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IN VITRO ANTICANCER POTENTIAL OF MANILKARA HEXANDRA (ROXB.) LEAF METHANOLIC EXTRACTS VIA SRB AND MTT ASSAYS AGAINST MCF-7 CELL LINE

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ABSTRACT
Background: Cancer causes millions of deaths worldwide, with cases expected to reach 28.4 million by 2040. Natural plant compounds offer safer alternatives for cancer treatment. **Aim:** This study tested the anticancer activity of *Manilkara hexandra* leaf extracts against MCF-7 breast cancer cells. **Materials and methods:** Methanolic extraction, followed by sequential fractionation via column chromatography, yielded bioactive fractions that underwent phytochemical and GC-MS characterization. Quantification of cytotoxicity was performed using sulforhodamine B (SRB) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays across a concentration gradient (10–80 μg/mL). **Result and Discussion:** Chemical screening found alkaloids, flavonoids, tannins, and other bioactive compounds. The petroleum ether-ethyl acetate (PE-EA) fraction contained quercetin (25.28%) and another major flavonoid (28.62%). This fraction exhibited strong dose-dependent cell killing, reducing cell survival to 31.8% (SRB) and 33.0% (MTT) at 80 μg/mL ($p < 0.001$). The IC_{50} was 55 μg/mL in both assays. **Conclusion:** The anticancer activity correlates with high flavonoid content, suggesting these compounds cause cell death through apoptosis or cell cycle arrest. *M. hexandra* PE-EA fraction shows promise as a natural anticancer agent for breast cancer treatment.

INTRODUCTION

Cancer represents a complex disease spectrum characterized by abnormal cellular proliferation with the potential for metastatic spread [1]. Despite significant advances in conventional therapeutic modalities, including surgery, radiotherapy, and chemotherapy, the global cancer burden continues to escalate. According to epidemiological data compiled by the International Agency for Research on Cancer (IARC) through its GLOBOCAN 2020 initiative, approximately 19.3 million incident cases and 10 million mortality events were recorded in 2020. Projections indicate a significant escalation by 2040, with 28.4 million new cases anticipated worldwide, reflecting demographic shifts and aging populations [2,3].

The limitations of conventional treatment approaches, including systemic toxicity, adverse effects, and the emergence of chemoresistance, have catalyzed the exploration of alternative

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and adjuvant oncotherapeutics. Plant-derived bioactive compounds have emerged as promising candidates in this pursuit, with approximately 60% of currently approved anticancer agents derived from natural sources [4]. Contemporary scientific discourse reflects a paradigm shift toward natural product drug discovery, driven by the imperative to identify biocompatible agents capable of modulating carcinogenic pathways while minimizing iatrogenic sequelae associated with conventional modalities. Empirical and preclinical studies corroborate the antineoplastic potential of plant secondary metabolites, including alkaloids, terpenoids, flavonoids, and polyphenols, which exhibit pleiotropic mechanisms of action encompassing anti-proliferative, pro-apoptotic, anti-angiogenic, and immunomodulatory pathways [5].

This resurgence in phytopharmacological research not only validates millennia of empirical utilization in traditional medicinal systems but also aligns with precision oncology objectives through enhanced therapeutic indices and chemopreventive efficacy [6,7]. *Manilkara hexandra* (Roxb.), commonly known as Khirni or Rayan, is a medicinal plant of the Sapotaceae family widely distributed in tropical India. Traditionally used in Ayurvedic medicine, its various parts are employed for treating menorrhagia, fever, digestive ailments, and inflammatory disorders. Phytochemical investigations have reported the presence of phenolics, tannins, alkaloids, flavonoids, terpenoids & saponins, which contribute to its antioxidant, antidiabetic, and anti-inflammatory activities. Despite its recognized ethnomedicinal importance, systematic evaluation of its anticancer potential remains limited, highlighting a key research gap addressed in the present study.

Despite its established ethnomedicinal significance and documented bioactive compounds, systematic evaluation of the anticancer potential of *M. hexandra* leaf extracts remains underexplored, representing a significant research gap in phytopharmacological cancer research. This study, therefore, aims to investigate the cytotoxic efficacy of methanolic extracts and their fractions against the MCF-7 cell line. MCF-7 cells were selected because they represent a well-characterized estrogen receptor-positive breast cancer model widely used for screening anticancer compounds and evaluating cytotoxic mechanisms. This breast adenocarcinoma cell line enables the correlation of bioactivity with phytochemical composition, thereby elucidating potential mechanisms of action and therapeutic applications.

