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Research Article

Phytochemical Investigation and Cytotoxic Potential of Leaf Extract of *Medinilla beddomei* C B Clarke in Breast Cancer Cell Lines

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ABSTRACT

Leaves of the plant *Medinilla beddomei* were extracted using soxhlet and ultrasound-assisted extraction methods using polar solvents, acetone, and methanol to find out the extraction efficiency. The profiling of the phytoconstituents was done by LC-Qtof-MS analysis. An *in-vitro* MTT study was used to test the antiproliferative property of *M. beddomei* acetone leaf extract in two breast cancer (MCF-7, MDA-MB-231) cells and a normal cell line (L929 cells). The scratch wound healing assay, which evaluated cell migration and cell death, was conducted using the double staining method of ethidium bromide and acridine orange. The apoptotic potential of the leaf extract was examined in a cancer cell line, and the observations were evaluated using flow cytometry. The findings of the studies indicate that ultrasound-assisted extraction using acetone as the solvent yields a higher amount of phytochemicals. According to LC-Qtof-MS analysis, phytocompounds, including phenolics, flavonoids, alkaloids, terpenoids, and glycosides, are present in the leaf extracts. The acetone extract showed significant anticancer properties when tested against MCF-7 and MDA-MB-231 cell lines. MDA-MB-231 cells had an IC_{50} value of 68.27 ± 0.83 µg/mL, while MCF-7 cells had the lowest cell viability with an IC_{50} value of 46.19 ± 1.58 µg/mL. However, the plant extract showed negligible toxicity towards normal cells (IC_{50}-386.12 ± 4.07 µg/mL). The acetone leaf extract of *M. beddomei* showed a more effective antimetastatic effect in MCF-7 cells. Since this extract demonstrated modulation of apoptosis at several stages of the MCF-7 cell cycle, it may be a promising target for medicinal research against breast cancer. The results of this investigation show that the acetone leaf extract of *M. beddomei* may have anticancer properties and that ultrasonic extraction is a more effective method for extracting phytochemicals.

INTRODUCTION

Cancer is a global threat to human survival. Even though due to the progress of healthcare treatment, the chances of survival of this malignancy have improved. At the same time, 70% of deaths related to cancer are from low and middle-income countries, which cannot manage the expenses of screening and treatment costs.[1] The most prevalent malignancies in women are those of the breast, colon, lung, and cervical regions. Breast cancer is the second most common cause of death for women globally and around 2,088,849 cases are associated with breast cancer. Among them, 626,679 ladies have lost their lives. Breast cancer frequently results from mutations in the BRCA1 and BRCA2 genes.[2,3] Apart from the person's genetic factors, Changes in lifestyle, such as eating junk food, insufficient exercise, drinking alcohol, and being exposed to chemicals and radiation, are the main causes of cancer prevalence.[4,5] The most accepted screening technique for breast cancer is mammography and widely adopted treatment methods are surgery with chemical drugs, radiation and immunotherapy. Tumors in breast cancer can spread through blood and lymph vessels at the advanced stage of breast cancer stage V1; at that time, tumors are insensitive to conventional treatment

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methods. These treatments have a number of negative impacts on healthy cells and are unable to distinguish between cancerous and healthy normal cells. Toxicity with pain and inflammation and tumor resistance are the main disadvantages of the conventional treatment methods.[6-8] As a result, women with breast cancer urgently require natural therapies.

Several studies have revealed that plant-derived compounds, including phenols, flavonoids, alkaloids, essential oils, and terpenoids, possess antioxidant, antimicrobial, anti-inflammatory, anticarcinogenic, and anti-aging properties. Phytochemicals or plant-derived compounds act on cancer cells without damaging or providing negligible toxicity to normal cells.[9-11] Genus *Medinilla* belongs to the family Melastomataceae and consists of 430 species. Most of them are epiphytic climbers and clump-forming subshrubs. Members of this genus are ornamental, and some are edible and consumed by humans, exhibiting potent pharmacological activities. *Medinilla beddomei* C B Clarke is an epiphytic shrub native to the South Western Ghats, India. They are climbers with woody stems. The leaves are orbicular to sub-orbicular and fleshly in nature. Flowers are solitary and white in color. Also, fruits are turbinate berries with green in color.[12,13] Fresh and fleshy leaves of this plant are an important ethnomedicine for fever and giddiness. The leaf juice of this plant refreshes tribes as they travel through the forest. [14,15] Previous studies reported that besides Soxhlet extraction methods, ultrasound-assisted extraction methods yielded high polyphenolic compounds and essential oils. Additionally, studies have shown that extraction methods and the polarity of extraction solvents play a significant role in extracting polyphenols from plant species.[16,17] Therefore, the present study deals with the best extractive methods for phytochemical identification and the ability of *M. beddomei* leaf extract to cause cytotoxicity in breast cancer cell lines and a non-cancerous cell line.

