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Research Article

Evaluation of *Acalypha indica* Aerial Part Ethanol Extract for Anticancer Activity in MDA-MB-231 Cells Supported by GC-MS Chemical Fingerprinting

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ABSTRACT

Background: Plant extracts, their parts, and active chemicals are used for many decades in cancer management. **Aim:** In this study, the aerial part (stems, leaves and flowers) of *Acalypha indica* (Family: *Euphorbiaceae*) was cold-extracted using ethanol solvent, and the anticancer activity was assessed using breast cancer cell line, MDA-MB-231. **Materials and Methods:** The work investigated the cytotoxic effect of *Acalypha indica* using various cytological methods like MTT assay, DNA ladder assay, AO/EB dual staining method and Mitochondrial Membrane Potential (MMP) assay in different extract concentrations ranging from 20 to 100 µg/mL. GC-MS analysis was performed to identify the compounds. **Results:** Changes in the shape and appearance of both untreated and extract-treated cells were viewed under a digital inverted microscope after 24 hr and recorded as images. The ethanol extract of *A. indica* showed toxic effects on cancer cells, reducing their survival in a dose-dependent way. IC_{50} value was calculated as 59.97 µg/mL. DNA ladder assay suggests extensive cytogenetic damage in the extract-treated cells. The AO/EB assay and MMP assay results show apoptosis of varying degrees in the extract-treated cells. 60 plant compounds were identified by GC-MS analysis. **Conclusion:** Thus, this preliminary study indicates that the ethanol-extracted *Acalypha indica* aerial parts can be used to treat breast cancer. Further pharmacological activities and phytochemical study is required to confirm its potential.

Keywords: AO/EB staining, Carcinoma, Medicinal plant, MTT, Phytochemicals.

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INTRODUCTION

Cancer is the second most common cause of death worldwide showing a 28% increase in mortality over the last decade.[1] In women, breast cancer makes up about one-fourth of all cancer cases and one-sixth of cancer-related deaths.[2] It is the most common cancer in women globally and can be cured in around 70-80% of early, non-spreading cases; but, advanced breast cancer that spreads to other organs is still very difficult to treat.[3] The risk of breast cancer increases with age, and more than 80% of diagnosed women are over 50 years old.[4]

Although many treatments are available to treat existing breast cancer, multiple studies have revealed an inverse connection between cancer and natural materials, such as plant extracts, their fractions, and active principles. These bioactive phytochemicals have synergistic or cumulative effects in cancer treatment. Natural plant products show promising results as anti-tumor and anti-cancer agents. Plant-based compounds are valued for showing lower toxicity and fewer chances of resistance compared to many hormone-targeting cancer drugs.[5,6] These benefits may come from their antioxidant, anti-inflammatory, and immune-modulating actions, along with their ability to slow cell growth and trigger cell death. Because of this, they may provide strong chemopreventive effects that can be useful for both prevention and long-term treatment.[7]

A common weed *Acalypha indica* (Family: *Euphorbiaceae*), locally called "kuppaimeni" in Tamil, is used traditionally to alleviate wide range of human diseases. In Ayurveda, the whole plant of *Acalypha indica* is used to treat bronchitis, cough, asthma, and digestive problems like flatulence. The leaf juice is given for cough, earache, headache, skin infections, and syphilitic sores. Leaf paste is applied externally to heal wounds, burns, itching, chest pain, and snake bites. The root paste is used to relieve fungal infections and certain skin diseases. Leaf decoctions are taken for cold, dysentery, intestinal cleansing, and joint pain. Root decoctions are used for liver and kidney cleansing, to treat intestinal worms, and to ease stomach discomfort. Stem decoction is recommended

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for hemorrhoids; and the whole plant decoction is a cure for earache, toothache, burns and wheezing.[8]

Considering its vast traditional claim in prevention and progression of many diseases, our study aimed at assessing the anticancer activity and analyzing the phytochemicals of *Acalypha indica* aerial part ethanol extract.