MATERIALS AND METHODS

Materials

Adriamycin (doxorubicin hydrochloride) was procured from Dheer Healthcare Private Limited, Mumbai, Maharashtra, India. Analytical grade solvents, including petroleum ether, ethyl acetate, and methanol, were acquired from PCL, India. All cell culture reagents, including RPMI-1640 medium, fetal bovine serum (FBS), L-glutamine, trichloroacetic acid (TCA), sulforhodamine B (SRB), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), were of cell culture grade.

Methods

Collection and Authentication of Plant Material

Fresh leaves of *Manilkara hexandra*(Roxb.) were obtained from Mahatma Phule Krishi Vidyapeeth (MPKV), Ahmednagar, Maharashtra, India. The plant material was authenticated by Dr. A.S. Wabale, Department of Botany, PVP College of Arts, Science and Commerce, Pravaranagar, Maharashtra, India (authentication reference: PVPC/Bot./2024-25/300 dated 09/09/2024, Specimen no. SLV 123).

Extraction and Fractionation of Plant Materials

The collected *M. hexandra* leaves were shade-dried for 15 days at ambient temperature to maintain phytochemical integrity, followed by mechanical grinding to obtain a coarse powder. One kilogram of the powdered material underwent Soxhlet extraction at 70°C using methanol as the extraction solvent [11]. The extract was filtered through Whatman No. 1 filter paper, and the filtrate was concentrated under reduced pressure using a rotary evaporator (Buchi Rotavapor R-210, Switzerland) to yield the crude methanol extract.

For preliminary phytochemical analysis, the crude extract was subjected to thin-layer chromatography (TLC) on silica gel 60 F254 plates (Merck, Germany) using various mobile phase systems: petroleum ether (10), petroleum ether: ethyl acetate (5:5), ethyl acetate (10), ethyl acetate: methanol (5:5), and methanol (10). The developed plates were visualized under UV light (254 nm and 366 nm) and subsequently sprayed with specific detection reagents to identify phytoconstituent classes [12,13]. Based on TLC profiles, the crude extract was subjected to column chromatography for fractionation. A glass column (60

cm × 3 cm) was packed with silica gel (60–120 mesh) and equilibrated with petroleum ether. The methanolic extract (25 g) was adsorbed onto silica gel, loaded onto the column, and eluted using a gradient solvent system of increasing polarity: petroleum ether (100%), petroleum ether: ethyl acetate (50:50), ethyl acetate (100%), ethyl acetate: methanol (50:50), and methanol (100%). Fractions (50 mL each) were collected, monitored by TLC, and pooled based on similar TLC profiles.

The combined fractions were concentrated under reduced pressure to yield five significant fractions: petroleum ether (P), petroleum ether-ethyl acetate (PE-EA), ethyl acetate (EA), ethyl acetate-methanol (EA-M), and methanol (M) [14].

Phytochemical Analysis

The crude methanolic extract and its fractions were subjected to qualitative phytochemical screening to identify major classes of secondary metabolites using standard procedures [14,15].
The following tests were performed:

- **Alkaloids**: Mayer's test, Wagner's test
- **Flavonoids**: Alkaline reagent, Shinoda's & HCl test
- **Tannins & phenols**: Ferric chloride test, alkaline reagent test
- **Steroids and terpenoids**: Salkowski & chloroform test
- **Saponins**: Foam test
- **Glycosides**: Alkaline reagent test, Fehling's test
- **Carbohydrates**: Benedict's test
- **Anthraquinones**: Borntrager's test
- **Phlobatannins**: HCl test

Gas Chromatography-Mass Spectrometry (GC-MS) Analysis

The PE-EA fraction, which demonstrated optimal cytotoxic activity in preliminary screenings, was subjected to GC-MS analysis using an ITQ 900 Model (Thermo Fisher Scientific) equipped with an HP-5 fused silica capillary column (30 m × 0.25 mm, 0.25 μm film thickness) [16].

Instrument calibration was performed using standard reference compounds, and peak identification was validated through mass spectral matching with the NIST library database (match quality >90%). Quality control standards were analyzed every 10 samples to ensure analytical precision and accuracy.

The analysis was performed under the following conditions: 1 μL sample was injected via programmed temperature. Vaporizing (PTV) injector at 275°C; the GC oven temperature was programmed at 60°C (5 min hold) with a ramp of 8°C/min to 300°C (10 min hold); helium was used as carrier gas at a constant flow rate of 1.5 mL/min. The mass spectrometer operated in electron impact (EI) mode with an ion source temperature of 200°C. Compounds were identified by comparing their fragmentation patterns with those in the NIST library [17,18]. Relative percentages of identified compounds were calculated based on GC peak areas without applying correction factors.

