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Anticancer, Antimicrobial, and Cytotoxic Potential of Melaleuca alternifolia Essential Oil: An In Vitro Evaluation

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ABSTRACT: The present study evaluated the anticancer, antioxidant, cytotoxic, and antimicrobial potential of *Melaleuca alternifolia* essential oil using in vitro models and GC–MS profiling. Its cytotoxic effects against HEK293 cells and antibacterial activity against selected pathogens were also assessed for potential therapeutic significance. Fresh leaves of *M. alternifolia* were collected, authenticated, shade-dried, and subjected to hydrodistillation using a Clevenger-type apparatus. The obtained essential oil was dried over anhydrous sodium sulfate and stored. GC–MS was employed for chemical profiling. Antioxidant activity was evaluated using the FRAP radical-scavenging assay, with ascorbic acid as the standard. Cytotoxic activity was determined by MTT assay using HEK293 cells, with doxorubicin as the reference drug. Antimicrobial activity against *Escherichia coli* and *Staphylococcus aureus* was evaluated by agar well diffusion. IC_{50} values were calculated using GraphPad Prism.GC–MS analysis identified several volatile bioactive constituents, including terpenoids, fatty acid derivatives, and alcohols. The essential oil exhibited concentration-dependent antioxidant activity. MTT analysis demonstrated concentration-dependent reductions in HEK293 cell viability, with an IC_{50} of 68.69 ± 0.05 µg/mL, whereas doxorubicin showed an IC_{50} of 48.62 ± 0.04 µg/mL. Microscopic examination revealed cell shrinkage, rounding, reduced cell density, and altered morphology. The essential oil also demonstrated antibacterial activity against *E. coli* and *S. aureus*. *M. alternifolia* essential oil exhibits promising antioxidant, cytotoxic, and antimicrobial properties, supporting its potential as a natural source of bioactive compounds. Further mechanistic and in vivo studies are warranted to establish its therapeutic efficacy and safety.

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1. INTRODUCTION

Cancer remains a major global health challenge, and the limitations of conventional chemotherapy, including drug resistance, systemic toxicity, and adverse effects, have stimulated interest in naturally derived anticancer agents [1]. Essential oils (EOs) are complex mixtures of volatile terpenes and other bioactive constituents that have demonstrated antiproliferative, pro-apoptotic, antioxidant, and antimicrobial activities across several experimental cancer models. Their reported mechanisms include oxidative stress modulation, mitochondrial dysfunction, cell-cycle arrest, apoptosis, and interference with tumor-associated signalling pathways. Breast cancer is one of the most extensively investigated malignancies in natural-product research, with several essential oils and their constituents showing inhibitory effects against breast cancer cell lines such as MCF-7, MDA-MB-231, and T47D. These effects have been associated with apoptosis, reactive oxygen species generation, mitochondrial dysfunction, and cell-cycle arrest. Beyond breast cancer, essential oils have also demonstrated promising activity against melanoma, lung, colorectal, cervical, and other cancer models [2]. *Melaleuca alternifolia* (tea tree) essential oil is particularly interesting because of its rich terpene composition, with terpinen-4-ol generally representing a major constituent. Experimental studies have reported that *M. alternifolia* essential oil and terpinen-4-ol inhibit tumor-cell proliferation and can induce necrotic/apoptotic responses and cell-cycle arrest. Antitumor effects have also been reported in melanoma models, including enhanced activity when combined with targeted therapy. Additionally, its established antimicrobial properties provide a complementary biological rationale for investigating this essential oil. Therefore, the present study evaluates the anticancer, antioxidant, and antimicrobial potential of *M. alternifolia* essential oil using in vitro experimental approaches [3].

2. MATERIALS AND METHODS

2.1 Plant material collection and identification: *Melaleuca alternifolia* leaves were employed in this investigation. The authors gathered the samples at Maharana Pratap College of Pharmacy's and approved the plant by the pharmacognosy department.