MATERIALS AND METHODS

Plant collection and extraction

Acalypha indica was collected in Tirunelveli, identified by a Botany Professor from St. Xavier's College, Palayamkottai and a voucher specimen is in the College Herbarium. The entire plant leaving the root was dried, pulverized and 50 g of the powder *A. indica* was transferred to 250 mL clean conical flask soaked with solvent ethanol, kept in a shaker for 72 hr. After 3 days, the extract was filtered and transferred to crucible kept in water bath at 60ºC for about 4 hr to get concentrated and purified crude *A. indica* ethanol extract.[9]

Cell culture

MDA-MB-231 cells were grown in T-flasks containing DMEM medium with 10% heat-inactivated FBS, and antibiotics (100 µg/mL penicillin and 100 µg/mL streptomycin). The cells adhere to the side of the flask by forming peptide bond. The media is removed; cell layer is taken and washed with PBS solution. After trypsinization, fresh medium is poured into the T-flask, and gently mixed the medium along with cells. The cells were then transferred into 96-well microplates and kept at 37ºC with 5% CO_2 in 95% air humidified incubator.[10]

MTT assay

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was performed with minor modifications.[11] 96-well microplates were taken from the incubator and 20 µL of MTT solution was added. The medium was changed, and the cells were exposed to different doses (20, 40, 60, 80, 100 µg) of *A. indica* ethanol extract for 24 hr. After 24 hr, both untreated and treated cells were viewed under a digital inverted microscope (20X) and photographed. The plates were incubated at 37ºC in the dark for 2 hr. Formazan crystals formed by the cells were dissolved in 100 µL of DMSO, and absorbance was measured (570 nm). Cell viability percentage was calculated from this absorbance reading:

$$\text{cell viability (\%)} = [\text{Absorbance of treated cells} / \text{Absorbance of control cells}] \times 100$$

DNA ladder assay

DNA fragmentation assay was performed with minor modifications.[12] MDA-MB-231 cells were grown overnight in 25 cm² flasks and then exposed to the IC_{50} dose for 24 hr. The cells were gently scraped, collected by centrifugation, and resuspended in 0.5 mL lysis buffer and incubated at 37ºC for 15 min. Proteinase K (20 mg/mL) was added to purify DNA. The mixture was incubated at 50ºC for 45 min, followed by DNA extraction using phenol:chloroform:isoamyl alcohol (25:24:1). DNA was precipitated with sodium acetate and washed with cold 70% ethanol. The banding pattern was visualized by gel electrophoresis.

AO/EB dual staining

Acridine orange/ethidium bromide assay was performed with minor modifications.[13] MDA-MB-231 cells were seeded in a 6-well plate (1×10^5 cells/well) and treated with the IC_{50} dose of *A. indica* extract for 24 hr, with untreated cells as the control. Cells were washed with PBS and stained with 20 µL AO/EB solution for 5 min. The stained cells were viewed under a fluorescence microscope (40X).

Mitochondrial membrane potential (Δψm)

Rhodamine-123 dye was used to measure mitochondrial membrane potential.[14] Cells (both control and treated) were washed with PBS, stained with 50 µL Rhodamine 123 (10 µg/mL), and incubated for 30 min. The differences in cell structure and membrane permeability were captured using a fluorescence microscope at 40X.

GC-MS analysis

The GC-MS analysis of the crude extract was performed using a GC-MS (Shimadzu GCMS-QP2020NX) model. An electron ionization system was operated in electron impact mode with ionization energy of 70 eV for GC-MS detection. Nitrogen gas (96.5%) was used as a carrier gas at a constant flow rate of 1 mL/min, and an injection volume of 1 µL was employed (a split ratio of 10:1). The injector temperature was maintained at 300ºC; oven temperature was programmed 60ºC hold time 5 min than at the rate of 10ºC per minute reaches upto 300ºC and hold for 15 min. GC-MS run was 39 min. The relative percentage of each compound was calculated by comparing its peak area to the total peak area. Mass detection was done using a Turbo-Mass Perkin-Elmer system, and the software matched unknown spectra with standard spectra from the NIST database.