In Vitro Anticancer Activity Assessment

Cell Culture

Human breast adenocarcinoma (MCF-7) cells were obtained from the cell repository of the Advanced Centre for Treatment, Research and Education in Cancer (ACTREC), Tata Memorial Centre, Mumbai, India. Cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum and 2 mM L-glutamine in a humidified atmosphere of 5% CO_2 at 37°C. For cytotoxicity assays, cells were seeded at a density of 5×10^3 cells per well in 96-well microtiter plates and allowed to adhere for 24 hours before treatment [19].

Sample Preparation

Stock solutions of test samples (crude extract and fractions) were prepared by dissolving 100 mg in 1 mL DMSO to obtain a concentration of 100 mg/mL. These were further diluted with complete medium to achieve a working concentration of 1 mg/mL and stored at –80°C until use. For assays, working solutions were diluted with complete medium to achieve final concentrations of 10, 20, 40, and 80 μg/mL in the culture wells. Adriamycin served as the positive control.

Sulforhodamine B (SRB) Assay

The SRB assay was performed according to the method described by Skehan et al. [20] with minor modifications [21,22]. After 48 h of treatment, cells were fixed with 50 μL of cold 30% trichloroacetic acid (final concentration 10%) and incubated at 4°C for 60 minutes. The supernatant was discarded, the plates were washed five times with tap water, and air-dried. Fixed cells were stained with 50 μL of 0.4% (w/v) SRB in 1% acetic acid for 20 minutes at room temperature.

Excess dye was removed by washing the plates five times with 1% acetic acid, and then they were air-dried. The protein-bound

dye was solubilized with 10 mM Tris base (pH 10.5), and absorbance was measured at 540 nm with 690 nm as the reference wavelength using a microplate reader (BioTek Synergy HT, USA). All experiments were performed in triplicate (n=3), and results were expressed as mean ± standard deviation (SD).

MTT Assay

The MTT assay was performed as previously described [23–25]. After 48 h of treatment, the medium was aspirated, and 50 µL of MTT reagent (5 mg/mL in PBS) was added to each well. Following incubation at 37°C for 2 h, the supernatant was carefully removed, and 100 µL of DMSO was added to dissolve the formazan crystals. Plates were agitated for 5 minutes at 300 rpm, and absorbance was measured at 570 nm using a microplate reader. All experiments were performed in triplicate (n = 3), and results are expressed as mean ± SD.

Data Analysis

Percent growth inhibition was calculated using the following formula:

$$\%\boldsymbol{Growth\ Inhibition} = \frac{(\boldsymbol{Control\ OD} - \boldsymbol{Test\ OD})}{\boldsymbol{Control\ OD}} \times \mathbf{100}$$

Where:

- *Control OD = Average absorbance of control wells*
- *Test OD = Average absorbance of test wells*

The IC_{50} value (concentration causing 50% growth inhibition) was determined by plotting percent growth inhibition versus log concentration and applying non-linear regression analysis.

Statistical Analysis

All experiments were performed in triplicate (n=3) and results expressed as mean ± SD. Statistical analysis was performed using GraphPad Prism version 8.0 (GraphPad Software, San Diego, CA, USA). Data normality was assessed using the Shapiro–Wilk test. One-way ANOVA followed by Dunnett's multiple comparison test was used to compare the treated groups with the control group. The significance level was set at $\alpha = 0.05$, with $p < 0.05$ considered statistically significant. The IC_{50} values were calculated using non-linear regression analysis with a four-parameter logistic curve fit. All statistical comparisons were two-tailed, with significance levels indicated as *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

RESULTS AND DISCUSSION

Phytochemical Analysis

Qualitative phytochemical screening of the crude methanolic extract revealed the presence of diverse classes of secondary metabolites (Table 1). The extract showed positive reactions for alkaloids, flavonoids, tannins, terpenoids, saponins, and carbohydrates, while tests for phlobatannins, steroids, anthraquinones, and glycosides were negative.

Table 1: Phytochemical constituents of the methanolic leaf extract of *M. hexandra* (Roxb.)

