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RESEARCH ARTICLE

Induction of Apoptosis by Methanolic Extract of *Rhizophora mucronata Lam* on MCF-7 Cell Line

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ABSTRACT:

The tropical red mangrove *Rhizophora mucronata (Lam.)* has been widely used as an astringent, antiseptic, antibacterial, anti-ulcerogenic, and anti-inflammatory agent in traditional Oriental medicine. Cancer is a concept to be characterized by differentiating features, uncontrolled proliferation of cells, invasion of abnormal cells into healthy cells, and their spread through the bloodstream to vital organs. The key programmed cell death mechanism for silently killing unwanted and detrimental cells during embryonic development, tissue homeostasis, and immune regulation is apoptosis. Our research investigated the activation of apoptosis by the methanolic extract of *R. mucronata* and the expression of apoptotic and anti-apoptotic proteins. The cell cycle and apoptosis were observed by the Annexin V/PI-flow cytometry technique. The MTT test showed a strong cytotoxic effect on the cell lines of MCF-7. In MCF-7 cells, the sample demonstrated successful cell cycle arrest. More number of MCF7 cells get arrested in Sub G0/G1. In our study, FITC Rabbit Anti-Active Caspase 3 is used as a fluorochrome. It can be inferred from our study that good therapeutic potential against Human Breast derived diseases can be compared to the std control and the compound samples.

KEYWORDS: Apoptosis, Caspase-3, Cell cycle, Flow Cytometry, *Rhizophora mucronata.*

INTRODUCTION:

Cancer is a term to identify with distinguishing characteristics, uncontrolled cell proliferation, invasion of abnormal cells to healthy cells, and their spread towards vital organs via bloodstream [1]. Breast cancer is becoming a leading cause of female mortality and new therapeutic compounds are being continuously identified [2]. However, the incidence of breast cancer has increased dramatically in recent decades in developed countries, where approximately 82% of the world's population lives, due to population growth and aging[3]. For their medicinal wealth, mangroves are renowned.

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Different sections of *Rhizophora mucronata* are used in the treatment of different diseases. Mangroves are salt-tolerant plants from intertidal tropical and subtropical regions of the world [4] . The best natural anti-diarrheal agent is shown in *Rhizophora mucronata* leaf extracts. The natural compounds used for human health enhancement in both traditional and modern therapies with fewer side effects. In folk medicine, different parts of the plants are used [5]. The tropical red mangrove *Rhizophora mucronata (Lam.)* has been widely used as an astringent, antiseptic, antibacterial, anti-ulcerogenic, and anti-inflammatory agent[6]in traditional oriental medicine.

The main programmed cell death mechanism for silently removing unwanted and detrimental cells during embryonic development, tissue homeostasis, and

immune regulation is apoptosis. In addition, apoptosis results from the collapse of the cellular infrastructure by some enzymes called caspases through internal proteolytic digestion, resulting in cytoskeletal disintegration, metabolic derangement, and genomic fragmentation [7,8,9].

In apoptotic cells the phospholipid phosphatidylserine membrane is moved from the inner region to the outer region of the plasma membrane, and its expose outside the external cellular environment. The earlier stages of apoptosis, FITC Annexin-V staining can identify apoptosis at an earlier stage than nuclear-based assays such as DNA fragmentation [10] .

In apoptosis and inflammation, the caspase family of cysteine proteases plays a key role. Caspase-3 is a key protease that is activated during the early stages of apoptosis and is synthesized as an inactive pro-enzyme like other members of the caspase family that are processed by self-proteolysis and/or cleavage by another protease in cells undergoing apoptosis. Caspase consist of two subunits one is large (17-22 kDa) and another one is small (10-12 kDa) subunits that are linked together to form an active enzyme. When the cell undergoing the apoptosis process, from the 32kDa proenzyme , caspase-3 is derived with heterodimer 17kDa and 12 kDa subunits.When this active caspase -3 undergoes the cleavage it activates the other caspases protein. Our current research was aimed at investigating the expression of apoptotic and anti-apoptotic proteins by using *Rhizophora mucronata* methanolic leaf extract as a test on MCF-7 respectively. We used fluorochromes conjugated with Annexin-V instead of DNA fragmentation to detect apoptosis using the flow cytometer, a novel method.