Data analysis

All data were reported as Mean ± Standard error. IC_{50} calculation and other computations were done using Microsoft Excel 2013.

RESULTS

Effect of *A. indica* on cell viability

The ethanol extract of *A. indica* at its starting dose of 20 µg/mL slowed down the growth of MDA-MB-231 cells and gradually caused them to die. The level of cell death increased as the extract concentration increased (Figure 1). Figure 2 shows how the cancer

cells looked after being treated with five different concentrations of the extract. The control cells looked normal with no noticeable changes. At 20 μg/mL, the cells began to shrink. At 40, 60, and 80 μg/mL, the changes in cell shape were much clearer. At 100 μg/mL, the cells became rounded or shrunken, and the cytoplasm showed granules or vacuoles. When the concentration increases, the cell viability decreases. The IC_{50} value for *A. indica* ethanol extract was found to be 59.97 μg/mL.

Effect of *A. indica* on DNA fragmentation

The gel documentation showing a typical DNA ladder pattern is observed when the cells undergo apoptosis, while DNA smear pattern is indicative of necrosis resulting from DNA fragmentation. *A. indica* extract-treated cells showed marked DNA breakage in our study.

Effect of *A. indica* on apoptotic fluorescent staining

MDA-MB-231 cells exposed to the IC_{50} dose of *A. indica* extract for 24 hr were examined under a fluorescence microscope after AO/EB staining. Four types of cells were identified based on their colour and the appearance of their chromatin, as shown in Figure 3: healthy cells with bright green, evenly shaped nuclei; early apoptotic cells with uneven green nuclei and bright green condensed chromatin or small fragmented bodies; late apoptotic cells with orange-red nuclei and highly fragmented chromatin; and necrotic cells with uniform orange-red nuclei.

Effect of *A. indica* on mitochondrial membrane potential

The MDA-MB-231 cells treated with IC_{50} concentration of *A. indica* aerial part ethanol extract was monitored for $\Delta\psi$ changes coupled with fluorescence. In extract-treated cells, the $\Delta\psi$ substantially dropped to a certain limit evidenced by decreased fluorescence, reporting on a decrease in its functionality corresponding to the dead breast-cancer cells. In control, the cells were normal, whereas the mitochondrial membrane potential was affected in treated cells accompanied by differences in morphology and membrane permeability.

Phytochemicals in *A. indica*

Table 1 is the GC/MS chromatogram derived from chromatography analysis representing the peak intensity of 60 different compounds present in the extract of *A. indica*. Table 1 lists the name, Retention Time (RT), area in % and height in % of individual compound identified in the crude extract. Among the major constituents, Oxirane, hexadecyl shows the highest abundance (33.45%), followed by various hydrocarbons and heterocyclic compounds such as Neophytadiene (5%) and 2-(Hydroxymethyl)-3-methoxy-2H-furan-5-one (8.08%).

DISCUSSION

Many studies show that plant-based chemicals can slow cancer development by influencing different cell-signalling pathways. Several natural compounds are known to control processes linked to cancer, such as cell death, cell growth, blood vessel formation, inflammation, invasion, and metastasis.[15]

Acalypha indica was chosen for this study because it has long been used in traditional medicine to treat several illnesses. An ethanol extract from its aerial parts was prepared and tested for anticancer activity. The extract was evaluated using the MDA-MB-231 breast cancer cell line. In the MTT test, living cells convert the tetrazolium dye into MTT formazan through specific enzymes. The amount of formazan formed reflects how many cells are still alive.[16] Cells that served as control did not receive extract treatment and had high cell viability, while *A. indica* ethanol extract-treated cells was cytotoxic to breast cancer cell line in a concentration-dependent manner, Cytotoxicity is noticed in breast cancer cell line while having no effect on primary cell cultures from healthy humans.[17]