Materials and Methods

Collection of the Plant Material

The study material, *M. beddomei,* was obtained from Mathikettan Shola National Park, Idukki, Kerala, in the summer season. The plant was identified and the herbarium sheet was lodged to KFRI, Thrissur, Kerala, under Voucher Number-18374 for further reference. Collected leaves were washed and dried in the shade for two weeks before being milled into a fine powder with a mechanical grinder. An airtight container was used to keep the powdered samples for later use.

Preparation of Plant Extracts

Ultrasound-assisted extraction and Soxhlet extraction techniques were used sequentially to extract phyto-compounds using acetone and methanol as solvents. For Soxhlet extraction, 25 g of the plant material was subjected to 6 hours in 250 mL of acetone and methanol solvents at a temperature of 10^0C above their boiling points of the respective solvents. During ultrasound-mediated extraction, approximately 25 g of powdered plant material was immersed in 250 mL of solvent and warmed in an oven for 2 minutes. The mixture was then subjected to sonication (Johnson Plastosinc, India) at a frequency of 20 kHz for 30 minutes, with intermittent cycles of 2 minutes. [18] A rotary evaporator was used to concentrate the extracts after they had been filtered through Whatman No. 1 filter paper.

Extractive Yield, Total Phenolic and Total Flavonoid Content

The soxhlet and ultrasound-assisted leaf extracts were dried then weighed to calculate the solvents' percentage extractive value. The extracts were then gathered and kept in glass bottles for future research. The Folin-Ciocalteu method, the aluminum chloride method, and the method described by Ghori et al. (2012) were used to measure the extract's total phenolic content (TPC), total flavonoid content (TFC), and total terpenoid content (TTC), respectively. Quercetin equivalent (QE mg/g) was used to quantify TFC, while gallic acid equivalent (GAE mg/g) was used to express TPC. Also, TTC was determined as linalool equivalents (LE mg/g) based on the calibration curve. [19-21]

Phytochemical Investigation by LC-Qtof-MS Analysis

The plant extract with a high extractive yield and polyphenol content, selected for LC-Qtof-MS analysis (Agilent, USA), was equipped with an electrospray ionization source. The column has been employed to separate 4.6 × 250 mm dimensions with a particle size of 5 µm at a flow rate of 0.5 mL/min. The elution was conducted with a mobile phase of a mixture of solvents, 95% A (water) and 5% B (acetonitrile) for 1 to 3 minutes linear from 5 to 95% A; 95 to 5% B. 30 minutes was the overall run time, and the injection volume was 5 µ. Eluted compounds were detected with an MS Q-TOF equipped with an electrospray ion source. The ionization mode was negative, and nitrogen functioned as the nebulizer gas. The temperature was 250°C, and the nozzle voltage was 1000 V. The back pressure was applied at a rate of 1500 psi. The obtained Qtof data was evaluated using Mass Lynx V 4.1 software. [22]

In-vitro Cell Viability Assay

The National Centre for Cell Science in Pune, India, provided the breast cancer cell lines MCF-7, MDA-MB-231, and L929, which were then cultured in DMEM media on a 96-well plate. Cells were cultured at 37°C in a humidified 5% CO2 incubator (Eppendorf Galaxy® 170 S, Germany) after being treated with 25, 50, and 100 µg/mL doses of the plant extracts. As a control, untreated cells were kept.

All test and control wells received 30 µL of MTT solution in PBS. After 4 hours of incubation, the plate was cleared of the supernatant, and 100 µL of DMSO, the solvent used to dissolve MTT, was applied to each well. The absorbance at 570 nm was measured following a mild shake. IC_{50} values were calculated as the concentration of the plant extract that induced 50% inhibition of cell growth. The morphological changes of cells were observed under an inverted phase contrast microscope (Labomed ^{TM} TCM 400, USA). The cell line showing the lowest IC_{50} value was chosen for further analysis. [23,24]

Wound Healing Assay

Cell migration inhibition was tested using the scratch wound healing assay. In a 24-well plate, MCF-7 cells were cultured for one day. The cells were exposed to the acetone leaf extract of *M. beddomei* at concentrations of 25 and 50 µg/mL, while the untreated cells served as the control. Then, using a sterile 200 µL pipette tip, a uniformly sized scratch was created. After two rounds of ice-cold PBS washing to get rid of any debris, a new medium was added to each well.[25] Using an inverted microscope, the wound gap was assessed right away (0 h) and observed for 12 and 24 hours time intervals. Photographs were taken from a camera coupled with a microscope. The percentage of wound healing was analyzed using the Image J analysis program.