MATERIALS AND METHODS:

Plant Extraction Preparation:

Rhizophora mucronata dried material was combined for seven days with methanol,ethanol, diethyl ether and ethyl acetate solvents. The extract was then filtered onto Whatman no. 1 paper and the extract was then dried using a rotor evaporator to get rid of the solvents to get the concentrated jelly extract. The extract was later stored at -20 ˚C in a sterile container. Out of four extracts, methanol extract shows the predominent number of phytoconstituents and thus the apotopsis studies were carried out using methanol extract.

Cytotoxic assay:

The MTT assay [11] was used to assess cytotoxicity. For anticancer research, the MCF7 cell line and VERO cell line were used. In a 96-well plate, 200µl of cell suspension was seeded and incubated for around 24 hours. 25µg/ml, 50µg/ml, 100µg/ml, 200µg/ml, 400µg/ml of leaf extract concentration was applied to the wells. At 37 ° C with 5 percent CO_2, the plate was incubated for 24 hrs. The absorbance was read at 570 nm and 630 nm using the ELISA scanner. And then IC_{50} value was calculated [12] .

Flow cytometric detection of apoptosis:

MCF-7 cells were treated in 2 ml of culture medium with the necessary concentration of *Rhizophora mucronata* methanolic leaf extract and Camptothecin control and incubated for 24 hours. With PBS, the cells were washed and then centrifuged. 200 µl of Trypsin-EDTA solution was applied to the remaining components and incubated for 4 minutes at 37 ℃. 2 ml of culture medium was applied and the cells were collected directly into polystyrene 12 x 75 mm tubes, centrifuged, and decanted with the supernatant. In the dark, 5 µl of FITC Annexin-V was added and incubated at RT (25°C) for 15 min. In each tube, 5 µl of Propidium Iodide (PI) and 400 µl of 1X Binding Buffer were added and gently vortexed. It was then analyzed immediately after the addition of PI by flow cytometry.

Analysis of cell cycle using Propidium iodide Assay:

This is a cell cycle analysis approach that uses Propidium Iodide (PI) to classify the proportion of cells in each of the three interphase stages of the cell cycle using the fluorescent nucleic acid dye PI. The lines of breast cancer cells were fixed after PBS washing in 1 ml of 70 percent ice-cold ethanol. The fixation was done and it was centrifuged for about 30 minutes. The centrifuged cells were washed twice with PBS and the cell pellet was treated with 50µl Ribonuclease A to get rid of RNA to ensure that only DNA was stained (PI stains all nucleic acids). Subsequently, the 400µl PI solution was directly applied to the RNase A suspension cells. The suspension of the cell was incubated and observed under flow cytometry for 5 to 10 mins.

Evaluation of Caspase 3 expression:

Fluorescein isothiocyanate, FITC, is a fluorochrome with a 389 Da molecular weight. FITC is susceptible to alterations in pH and photo bleaching. FITC and Alexa Fluor® 488 should not be used simultaneously due to virtually identical excitation and emission properties but different spill over characteristics. FITC is relatively dim and should be reserved wherever possible for strongly articulated markers. In our study, FITC Rabbit Anti-Active Caspase 3 is used as a fluorochrome. MCF-7 cells were treated with 5 µl FITC Caspase 3 antibodies and incubated for 30 mins at room temperature in the dark. It was then washed with 0.1% sodium azide and 0.5 ml PBS for cytometric flow analysis.

RESULTS AND DISCUSSION:

Cytotoxic Assay:

After an exposure period of 24 hours, the cell cytotoxicity of methanolic leaf extract against human breast carcinoma cells indicates IC 50 at 76.52µg/ml. The viability percentage concluded that the extract of the leaf is more toxic than normal VERO cells to MCF-7 cancer cells. The extract can be considered as a possible compound against cancer [13]. Plant compounds were found to be inhibiting agent for the cancer cells and promotes the apoptosis process in MCF-7 breast cancer cells [14].

