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Phytochemical Profiling and Evaluation of Anticancer Activity of Ethanolic Extract of *Alpinia calcarata* Against MCF-7 Breast Cancer Cells

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ABSTRACT

Background: This study aimed to examine the phytochemical constituents of the medicinal plant *Alpinia calcarata* and evaluate the efficacy of the ethanolic extract of *A. calcarata* in the treatment of the MCF-7 breast cancer cell line.
Materials & Methods: Fresh samples were taken for maceration extraction. A magnetic laboratory shaker was used to extract dried powdered ginger rhizome with 96% ethanol for 4 hours. The extract was balanced and dissolved in DMSO (dimethylsulfoxide). The prepared extracts underwent phytochemical screening and analysis by gas chromatography- mass spectrometry (GC-MS). MTT assay was used to assess cell viability in the presence of different concentrations of *A. calcarata* extract (12.5, 25, 50, 100, and 200 µg/mL) over a 24-hour period.
Findings: Preliminary phytochemical screening and GC–MS analysis of the ethanolic extract of *A. calcarata* showed the presence of several bioactive phytocompounds like terpenoids, phenolic compounds, fatty acids, and aromatic derivatives, which are recognized for their antioxidant, antimicrobial, and anticancer activities. The IC_{50} value of the ethanolic extract of *A. calcarata* against MCF7 cells after 24 hours of treatment was 47.25 µg/mL.
Conclusion: The study revealed the presence of significant phytochemical constituents in the ethanolic extract of AC, having promising anticancer activity against MCF-7 breast cancer cells. The overall findings indicate that the bioactive compounds found could serve as a valuable foundation for novel plant-derived cancer treatments.

Keywords: *Alpinia calcarata*, Breast cancer, Cytotoxicity, Phytochemical analysis, MTT assay

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Introduction

Cancer is one of the deadliest illnesses. Cancer statistics in 2022 estimated the incidence of cancer at 1,918,030 cases and cancer-related deaths at 609,360 cases. Of these, 287,850 (31%) were estimated to be new breast cancer cases [1]. All cancers are diseases that occur when abnormalities in a group of healthy cells in the body become uncontrollable, forming a lump called a tumor.

It is a complex genetic disease, but the origin and progression of tumors are influenced by environmental, chemical, physical, metabolic, and genetic factors [2]. There are many reasons that cause cancer, but one of the prominent reasons is an imbalance between cell propagation and cell death. This phenomenon increases cell reproduction, while damaged cells are not eliminated by apoptosis [3]. Breast cancer is the most common cancer diagnosed in women. The abnormal multiplication of cells in the milk-producing glands of the breast or in the channels (ducts or passages) that carry milk to the nipple is termed breast cancer [4]. The Global Cancer Observatory (GLOBOCAN 2022) has estimated that 2.3 million new cases of breast cancer occur annually, and it is a significant cause of cancer-related mortality. The high incidence and mortality rates highlight the need for innovative therapeutic approaches that target the molecular processes of tumor growth, despite advancements in diagnosis and treatment [5]

A distinctive feature of cancer is the capacity of malignant cells to avoid apoptosis, the programmed cell death process that maintains tissue homeostasis by eliminating damaged or abnormal cells. Defects in apoptotic pathways promote uncontrolled cell proliferation, resistance to treatment, and tumor progression. As a result, induction of apoptosis has become a fundamental target in anticancer drug research, because many conventional and natural anticancer drugs exert their effects by activating intrinsic or extrinsic apoptotic pathways [6]. Apoptosis-related proteins such as Bcl-2, Bax, p53, and caspases are dysregulated in breast cancer, leading to tumor growth, metastasis, and treatment resistance. In order to find safer and more potent anticancer treatments, assessing the apoptotic potential of natural products and phytochemicals has attracted much attention. Research on the induction of apoptosis in breast cancer cells supports the development of plant-derived medicines and offers important insights into the molecular mechanisms behind their anticancer action [7].

Several essential medications with diverse structures and mechanisms of anti-tumor activities are no longer effective due to drug resistance [8], and the failure of traditional chemotherapies to achieve a significant reduction in mortality suggests that novel techniques are critical [9]. It has been established that a wide range of medicinal drugs could inhibit the growth of cancer cells by triggering apoptosis. However, resistance to chemotherapeutic medicines is a typical challenge in the treatment of several types of malignancies, including breast cancer. Identification of hormone receptors, including estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), is considered a biomarker in breast cancer patients because of their diagnostic and prognostic implications [10].

The HER2 subtype tumors are clinically and biologically heterogeneous, making therapeutic options complicate and diverse. However, they are essential for targeted therapies [11]. Approximately 70% of human breast tumors are hormone receptor–positive, and hormone therapy following surgery, radiotherapy, or chemotherapy leads to a considerable improvement in

post- treatment survival and a reduction in disease relapse [12]. Over the last few decades, various chemotherapy drugs have been developed, most of which have different adverse effects. Finding medications with high efficacy and minimal side effects is the goal of current efforts toward anticancer drug development and research in order to reduce the suffering of patients undergoing chemotherapy and improve their survival rates. Bioactive substances derived from natural sources are of particular interest. Drugs derived from natural products have been the only source of medications for thousands of years and have improved human health. In the 1950s, the US National Cancer Institute (NCI) recognized the potential of natural compounds as anticancer drugs [13]. The plant family *Zingiberaceae* is one of the major dietary herb/spice families, which has been found to have diverse biological activities, including anticancer, antibacterial, and anti-inflammatory properties. *Alpinia calcarata (*AC) is a perennial medicinal herb widely found in Sri Lanka and India. It is a member of the *Zingiberaceae* family. Traditional medicine has made extensive use of AC rhizomes to treat digestive problems, respiratory conditions, inflammation, and microbiological infections [14]*. A. calcarata* is rich in a variety of bioactive phytochemicals, including flavonoids, phenolic compounds, alkaloids, terpenoids, tannins, saponins, and essential oils. These constituents are responsible for its diverse pharmacological activities, such as antioxidant, antimicrobial, anti-inflammatory, and anticancer effects. The rhizomes are particularly rich in terpenoid compounds, including 1,8-cineole (eucalyptol), camphene, α-pinene, β-pinene, limonene, and other monoterpenes and sesquiterpenes, which contribute to its biological properties.