Sr. No.	Constituents	Tests	Observation
1	Tannins	$FeCl_3$, Alkaline reagent	+
2	Terpenoids	Salkowski test	+
3	Alkaloids	Mayer's, Wagner's	+
4	Phlobatannins	HCl	–
5	Flavonoids	Alkaline test, Shinoda's, HCl	+
6	Saponins	Foam test	+
7	Carbohydrates	Benedict's test	+
8	Steroids	Chloroform test	–
9	Anthraquinones	Borntrager's test	–
10	Glycosides	Alkaline reagent, Fehling's	–

+ Present; – Absent

GC-MS Analysis

The PE-EA fraction, which displayed optimal cytotoxic activity in preliminary assays, was further analyzed by GC-MS. The chromatographic profile revealed 10 distinct bioactive compounds (Table 2). Identified metabolites comprised terpenoids, flavonoids, and aromatic compounds, reflecting a chemically diverse profile. Notably, quercetin (3,3′,4′,5,7-pentahydroxyflavone) was identified at a retention time of 21.00 min, representing 25.28% of the fraction. Another prominent flavonoid eluted at 42.29 min, accounting for 28.62% of the total composition. Additional bioactive constituents included eucalyptol (12.87%), various aromatic compounds (collectively,

20.73%), and terpenoids such as camphor and phytol derivatives (Figure 1).

In Vitro Anticancer Activity

The cytotoxic potential of *M. hexandra* leaf extract and its fractions against MCF-7 cells was evaluated using complementary Sulforhodamine B (SRB) and MTT assays. Results from both assays demonstrated consistent dose-dependent trends, validating the reliability of the observed effects (Tables 3 and 4). Among all tested fractions, the petroleum ether–ethyl acetate (PE-EA) fraction exhibited the most potent dose-dependent cytotoxicity. At the highest tested concentration (80 µg/mL), the PE-EA fraction reduced cell viability to 31.8% and 33.0%, as determined by SRB and MTT assays, respectively ($p < 0.001$ compared to the control). Progressive inhibition was observed with increasing concentrations, with viability metrics of 86.4%, 79.3%, 57.7%, and 31.8% at concentrations of 10, 20, 40, and 80 µg/mL in the SRB assay, and 86.7%, 79.6%, 58.7%, and 33.0% at the corresponding concentration in the MTT assay.

In contrast, other fractions (P, EA, EA-M, and M) demonstrated minimal cytotoxic effects, maintaining cell viability above 90% even at the highest tested concentration. Adriamycin, used as a positive control, demonstrated potent cytotoxicity with cell viability reduced to approximately 5.5% across all concentrations.

Table 2: GC-MS profile of PE-EA fraction of *M. hexandra* leaves showing retention time (RT), peak area (%) & compound identity.

Peak	RT (min)	Area (%)	Compound	Class
1	8.19	4.60	Sabinene (Bicyclo[3.1.0]hexane, 4-methylene-1-(1-methylethyl)-)	Monoterpene (Terpenoid)
2	9.98	12.87	Eucalyptol	Terpenoid
3	13.05	2.61	Camphor	Terpenoid
4	21.00	25.28	Quercetin (3,3′,4′,5,7-pentahydroxyflavone)	Flavonoid
5	22.78	1.89	(1S,4aR,7R)-1,4a-Dimethyl-7-(prop-1-en-2-yl) decahydro-1-naphthalenol	Flavonoid
6	25.11	4.90	1,3,6,10-Cyclotetradecatetraene, 3,7,11-trimethyl	Aromatic chemical
7	36.23	15.58	7-Isopropyl-1,1,4a-trimethyl-1,2,3,4,4a,9,10,10a-octahydrophenanthrene	Aromatic chemical
8	36.19	0.25	(4aS,4bR,10aS)-7-Isopropyl-1,1,4a-trimethyl-decahydrophenanthrene	Aromatic chemical
9	39.33	3.39	Phytol (3,7,11,15-Tetramethyl-2-hexadecen-1-ol)	Terpenoid
10	42.29	28.62	1-Phenanthrenemethanol, 1,2,3,4,4a,9,10,10a-octahydro-	Flavonoid