2.2 Extraction of Essential Oil from *Melaleuca alternifolia* Leaves (Hydro distillation Method): Fresh, healthy leaves of *Melaleuca alternifolia* were collected, washed thoroughly with distilled water to remove dust and foreign matter, and shade-dried at room temperature (25–30 °C) for 5–7 days until a constant weight was achieved. The dried leaves were coarsely ground using a laboratory grinder. Approximately 500 g of the powdered leaves were transferred into a 5 L round-bottom flask containing 3 L of distilled water. The essential oil was extracted by hydrodistillation using a Clevenger-type apparatus for 3–4 hours, following the procedure described in the European Pharmacopoeia. During distillation, the volatile oil vapors condensed and separated from the aqueous phase in the graduated arm of the Clevenger apparatus. The collected essential oil was separated carefully using a micropipette and dried over anhydrous sodium sulfate to remove residual moisture. The dried oil was filtered through Whatman No. 1 filter paper and stored in amber-colored glass vials at 4 °C until further phytochemical and biological analyses [4]. The percentage yield of essential oil was calculated based on the dry weight of the plant material using the following equation:

$$\textbf{Essential Oil Yield (\%, w/w)} = \frac{\text{Weight of Essential Oil Obtained (g)}}{\text{Weight of Dried Plant Material (g)}} \times 100$$

Figure-1 Extraction Essential Oil from *Melaleuca alternifolia*

2.3 Qualitative Phytochemical Screening: To confirm the existence of different bioactive components, a standard qualitative investigation of the Essential Oil from *Melaleuca alternifolia* Leaves was performed.

2.3.1 Liebermann-Burchard Test: Two millilitres of essential oil was mixed with 2 mL of acetic acid and concentrated sulfuric acid. The development of initial red coloration followed by blue and finally green colour indicated steroid presence.

2.3.2 Salkowski-Reaction: The presence of steroids in the plant extract was determined using the Salkowski reaction. Approximately 2 mL of the essential oil was mixed with 2 mL of chloroform, followed by the careful addition of concentrated sulfuric acid along the side of the test tube to form a separate layer. The appearance of a red or reddish-brown coloration in the chloroform layer, accompanied by a greenish-yellow fluorescence in the sulfuric acid layer, indicated the presence of steroidal compounds. This colour change occurs due to the reaction of sterol molecules with concentrated sulfuric acid, making the Salkowski test a reliable preliminary method for steroid detection.

2.3.3 Lead Acetate Test: To detect tannins, 2 mL of the essential oil was treated with a few drops of 10% lead acetate solution. The formation of a white or cream-colored precipitate indicated the presence of tannins. This reaction occurs because tannins form insoluble complexes with lead ions, resulting in visible precipitation.

2.3.4 Ferric Chloride Test: For the detection of phenolic compounds, 2 mL of the essential oil was treated with a few drops of 5% ferric chloride solution. The development of a bluish-black, dark green, or violet coloration confirmed the presence of phenolic constituents. The ferric ions react with the hydroxyl groups of phenolic compounds to produce colored complexes, making this test a widely accepted qualitative method for identifying phenolics.

2.3.5 Alkaline Reagent Test: The presence of flavonoids was evaluated by adding a few drops of 10% sodium hydroxide solution to 2 mL of the essential oil. The immediate appearance of an intense yellow coloration indicated the possible presence of flavonoids. Upon the addition of dilute hydrochloric acid, the yellow colour disappeared, confirming the presence of flavonoid compounds. This reversible colour change is attributed to the formation and subsequent neutralization of flavonoid sodium salts under alkaline and acidic conditions, respectively.

2.3.6 Foam Test: To identify saponins, 2 mL of the essential oil was diluted with approximately 5 mL of distilled water and shaken vigorously for several minutes. The persistence of a stable froth or foam for at least 10 minutes indicated the presence of saponins. The foam-forming property results from the surfactant nature of saponins, which reduce the surface tension of water.

2.3.7 Froth Test: In a separate assay, essential oil was diluted with distilled water in a graduated cylinder and shaken vigorously for about 2–3 minutes. The formation of a stable froth layer measuring approximately 1 cm in height that persisted for at least 10 minutes confirmed the presence of saponins. The stability and persistence of the froth provide qualitative evidence of saponin-containing phytoconstituents in the extract [5].