Apoptosis is programmed cell death, that is pivotal in preventing the growth and spread of cancer cells. Thus, it can be very important to detect the signs of apoptosis in the study of cancer cellular metabolism. From the gel electrophoresis one can infer that morphological and biochemical changes has occurred subsequent to the inter-nucleosomal cleavage of DNA in the event. This implies that the dying breast cancer cells degrade their DNA into small fragments.[18]

The AO/EB double-staining method is a simple and cost-effective way to identify apoptotic damage in extract-treated cancer cells. Living and dead cells are distinguished based on the colours they emit under fluorescence. Early apoptotic cells show bright green fluorescence because their DNA breaks into fragments and becomes condensed. Necrotic cells with damaged membranes are red due to EB entry. Cells showing orange colour with DNA fragments or condensed material are considered late apoptotic because EB has entered the dying cell. Orange cells without apoptotic features indicate necrosis. EB produces red fluorescence when it inserts into the DNA of cells that have lost the integrity of their membranes.[19] The *A. indica* extract must have increased the reactive oxygen species level of the cells causing it to burst out resulting in the manifestation of varied coloured apoptotic and necrotic cells.[20]

Mitochondria are essential for many cell functions, including ion balance, redox control, the handling of radicals, movement of metabolites, protein import, and the metabolism of lipids and amino acids. They also play a major role in regulating cell death. All these functions rely heavily on the mitochondrial membrane potential ($\Delta\psi$), the electric charge across the mitochondrial membrane.[21] The decreased mitochondrial fluorescence is in accordance with a previous study which reported the reduction in Rhodamine 123 uptake following treatment of mouse

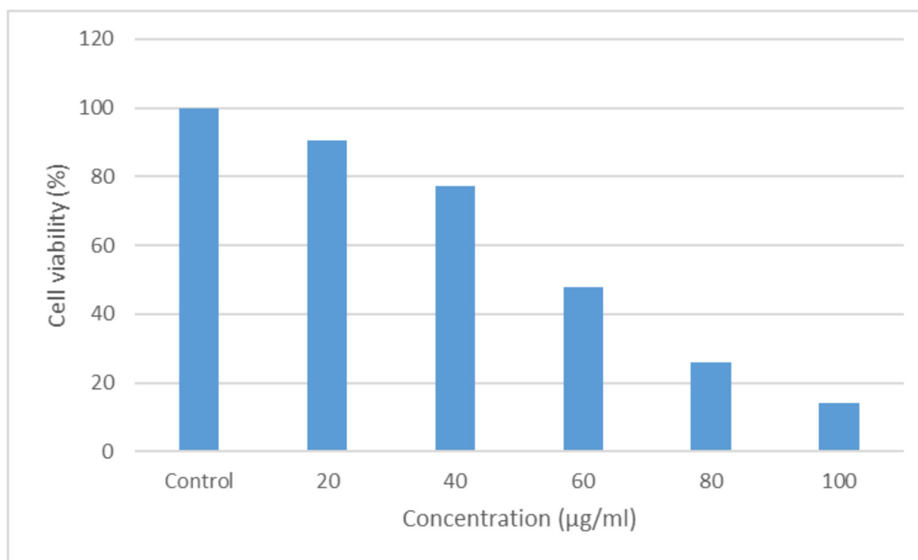

Figure 1: Percentage of cell viability in different concentrations of the extract.