Owing to its rich phytochemical compounds, *A. calcarata* has emerged as a promising medicinal plant for the development of novel therapeutic agents against chronic diseases, including cancer [15]. Natural products have attracted much attention as potential anticancer treatments because they could decrease cancer cell development and trigger apoptosis while causing minimal damage to normal cells.

Objectives: The current work intended to analyze the cytotoxic activity of *A. calcarata* ethanolic extract against the human breast cancer (MCF-7) cell line and to investigate its potential as a natural source of anticancer compounds.

Materials and Methods

Preparation of *A. calcarata* ethanolic extract: Fresh rhizome (50 g) of *A. calcarata* plant was collected from ICAR IISR–Indian Institute of Spices Research, Kozhikode, Kerala, India. Herbarium was prepared for taxonomic authentication. It was authenticated by Dr. Mary Kensa S, Associate Professor, Department of Botany, ST Hindu College, Nagercoil. Fresh samples were taken for maceration extraction. After drying, they were ground into a fine powder without debris. A magnetic laboratory shaker was used to extract dried powdered ginger rhizome with 96% ethanol (10:1; volume: weight) for 4 hours. The alcohol was evaporated at 65 °C to prepare *A. calcarata* ethanolic extract [16]. The prepared extracts were evaporated to dryness using a rotary evaporator and dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). Then different concentrations of *A. calcarata* extract (12.5, 25, 50, 100, and 200 µg/mL) were prepared.

Cell culture medium/reagents: The human breast adenocarcinoma cell line MCF-7 was obtained from the National Centre for Cell Science (NCCS) in Pune, India, and cultured under normal conditions. The cells were cultured in high-glucose Dulbecco's modified

Eagle medium (DMEM) supplemented with fetal bovine serum (FBS). MTT reagent (5 mg/mL) was used to test cell viability, and DMSO was used to dissolve formazan crystals. Dulbecco's phosphate buffered saline was used to wash the cells, and doxorubicin served as a positive control. Cell culture was carried out in sterile T25 flasks and 96-well tissue culture plates using standard sterile laboratory supplies.

Laboratory equipment: The equipment used in this study included a centrifuge (Remi: R-8oC), multiple pipettes (2-10, 10-100, and 100-1000 µL), an inverted binocular biological microscope (Biolinkz, India), a biosafety hood (Biobase, China), a 37 °C incubator with a humidified atmosphere and 5% CO_2 (Healforce, China), and a 96-well plate reader (ELX-800, BioTek, CA, USA).

Maintenance of cell line: The human breast cancer cell line MCF-7 was obtained from the National Center for Cell Science (NCCS) in Pune, India. The cells were cultured in high-glucose DMEM supplemented with 10% FBS and 1% antibiotic-antimycotic solution. Cell cultures were incubated at 37 °C in a humidified environment with %5 CO_2. The culture medium was replenished every 2-3 days, and the cells were sub-cultured when they reached about 70-80% confluence. The current study used passage number 30.

MTT cell viability assay: The cytotoxicity of the test compounds was assessed using the MTT cell viability assay. In order to facilitate cell adhesion, a 96-well plate was seeded with 200 µL of cell suspension (about 20,000 cells per well) and incubated at 37 °C for 24 hours in a humidified environment with 5% CO_2. The cells were cultured for an additional 24 hours following treatment with proper amounts of test compounds prepared in complete culture medium. After treatment, the spent medium was removed, and MTT reagent was added to reach a final concentration of 0.5 mg/mL. The plate was then covered with aluminum foil to keep out light and incubated for three hours. The MTT solution was then removed, and 100 µL of DMSO was added with moderate shaking to completely dissolve the resulting formazan crystals. Cell viability was then assessed by comparing the absorbance of treated and untreated control cells at 570 nm using a microplate ELISA (enzyme-linked immunosorbent assay) reader. Cell viability was computed using the following formula.

% cell viability = [Mean abs of treated cells/ mean abs of untreated cells] x 100 (1)

Preliminary phytochemical screening of study ginger: The ethanolic extract of AC plant was subjected to preliminary phytochemical screening using standard qualitative tests to identify the presence of various bioactive compounds, including carbohydrates, alkaloids, flavonoids, saponins, glycosides, tannins, phenolic compounds, and terpenoids [17].

Phytochemical screening by GC-MS: The AC ethanolic extract was screened for GC-MS analysis to identify key bioactive compounds. The prepared samples were dissolved in the respective solvents and filtered before analysis. GC-MS analysis was performed on an Agilent Technologies GC-MS system, including a 7820A GC system and a 5977E mass selective detector (MSD). The separation of compounds was carried out using a DB-5 capillary column. Helium was used as a carrier gas at a constant flow rate of 1.2 mL/min. The oven temperature was initially maintained at 100 °C and then increased stepwise to 270 °C at a rate of 10 °C/min. The separated compounds were detected by a mass spectrometry (MS) detector. The chromatograms and mass spectra obtained were analyzed to evaluate the presence of phytochemical constituents within the extract [18-22].