2.4 IN VITRO ANTIOXIDANT ACTIVITY

2.4.1 Antioxidant Assay FRAP: 10µl of different stock of the test compound and standard (Ascorbic Acid – (SRL, Cat no-23006) was added to the wells of the 96-well plate in treatment and treatment blank while 10 µl of demineralized water was added to the wells of control and control blank. Then, 50 µl of Solution A- potassium ferricyanide [K3Fe (CN) 6] (SRL, Cat no- 15766) was added to the wells in treated and control, and 50 µl of demineralized water was added to the blank wells of control and treated. The wells without treatment were considered as control. The plate was then incubated at 50 °C for 20 minutes. After incubation, 50 µl of Solution B- trichloroacetic acid (SRL, Cat no-92390) was added to all wells of the 96-well plate. Then 40µl of demineralized water and 50µl of Solution C-ferric chloride (Fischer scientific – Cat no. 23585) were added. The colored solution was read at 700 nm against the blank using microplate reader (iMark, BioRad). IC50 was calculated by using software Graph Pad prism 6. Graph was prepared between X axis (Sample Concentration) Vs. Y axis (% inhibition wrt control) [6-8].

CALCULATIONS

% FRAP = (A Sample - A Control /A Control) ×100

A Control = Absorbance of control

A Sample = Absorbance of sample

2.5 GC-MS Analysis: GC-MS analysis of Essential Oil from *Melaleuca alternifolia* was performed using a PerkinElmer Clarus 600 system equipped with an Rtx-5MS capillary column. Helium (99.99%) was used as the carrier gas at a constant flow rate of 1.0 mL/min. Injection volume: 1 µL, split ratio: 10:1, ionization energy: 70 eV, scan range: 40–650 m/z, scan time: 0.2 sec. Injector temperature: 260°C. Oven temp held at 50°C for 3 min, then ramped to 300°C at 10°C/min. Compounds were identified by comparing retention times and mass spectra with those in the NIST and Wiley-8 libraries [9-10].

2.6 In *Vitro* Cytotoxicity Evaluation of the Compounds- HEK293

2.6.1 MTT assay method: Cytotoxicity of the samples provided on HEK293 (Procured from NCCS Pune, India, Passage No-29, RRID- CVCL_0045) cell line was determined by MTT Assay. The cells (10000 cells/well) were cultured in 96 well plate for 24 h in Ham's F12k medium supplemented with 10% FBS (Fetal Bovine Serum - HIMEDIA-RM 10432) and 1% antibiotic solution (Penicillin Streptomycin-Sigma-Aldrich P0781) at 37°C with 5% CO2. Next day cells were treated from different concentrations of the samples (concentration as per mentioned in excel sheet). Stock solution of samples was prepared in DMSO and further diluted to get different concentrations in incomplete Cell culture Medium (Without FBS). After incubation for 24 hours, MTT Solution (5 mg/ml) was added to cell culture and further incubated (Air- jacketed CO2 incubator-Heal force HF90) for 2 h. Cells without treatment were considered as Control and cells without MTT were considered as Blank. At the end of the experiment, culture supernatant was removed, and cell layer matrix was dissolved in 100 µl Dimethyl Sulfoxide (DMSO–SRL- Cat no.- 67685) and read in an Elisa plate reader (iMark, Biorad, USA) at 540 nm. IC50 was calculated by using Graph Pad Prism -6. Images were captured under inverted microscope (Olympus ek2) using Camera (AmScope digital camera 10 MP Aptima CMOS). 50% inhibitory concentration (IC50) was presented as Mean ± SEM (Standard Error of Mean) [11-12].

