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Original Article

ANTIBACTERIAL POTENTIAL OF ESSENTIAL OILS DERIVED FROM NATURAL, CALLUS AND *IN-VITRO* PROPAGATED SOURCES OF MELALEUCA ALTERNIFOLIA AGAINST COMMON BACTERIAL PATHOGENS

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ABSTRACT

Objective: *Melaleuca alternifolia (M. alternifolia)* and its essential oil (EO) fractions have been used widely and traditionally in the treatment of various infectious diseases and hence its antibacterial potential is investigated in the present study.

Methods: The antibacterial activity was studied through the agar disc diffusion method and broth dilution method, DNA fragmentation studies and confocal microscopy morphological studies were done. *In-silico* molecular interaction was studied against microbial target using docking software.

Results: The inhibitory concentration of the EOs was recorded at 75% dilution with larger inhibition zones. The DNA fragmentation analyzed in the essential oil derived from in-vitro propagated leaves (EOIPL) of *M. alternifolia* treated bacterial cultures was compared with negative and positive controls. In Minimum Inhibitory Concentration (MIC) of EOIPL treated *Staphylococcus aureus* (*S. aureus*) showed time-dependent growth inhibition. The DNA content in the EOIPL treated bacterial cultures was comparatively less than in control cultures. The cell morphology changes of *S. aureus* cells were studied through confocal laser scanning microscopic analysis which showed a significant decrease in viable bacterial cells. The active component, terpinen-4-ol docked to autolysin receptor revealed stable interaction with the microbial target.

Conclusion: Thus EOIPL was explored to possess bactericidal activity against common infectious bacteria and could in incorporated in therapeutic natural antibiotic formulations as with future studies.

Keywords: *M. alternifolia*; EOIPL, Minimum inhibitory concentration, Terpinen-4-ol, Molecular docking

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INTRODUCTION

Since ancient times, medicinal plants have been used to maintain human healthcare [1]. Phytotherapy is a plant-based medicinal practice and the phytochemical constituents derived from these medicinal plants serve as alternative sources to synthetic drugs in treating different infections and ailments. WHO has also recommended the development and use of environment-friendly alternative methods to control diseases [2]. The phytocompounds have a history of clinical use, better patient tolerance and acceptance. Many plant extracts and essential oils isolated from plants have been shown to exert biological activity *in-vitro* and *in-vivo*, which justified research on traditional medicine focused on the characterization of antimicrobial activity of these plants [3]. Thus the development of new antibiotics depends on the strategies such as bioavailability, targeting sites, route of administration, half-life period, spectrum of resistance. *Melaleuca alternifolia* (*M. alternifolia*) commonly known as Tea tree, *M. alternifolia* belongs to the family of Myrtaceae. Tea tree oil (TTO) of *M. alternifolia* contains various mono and sesquiterpenes, aromatic compounds. The monoterpenes, terpinen-4-ol, γ-terpinene,α-terpinene, 1,8-cineole, pcymene, α-terpineol, α-pinene, terpinolenes, limonene and sabinene account for 80-90% of the oil. The capabilities of the unique and demonstrated anti-bacterial, anti-fungal, anti-viral, anti-inflammatory, and anti-septic, essential oil were first scientifically documented in 1905. The TTO is considered as a universal treatment for acne, eczema, and skin infections like herpes, warts, wounds, burns, nail mycosis, insect bites, colds, sore throat, gingival infections, haemorrhoids and vaginal infections [4].

MATERIALS AND METHODS

Sources of essential oils

The essential oils (EO) used in the present study was obtained from the natural leaves, callus and in-vitro propagated leaves sources of *M. alternifolia* through steam distillation process.