Statistical analysis: Cell viability formula

used was as follows:

% cell viability = (Mean OD of test-treated cells/mean OD of control cells) x 100 (2)

The following linear regression equation was utilized to determine the IC_{50} value:

$Y = Mx + C$ (3)

Where Y is equal to 50, and M and C are derived from the viability graph. All results were expressed as mean± SEM. The significance of differences was evaluated by one-way analysis of variance (ANOVA), followed by Dunnett's multiple-comparison test. The significance of the results was represented by * for p values < .05, ** for p values < .01, and *** for p values < .001.

Findings

Table 1 presents the results of the preliminary phytochemical study of the ethanolic extract of AC, including the presence of different bioactive secondary compounds. The results revealed the presence of carbohydrates, alkaloids, flavonoids, saponins, phenols, tannins, terpenoids, and steroids in the extract, whereas proteins were absent.

Table 2 shows the GC–MS analysis results of the ethanolic extract of AC, revealing the presence of several bioactive phytochemicals like terpenoids, phenolic compounds, fatty acids, and aromatic derivatives. The major compounds identified were benzenemethanol, α-1-propenyl- (16.37%), n-hexadecanoic acid (15.29%), eucalyptol (10.23%), oleic acid (8.5%), and α-terpineol. The presence of these phytoconstituents indicates that *A. calcarata* has great pharmacological potential, especially due to the abundance of phenolic and flavonoid compounds exhibiting antioxidant, antimicrobial, and anticancer activities.

Figure 1 displays the GC-MS chromatogram of *A. calcarata* ethanolic extract. This chromatogram shows numerous phytochemical constituents as multiple peaks at different retention times. The major peaks are observed at retention times of around 8.32, 12.54, 16.59, 21.93, and 24.83 min, indicating the abundance of different bioactive compounds in the extract. Variations in peak intensity and retention time indicate the existence of compounds with different chemical properties and concentrations. This chromatogram confirms the complex phytochemical nature of the ethanolic extract of AC.

Table 3 describes the experimental design used to test the cytotoxicity of the test compounds on MCF-7 breast cancer cells. The

Table 1) Preliminary phytochemical screening of the ethanolic extract of AC

Sl. No.	Phytoconstituent	Tests Conducted	Result
1	Carbohydrates	Molisch's test	Positive
2	Alkaloids	Mayer's test	Positive
3	Flavonoids	Lead acetate test	Positive
4	Saponins	Foam test	Positive
5	Phenols	Ferric chloride test	Positive
6	Tannins	Ferric chloride test	Positive
7	Terpenoids	Salkowski test	Positive
8	Steroids	Liebermann–Burchard test	Positive
9	Proteins	Biuret test	Negative

Table 2) GC-MS spectral peaks used for phytoconstituent analysis

Peak#	R.Time	Area	Area%	Height	Height%	Name
1	6.648	145092568	2.38	33951488	1.97	Methanol, triethylsilyl-
2	8.327	622815500	10.23	183617082	10.65	Eucalyptol
3	10.617	40962850	0.67	16397186	0.95	Bicyclo[2.2.1]heptan-2-one, 1,7,7-trimethyl-, (1S)-
4	10.736	17835690	0.29	8496919	0.49	(+)-2-Bornanone
5	10.873	91376354	1.5	33623243	1.95	Benzenepropanal
6	11.037	350518743	5.76	108454251	6.29	2-Propen-1-ol, 3-phenyl-, (E)-
7	11.237	49319177	0.81	22499913	1.31	Terpinen-4-ol
8	11.379	32002719	0.53	14916650	0.87	3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)-, (R)-
9	11.503	260291275	4.28	80940485	4.69	Alpha.-Terpineol
10	11.66	208703858	3.43	85806469	4.98	Alpha.-Terpineol
11	12.152	38607219	0.63	18109971	1.05	4-Vinylphenol
12	12.274	198397885	3.26	60374289	3.5	2-Butanone, 4-phenyl-
13	12.543	996680332	16.37	178790525	10.37	Benzenemethanol, .alpha.-1-propenyl-
14	13.571	44413164	0.73	14606140	0.85	2-Methoxy-4-vinylphenol
15	14.119	11729268	0.19	5224693	0.3	2,6-Dimethoxyphenyl-.beta.-phenylpropionate
16	14.702	21379752	0.35	7463789	0.43	4-(7-Methoxy-3,3,7-trimethyl-oxepan-2-ylidene)-butan-2-one
17	15.296	32192288	0.53	18702755	1.08	Caryophyllene
18	15.429	52816169	0.87	29109309	1.69	Trans-.alpha.-bergamotene
19	15.807	19759867	0.32	11072580	0.64	Humulene
20	16.366	95934685	1.58	36394486	2.11	Naphthalene, 1,2,3,5,6,7,8,8a-octahydro-1,8a-dimethyl-7-(1-methylethenyl)-, [
21	16.453	37692475	0.62	17272251	1	Beta.-Bisabolene
22	16.589	134533572	2.21	75069508	4.35	Alloaromadendrene
23	17.452	21682378	0.36	9539010	0.55	Diethyl Phthalate
24	17.544	19424154	0.32	10970137	0.64	Caryophyllene oxide
25	17.61	8070321	0.13	5588635	0.32	Azulene, 1,2,3,3a,4,5,6,7-octahydro-1,4-dimethyl-7-(1-methylethenyl)-, [1R-(1
26	17.693	10419875	0.17	5639377	0.33	4a(2H)-Naphthalenol, 1,3,4,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-
27	17.935	55794227	0.92	17575678	1.02	4a(2H)-Naphthalenol, 1,3,4,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methylethyl)-
28	18.193	95251418	1.56	40579131	2.35	2-Butanone, 4-(4-hydroxy-3-methoxyphenyl)-