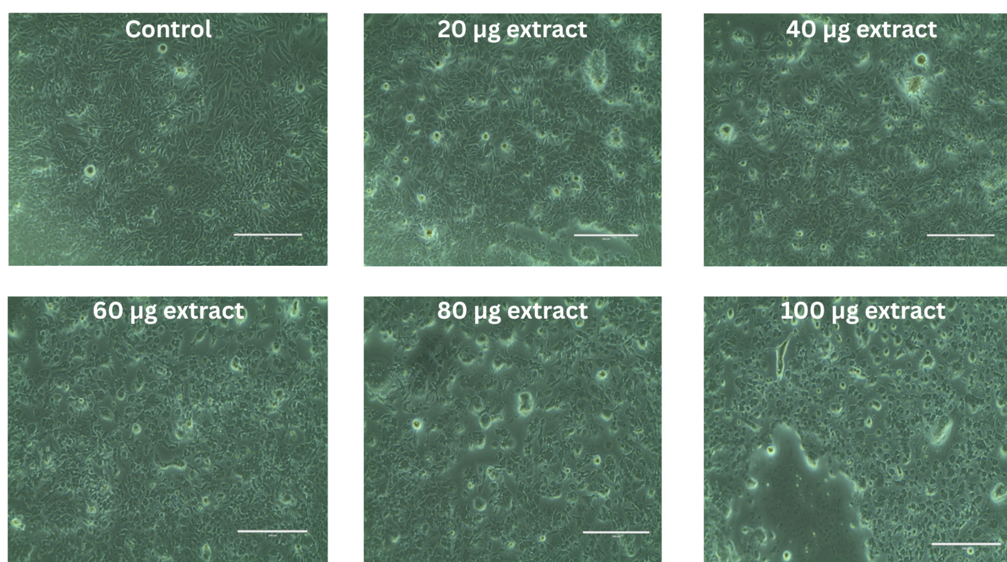

Figure 2: Morphological changes in MDA-MB-231 cells with *A. indica* extract.

leukemic cells with anticancer drugs.[22] In the control group, the cells show strong, uniform green fluorescence, indicating intact mitochondrial membrane potential. In contrast, the extract-treated cells display noticeably reduced fluorescence intensity, suggesting a loss of mitochondrial membrane integrity. This reduction reflects mitochondrial depolarization, a key early event in apoptosis.[23]

GC/MS method is a simple screening technique applied to quickly analyze the volatile composition. The method can be considered complementary to commonly used methods such as solvent extraction.[24] The presence of major phytochemicals such as neophytadiene and hexadecyl epoxide (oxirane) in *Acalypha indica* extract explain its anticancer potential. Neophytadiene, a diterpene, is linked to inhibition of pro-tumor inflammatory signaling via NF-κB, which is often hyperactivated in cancer.[25] Long-chain epoxide structures, similar to the oxirane (hexadecyl) is shown to induce apoptosis and oxidative damage in cancer cells.[26] Terpenes such as phytol inhibit the PI3K/Akt pathway and suppress cell migration, further supporting anticancer mechanisms.[27] This combination might contribute to *A. indica* extract anticancer activity by targeting multiple cancer-relevant pathways.

Table 1: GC-MS qualitative report of *Acalypha indica* aerial part ethanol extract.