Double Staining Method

The effects of plant extract-induced apoptosis on MCF-7 cells were analyzed by using the acridine orange (AO) and ethidium bromide (EtBr) double staining method. Typically, a combination of DNA-binding stains is employed to identify necrotic and apoptotic cells. After treating the cells with the acetone leaf extract of *M. beddomei* at the IC_{50} concentration determined by the MTT experiment, the cells were incubated for a whole day. EtBr and AO solutions were used to stain the cells following PBS washing. The stained cells were periodically rinsed with PBS after being treated for 15 minutes at 37°C. Viable cells stained by EtBr and AO emit green fluorescence and apoptotic cells stained by EtBr produce red fluorescence. [26] A digital camera was connected to a microscope (Olympus CKX41, Japan) to examine fluorescence and capture photographs.

Cell Cycle Analysis

Using flow cytometry, the amount of DNA in each cell cycle phase was determined. The IC_{50} values of the plant extract derived from the MTT assay were used to incubate and treat the cells, while untreated cells served as a control for a whole day. Cells were gathered and subjected to centrifugation for five minutes at 3000 rpm following treatment. Pellets were incubated for 24 hours after being cleaned with ice-cold 70% ethanol and again centrifuged for five minutes at the same speed in order to obtain a white, transparent, or clear pellet. The pellet was mixed with 250 µL of fresh PBS after the supernatant was removed. Once 100 µL of Muse TM Annexin V & Dead cell reagent was added, the mixture was incubated in the dark for 30 minutes. [27] Following treatment, a flow cytometer equipped with Muse flow cytometry software (Muse FCS 3.0) was used for analysis and interpretation.

Statistical Analysis

Triplicate trials were conducted. By using SPSS (Statistical Package for Social Science) 16.0 software, the results are presented as mean ± Standard Deviation. One-way Analysis of Variance (ANOVA) and an unpaired t-test were applied to the data. The results' mean values were estimated as significant when $*p < 0.05$ and $**p < 0.01$ were present.

Results

Optimization of the Extraction of *M. beddomei* Leaves

The current study demonstrated the efficient extraction methods for the plant sample *M. beddomei*. It was observed that ultrasound-assisted extraction yielded a high amount of active ingredients in the leaves of this plant and reduced the time duration for the extraction techniques in selected solvents. Comparing the efficiency of chosen solvents like acetone and methanol, Acetone leaf extract had high extractive yield (11.75 ± 1.38% w/w), phenolic (224.19 ± 4.02 mg GE/ g DW) flavonoid (351.04 ± 4.03 mg QE/g DW) and terpenoid (275.43 ± 2.85 mg LE/g DW) contents than that of methanolic leaf extracts obtained from ultrasound-assisted extraction method (Table 1). The high extractive yield of polar solvents indicated the amount of polar-soluble contents. We found that the amount of phytochemical compounds in plants can vary based on the organic solvents and extraction techniques used in the study.

LC Qtof MS analysis of leaf extract of *M. beddomei*

The phytochemical investigation of the acetone leaf extract of *M. beddomei* was identified by LC Qtof MS analysis (Fig. 1). Around 26 compounds were detected from this

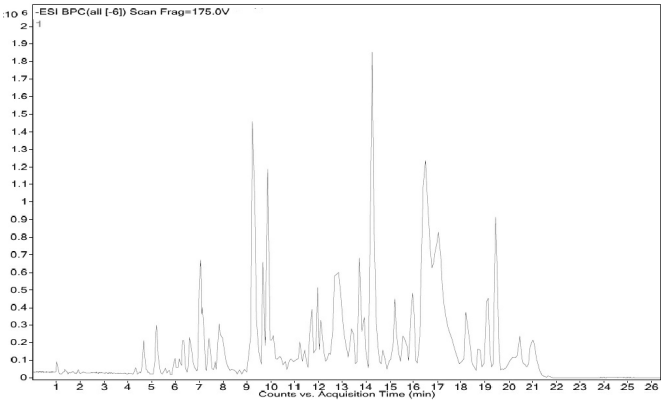

Fig. 1: Liquid chromatogram of acetone leaf extract of *M. beddomei*

Table 1: The extractive yield, total phenol, flavonoid, and terpenoid content of acetone and methanol leaf extracts of *M. beddomei* prepared by Soxhlet and Ultrasound-assisted extraction techniques