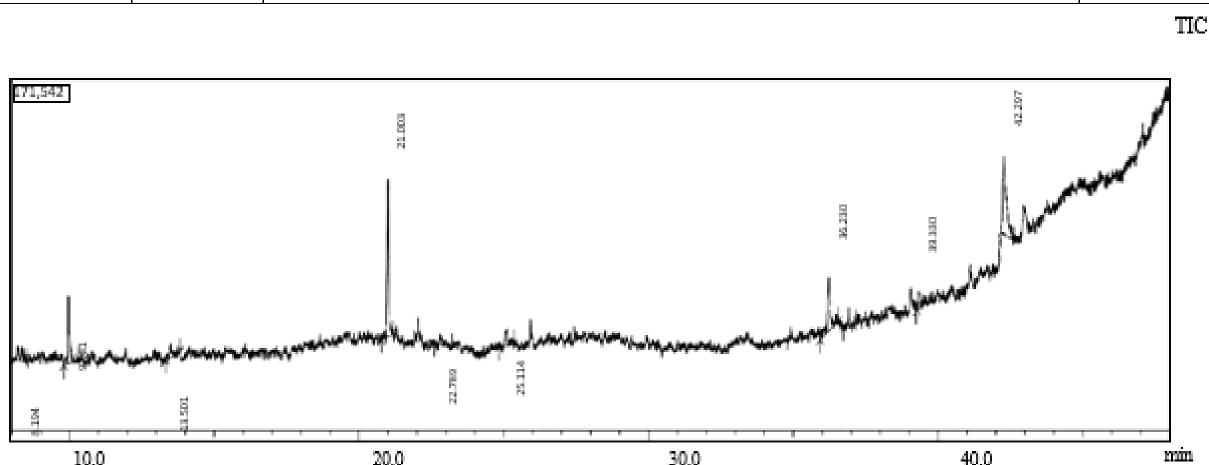

Figure 1: GC-MS chromatogram of PE-EA fraction of *M. hexandra* (showing peaks corresponding to identified bioactive compounds).

Table 3: SRB assay on human breast cancer cell line (MCF-7).

Fraction	% Control Growth (Mean ± SD)	%ControlGrowth		
	10 µg/mL	20 µg/mL	40 µg/mL	80 µg/mL
P	102.4 ± 1.9	96.4 ± 1.0	97.5 ± 2.8	102.0 ± 2.0
PE-EA	86.4 ± 4.7	79.3 ± 5.3	57.7 ± 4.2**	31.8 ± 7.5***
EA	92.8 ± 2.4	94.8 ± 2.3	92.7 ± 4.5	101.3 ± 11.5
EA-M	92.3 ± 1.8	99.3 ± 1.7	92.6 ± 2.0	101.0 ± 9.3
M	89.9 ± 6.0	94.3 ± 2.9	94.4 ± 7.3	99.6 ± 7.5
ADR	6.4 ± 0.5***	5.3 ± 1.8***	-1.0 ± 5.2***	5.5 ± 4.8***

PE = Petroleum ether fraction; PE-EA = Petroleum ether:Ethyl acetate fraction; EA = Ethyl acetate fraction; EA-M = Ethyl acetate:Methanol fraction; M = Methanol fraction; ADR = Adriamycin (positive control). Statistical significance vs. control: PE-EA at 40 µg/mL (p < 0.01) and 80 µg/mL (***p < 0.001); ADR at all concentrations (**p < 0.001).*

Table4:MTTassayonHumanBreastCancerCellLineMCF-7

Fraction	% Control Growth (Mean ± SD)	%ControlGrowth		
	10 µg/mL	20 µg/mL	40 µg/mL	80 µg/mL
P	101.1 ± 0.5	95.5 ± 0.4	96.3 ± 4.2	101.6 ± 3.2
PE-EA	86.7 ± 5.0	79.6 ± 4.9	58.7 ± 4.9**	33.0 ± 6.0***
EA	93.2 ± 1.4	95.5 ± 1.2	94.5 ± 4.5	101.9 ± 11.0
EA-M	92.9 ± 1.9	99.0 ± 2.6	93.0 ± 2.0	101.0 ± 8.2
M	90.0 ± 6.1	95.0 ± 1.4	94.3 ± 7.7	100.1 ± 7.6
ADR	6.4 ± 0.5***	5.3 ± 1.8***	-1.0 ± 5.2***	5.5 ± 4.8***

PE = Petroleum ether; PE-EA = Petroleum ether:Ethyl acetate; EA = Ethyl acetate; EA-M = Ethyl acetate:Methanol; M = Methanol; ADR = Adriamycin (positive control). Statistical significance vs. control: PE-EA fraction at 40 µg/mL (*p < 0.01) and 80 µg/mL (*p < 0.001); ADR at all concentrations (**p < 0.001).*

Dose–Response and GI_{50} Analysis

The dose–response curves (Figures 2 and 3) clearly demonstrated that among all tested fractions, only the PE-EA fraction exhibited significant cytotoxic activity against MCF-7 cells. Growth curves for PE-EA in both SRB and MTT assays exhibited characteristic sigmoidal inhibition patterns, with a steep decline in viability between 20 and 80 µg/mL. The estimated IC_{50} values for PE-EA were approximately 45–50 µg/mL in both assays, indicating consistent potency. In contrast, all other fractions (P, EA, EA-M, and M) displayed nearly flat, horizontal curves, with cell viability maintained above 90% across all concentrations, indicating a minimal to no cytotoxic effect.

The positive control Adriamycin showed complete cytotoxic activity, with viability plateauing at ~5%, thereby confirming the sensitivity & reliability of both assays. The parallel dose-response patterns observed in SRB & MTT assays validate the reproducibility of the results and reinforce PE-EA as the most promising cytotoxic fraction, warranting further investigation.