Quantitative estimation of EO components by GC-MS/MS

The GC-MS/MS analysis was performed on a combined GC-MS/MS instrument (ITQ 900 Model of Thermo Fisher Scientific make) using a HP-5 fused silica gel capillary column. The method to perform the analysis was designed for both GC and MS. 1µl aliquot of sample was injected into the column using a PTV injector whose temperature was set at 275 °C. The GC program was initiated by a column temperature set at 60 °C for 5 min, increased to 300 °C at a rate of 8 °C/min, held for 10 min. Helium was used as the carrier gas (1.5 ml/min). The mass spectrometer was operated in EI mode with mass source was set at 200 °C. The chromatogram and spectrum of the peaks were visualized. The particular compounds present in the samples were identified by matching their mass spectral fragmentation patterns of the respective peaks in the chromatogram with those stored in the National Institute of Standards and Technology Mass Spectral database library.

Antibacterial studies

Preparation of bacterial inoculums

The bacteria used in this experiment were *Staphylococcus aureus (S. aureus)* (ATCC 43957), *Escherichia coli (E. coli*) (ATCC 23849), *Pseudomonas aeruginosa (P. aeruginosa)* (ATCC 33363), *Bacillus subtilis (B. subtilis)* (ATCC 21833), were procured from the Department of Clinical Microbiology, K. A. P. V. Government Medical College, Tiruchirappalli. Bacterial inoculums were prepared by sub culturing the strains under aseptic condition. A loop full of test organisms were taken and inoculated into 5 ml of Muller Hinton Broth (MHB) and incubated at 37°C for 3 to 5h till a moderate turbidity was developed.

Agar disc diffusion method

Antibacterial activity of the three types of EOs namely essential oil from natural leaves (EONL), essential oil from in-vitro propagated

leaves (EOIPL), essential oil from callus (EOC) of *M. alternifolia* leaves were determined by using agar disc diffusion method. A small aliquot of the bacterial culture was swabbed over the Muller Hinton agar plates aseptically. The required sterile discs were placed on the agar medium. Using sterile tips filled with the discs in different weight percentage concentrations (10%, 25%, 50% and 75%) of the EOs prepared and allowed to diffuse into the medium for 30 min at room temperature. Plates were incubated at room temperature (37 °C) for 24h. The zone of inhibition was recorded as the mean ± standard deviation (SD) of triplicate experiments. Tetracycline was used as reference antibiotics [5].

Broth dilution method–determination of MIC

The EOIPL was subjected to antibacterial susceptibility testing by broth microdilution method [5]. The 96-microtiter well was prepared by dispensing 45µl of Muller-Hinton broth and then a series of two-fold dilutions of each oil (50µl), ranging from 2% (v/v) to 0·03% (v/v), was prepared with 0·5% (v/v) Tween-20, followed by the addition of 5µl of the *S. aureus, E. coli, B. subtilis* and *P. aeruginosa* suspensions into each well. The microtitre plate was placed in a sterile plate shaker at 300rpm for 20s and then incubated at 37 °C for 24h. At the end of the incubation period, the plate was evaluated for the presence or absence of bacterial growth from the optical densities recorded.

Statistical analysis

All data were expressed as mean ± SEM for control and experimental groups. The data were analyzed using one-way Analysis of Variance (ANOVA) on Statistical Package for Social Sciences (SPSS) (Version 17.0) and the group means were compared by Duncan's Multiple Range Test [6]. The results were considered statistically significant if the calculated 'p' value was less than 0.05.

DNA fragmentation analysis

The ability of EOIPL (75%) to cause DNA fragmentation was tested *S. aureus, E. coli*. ½ MIC, MIC and 2MIC for the tested oil was added to 10 ml LB broth containing S. *aureus, E. coli,* and that was incubated for 4h at room temperature. One ml of each EOIPL-bacteria combination was withdrawn and submitted to centrifugation at 10,000rpm for 20 min followed by DNA extraction. For control, genomic DNA was isolated from non-treated *S. aureus, E. coli*. The extracted DNA was checked for fragmentation using agarose gel electrophoresis.