Parameters / *Extraction*	*Soxhlet Extraction*		*Ultrasound-assisted extraction*	
	Acetone	*Methanol*	*Acetone*	*Methanol*
Extractive yield (% w/w)	8.01 ± 1.22	6.82 ± 0.87	11.75 ± 1.38	9.82 ± 0.96
Total Phenolic content (mg GE/g DW)	126.32 ± 0.99	117.95 ± 1.87	224.19 ± 4.02	198.24 ± 3.45
Total Flavonoid content (mg QE/g DW)	272.69 ± 1.04	266.50 ± 2.05	351.04 ± 4.03	349.38 ± 2.52
Total Terpenoid content (mg LE/g DW)	126.39 ± 4.27	104.29 ± 4.08	275.43 ± 2.85	209.79 ± 1.27

Values are expressed as mean ± SD, GE- Gallic acid Equivalent, QE- Quercetin Equivalent, LE- Linalool Equivalent, DW- Dry weight

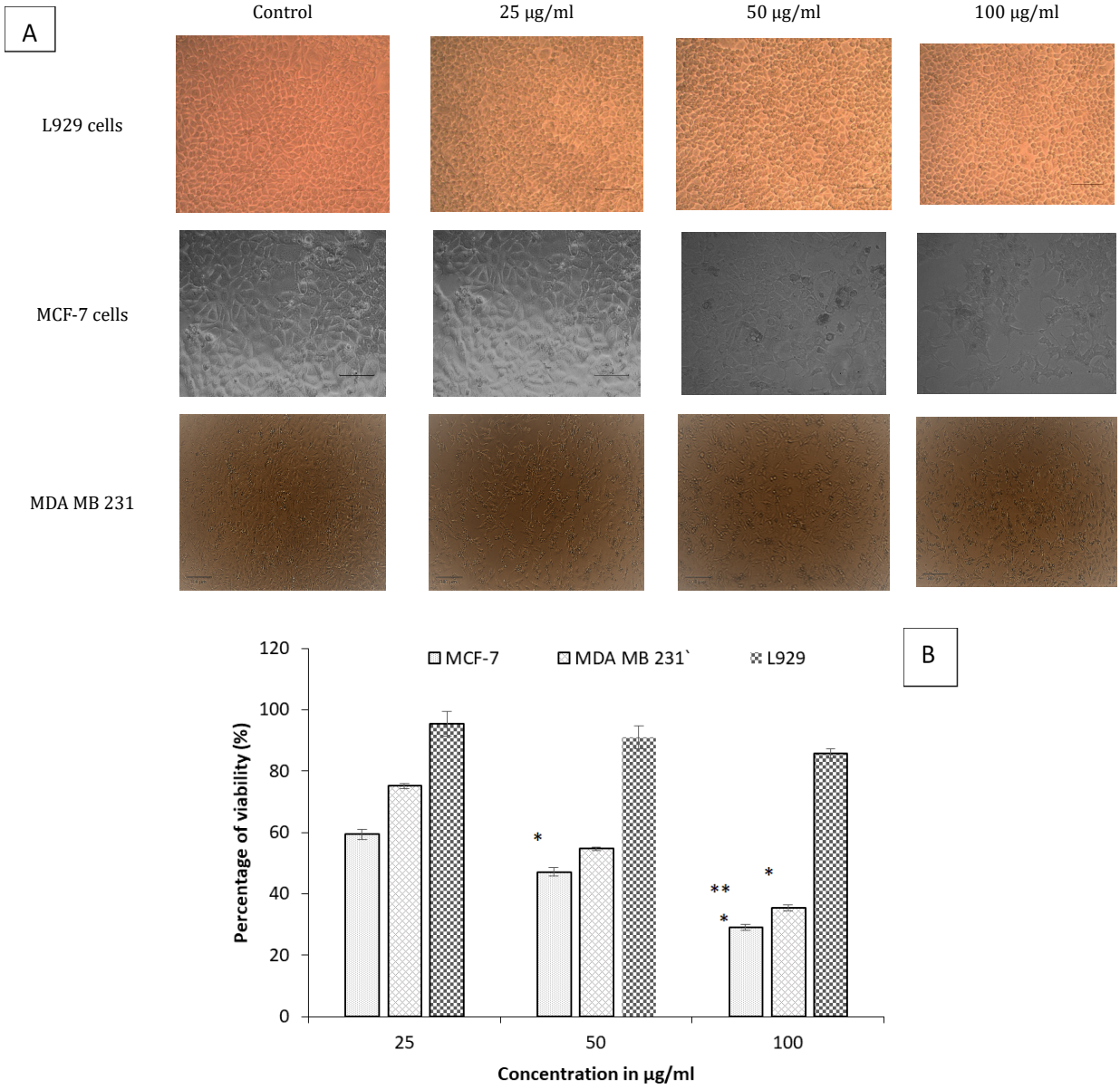

Fig. 2: Cytotoxic impact of M. beddomei leaf extract in acetone at three different doses on several cell lines compared to untreated control cells. A. After 24 hours, morphological alterations in L929, MCF-7, and MDA-MB-231 treated with th acetone leaf extract of *M. beddomei* were seen under an inverted microscope (×400 magnification). B. Cell viability percentage of MCF-7, MDA-MB-231, and L929 cells treated with varying acetone leaf extract concentrations after 24 hours and results are expressed as mean ± SD (n=3). Significant differences are shown by *p<0.05 and **p<0.01 when compared to untreated cells

Table 2: Phytocompounds of acetone leaf extract of *M. beddomei* identified by LC Qtof MS analysis

S. No.	*Retention time (RT)*	*Phytocompounds*	*Classes*
1	1.361	1-O-Caffeoyl-(b-D-glucose 6-O-sulfate)	Glycosides
2	4.917	Gallic acid 4-O-(6-galloylglucoside)	Phenolic glycosides
3	5.81	Tercatain	Hydrolyzable tannin
4	5.847	1,3,6-Tri-O-galloylglucose	Hydrolyzable tannin