Peak#	R.Time	Area	Area%	Height	Height%	Name
29	19.05	35373962	0.58	18791215	1.09	Bergamotol, Z-.alpha.-trans-
30	19.598	130344790	2.14	39484980	2.29	Tetradecanoic acid
31	20.023	16725366	0.27	9077393	0.53	1-(2-Furyl)-3-phenylpropane
32	20.099	15061913	0.25	7875488	0.46	Cubenol
33	20.237	9348299	0.15	5561217	0.32	Ambrial
34	21.178	47517604	0.78	15281916	0.89	(3S,3aS,6R,7R,9aS)-1,1,7-Trimethyldecahydro-3a,7-methanocyclopenta[8]ann
35	21.368	5144787	0.08	2514944	0.15	Triacontanoic acid, methyl ester
36	21.925	930723267	15.29	146461510	8.5	n-Hexadecanoic acid
37	22.101	14282591	0.23	5844707	0.34	Hexadecanoic acid, ethyl ester
38	22.189	17093840	0.28	8217800	0.48	(E)-15,16-Dinorlabda-8(17),11-dien-13-one
39	22.584	13956578	0.23	5806615	0.34	Tetradecane, 1-chloro-
40	22.822	6418993	0.11	3442349	0.2	Eicosanoic acid
41	23.371	48784382	0.8	25140324	1.46	1-Naphthalenemethanol, .alpha.-methyl-.alpha.-(phenylmethyl)-
42	23.68	48784382	0.8	25140324	6.5	Methanone, [4-(2-methoxy-1-naphthylmethyl)-1-piperazinyl](phenyl)
43	23.677	517194194	8.5	78566422	4.56	Oleic Acid
44	23.87	79734764	1.31	31990149	1.86	Octadecanoic acid
45	24.756	218172748	3.58	50983167	2.96	Dihydroyashabushiketol
46	24.883	267421300	4.39	118221884	6.86	1-Hydroxy-5-methoxy-3-phenylpentane

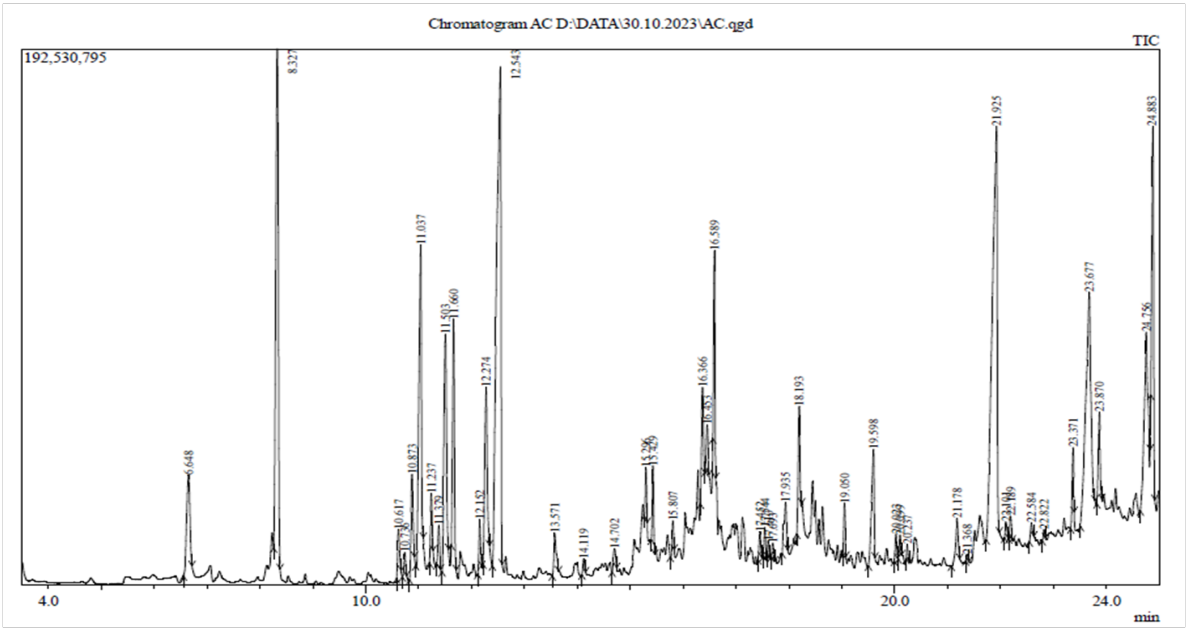


Figure 1) GC-MS chromatogram of *A. calcarata* ethanolic extract

Table 3) Details of the treatment of cell lines studied

Sl.No	Culture Condition	Cell Lines	Concentration Used
1	Untreated	MCF7	No treatment
2	Blank	-	Cell-free medium
3	Std control (Dox)	MCF7	1 µM
4	Test compounds	MCF7	12.5, 25, 50, 100, 200, µg/mL

Table 4) IC_{50} value of the given DARC against MCF7 cells after a 24-hour treatment period and percentage of cell viability

