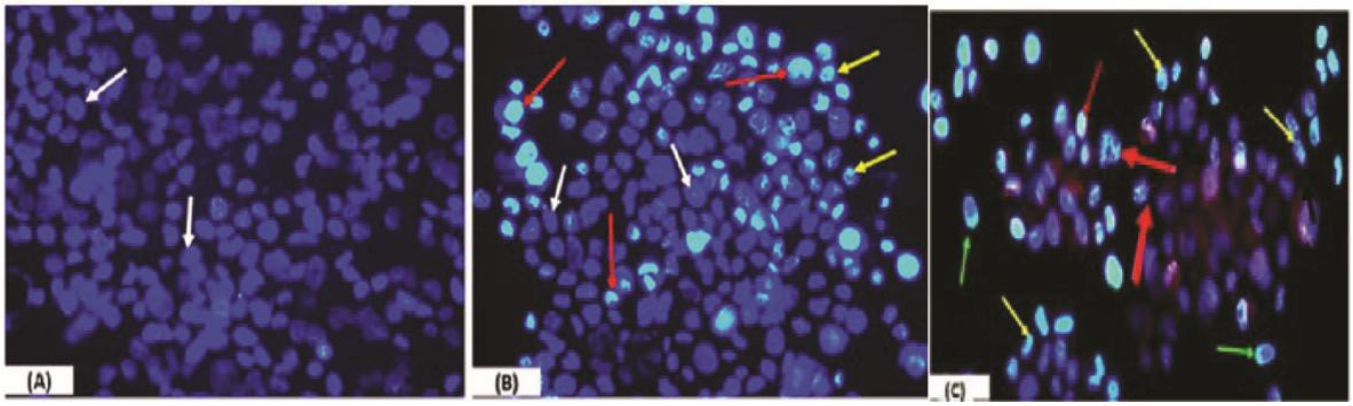

Fig. 3. Morphological changes of MDA-MB-231 cells by Hoechst staining, 40X. A- Control cells; B- Cells treated with doxorubicin 0.58 µg/mL; C- cells treated with MBA at IC_{50} concentration. White arrow – live cells, Red arrow- fragmentation of nuclei, Yellow arrow- chromatin condensation, Green arrow – marginalization of nucleus

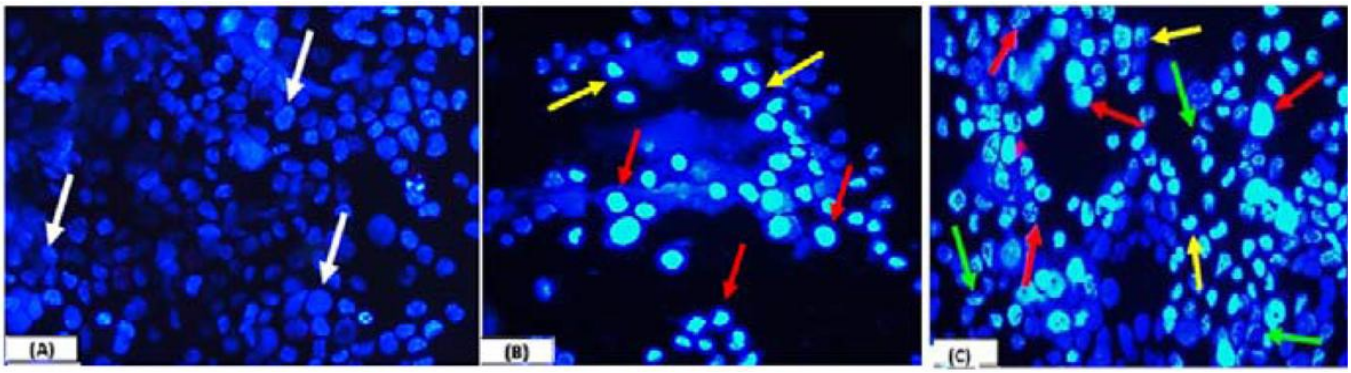

Fig. 4. Morphological changes of MCF-7 cells by Hoechst staining, 40X. A- Control cells; B- Cells treated with doxorubicin 0.58 µg/mL; C- Cells treated with MBA at IC_{50} concentration. White arrow – live cells, red arrow- fragmentation of nuclei, Yellow arrow- chromatin condensation, Green arrow – marginalization of the nucleus