Calculation

% Viable cells = (A_{test} / $A_{Control}$) *100

(A_{test} = Absorbance of test sample)

($A_{Control}$ = Absorbance of Control)

2.7 Antimicrobial Tests

2.7.1Anti-Microbial-Zone Inhibition Test (Well diffusion Method) –*Staphylococcus aureus*: The Antibacterial activity was checked by following Zone Inhibition Method (Kirby-Bauer method). The MHA plates were inoculated by spreading with 100 µl of Bacterial culture, *Staphylococcus aureus* (Inoculum was prepared by adjusting 0.5 McFarland Unit - Approx. cell density (1.5 X 10^8 CFU/mL from Mueller- Hinton Broth) and followed by making the wells containing 10 µl of different concentration (0 to 100 %). 10 % of the sample was taken and serially diluted to achieve the required amount to be loaded in the well. One well in each plate was loaded with solvent alone which served as vehicle control and Ciprofloxacin well (3 µg) was taken as positive control. The plates of *Staphylococcus aureus* were incubated in incubator (Basil Scientific Corp. India) at 37 °C for 24 hrs. The clear zones created around the well were measured and recorded [13].

2.7.2 Anti-Microbial-Zone Inhibition Test (Well diffusion Method) – *Escherichia coli:* The Antibacterial activity was checked by following Zone Inhibition Method (Kirby-Bauer method). The MHA plates were inoculated by spreading with 100 µl of Bacterial culture, *Escherichia coli* (Inoculum was prepared by adjusting 0.5 McFarland Unit - Approx. cell density (1.5 X 10^8 CFU/mL from Mueller- Hinton Broth) and followed by making the wells containing 10 µl of different concentration (0 to 100 %). 10 % of the sample was taken and serially diluted to achieve the required amount to be loaded in the well. One well in each plate was loaded with solvent alone which served as vehicle control and Ciprofloxacin well (3 µg) was taken as positive control. The plates of *Escherichia coli* were incubated in incubator (Basil Scientific Corp. India) at 37 °C for 24 hrs. The clear zones created around the well were measured and recorded [14].

2.7.3 Anti-fungal -Zone Inhibition Test (Well diffusion Method) -*Aspergillus Niger:* The Antifungal activity was checked by following Zone Inhibition Method (Kirby-Bauer method). The SDA plates were inoculated by spreading with 100 µl of fungal culture, *Aspergillus niger* (Inoculum was prepared by adjusting 0.5 McFarland Unit - Approx cell density (1.5 X 10^8 CFU/mL from Sabouraud dextrose broth) and followed by placing the wells containing 10 µl of different concentration (0 to 100 %). 10 % of the sample was taken and serially diluted to achieve the required amount to be loaded in the well. One well in each plate was loaded with solvent alone which served as vehicle control and Fluconazole (40µg) was taken as positive control. The plates of *Aspergillus Niger* were incubated in incubator at 28 °C for 24 hrs. The clear zones created around the well were measured and recorded [15].

2.7.4 Anti-Fungal-Zone Inhibition Test – (Well diffusion Method) *Candida albicans:* The Antifungal activity was checked by following Zone Inhibition Method (Kirby-Bauer method). The PDA plates were inoculated by spreading with 100 µl of Fungal culture, *Candida albicans* (Inoculum was prepared by adjusting 0.5 McFarland Unit - Approx cell density (1.5 X 10^8 CFU/mL from Sabouraud dextrose broth) and followed by the wells containing 10 µl of different concentration (0 to 100 %). 10 % of the sample was taken and serially diluted to achieve the required amount to be loaded in the well. One well in each plate was loaded with solvent alone which served as vehicle control and Fluconazole well (40 µg) was taken as positive control. The plates of *Candida albicans* were incubated in incubator at 37 °C for 24 hrs. The clear zones created around the well were measured and recorded [16].

2.8 Statistical investigation: All data were investigated by means of the analysis of variance (ANOVA) followed by the Post Hoc Tukey's test (Graph Pad Prism Software-6). All data represent three independent values and results are presented as mean ± S.E. Statistical significance was considered as P value ≤ 0.05.