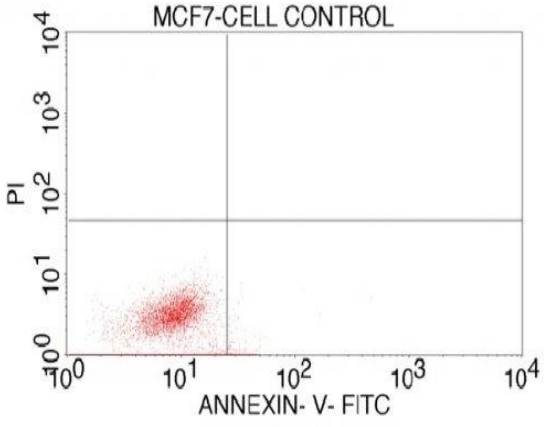
Flow cytometric detection of apoptosis:

FITC Annexin-V is employed to quantitatively evaluate the percentage of cells actively undergoing apoptosis within the population. Annexin-V could be a phospholipid-binding protein and it's useful for the detection of apoptotic cells. The apotic status of the methanol extract treated MCF-7 cells are assessed by pro-cleavage caspase expression .The gene expression of the apoptotic regulators and p53 tumour supressor are upregulated by DNA damage [15].

In this study, the compound concentrations used to treat the cells are as follows in Table 1. Figure 1 shows the quadrants expression of given (A) control MCF7 Cells, (B) Standard drug (Camptothecin) treated MCF7 cells and (C) methanolic extract of *Rhizophora mucronata* leaves treated MCF7 cells beside the stain Annexin V-FITC and Propidium Iodide . Table 2 enlist the percentage of Cells of undergone Apoptosis in the Untreated, Standard and leaf extract treated MCF7 Cells.

Table 1: Details of Drug Treatment to respective Cell line used for the study

Sl. no	Test Compounds	Cell Line	Concentration treated to cells
1	Untreated cells	MCF7	No treatment
2	Standard Camptothecin treated cells	MCF7	25µM
3	*Rhizophora mucronata* methanolic leaf extract treated cells	MCF7	76.52µG



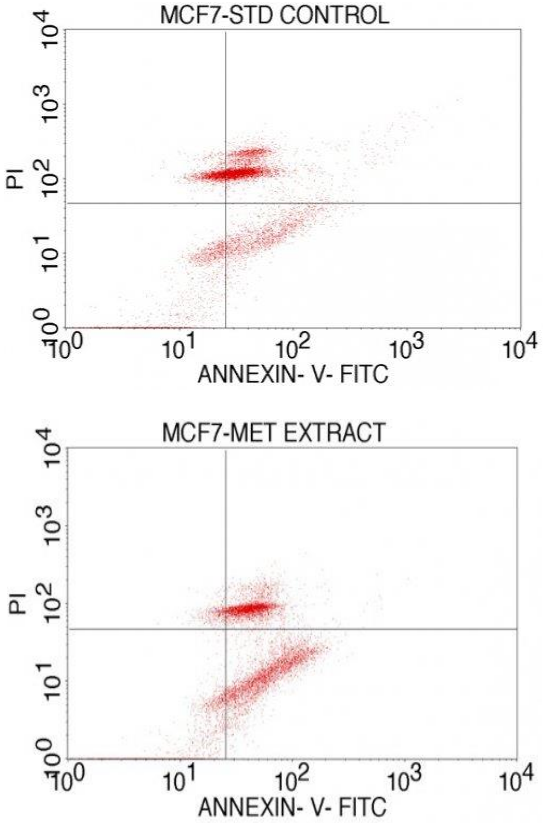


Figure 1: Annexin V-PI expression Study of the Test Compound against the MCF 7 Cell line

Table 2: Table showing the % of Cells of undergone Apoptosis in the Untreated, Standard and Leaf extract treated MCF7 Cells.

Quadrant	% of Necrotic Cells	% Late Apoptotic Cells	% Viable Cells	% of Early apoptotic cells
Untreated Cell Control	0	0	94.17	5.83
Standard Camptothecin control	18.33	52.28	13.59	15.8
Leaf extract	3.85	39.19	17.84	39.12

Analysis of cell cycle using Propidium iodide staining method:

Propidium Iodide (PI), which has red fluorescence and can be excited at 488 nm, is the most commonly used dye. In order to determine the cell cycle distribution, the compound concentrations used to treat the cells are as follows in Table 1. Figure 2 shows the PI -Propidium Iodide histogram of the gated MCF 7 cell singlets distinguishes cells at the Sub G0/G1, G0/G1, S, and G2/M cycle phases. Table 3 shows the percentage of cells get arrested after the control, standard and methanol extract treated at the different stages of their life cycle. The cell cycle study of MCF7 cells compared to cell control and methanol extract treated with the IC_{50} concentration, it shows more number of cells get arrested in the Sub Go/G1 phase (16.78%) and in G0/G1 phase (52.93%).