Cell morphological studies-confocal laser scanning microscopic analysis

The *S. aureus* control culture and EOIPL (75%) treated culture were prepared for CLSM analysis. One ml of MHB was taken in microcentrifuge tubes inoculated with both bacterial cultures separately and then 30µl of the EO was added to each of the tube and incubated overnight at 37 °C. Tetracycline (30 µg/ml) treated bacterial cultures were taken as positive control. 0.1% Acridine orange (AO) stain was prepared and 10µl of AO stain was added to each tube and incubated for 30 sec. The tubes were then centrifuged to collect the pellets. The pellets were washed with water twice and air-dried. The stained bacterial cultures were smeared on the glass slides. On viewing under the microscope 15% glycerol was added to the thin smear and covered with a coverslip [7].

***In-silico* analysis**

The exact interaction of the compound with the microbial target was analyzed using *in-silico* molecular docking analysis.

Molecular docking

The structure of Autolysin (PDB id- 2B0P) was retrieved from the Protein Data Bank (PDB) in. pdb text format [8]. The 3D structures of terpinen-4-ol were developed using ACD/ChemSketch software. In molecular docking, the software allows us to virtually screen a database of compounds and predict the strongest binders based on various scoring functions. It explores the ways in which the molecules and the receptors fit together and dock to each other well. Thus docking analysis of the terpinen-4-ol with Autolysin was carried out by Libdock module of Discovery studio (Version 2.1, Accelry's Software Inc.), a licensed life science modeling and simulation suite of application focused on optimizing the drug discovery process by identifying the specific amino acid residues to which the compound fitted together.

RESULTS

Essential oil extraction

The EOs were extracted from all three sources of *M. alternifolia* (natural leaves, callus and *in-vitro* propagated leaves) through the steam distillation process and the oil content was comparatively analyzed. The results tabulated (table 1) showed that, the maximum level of oil recovery was obtained in *in-vitro* propagated leaves of about 3.79 ml and the influence of moisture was 10.34%.

Table 1: Essential oil extraction from natural leaves, callus and *in-vitro* propagated leaves of *M. alternifolia*

Steam distillation			Natural leaves	Callus	*In-vitro* propagated leaves
Raw material input (g)	Vapour seed (g/h)	Time extract (min)	Oil volume (ml)	Oil volume (ml)	Oil volume (ml)
100	200	200	1.4	1.5	1.64
100	200	250	1.4	1.9	2.2
100	200	300	2.4	2.5	2.79
100	200	350	2.6	2.8	3.4
100	200	400	3.12	3.3	3.79

Table 2: Component analysis of EONL, EOIPL and EOC derived from *M. alternifolia* through GC-MS/MS analysis

S. No.	Component	Max %	EONL	EOC	EOIPL
1	Terpine-4-ol	30-48	32.32	33.01	35
2	y-terpinene	10-28	12.5	12.8	14
3	α-terpinene	5-13	5	5.43	6
4	1-8-cineole	0-15	8.9	9.98	12.7
5	Terpinoeol	1.5-5	1.73	1.8	2.4
6	α-terpineol	1.5-8	3.46	3.53	4.76
7	α-pinene	1-6	2.34	3.11	3.5
8	p-cymene	0.5-8	3.8	4	4.4
9	Limonene	0.5-1.50	0.52	0.76	0.99

GC-MS/MS

The GC-MS/MS performed on EONL, EOIPL and EOC reported the presence of 9 major components (fig. 1a-1c). These components were found to be in abundance and the nature of them was identified with the peaks obtained in the GC graph in comparison to the library of compounds. The library search confirmed the presence of compounds such as limonene, α-pinene, α-terpinene, p-cymeme,

1,8-cineole, terpinene, α-terpinolene, terpinen-4-ol, and terpineol. The maximum percentages of the components obtained in the essential oils are depicted in table 2. Of these components, terpin en-4-ol is identified as the abundant component in all three essential oils.