The growth inhibitory potential was further quantified using GI_{50} values (Table 5). The PE-EA fraction exhibited a GI_{50} of 55 µg/mL, reflecting strong antiproliferative activity. In comparison, all other fractions demonstrated GI_{50} values exceeding 80 µg/mL, highlighting their relatively low cytotoxic potential. Adriamycin, as expected, showed potent activity with a GI_{50} below 10 µg/mL.

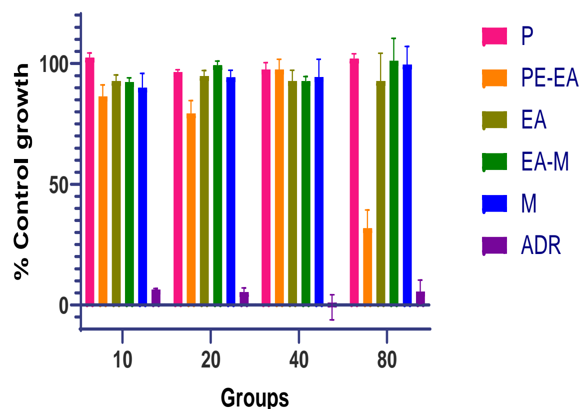

Figure 2: Graphical representation of the SRB assay

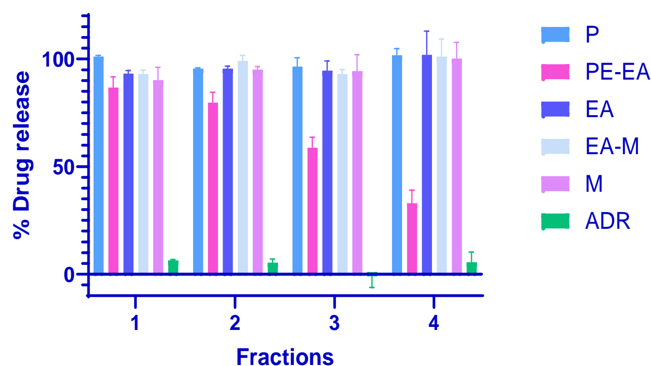

Figure 3: Graphical representation of MTT assay

Morphological Assessment

In addition to growth inhibition, morphological changes in MCF-7 cells were observed microscopically following treatment. Control cells (A) exhibited normal morphology with intact membranes and confluent monolayer growth. Cells treated with Adriamycin (B) and extract fractions (C–G) showed hallmark apoptotic features, including cell shrinkage, membrane blebbing, chromatin condensation, and reduced cell density.

Cytotoxic effects were concentration-dependent, with higher extract concentrations producing more pronounced structural alterations. These findings confirm the extract's anti-cancer potential and suggest apoptosis induction as a possible mechanism (Figures 4 and 5).

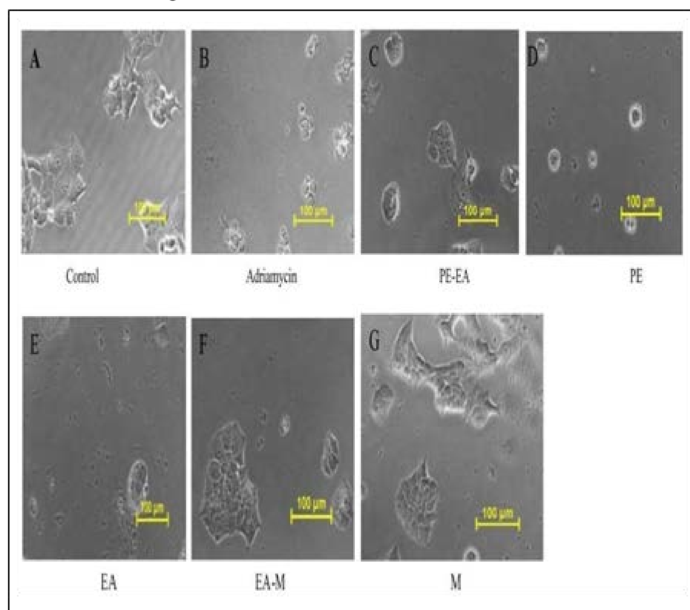

Figure 4: SRB assay micrographs of MCF-7 cells showing morphological changes post-treatment. A: Control; B: Adriamycin; C–G: Extract fractions. Scale bar: 100 µm; Magnification: 40×.