5	6.71	Ellagic acid	Polyphenol
6	7.082	2,3-Dihydro-5,5',7,7'-tetrahydroxy-2-(4-hydroxyphenyl) [3,8'-bi-4H-1-benzopyran]-4,4'-dione	Isoflavonoid
7	7.458	3-Methylellagic acid 8-rhamnoside	Hydrolyzable tannin
8	7.696	L-ascorbic acid-2-phosphate	Vitamin C derivative
9	9.09	2,8-Di-O-methylellagic acid	Hyrolyzable Tannin
10	9.865	Phloionolic acid	Fatty acid
11	11.002	Corchorifatty acid F	Fatty acid
12	11.544	Afrormosin	Methoxy isflavone
13	12.189	Esculentic acid	Triterpenoid
14	12.306	Dolicholide	Brassinosteroid
15	12.597	Madasiatic acid	Triterpenoid
16	12.759	3,3'-Bisjuglone	Naphthoquinone
17	13.589	(3beta,15alpha,22S,24E)-3,15,22-Trihydroxylanosta-7,9(11),24-trien-26-oic acid	Diterpenoid
18	13.941	Bryodulcosigenin	Triterpenoid
19	14.24	Ophiopogonone B	Steroid saponin
20	15.206	Delta-Maslinic acid	Triterpenoid
21	15.639	Kukoamine B	Alkaloids
22	15.88	Nb-Stearoyltryptamine	Alkaloids
23	16.566	Liquiritic acid	Triterenoid
24	17.232	Ganoderiol H	Triterpenoid
25	17.951	6-Hydroxy-4-tricosanone	Fatty alcohols
26	19.858	Ursolic acid	Triterpenoid

analysis. The names of these identified compounds, the class of the compounds, and the retention time are listed in Table 2. Polyphenols, alkaloids, glycosides, fatty acids, and terpenoids were detected in the leaf extract. Polyphenol, ellagic acid, and, phenolic glycoside, gallic acid 4-O-(6-galloylglucoside) were found in the leaf extract. Additionally, the leaf extract contains isoflavonoids, methoxyflavones, Afrormosin, and various valuable hydrolyzable tannins. Several terpenoids and hydrolyzable tannins enrich the leaf extract's bioactive properties.