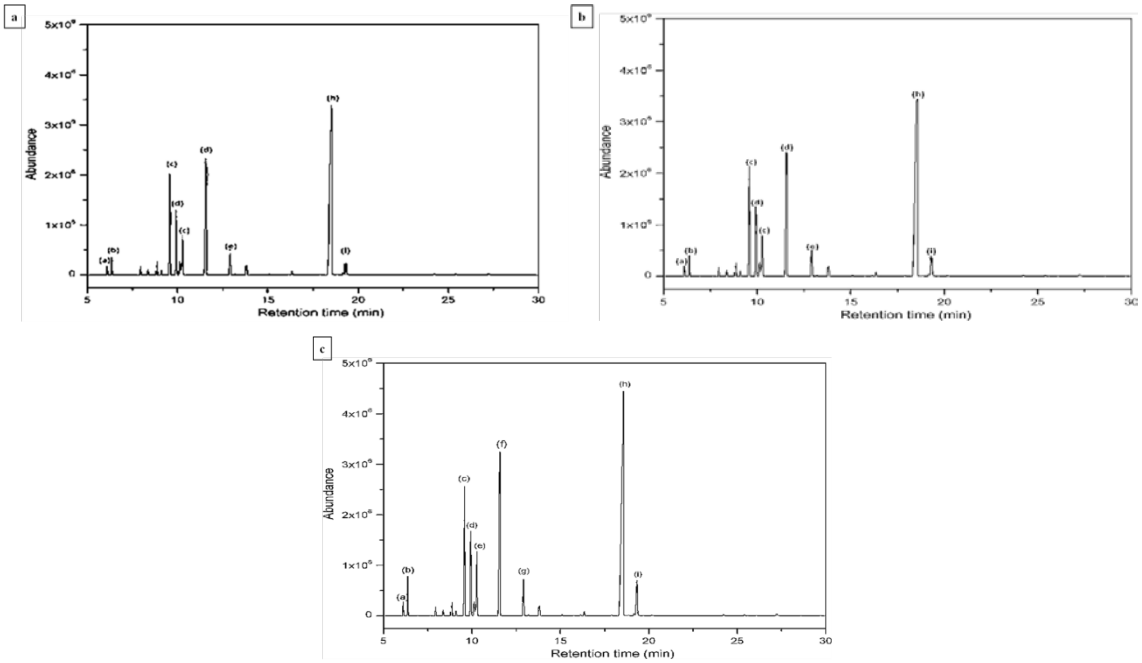

Fig. 1a-1c: Chromatograms of the EONL, EOC and EOIPL derived from *M. alternifolia.* The GC graph indicates the presence of nine major compounds (a) limonene, (b) α-pinene, (c) 1,8-cineole, (d) α-terpinene, (e) p-cymene, (f) γ-terpinene, (g) α –terpineol, (h) terpinen-4-ol, (i) α-terpinolene