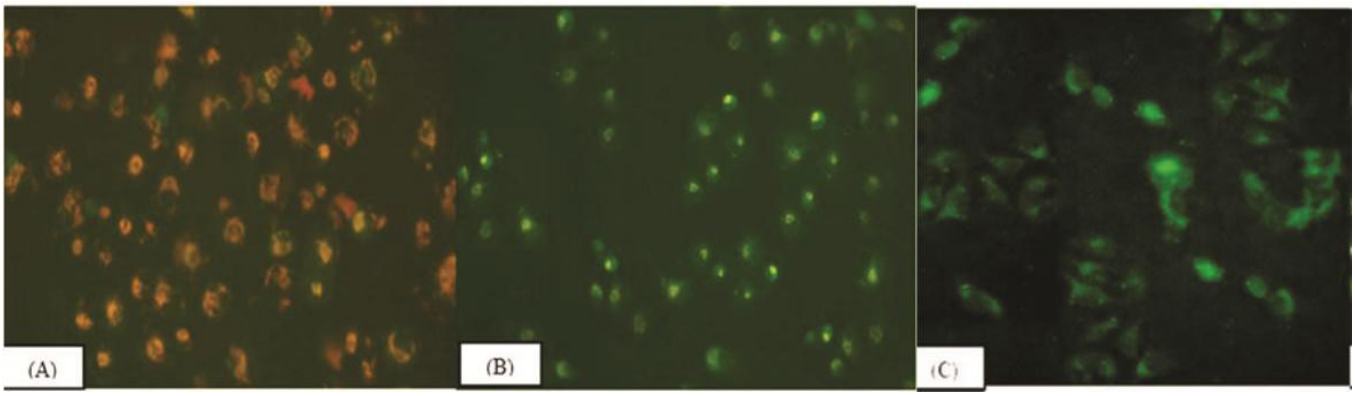

Fig. 5. Morphological changes of MDA-MB-231 by JC-1 staining, 40X. A- Control cells; B- Cells treated with doxorubicin 0.58 µg/mL; C- cells treated with MBA at

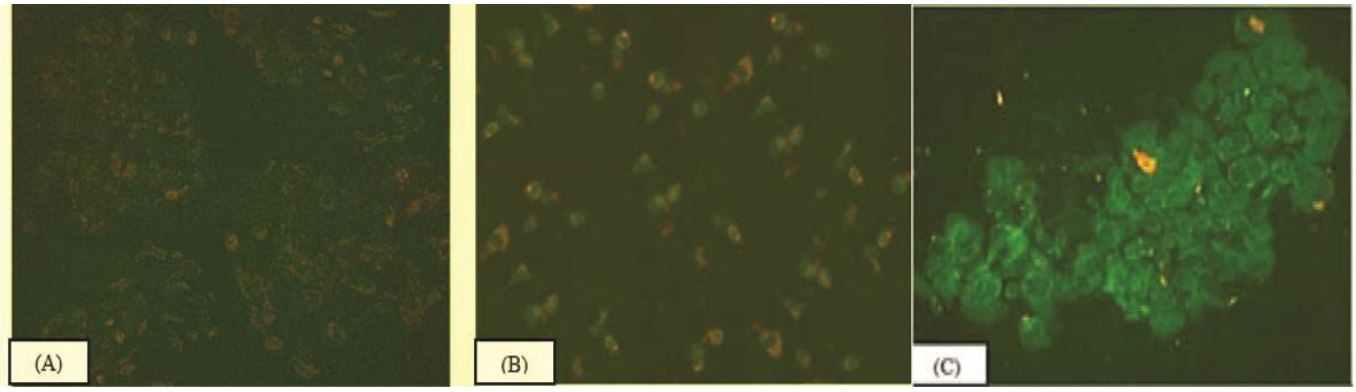

Fig. 6. Morphological changes of MCF-7 cells by JC-1 staining. A- control cells; B- Cells treated with doxorubicin 0.58 µg/mL; C- cells treated with MBA at IC_{50}