Cytotoxic Effect of the Acetone Leaf Extract of *M. beddomei*

In the present MTT test, the leaf extract of *M. beddomei* demonstrated dose-dependent cell damage against breast cancer cell lines, such as MCF-7 and MDA-MB-231, but showed less toxicity against normal L929 cells. MCF-7, MDA-MB-231, and L929 cells had IC_{50} values of 46.19 ± 1.58, 68.27 ± 0.83, and 386.12 ± 4.07 µg/mL, respectively, for the plant's acetone leaf extracts. Acetone leaf extract's cytotoxic capability was validated by comparing the morphology of treated cancer cells to control cells. The treated cells showed apoptotic bodies, nuclear condensation, and cell shrinkage (Fig. 2). The results showed that malignant cells were significantly more susceptible to the cytotoxic effects of acetone leaf extract (p <0.05) than normal cells. Furthermore, acetone leaf extract was more effective against MCF-7 cells than MDA-MB 231 cells, which were chosen for additional anticancer research.

Scratch Wound Healing Assay

The plant's considerable ability to prevent cell migration in MCF-7 cells in comparison to control cells was validated by an antimetastatic investigation using the acetone leaf extract. The experiment was conducted using two

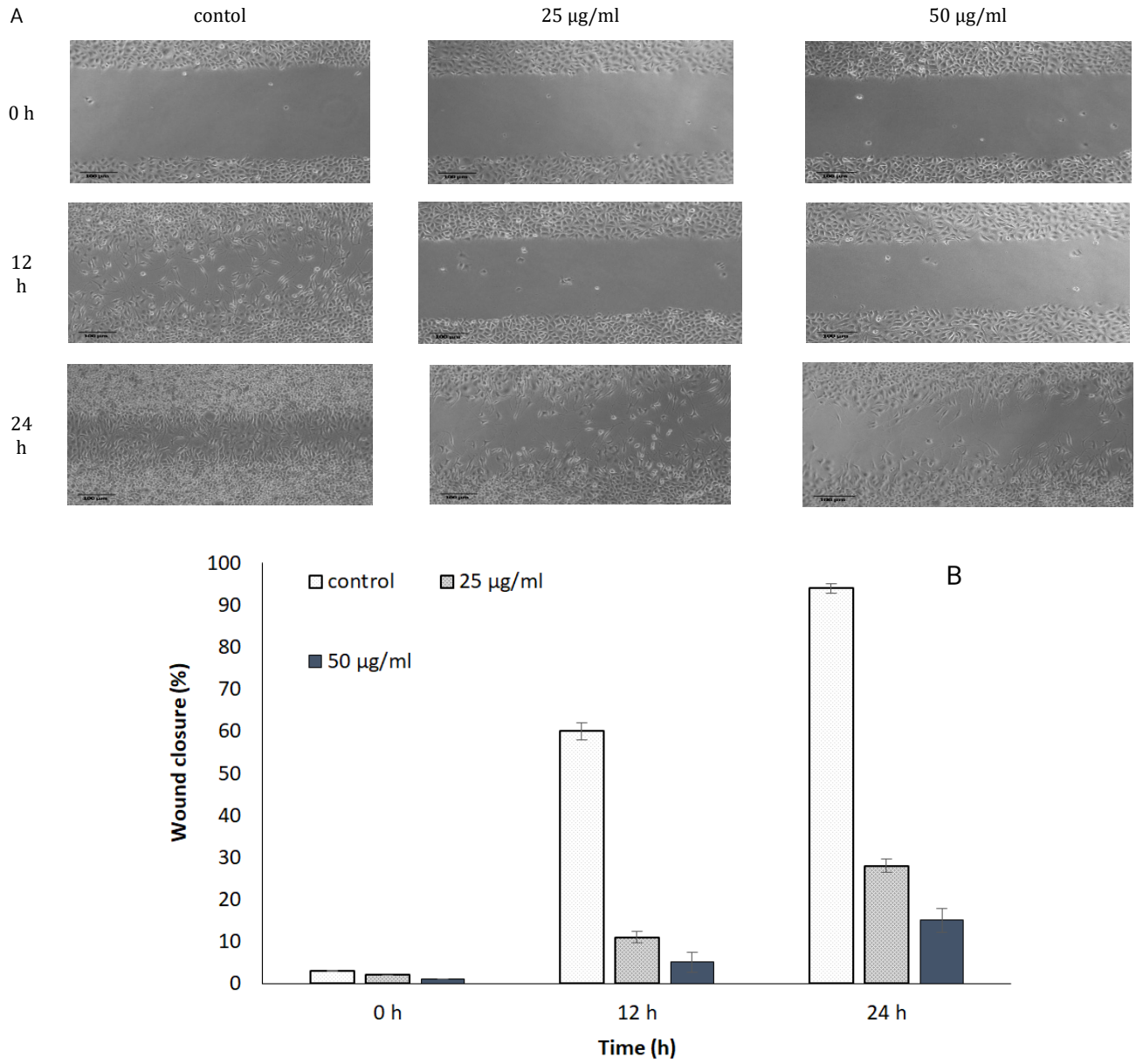

Fig. 3: *In-vitro* cell migration of acetone leaf extract of *M. beddomei* on breast cancer cell, MCF-7 was screened (A) Microscopic images of MCF-7 cells after treatment with plant extract as compared to the untreated cells (×10 magnification) (B) Percentage of wound gap closure induced by the leaf extract of different concentration at different time intervals

concentrations of the leaf extracts: 25 and 50 µg/mL. This extract inhibited the scratch wound's gap closure in a time-bound manner. The scratch wound healing capacity of non-treated cells is 94.22 ± 1.06% after 24 hours of treatment. Nonetheless, the cells that were exposed to 50 µg/mL of acetone leaf extract at that time demonstrated 15.04 ± 1.52% wound healing (Fig. 3). It was found that *M. beddomei's* acetone leaf extract may inhibit the growth and spreading of MCF-7 breast cancer cells.