Culture Condition	% cell viability	IC_{50} (µg/mL)
Untreated	100.00	47.25 µg/mL
Dox-1 µM	43.53	
AC-12.5 µg	87.85	
AC-25 µg	73.34	
AC-50 µg	47.76	
AC-100 µg	26.45	
AC-200 µg	5.76	

experiment was performed with a negative control group (untreated cells), a blank (culture medium without cells), a standard positive control group (treatment with 1 µM doxorubicin), and experimental groups [treatment with the test compounds (DARC: Duffy Antigen Receptor for Chemokines/AC) at concentrations of 12.5 to 200 µg/mL]. Table 4 displays the percentage of cell viability and IC_{50} value after 24 hours of treatment. Untreated cells showed 100% viability, indicating normal cell growth. Treatment with the standard drug doxorubicin (1 µM) significantly reduced cell viability to 43.53%, confirming its strong cytotoxic effect. The test compounds (AC) showed a concentration-dependent reduction in cell viability, with cell viability decreasing to 87.85% at 12.5 µg/mL, 73.34% at 25 µg/mL, 47.76% at 50 µg/mL, 26.45% at 100 µg/mL, and %5.76 at 200 µg/mL, indicating an increase in cytotoxic activity with increasing concentration.

Table 5 shows that doxorubicin (1 µM) reduced cell viability to 43.53 ± 0.64%, demonstrating the assay's responsiveness. The untreated control group maintained 100% cell viability. The extract had a concentration-dependent cytotoxic impact, reducing cell viability from 87.85 ± 1.52% at 12.5 µg/mL to 5.76 ± 0.82% at 200 µg/mL. This resulted in an increase in growth inhibition from 12.15 to 94.24%. Cell viability was significantly reduced at doses of 50 µg/mL or higher, showing significant inhibition of MCF-7 cell proliferation.

One-way ANOVA followed by Dunnett's multiple-comparison test was performed with doxorubicin as a control, the results are displayed in Table 6. The results indicated that the extract at 12.5 and 25 µg/mL doses exhibited substantially higher cell viability than doxorubicin and therefore weaker MTT inhibition, and the viability at a 50 µg/mL dose was only 4.23% higher than that of doxorubicin (Dunnett-adjusted p= .0125) at the closest tested concentration. In addition, the extract at 50 and 200 µg/mL doses exhibited significantly lower viability than doxorubicin, indicating that higher doses lead to a greater decrease in MTT viability under the tested conditions

Figure 2 shows microscopic observations of MCF7 cells after a 24-hour treatment period based on the percentage of cell viability. Microscopic observations showed a dose-

Table 5) MCF-7 cell viability and growth inhibition after 24 hours of treatment with A. calcarata ethanolic extract (12.5–200 µg/mL).

Treatment	Reading 1	Reading 2	Raw OD Mean	Corrected OD Mean	Viability Mean (%)	Viability SD	Inhibition Mean (%)
Untreated control	1.25	1.236	1.245	1.206	100	1	0
Doxorubicin 1 µM	0.57	0.558	0.564	0.525	43.53	0.64	56.47
Extract at 12.5 µg/mL	1.09	1.111	1.098	1.0595	87.85	1.52	12.15
Extract at 25 µg/mL	0.92	0.929	0.923	0.885	73.34	0.7	26.66
Extract at 50 µg/mL	0.61	0.619	0.615	0.576	47.76	0.53	52.24
Extract at 100 µg/mL	0.365	0.35	0.358	0.319	26.45	0.88	73.55
Extract at 200 µg/mL	0.101	0.115	0.108	0.0695	5.76	0.82	94.24

Table 6) Dunnett-adjusted comparisons of each actual extract dose with 1 µMdoxorubicin

Extract Dose	Viability Mean ± SD (%)	Difference vs Doxorubicin	Dunnett 95% CI	Dunnett Adjusted *P*-Value	Key Findings
12.5 µg/mL	87.85 ± 1.52	44.32 points	41.24 to 47.40	< .01**	Higher viability than doxorubicin
25 µg/mL	73.34 ± 0.70	29.81 points	26.73 to 32.89	< .01**	Higher viability than doxorubicin
50 µg/mL	47.76 ± 0.53	4.23 points	1.15 to 7.31	.0125*	Higher viability than doxorubicin
100 µg/mL	26.45 ± 0.88	-17.08 points	-20.16 to -14.00	< .01**	Lower viability than doxorubicin
200 µg/mL	5.76 ± 0.82	-37.77 points	-40.85 to -34.69	< .01**	Lower viability than doxorubicin

dependent cytotoxic effect of AC ethanolic extract on MCF7 cells. In the presence of1 µM doxorubicin, the cells showed severe morphological changes with pronounced cell shrinkage, rounding, and detachment, indicating the strong cytotoxic activity of doxorubicin. In the untreated group, MCF7 cells were healthy and showed normal epithelial morphology, intact cell membranes, and dense cell distribution. In the presence of the extract at 12.5 µg/mL, the cells showed slight morphological changes, mild reduction in cell density, and early signs of cytotoxicity. At 25 µg/mL, the cells showed moderate cellular changes like cell shrinkage and partial detachment, indicating dose-dependent cytotoxic effects. At 50 µg/mL, a significant decrease in viable cells was observed along with increased rounding and disruption of normal cell