Table 2. The relative *Bcl-2* gene expression in MDA-MB-231 and MCF-7 cells in response to treatment with MBA

Cells		Fold change in *Bcl-2* RNA expression
	Control cells	1
MDA-MB-231 cells	MBA	0.30 ± 0.10**[a]
MCF-7 cells	MBA	0.56 ± 0.26**[b]

Note. Values are expressed as Mean ± SE (n = 3); ** denotes a significant ($p < 0.01$) difference compared with control. Means carrying different superscripts (a,b) differ significantly ($p < 0.05$).

MCF-7 cells. After treatment with the plant extract, expression of the Bcl-2 gene was considerably reduced ($p<0.05$) and it was more significant in MCF-7 cells than in MDA-MB-231 cells.

Western blot analysis

Fig. 7 depicts western blot images illustrating β- actin and Bcl-2 proteins in MDA-MB-231 and MCF-7 cells. The Bcl-2 protein expression in the control cells was set normalized to one. When compared with control cells, western blotting results showed significant ($p<0.01$) down regulation in Bcl-2 protein level for the plant extract treated with MDA-MB-231 and MCF-7 cells (Table 3).

Discussion

Breast cancer, a complicated, diverse and invasive disease, is one of the most prevalent cancer subtypes and ranks second in cancer-related fatalities only to lung cancer.

Table 3. The relative Bcl-2 protein expression in MDA-MB-231 and MCF-7 cells in response to treatment with MBA

Cells		Normalized protein levels
	Control cells	1
MDA-MB-231 cells	MBA	0.72 ± 0.10**
MCF-7 cells	MBA	0.76 ± 0.07**

Note. Values are expressed as Mean ± SE (n = 3); ** denotes significant ($p<0.01$) difference compared with control