Apoptotic Detection by Double Staining Method

An IC_{50} concentration of acetone leaf extract obtained from the MTT assay on MCF-7 cells was selected for a double-staining method. Living cells with normal nuclei take EtBr and AO stains and emit green fluorescence. Otherwise, apoptotic and necrotic cells with fragmented or condensed nuclei emitted orange to red fluorescence (Fig. 4). This fluorescence was a sign of cell death and confirmed the apoptotic potential of the extract in the breast cancer cells.

Effect of Acetone Leaf Extract on Cell Cycle

The cell cycle arrest induced by the acetone leaf extract was analyzed using flow cytometry. Here, the IC50 value of acetone leaf extract obtained from the MTT assay was also taken to treat MCF-7 cells. Fig. 5 indicates that the cell count increased in S and G2/M phases in contrast to non-

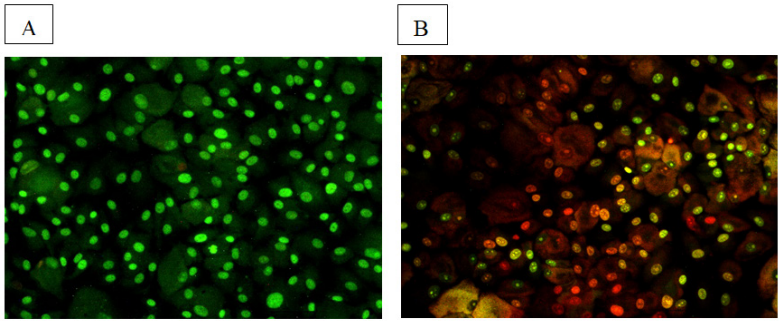

Fig. 4: Apoptotic effect of IC_{50} value of acetone leaf extract on MCF-7 cell after 24 hours treatment. (A) fluorescence of untreated MCF-7 cells, (B) apoptotic MCF-7 cells after the treatment of 46.19 ± 1.58 µg/ml acetone leaf extracts at ×40 magnification

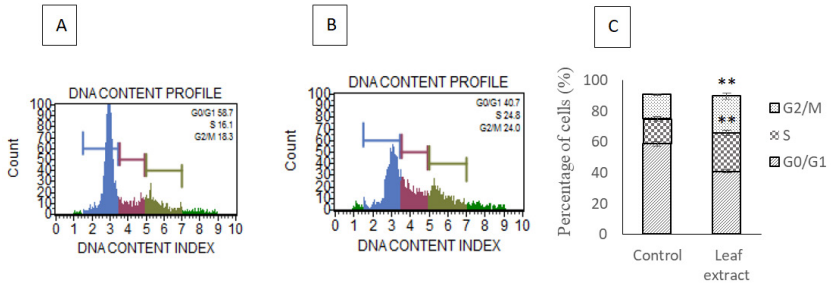

Fig. 5: Percentage of cell count by flow cytometry in breast cancer MCF-7 cells after the administration with an IC_{50} concentration of *M. beddomei* acetone leaf extract (A) Cell cycle distribution in reference cells (B) Percentage of MCF-7 cells treated with 46.19 ± 1.58 µg/ml of acetone leaf extract of *M. beddomei* (C) Distribution of cells in different phases of the cell cycle of control and treated cells expressed in the histogram as mean ± SD at a significant level of $^{*}p<0.05$ and $^{**}p<0.01$ compared with the control