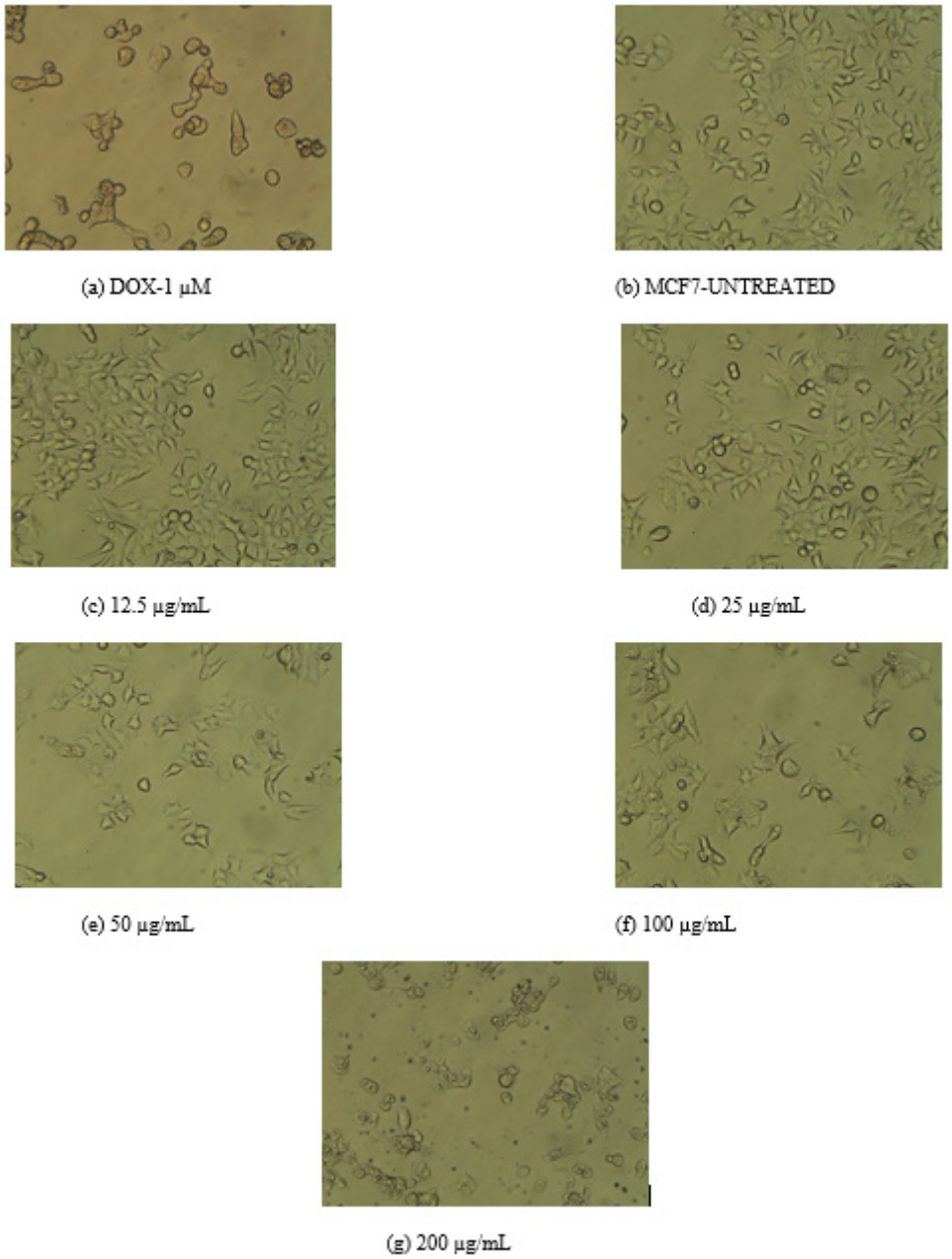


Figure 2) Microscopic observations of MCF7 cells after a -24hour treatment period according to the percentage of cell viability

morphology. Significant morphological damage, reduced confluency, and increased floating cells were observed at 100µg/mL, indicating increased cytotoxicity. At 200 µg/mL, severe cytotoxic effects were observed with marked cell shrinkage, detachment, and loss of normal cellular architecture. Maximum cytotoxicity was noticed at the highest extract concentration (200 µg/mL), which was similar to the standard drug doxorubicin.

Figure 3 presents cell viability percentage of MCF7 breast cancer cells treated with different concentrations of AC extract compared to untreated cells along with a standard drug control (doxorubicin). The results showed that the viability of MCF7 cells was gradually reduced with increasing AC concentration. At lower concentrations, mild cytotoxicity was observed, and at higher concentrations, strong inhibition of cell growth was observed. Maximum cytotoxic