3. RESULTS AND DISCUSSION

3.1Identification of components*:* Identification was done using the calculated fragments, molecular mass, and molecular structure. The NIST database and the Willy8-library, which has more than 62,000 pattern entries, were used to interpret the GC-MS spectra. Together with their molecular weights and chemical structures, the components of the test materials were determined. The percentage amounts discovered in the sample were contrasted with component spectra from the Willy8 collection and the NIST library. This is done in order to determine whether this plant species has any chemicals or a combination of them that could support its traditional and commercial therapeutic purposes. It also helps determine the most effective methods for removing harmful compounds. The test materials' constituent parts were determined (Table 1, and Figure 2).

Table 1- Peak Report of Essential Oil from *Melaleuca alternifolia*

Peak	R. Time	Area	Area %	Name	Common Names
1	17.59	16414046	22.01	N-Hexadecanoic acid	Palmitic acid (Hexadecanoic acid)
2	17.891	757749	1.02	Hexadecanoic acid, ethyl ester	Ethyl palmitate
3	17.976	463018	0.62	Octadecane	Octadecane
4	18.354	256564	0.34	Hexadecane-1,2-diol	Hexadecanediol
5	18.92	1086185	1.46	8,11,14-Docosatrienoic acid, methyl ester	Docosatrienoic acid methyl ester
6	19.022	2377485	3.19	Phytol	Phytol
7	19.153	694246	0.93	Methyl stearate	Methyl stearate
8	19.279	10781071	14.46	(Z, z)-6,9-cis-3,4-epoxy-nonadecadiene	Epoxy nonadecadiene derivative
9	19.488	10240763	13.73	Octadecanoic acid	Stearic acid (Octadecanoic acid)

10	22.263	3466263	4.65	Palmitic acid	Palmitic acid
11	22.454	5389089	7.23	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl est	Glyceryl hexadecanoate derivative (monopalmitin-type)
12	23.064	653726	0.88	Docosanoic acid, ethyl ester	Ethyl docosanoate
13	23.59	133283	0.18	Acetic acid n-octadecyl ester	Stearyl acetate (acetic acid octadecyl ester)
14	23.683	390518	0.52	1h-indole-3-ethanamine	Tryptamine derivative (indole ethylamine)
15	24.032	2718868	3.65	Octadecanoic acid, 2,3-dihydroxypropyl ester	Diglyceride of stearic acid
16	27.07	812326	1.09	Stigmast-5-en-3-ol, oleat	Stigmasterol oleate (plant sterol ester)
17	29.556	861249	1.15	25-homo-24-ketocholesterol	Oxygenated cholesterol derivative
18	30.923	1016859	1.36	Lupeol	Lupeol (plant triterpenoid)