Table 5. GI_{50} values of *M. hexandra* fractions and Adriamycin against MCF-7 cells

Fraction	GI_{50} (µg/mL)
P	>80
PE-EA	55
EA	>80
EA-M	>80
M	>80
ADR	<10

PE = Petroleum ether; PE-EA = Petroleum ether: Ethyl acetate; EA = Ethyl acetate; EA-M = Ethyl acetate: Methanol; M = Methanol; ADR = Adriamycin (positive control).

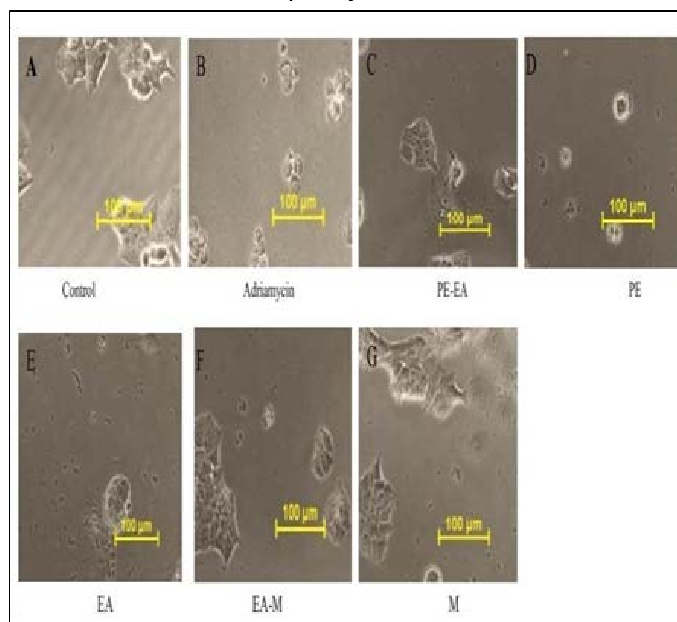

Figure 5: MTT assay micrographs of MCF-7 cells showing morphological changes post-treatment. A: Control; B: Adriamycin; C–G: Extract fractions. Scale bar: 100 µm; Magnification: 40×.

These morphological observations, together with quantitative cytotoxicity data, strongly support the therapeutic potential of the PE-EA fraction of *M. hexandra* in breast cancer treatment.

DISCUSSION

This study demonstrates that the petroleum ether–ethyl acetate (PE-EA) fraction of *Manilkara hexandra* leaves exhibits the highest cytotoxicity against MCF-7 breast cancer cells, with an IC_{50} of approximately 55 µg/mL in both SRB and MTT assays. GC-MS analysis revealed quercetin (25.28%) and another major flavonoid (28.62%) as the predominant constituents, suggesting that flavonoids are key contributors to the observed anticancer activity. Morphological analysis confirmed apoptotic features,

and statistical evaluation indicated significant dose-dependent growth inhibition, highlighting the PE-EA fraction as the most promising candidate for further mechanistic and in vivo studies. The present investigation represents a comprehensive evaluation of the anticancer potential of *Manilkara hexandra (Roxb.)* leaf extracts against the MCF-7 breast adenocarcinoma cell line, employing a systematic approach that integrates traditional ethnopharmacological knowledge with contemporary analytical and bioassay methodologies. The findings demonstrate significant cytotoxic activity in the petroleum ether-ethyl acetate (PE-EA) fraction, establishing a compelling correlation between bioactivity and phytochemical composition, particularly flavonoid content [31,32].

The methanolic extraction process yielded a substantial 25.5% (w/w), indicating the presence of significant quantities of polar and moderately polar bioactive compounds in M. hexandra leaves, which aligns with the plant's traditional medicinal applications. Comprehensive phytochemical profiling revealed a diverse array of secondary metabolites, including alkaloids, flavonoids, tannins, terpenoids, and saponins, chemical classes that have been extensively documented in the contemporary literature for their anticancer properties. This phytochemical diversity not only corroborates previous reports on M. hexandra but also establishes a robust compositional foundation for the observed bioactivity. The GC-MS analysis of the bioactive PE-EA fraction revealed a complex phytochemical profile dominated by flavonoids, with quercetin (25.28% relative abundance)and a structurally analogous flavonoid (28.62%) representing the predominant constituents. This flavonoid predominance is particularly significant given the extensive documentation of these compounds' antiproliferative, pro-apoptotic, antiangiogenic, and anti-inflammatory properties across various cancer models. Quercetin, specifically, has been established as a potent anticancer agent through multiple mechanisms, including cell cycle arrest, induction of apoptosis, and inhibition of tumor angiogenesis [33-36].