Peak	R. Time	Name	Area %	Height %
1	4.091	1H-Pyrrole, 3-methyl-	0.03	0.16
2	4.411	Pyridine, 3-methyl-	0.10	0.10
3	4.978	beta.-d-Glucofuranosiduronic acid, methyl, .gamma.-lactone	0.53	0.26
4	5.204	2-Norbornane carbonyl chloride	0.34	0.38
5	5.350	2-Norbornane carbonyl chloride	0.16	0.23
6	5.592	4-Bromo-2-methylbutenal	0.12	0.17
7	6.856	1-Pyrrolidinylacetonitrile	1.78	1.52
8	7.235	2-Cyclohexen-1-one	0.62	0.36
9	7.855	1-(1'-Pyrrolidinyl)-2-propanone	1.18	1.01
10	8.298	2-Oxepanone, 7-butyl-	3.26	3.01
11	8.567	2,4-Heptadiene, 2,6-dimethyl-	0.49	0.43
12	8.759	Bicyclo[2.2.2]octane, 1,2,3,6-tetramethyl-	2.26	2.43
13	8.955	1,3-Heptadiene, 2,3-dimethyl-	3.86	3.00
14	9.264	2-Naphthol, 1,2,3,4,4a,5,6,7-octahydro-4a-methyl-	0.13	0.21
15	9.381	Cyclobutanecarboxylic acid, undec-2-enyl ester	0.30	0.44
16	9.705	1,3-Dimethyl-3,4,5,6-tetrahydro-2(1H)-pyrimidinone	1.54	0.63
17	10.338	2(5H)-Furanone, 5-(1-methylethyl)-	0.26	0.36
18	10.584	2-Methoxyethyl heptanoate	2.21	1.30
19	10.781	2-Methoxyethyl heptanoate	0.29	0.31
20	11.308	2-(Hydroxymethyl)-3-methoxy-2H-furan-5-one	8.08	4.50
21	11.626	trans-Carveol	0.16	0.18
22	11.749	9-Hydroxy-7-nonanal	0.15	0.13
23	11.942	8-Azabicyclo[3.2.1]oct-6-en-3-one, 8-methyl	0.13	0.23
24	12.124	Ethyl oxo(pyrrolidin-1-yl)acetate	0.16	0.26
25	12.346	2-Isobutylideneamino-3-methylbutyronitrile	0.48	0.50
26	12.565	2,4-Heptadiene, 2,4-dimethyl-	2.31	1.04
27	12.838	2,6-Heptadienal, 2,4-dimethyl-	0.07	0.17
28	12.941	Pyrrolidine, 1-(1-propenyl)-	-0.03	0.06
29	13.004	2-Aminoacetanilide	-0.01	0.15
30	13.082	3-Oxatricyclo[4.1.1.0(2,4)]octane, 2,7,7-trimethyl-	0.20	0.37
31	13.354	12-Methyl-E,E-2,13-octadecadien-1-ol	1.88	1.20
32	13.587	Indole	0.17	0.15
33	13.771	Dopamine, N,N-dimethyl-, dimethyl ether	0.66	0.70
34	14.007	N-[4-(Aminosulfonyl)phenyl]-2-(1-pyrrolidinyl)acetamide	0.16	0.27
35	14.411	Naphthalene, 1,2,3,4-tetrahydro-1,4,6-trimethyl-	0.71	0.50
36	14.656	[(2,5-Dimethylpyrazol-3-yl)methyl][(2,5-dimethylpyrazol-3-yl) methylidene]am	1.47	2.38
37	14.852	1-Undecene, 9-methyl-	1.37	0.88
38	14.979	Benzonitrile, 4-hydroxy-3-methoxy-	0.40	0.69
39	15.255	Pyrrolidine, 1-(1-cyclohexen-1-yl)-	3.68	6.08
40	15.518	N-(2,6-Dimethylphenyl)-1-methylpyrrolidine-2-carboxamide	0.22	0.24
41	15.675	Cyclopentane, 1-methyl-3-(1-methylethyl)-	0.12	0.13

Peak	R. Time	Name	Area %	Height %
42	15.815	2-Hydroxy-1-(1'-pyrrolidiyl)-1-buten-3-one	0.31	0.24
43	16.138	4-(2,6,6-Trimethylcyclohexa-1,3-dienyl)but-3-en-2-one	0.36	0.52
44	16.860	Phosphoric acid, diethyl octyl ester	0.25	0.72
45	17.021	2,6-Nonadienal, (E,Z)-	0.63	0.64
46	18.104	1,2-Benzisothiazol-3(2H)-one, 2-methyl-, 1,1-dioxide	0.98	0.52
47	18.437	9-Borabicyclo[3.3.1]nonane, 9-(3-aminopropyl)-	0.68	0.82
48	18.579	8-Pentadecanone	0.21	0.48
49	19.998	1-Heptadecene	0.63	1.20
50	20.414	3,7,11,15-Tetramethylhexadec-2-ene	0.48	0.69
51	20.500	Neophytadiene	5.00	8.39
52	20.500	Neophytadiene	4.74	8.24
53	20.972	Neophytadiene	1.50	2.67
54	21.269	Lidocaine	2.92	3.28
55	21.463	Pentadecanoic acid, 13-methyl-, methyl ester	0.44	0.95
56	22.761	Phytol	1.33	0.78
57	23.288	11,14,17-Eicosatrienoic acid, methyl ester	2.32	3.23
58	23.506	Oxirane, hexadecyl-	33.45	25.21
59	24.130	Octadecanoic acid	0.08	0.53
60	24.390	2-Hexadecen-1-ol, 3,7,11,15-tetramethyl-, acetate, [R-[R*,R*-(E)]]-	1.72	3.77