treated reference cells. Treated cells showed a percentage of cell population of 16.11 ± 1.32 in S and 15.9 ± 0.43 in G2/M phases. Nevertheless, in the case of control cells, S and G2/M phases showed cell population percentages of 24.8 ± 1.69 and 24.01 ± 1.98, respectively. A significant increase in the number of cell clusters indicated cell cycle arrest. Defects in the S and G2/M phases of cells screen the checkpoint cell repair mechanisms, which allow the cells to enter apoptosis and mitosis. In addition to S and G2/M phases, the cell population was considerably reduced ($p < 0.05$) during the G0/G1 phase.

Discussion

The plant *M. beddomei* has been reported to have ethnomedicinal importance among the tribal people of Kerala. Even though the reports on the scientific evaluation of this plant are limited. [28] So, the current study dealt with the phytochemical and cytotoxic potential of the plant *M. beddomei.* Initially, the study focused on optimizing the extraction methods of *M. beddomei* leaves, which was carried out using soxhlet and ultrasound-assisted extraction using polar solvents like acetone and methanol. Based on the results of the work, ultrasound-assisted extraction is a better method for yield and stability of phytochemicals than the conventional soxhlet method. [29] Also, there is a positive correlation between the percentage of extractive yield, total phenol content, total flavonoid content, and total terpenoid content due to the solubility of the compounds in the selected solvents and in the extraction process. [30] The present study revealed that the solvent acetone has a high extractive yield of phytocompounds. Phytochemical profiling of acetone leaf extract of *M. beddomei* by LC Qtof MS analysis revealed the occurrence of polyphenols, glycosides, terpenoids and alkaloids. The synergetic action of these phytochemicals makes the plant sample more safe and has specific therapeutic properties. According to numerous studies, ellagic acid derived from various plant species may prevent cancer cells from proliferating and dying. [31] Terpenoid ursolic acid is a potent candidate against breast cancer in women, and also steroid saponin ophiopogonone B possesses cytotoxic potential against different cancer cells. [32,33] It is clear that the unique structural diversity and chemical properties of these phytocompounds make the plant extract more potent against cancer cells, especially in breast cancer cells. The work evaluated the anticancer properties of the leaf extract of the plant in breast cancer (MDA-MB-231 and MCF-7) cells and non-cancerous cells (L929). The acetone leaf extract of *M. beddomei* showed significant cytotoxic potential in MCF-7 and MDA-MB-231 cells. It showed minimum toxicity towards normal L929 cells with an IC_{50} value of 386.12 ± 4.07 µg/ml. Abnormal

cell proliferation and cell migration are important causes of the increase in the mortality rate of cancer. So we screened the antimetastatic property of two different concentrations (25 and 50µg/ml) of acetone leaf extract of *M. beddomei* in MCF-7 cells, which showed a high cytotoxic activity in the MTT assay, indicating a decline of wound closure over time. IC_{50} concentration of leaf extract lies within these selected concentrations, indicating that this concentration also has significant potential to inhibit cell migration for metastasis. The IC_{50} concentration of the leaf extract, as determined by the cell viability assay in MCF-7 cells, revealed non-viable apoptotic characteristics and cell cycle arrest, following the detection of apoptosis using the double staining approach with EtBr and AO dyes. The findings obtained from flow cytometry indicated that the MCF-7 cells' cell death was the cause of the increased cell cluster in the S and G2/M phases. These data also correlate with other studies on different plant species in various cancer cells. [34,35] The results of the current work ensure that this plant has antiproliferative and antimetastatic effects and can be considered as an adjuvant nutraceutical source to target dreadful diseases such as breast cancer.

Conclusion

According to the study, the acetone leaf extract of *M. beddomei* leaves has significant anticancer properties when tested against breast cancer cell lines. The phytochemical composition of this plant extract might be responsible for this activity. However, further study is needed to explore the mechanism and the phytochemicals behind this property.

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