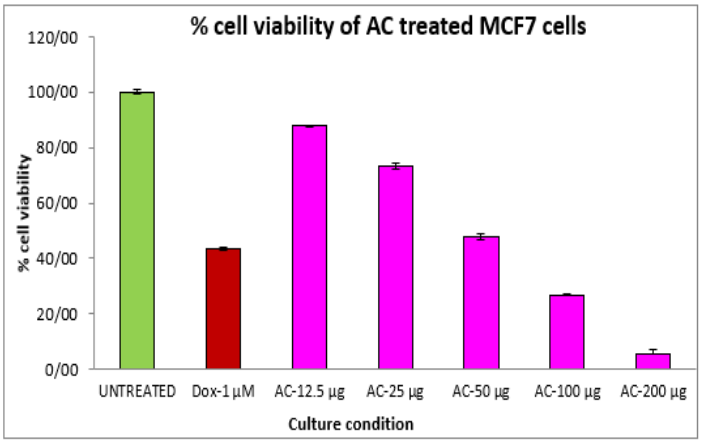


Figure 3) Cell viability percentage of AC-treated MCF7 cells

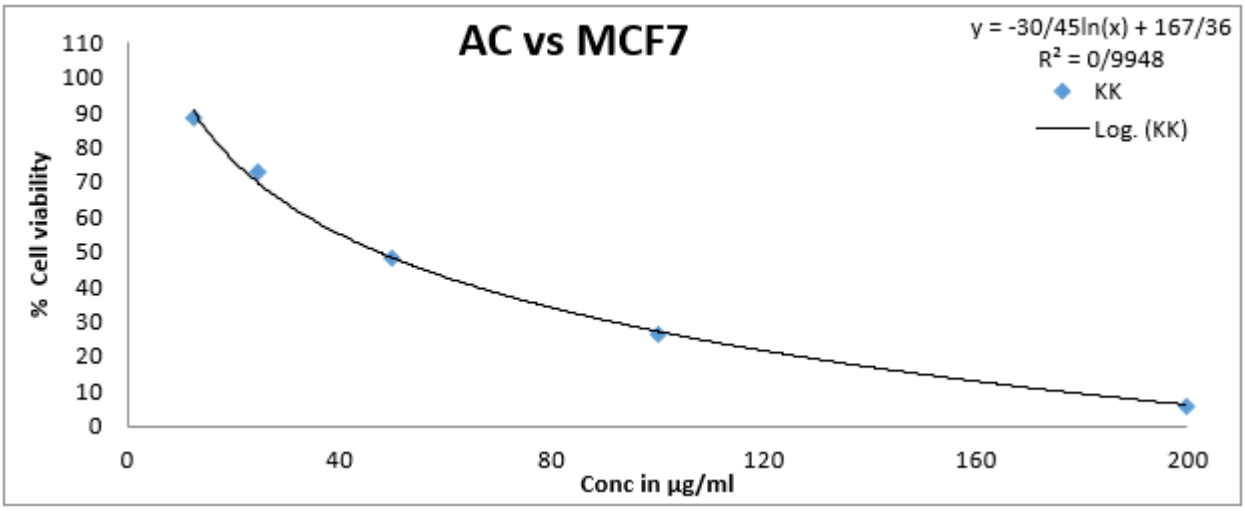


Figure 4) Logarithmic dose–response curve of *A. calcarata* treated MCF-7 cells

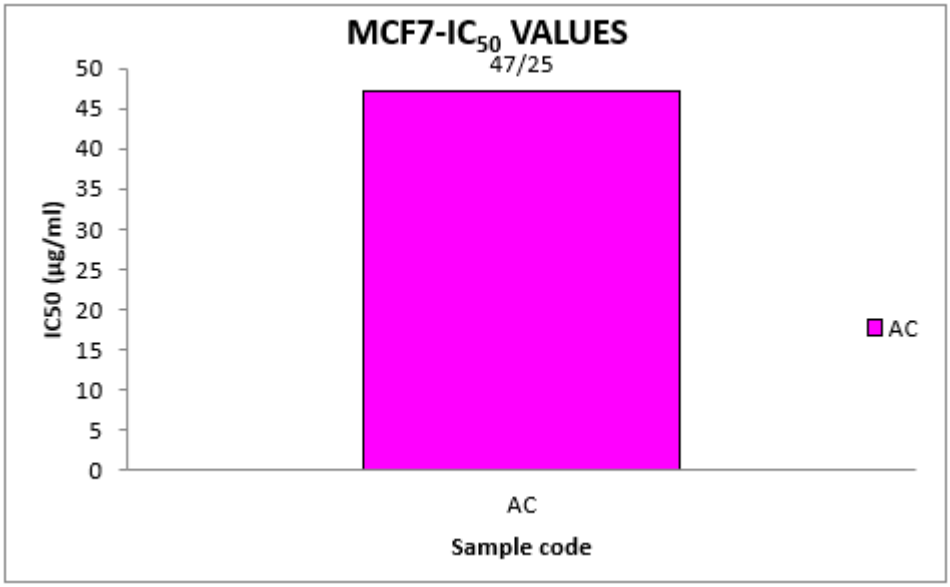


Figure 5) IC_{50} value of *A. calcarata* extract against MCF-7 breast cancer cell line

activity was observed at the highest extract concentration tested (200 µg/mL), which reduced cell viability to below 10%.

Figure 4 displays the relationship between AC concentration (µg/mL) and the percentage of cell viability of MCF7 breast cancer cells. The results show a clear dose-dependent cytotoxic effect of AC on cancer cells. AC concentration (µg/mL) is plotted on the x-axis and the percentage of viable MCF7 cells on the y-axis. The results indicate that AC has strong cytotoxic potential against MCF7 breast cancer cells, and higher concentrations lead to greater inhibition of cell growth. The good R^2 value obtained further confirms the reliability of the observed dose-response relationship.

Figure 5 shows a bar chart illustrating the IC_{50} value of an AC-labeled sample against the MCF7 cell line. The IC_{50} in µg/mL is plotted on the y-axis from 0 to 60, and the sample code, which is AC, is plotted on the x-axis. The figure shows that the sample AC has an IC_{50} value of 47.25 µg/mL against the MCF7 cell line.

Discussion

The present study identified the phytochemical composition and anticancer activity of the ethanolic extract of *A. calcarata* (AC). Preliminary phytochemical screening confirmed the absence of proteins and the presence of many important secondary metabolites such as carbohydrates, alkaloids, flavonoids, saponins, phenols, tannins, terpenoids, and steroids. These bioactive compounds are known for their various pharmacological properties like antioxidant, antimicrobial, anti-inflammatory, and anticancer activities.