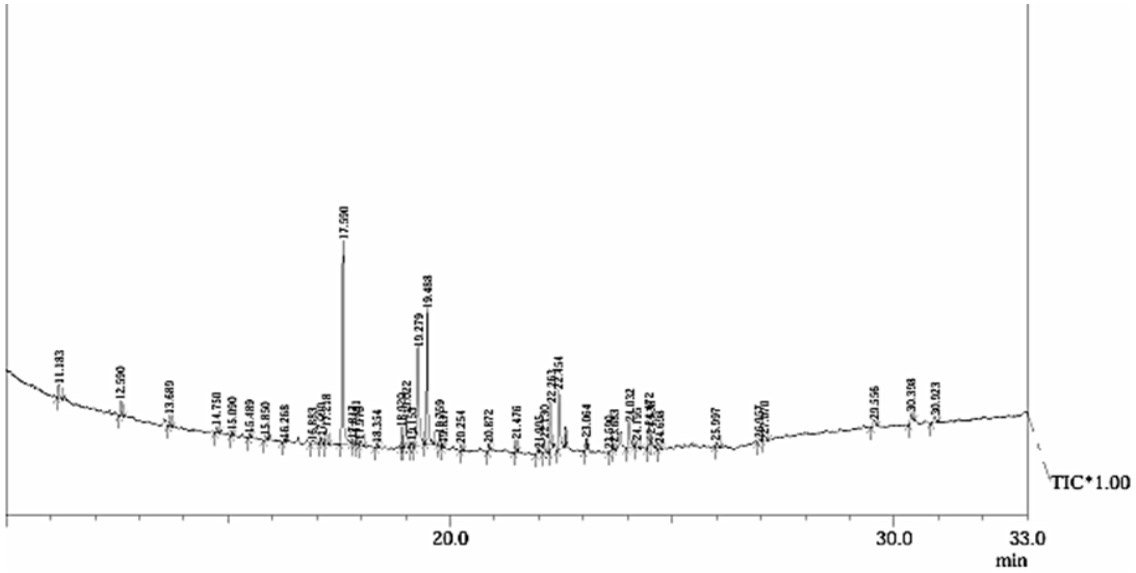

Figure-2 Chromatogram of Essential Oil from *Melaleuca alternifolia*

Numerous chemicals were found in the Essential Oil from *Melaleuca alternifolia* during GCMS analysis. 18 compounds were found in samples, which were already included in the table above.

3.2 Activities of antioxidants: Based on the results obtained from the experimental work, Antioxidant activity was estimated in the sample, and 50% inhibitory concentration (IC_{50}) was mentioned in table 2. Samples – *Melaleuca alternifolia* essential oil was found to be less active. 982 µg of sample- *Melaleuca alternifolia* essential oil was found equivalent to 21.89 µg of standard Ascorbic acid.

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Table-2 Scavenging Assay Report

Compounds code	IC_{50} value (microgram/millilitre)
Ascorbic Acids	21.89±0.02
Melaleuca alternifolia essential oil	982.6±0.02

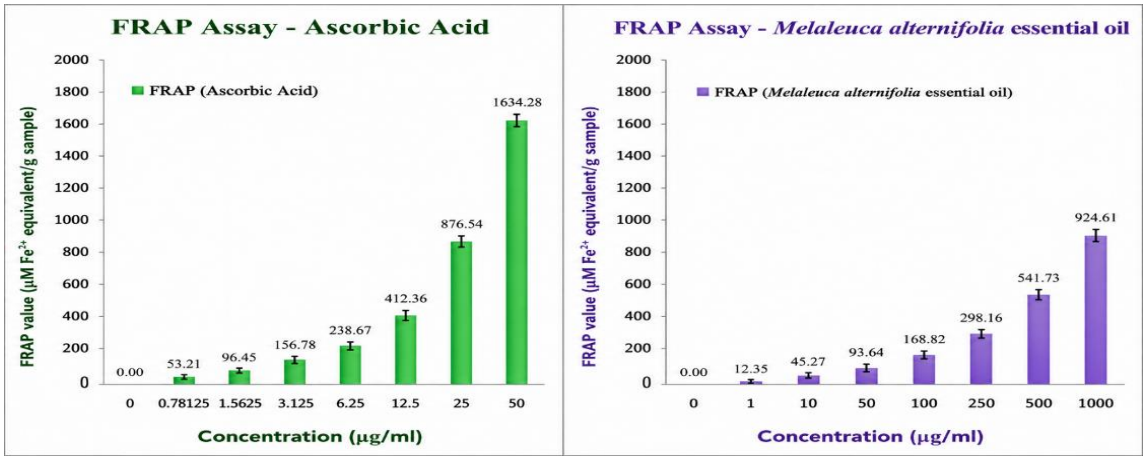

Figure-3 Graph of Scavenging Assay of Essential Oil from *Melaleuca alternifoli*

Table-3 Testing Compound Toxicity in Cells Using the HEK293 MTT Assay

Sample code	IC_{50} value
Doxorubicin	11.26±0.20 (µg/ml)
Melaleuca alternifolia essential oil	75.54±0.08 (µM)

Based on the results obtained from the MTT assay, it was observed that when the HEK293 cell line was exposed to different concentrations of the sample, cytotoxic activity was estimated for the samples and 50% inhibitory concentration as mentioned in table 3. Sample – *Melaleuca alternifolia* essential oil was found to be highly cytotoxic. IC_{50} is the concentration of an inhibitor/sample/ formulation at which the viable cells are reduced by half.