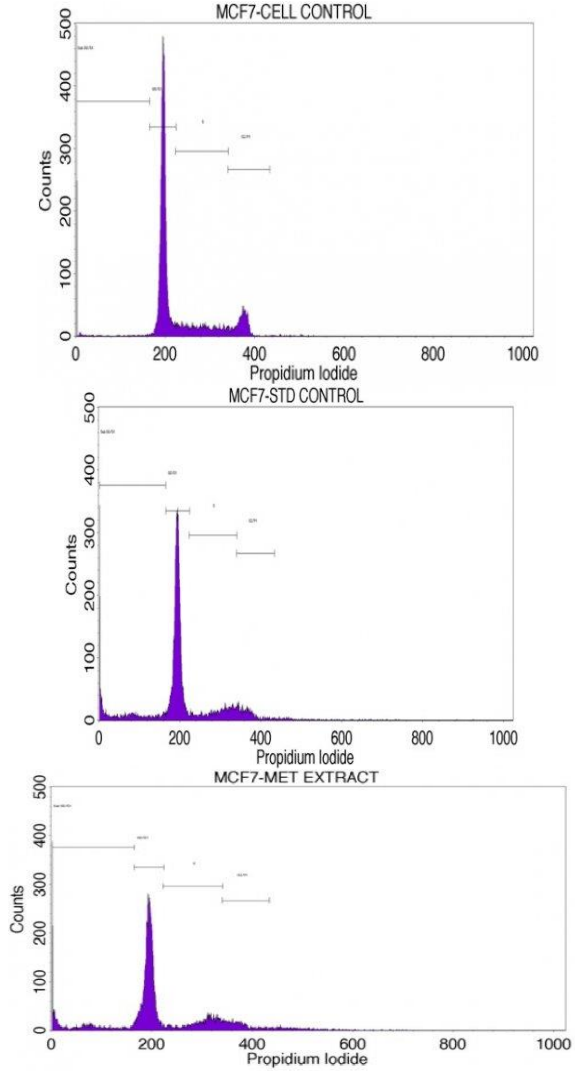


Figure 2: Cell cycle analysis using PI stain using A control, B standard control and C crude methanol extract against MCF7 cells

Table 3: Showing the percentage of Cells get arrested in different phases and overlay

Overlay of Cell Cycle Study vs MCF7				
Sl No	**Cell Cycle Stage**	**Untreated-Cell Control %**	**Standard Control-25µM camptothecin %**	**Test-76.52uG Methanol Extract %**
1	Sub G0/G1	11.24	16.97	16.78
2	G0/G1	60.12	57.79	52.93
3	S	17.33	15.31	16.69
4	G2/M	11.89	8.69	9.9
Total Events Selected (cells)		10000	10000	10000

The samples showed significant cell cycle arrest by reducing the percentage of G0/G1 phase cell cycle arrest compared to the controls and also increasing the percentage of G0/G1 phase cell cycle arrest compared to the controls. It is clearly confirmed that Good Cell Cycle arrest against the MCF-7 Cells is shown by the extract. Compared to the Camptothecin standard control, more MCF7 cells are arrested in the Sub G0/G1 phase and the extract can have good therapeutic potential against diseases derived from the human breast. Apoptotic cells are detectable with Propidium Iodide it is a DNA binding dye which are penetrable inside the dead cells.The apoptotic cell death in MCF-7 cells forms the fragmented DNA in early apoptotic period. There is an increase in apoptosis by caspase dependent pathway in the anticancer drug initiated cell death [16].