Subsequent GC–MS analysis confirmed that the ethanolic extract of AC was rich in bioactive compounds. Several phytochemical constituents were identified as terpenoids, phenolic compounds, fatty acids, and aromatic derivatives. The major compounds identified were benzenemethanol, α-1-propenyl- (16.37%), n-hexadecanoic acid (15.29%), eucalyptol (10.23%), oleic acid (8.5%), α-terpineol, alloaromadendrene, tetradecanoic acid, caryophyllene, and bergamotene. The chromatogram showed several peaks at different retention times, indicating the complex phytochemical nature of the extract and the presence of compounds with different concentrations and chemical properties. The abundance of phenolic and terpenoid compounds suggests that AC is highly promising for therapeutic applications.

AC ethanolic extract was tested against the MCF7 breast cancer cell line to find the cytotoxic effect of its various concentrations. The methodology of the experiment consisted of untreated control cells, blank culture medium, standard drug control with doxorubicin (1 µM), and AC-treated groups at concentrations of 12.5 to 200 µg/mL. The untreated cells showed 100% viability, indicating healthy cell growth, whereas the treatment of cells with doxorubicin reduced cell viability to 43.53%, confirming its potent cytotoxic activity. AC extract showed a clear concentration-dependent reduction in MCF7 cell viability. The extract exhibited a concentration-dependent cytotoxic impact, reducing cell viability from 87.85 ± 1.52% at 12.5 µg/mL to 5.76 ± 0.82% at 200 µg/mL. Cell viability was significantly reduced at doses of 50 µg/mL or higher, showing significant inhibition of MCF-7 cell proliferation. This resulted in an increase in growth inhibition from 12.15 to 94.24%. The extract demonstrated an IC_{50} value of 47.25 µg/mL, which shows promising cytotoxic efficacy against the MCF7 breast cancer cell line.

The viability at a 50 µg/mL dose of the extract was only 4.23% greater than that of doxorubicin (Dunnett-adjusted p = .0125) at the closest tested concentration, but the

extract at 12.5 and 25 µg/mL doses exhibited much higher viability than doxorubicin and thus weaker MTT inhibition. Furthermore, the viability at 100 and 200 µg/mL doses was considerably lower than that of doxorubicin, confirming that higher doses cause a greater decline in MTT viability under the studied conditions.

Microscopic observations were consistent with the cytotoxicity test results and showed concentration-dependent morphological changes in AC-treated MCF7 cells.

Control cells (untreated) showed normal epithelial morphology, intact membranes, and dense distribution. Whereas AC-treated cells showed progressive cellular damage, including cell shrinkage, rounding, detachment, reduced confluency, and loss of normal cellular architecture with increasing concentrations. Under the same experimental conditions, doxorubicin (1 µM) remained much more powerful, even when the maximum extract concentration demonstrated a considerable drop in cell survival. The percentage cell viability graph and the logarithmic dose-response curve further confirmed the strong dose-dependent cytotoxic efficacy of AC extract. Higher extract concentrations resulted in reduced cancer cell growth. The good R^2 value obtained indicated a reliable dose-response relationship. Overall, the research findings suggest that the ethanolic extract of *A. calcarata* has significant anticancer potential against MCF7 breast cancer cells due to the presence of various bioactive phytochemicals. The cytotoxic action observed may be associated with the presence of bioactive phytochemicals, including phenolic compounds, terpenoids, and fatty acids detected by GC–MS analysis. However, the individual contributions and potential interactions of these chemicals were not examined in the present investigation; thus, their precise involvement in the reported cytotoxic effects remains to be clarified. These results indicate that *A. calcarata* is a promising source of bioactive chemicals for more research and offer preliminary evidence for its anticancer potential. To confirm the compounds responsible for the observed effects and determine their therapeutic potential, more research incorporating bioactivity-guided fractionation, isolation of active ingredients, mechanistic analysis, and in vivo evaluation is needed.

Conclusion

In conclusion, the present study confirmed the presence of a wide range of bioactive phytochemicals including flavonoids, phenols, alkaloids, terpenoids, and steroids in the ethanolic extract of *A. calcarata.* This finding was further validated by GC–MS analysis. The extract exhibited a significant dose-dependent cytotoxic activity against the MCF7 breast cancer cell line, yielding an IC_{50} value of 47.25 µg/mL. Morphological changes observed in the treated cells also confirmed its anti-cancer potential. The identified biological activity may be due to the presence of key phytochemicals, such as eucalyptol, α-terpineol, oleic acid, and n-hexadecanoic acid. The findings provide preliminary scientific data supporting the anticancer potential of *A. calcarata* and are compatible with its reported therapeutic relevance. The overall findings indicate that the bioactive phytochemicals found in *A. calcarata* could serve as a valuable foundation for novel plant-derived cancer treatments. Further in vivo and molecular studies are recommended to confirm its therapeutic efficacy and safety; thus, it is necessary to conduct studies including apoptosis assays, ROS generation studies, cell-cycle analysis, gene expression studies, and in vivo studies. Therefore, even though *A. calcarata* is a promising source of bioactive

compounds for anticancer research, more research is needed to determine its safety, efficacy, and therapeutic potential using a variety of cancer and normal cell lines, in-depth molecular analyses, animal models, and carefully-planned clinical trials.

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Declaration of Artificial Intelligence (AI) Use: The author confirms that no artificial intelligence (AI) generated content used in the preparation of this manuscript except for plagiarism checking. All text analysis, results and conclusion are the result of the authors' original work.

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