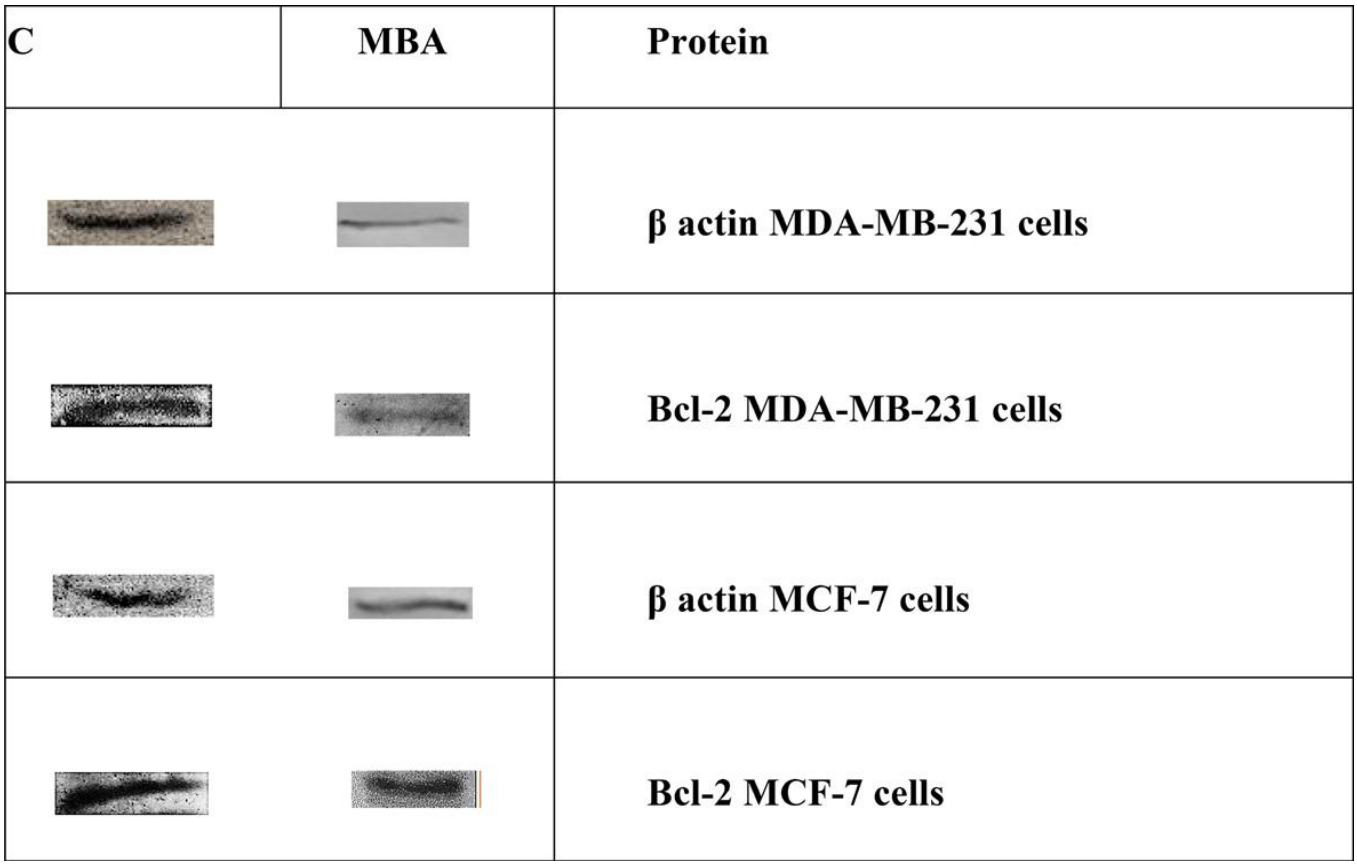

Fig. 7. Western blot images of β- actin and Bcl-2 proteins in MDA-MB-231 and MCF-7 cells. C - control cells, MBA denotes cells after treatment with MBA at IC_{50} concentration.

Breast cancers are classified into hormone-sensitive and insensitive types based on the presence of the hormone receptors, which are applied in the utility of hormones and their derivatives in the therapy. Of the 2 types of breast cancers, insensitive kinds are characterized by poor prognosis, high recurrence rate and metastatic potential (28). These factors led to the selection of MDA-MB-231 and MCF-7 cells for the purpose of research. MDA-MB-231 cells are highly aggressive and invasive and lack oestrogen, progesterone and HER2/neu receptors, whereas MCF-7 cells are non-invasive with both luminal and ductal origin and lack HER2/neu receptors (29). Hence, the present study suggests the anticancer potential of MBA in hormone -dependent and independent breast carcinomas. *Basella alba*, although it is known for its medicinal properties against cancer both traditionally and scientifically, a complete understanding of the mode of activity was not explored to date (22).

NADPH-dependent oxidoreductases in viable cells reduce yellow tetrazolium dye/ MTT to insoluble purple formazan crystals. DMSO dissolves these crystals, and their absorbance is quantified using an ELISA plate reader (30). MBA reduced the cellular proliferation of MDA-MB-231 and MCF-7 cells in the current investigation, as evidenced by a decrease in purple formazan crystals. It was observed from a similar study that MTT assay indicated that, recipes from Nigerian and African medicinal plants were able to inhibit the growth and proliferation of MCF-7 and MDA-MB-231 in a concentration-dependent manner (31). Hence, the results suggest that MBA have cytotoxic potential against breast cancer cell lines *in vitro*.