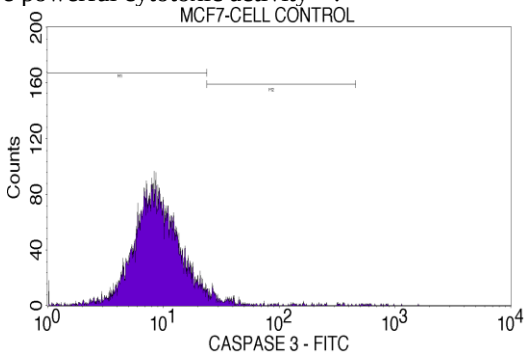
Evaluation of Caspase 3 expression:

The expression of CASPASE 3 - cysteine dependent aspartate specific proteases 3 in MCF 7 cell line during apoptosis was studied using methanolic extract of *Rhizophora mucronata* leaves. Flow cytometry was used to examine the cells after incubated with rabbit anti-active caspase antibodies. Table 1 gives the details of concentration used for the study.

Figure 3 shows the caspase 3-FITC histogram of the gated MCF7 singlets distinguishes cells at the M1-negative expression region and M2- positive expression region of (A) Cell Control, (B) Standard Control (B) and (C) methanol extract against MCF7 cells. Flow cytometric analysis shows that the treatment of MCF 7 cells with methanol extract results in a significant shift in the peak towards the right, suggesting activation of caspase-3.

Table 4 shows the mean fluorescence intensity and positive expression of caspase- 3 against the cell control , standard control and methanol extract treated MCF7 cells. Activation of caspase-3 was involved in the induction of apoptosis upon methanol extract treatment of MCF7cells. Therefore, it was found that in MCF-7 cells induction of apoptosis was through caspase dependent pathway that contributed to its anti-cancerous property.

Under the stress condition or diseased condition caspase-3 expression takes place [17] . In retorting the immune cells of malignant tumours, Natural killer cells have powerful cytotoxic activity [18].



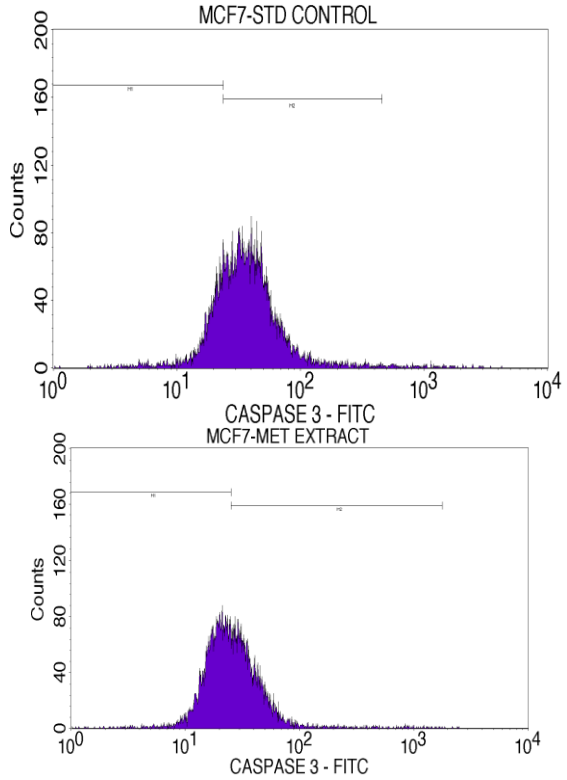


Figure 3: Caspase 3 expressionof the Test Compound (ME) against MCF7 Cell line

Table 4: Showing the Mean Fluorescence Intensity and Positive cell Expression of CASPASE 3 against MCF7 Cells

Caspase 3	Relative Mean Fluorescence Intensity	Positive Expression (%)
Untreated Cell Control	13.02	4.52
Standard Camptothecin control	54	77.78
Leaf extract	34.88	52.64

It can be inferred from the above analysis that the methanolic extract of *Rhizophora mucronata* leaf methanol extract may have possible therapeutic potential against diseases derived from Human Breast Cancer.

CONCLUSION:

In conclusion, human MCF-7 breast cancer cells have dose dependent effects with *Rhizophora mucronata* leaf methanol extract. In rapid cell death in the MCF-7 cells via apoptosis induction, the antiproliferative property of the plant extracts was revealed. Further studies will be carried out to classify and extract potent bioactive phytochemicals and to explore the production of possible breast cancer chemotherapeutic or chemopreventive agents.

CONFLICT OF INTEREST:

In this research study,the authors have no conflicts of interest.

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