The presence of complementary terpenoids (eucalyptol, camphor) and aromatic compounds as secondary constituents suggests potential synergistic interactions that may enhance the overall cytotoxic efficacy through additive or multiplicative effects. The cytotoxicity assays demonstrated pronounced antiproliferative activity of the PE-EAfraction against MCF-7 cells, with remarkably consistent results across both SRB and MTT methodologies, thereby validating the reliability and reproducibility of the observed effects. The dose-dependent response profile (GI_{50} = 55 µg/mL) indicates a concentration-dependent mechanism of action, potentially involving modulation of multiple cellular targets and pathways.

This study demonstrates that *M. hexandra* PE-EA fraction exhibits significant cytotoxicity against MCF-7 breast cancer cells (IC_{50} = 55 µg/mL), with quercetin identified as the major bioactive component (25.28% relative abundance), providing scientific validation for traditional anticancer applications. The superior cytotoxic efficacy of the PE-EA fraction compared to other fractions directly correlates with its distinctive phytochemical signature, particularly its high flavonoid content, suggesting that these compounds serve as the primary mediators of the observed anticancer activity.

The morphological alterations observed in treated cells, including characteristic apoptotic features such as membrane blebbing, chromatin condensation, and nuclear fragmentation, strongly suggest apoptosis as the underlying mechanism of cytotoxicity. This hypothesis aligns with established literature demonstrating that flavonoids induce apoptosis through multiple pathways, including the generation of reactive oxygen species, disruption of mitochondrial membrane potential, activation of caspases, and modulation of the cell cycle checkpoint. The differential cytotoxicity observed among fractions underscores the critical importance of bioactivity-guided fractionation in natural product research. The enhanced potency of the PE-EA fraction suggests a concentration effect of bioactive principles through the fractionation process, highlighting the potential therapeutic advantages of semi-purified extracts over crude preparations.

From an ethnopharmacological perspective, these findings provide substantial scientific validation for the traditional medicinal applications of *M. hexandra,* establishing a compelling correlation between documented traditional uses and experimentally demonstrated cytotoxic properties. However, several limitations must be acknowledged: in vitro cytotoxicity assays, while providing valuable preliminary data, cannot fully recapitulate the complexity of tumor microenvironments or systemic physiological responses; the current study focuses on a single cancer cell line, which may not reflect the broader spectrum of cancer types; and thelack of selectivity assessment

against normal cell lines limits understanding of the therapeutic index. Several limitations must be acknowledged: (1) in vitro assays cannot fully replicate tumor microenvironment complexity, (2) evaluation was limited to a single cancer cell line, potentially limiting broader applicability, (3) lack of selectivity assessment against normal cell lines restricts therapeutic index understanding, and (4) absence of in vivo validation limits clinical translation potential. Future studies should address these limitations by determining the selectivity index using normal cell lines, evaluating the approach across multiple cancer types, conducting comprehensive mechanistic studies, and assessing in vivo efficacy in appropriate animal models. Future research directions should encompass isolation and structural characterization of individual bioactive compounds, comprehensive elucidation of molecular mechanisms underlying the observed cytotoxicity, evaluation of in vivo efficacy in appropriate animal models, assessment of selectivity profiles against normal cell lines, and investigation of potential synergistic interactions with conventional chemotherapeutic agents to explore adjuvant applications in integrative oncology [37–39]. These findings collectively position *M. hexandra* as a promising source of novel phytotherapeutic candidates for breast cancer management, with the flavonoid-rich PE-EA fraction demonstrating significant potential for development as a targeted adjuvant in comprehensive cancer treatment regimens [40].

CONCLUSION

M. hexandra is a promising source of novel phytotherapeutic candidates for breast cancer management, with the flavonoid-rich PE-EA fraction demonstrating significant potential for development as a targeted adjuvant in comprehensive cancer treatment regimens. Future research directions should encompass isolation and structural characterization of individual bioactive compounds, comprehensive elucidation of molecular mechanisms underlying the observed cytotoxicity, evaluation of in vivo efficacy in appropriate animal models, assessment of selectivity profiles against normal cell lines, and investigation of potential synergistic interactions with conventional chemotherapeutic agents to explore adjuvant applications in integrative oncology. The study was limited to one cancer cell line (MCF-7), without testing on normal cells or confirming mechanisms of action, and GC-MS identifications lacked standard confirmation. Future work should include multiple cancer and normal cell lines, mechanistic assays, isolation of active compounds, in vivo validation, and confirmation of GC-MS findings with authentic standards.

FINANCIAL ASSISTANCE

NIL

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTION

Sunayana Vikhe conceptualized the study, designed it, developed the software, validated the investigation, contributed resources, and wrote the original draft. Sarika Vikhe and Vaibhav Bhamare contributed to formal analysis, investigation, data curation, visualization, supervision, and project administration.

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