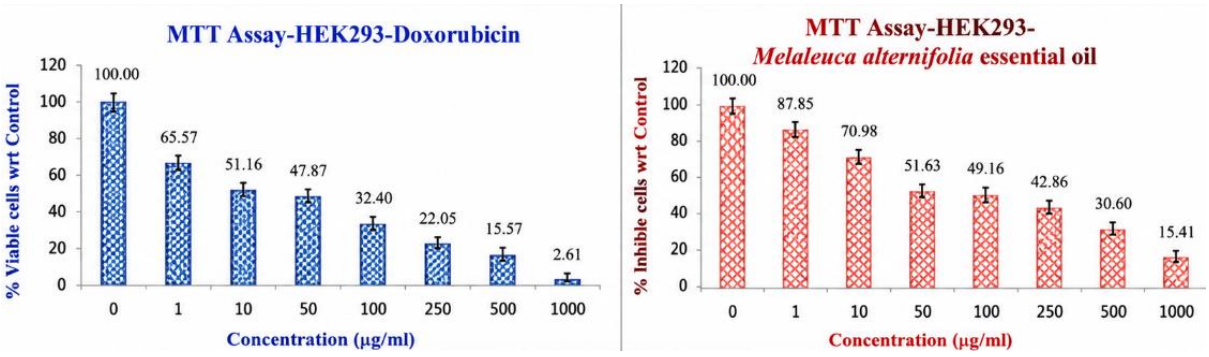

Figure 4. Concentration-dependent cytotoxic effects of doxorubicin and *Melaleuca alternifolia* essential oil on HEK293 cells assessed by MTT assay

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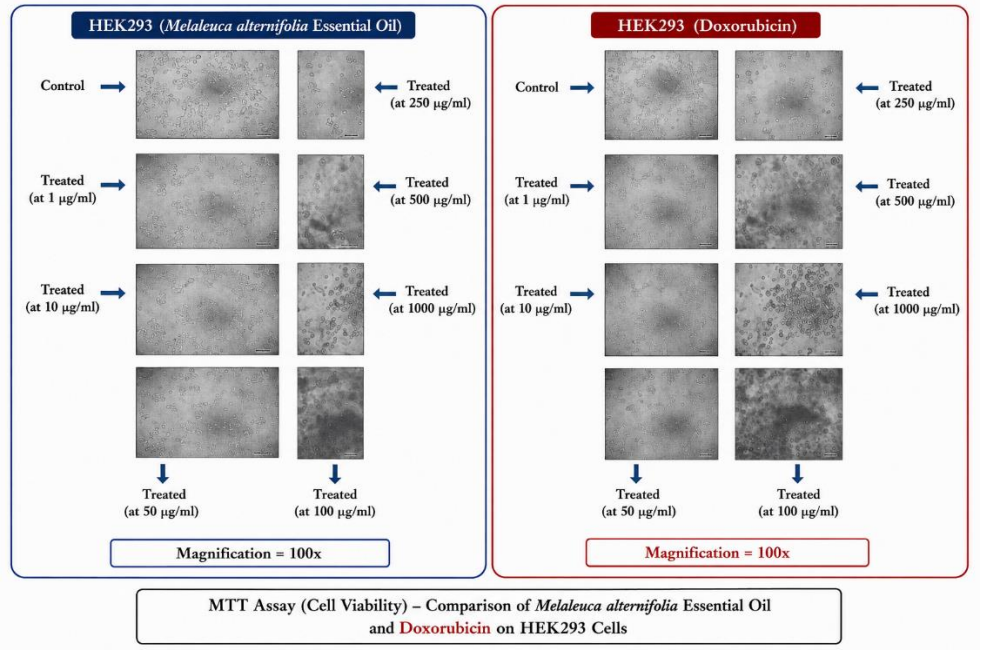

Figure-5 Cytopathic effects on the HEK293 cell line treated by *Melaleuca alternifolia* essential oil and Doxorubicin

3.3 Antimicrobial activity – Table-4 Summarize the microbial growth inhibition Essential Oil from *Melaleuca alternifolia* Against Different Microorganisms (Zone of Inhibition, mm)

on E. coli, *Staphylococcus aureus*, *Aspergillus Niger*, *Candida albicans* which showed good antibacterial activities.