Cancer cells are destroyed by drugs mainly through the process of apoptosis (32). Dual AO/EB staining is widely accepted as an easy and economical tool to assess apoptosis occurring as a mode of cellular destruction (33). Live cells, early apoptotic, late apoptotic and necrotic cells could be profoundly distinguished. In AO/EB staining, acridine orange penetrates intact live cells and emits green fluorescence as a result of intercalation in DNA. At the same time, ethylene blue enters only the cells with the damaged cell membrane and emits red fluorescence (34). In the present study, MBA showed early and late apoptotic changes as evidenced by emission of yellowish-green and red fluorescence in MDA-MB-231 and MCF -7 cells respectively.

Hoechst 33258 stain is a popular DNA-specific dye that intercalates adenine and thymine to produce uniform blue fluorescence. Nuclear morphological changes in apoptotic cells, such as chromatin condensation, nuclear marginalization and disintegration, result in the emission of brilliant blue fluorescence (35). Nuclear staining by Hoechst 33258 showed bright blue fluorescence with apoptotic morphology like shrunken cells, apoptotic bodies with nuclear fragments, condensed and marginalized chromatin and lytic, shrunken nuclear membrane (36, 37).

In the early apoptosis stage, the mitochondrial transmembrane potential (MMP) reduces; the membrane depolarizes, leading to DNA fragmentation and chromatin condensation. JC-1, fluoroprobe targeting MMP, has been an excellent tool for detecting such apoptotic cells. JC-1 accumulates in healthy mitochondria, forming J-aggregates with orange/red fluorescence, but in apoptotic cells, JC-1 aggregates convert to JC-1 monomer

(green), indicating loss of membrane potential (25). In JC-1 staining, after 48 h of MBA treatment, cells displayed a fluorescence shift from red to green, indicating a reduction in mitochondrial membrane potential (38).

B-cell lymphoma 2 (*Bcl-2*) belongs to the Bcl-2 family of proteins and it is found in humans as a compressed form of the *Bcl-2* gene. Chromosomal rearrangement between the 14^{th} and 18^{th} chromosomes induces strong transcriptional *Bcl-2* expression, which further gives rise to tumorigenesis by ensuring the survival of cells (10). Oestrogen regulates *Bcl-2* gene expression in breast epithelial cells and ER+ve breast cancer cell lines. The *Bcl-2* gene is expressed at around 81 and 29 % in triple -negative and other breast cancers respectively (39). B-cell lymphoma 2, one of the antiapoptotic proteins from the Bcl-2 family, is generally located on mitochondria, endoplasmic reticulum (ER) and nuclear membranes. It fuses explicitly with the outer membrane of mitochondria and is so involved in the intrinsic pathway of apoptosis. Bcl -2 prevents lethal pore formation on the outer membrane of mitochondria (permeabilization), thus inhibiting cytochrome C release and caspase activation, culminating in apoptosis (40). It obstructs the mitochondrial cytochrome c exocytosis and caspase activation thereby preventing apoptosis (41). Protein and *Bcl-2* gene expression were significantly down regulated due to MBA in MCF-7 and MDA-MB-231 cells (42, 43).

Conclusion

The research work investigated the cytotoxic effects of MBA on MDA-MB-231 and MCF-7 cells. Staining methods unveiled the intrinsic apoptotic mechanism triggered by MBA treatment. Gene expression analysis showed a significant decrease in the *Bcl-2* gene expression in MCF-7 cells compared to MDA-MB-231 cells following MBA treatment. These findings strongly indicate the anticancer potential of MBA against both MDA-MB-231 and MCF-7 cells.

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Authors' contributions

HMS carried out the research work. BJK conceptualized and designed the experiment. RJ provided the technical help for the western blot studies during the conduct of the experiment. RJ and PMK drafted the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: : Authors do not have any conflict of interests to declare.

Ethical issues: None.

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