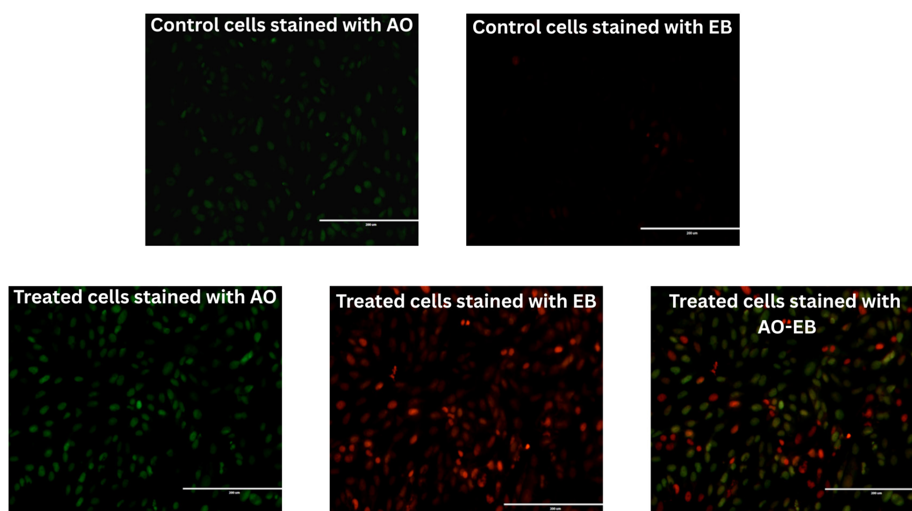

Figure 3: AO/EB-stained control and *A. indica* extract-treated cells.

CONCLUSION

To conclude, the medicinal herb *Acalypha indica* aerial part ethanol extract could be further researched and exploited for the treatment of breast cancer. The IC_{50} values from cell viability assay, the DNA fragmentation study, AO/EB dual staining for apoptosis detection and mitochondrial membrane permeability changes clearly indicate that the ethanol extract of *Acalypha indica* aerial part exhibits significant anticancer activity against the MDA-MB-231 cell line, supported by the major phytocompounds identified in GC/MS analysis. Further studies will have to be carried *in vivo* in the laboratory animal models for evaluation of the bioactive leads.

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ABBREVIATIONS

MTT: 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; **DMEM:** Dulbecco's Modified Eagle Medium; **FBS:** Fetal Bovine Serum; **PBS:** Phosphate-Buffered Saline; **DMSO:** Dimethyl Sulfoxide; **AO/EB:** Acridine Orange/Ethidium Bromide; **MMP:** Mitochondrial Membrane Potential; **GC-MS:** Gas Chromatography-Mass Spectrometry; **IC_{50}:** Half Maximal Inhibitory Concentration; **RT:** Retention Time.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ETHICAL STATEMENT

Experiments were performed on established human breast cancer cell line MDA-MB-231, obtained from accredited source. As no primary human tissue or animal models were used, the study was exempt from ethical approval under institutional guidelines.

AUTHORS CONTRIBUTION

R. Ancy performed the experiments, analyzed the data, and prepared the first draft of the manuscript. S. Mabel Parimala designed the study, interpreted results, reviewed, edited and approved the final version of the manuscript.

SUMMARY

The study evaluated ethanol extract of *Acalypha indica* aerial parts for anticancer activity against MDA-MB-231 breast cancer cells. The extract showed strong dose-dependent cytotoxicity with an IC_{50} of 59.97 µg/mL, confirmed through MTT, DNA ladder, AO/EB, and MMP assays indicating apoptosis-mediated cell death. GC-MS profiling revealed 60 bioactive compounds. This work provides the first integrated cytotoxic and mechanistic evidence for *A. indica* aerial parts against triple-negative breast cancer.

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