Table 4. Antimicrobial Activity of Essential Oil from *Melaleuca alternifolia* Against Different Microorganisms (Zone of Inhibition, mm)

Microorganism	Loaded Volume (µL)	0%	6.25%	12.50%	25%	50%	100%
Staphylococcus aureus	10	0	6.67	9.67	10.33	11.67	12.67
Escherichia coli	10	0	9	10	11	14	15
Aspergillus niger	10	0	16	16	27	28	29
Candida albicans	10	0	19	21	26	28	28

3.4 Discussion

The present study demonstrated that *Melaleuca alternifolia* essential oil possesses a combination of antioxidant, cytotoxic, and antimicrobial activities, supporting its potential as a source of biologically active phytoconstituents. GC–MS analysis identified 18 compounds, including fatty acids, esters, phytol, oxygenated derivatives, and triterpenoid constituents, which may collectively contribute to the observed biological effects. The antioxidant evaluation using the FRAP-based assay indicated relatively weak activity of the essential oil compared with ascorbic acid. The IC_{50} of *M. alternifolia* essential oil was 982.6 ± 0.02 µg/mL, whereas ascorbic acid showed an IC_{50} of 21.89 ± 0.02 µg/mL. This comparatively higher IC_{50} suggests limited reducing/free-radical-scavenging activity under the experimental conditions. In contrast, the MTT assay demonstrated cytotoxic effects against HEK293 cells, with an IC_{50} of 75.54 ± 0.08 µM, while doxorubicin exhibited greater cytotoxic potency with an IC_{50} of 11.26 ± 0.20 µg/mL. Microscopic observations further supported concentration-associated cellular morphological changes. The antimicrobial results showed concentration-dependent inhibition against *S. aureus*, *E. coli*, *A. niger*, and *C. albicans*, with

particularly pronounced activity against the fungal organisms. Overall, these findings indicate that *M. alternifolia* essential oil has promising antimicrobial and cytotoxic properties, although further mechanistic and in vivo studies are required.

4. CONCLUSION

The present study concludes that *Melaleuca alternifolia* essential oil exhibits promising biological activities, particularly cytotoxic and antimicrobial effects. GC–MS analysis revealed 18 chemical constituents, including fatty acid derivatives, phytol, esters, oxygenated compounds, and triterpenoid constituents, which may contribute to its observed pharmacological properties. The essential oil showed comparatively weak antioxidant activity, with an IC_{50} of 982.6 ± 0.02 µg/mL compared with 21.89 ± 0.02 µg/mL for ascorbic acid. In the HEK293 MTT assay, the essential oil demonstrated concentration-dependent cytotoxicity, with an IC_{50} of 75.54 ± 0.08 µM, while doxorubicin showed greater cytotoxic potency. Furthermore, the essential oil exhibited concentration-dependent antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Aspergillus niger*, and *Candida albicans*. Overall, these findings support further investigation of *M. alternifolia* essential oil through mechanistic, toxicity, and in vivo studies.

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AUTHOR CONTRIBUTIONS

Dr. Anubhav Dubey: Conceptualization, Investigation.

Dr. Sanyogita Shahi: Methodology, Laboratory Analysis.

Dr. Kumara Swamy Samanthula: Data Curation, Formal Analysis.

Dr. Sribatsa Lanchhana Dash and Soubhagya Lenka: Supervision, Writing—Review & Editing.

CONFLICT OF INTEREST

The Authors Declare That There is No Conflict of Interest.

ETHICS STATEMENT

Ethical Approval Was Not Required for This Study.

DATA AVAILABILITY STATEMENT

Data Supporting the Findings of This Study Are Available from The Corresponding Author Upon Reasonable Request.

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