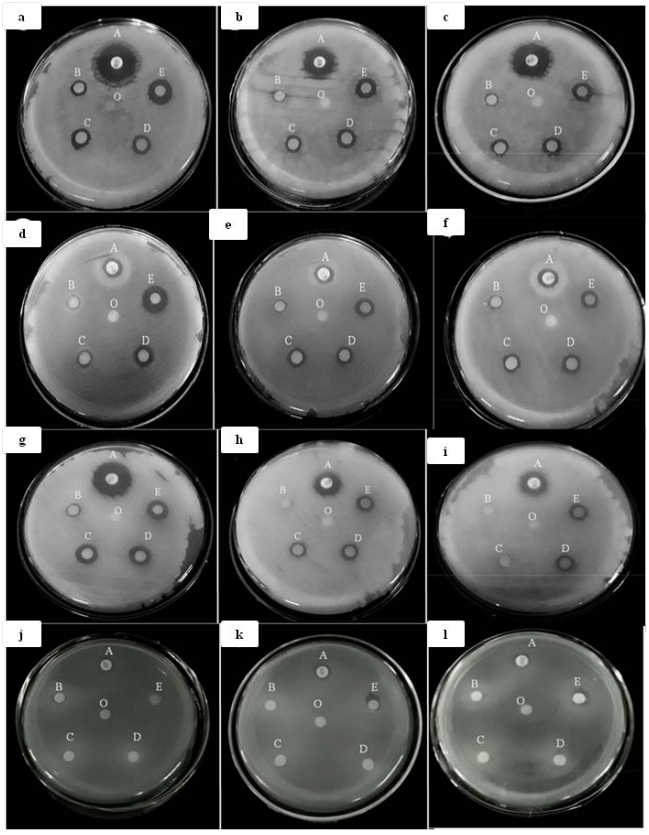

Fig. 2: Antibacterial activity of essential oils a) EONL against *E. coli*, b) EOIPL against *E. coli*, c) EOC against *E. coli*, d) EONL against *S. aureus*, e) EOIPL against *S. aureus*, f) EOC against *S. aureus*, g) EONL against *B. subtilis*, h) EOIPL against *B. subtilis*, i) EOC against *B. subtilis*, j) EONL against *P. aeruginosa*, k) EOIPL against *P. aeruginosa*, l) EOC against *P. aeruginosa*. A- Tetracycline (30 µg), B- 10% EO, C- 25% EO, D-50% EO, E-75% EO, O-Control

Determination of zone of inhibition

The antimicrobial activity of EONL, EOC and EOIPL of *M. alternifolia* at varying concentrations (10%, 25%, 50%, 75%) was compared with a standard antibiotic drug, tetracycline (30 µg/ml) against *E. coli*, *S. aureus, B. subtilis*, and *P. aeruginosa*. Among these concentrations from all the above three sources, 75% of EOs produced a clear zones around the discs (fig. 2) and are comparatively similar to antibiotic drug (Tetracycline (30 µg/ml). The zone of inhibition diameters were ranged from 2 to 6.5 mm, 2 to 7 mm, 0.5 to 6 mm and 0.5 to 3.5 mm for *E. coli*, *S. aureus, B. subtilis*, and *P. aeruginosa* respectively (table 3).

Table 3: Antibacterial activity of EONL, EOIPL and EOC obtained from *M. alternifolia* against bacterial pathogens

Source of essential oil	Concentration of essential oil (in weight percentage)	Bacterial pathogens			
		S. aureus	*E. coli*	*B. subtilis*	*P. aeruginosa*
EONL	10%	2±0.29	3±0.29	3±0.33	0.5±0.29
	25%	3±0.29	4±0.5	4.5±0.29	1±0.5
	50%	5±0.33	5±0.33	5±0.29	1±0.33
	75%	7±0.29	6.5±0.29	6±0.33	1±0.33
	Tetracycline	5±0	12±0	10±0	8±0
EOIPL	10%	2±0.33	2±0.29	1.5±0.29	1±0.29
	25%	3±0.29	3.5±0.33	2.5±0.29	1.5±0.29
	50%	4±0.29	5±0.29	3.5±0.29	2±0.33
	75%	5.5±0.29	6±0.33	4.5±0.33	3±0.33
	Tetracycline	5±0	12±0	10±0	8±0
EOC	10%	2±0.29	2±0.29	0.5±0.29	2±0.29
	25%	3±0.29	3±0.33	1±0.5	3±0.29
	50%	4±0.33	4.5±0.33	1.5±0.5	3±0.29
	75%	5±0.33	5±0.29	3±0.29	3.5±0.29
	Tetracycline	5±0	12±0	10±0	8±0

*Each value represents mean±SD done in triplicates.

Determination of minimum inhibitory concentration

In order to estimate the susceptibility of the bacteria towards EOIPL, the broth dilution method was carried out in two-fold dilution. EOIPL treated bacterial culture showed better results with an MIC value of 75×2^{-2}% (i. e. 18.75%) essential oil. The results also showed that maximum dilution of the sample produced least inhibition on bacterial growth or vice-versa an exponential increase in the bacterial population (fig. 3).

EOIPL-induced DNA fragmentation

The bacterial cultures were treated with the standard concentration (75%) of EOIPL and the subsequent change in the DNA pattern due to the induction of DNA fragmentation upon EO treatment for 24h was analyzed through agarose gel electrophoresis. The DNA patterns of the EOIPL treated *E. coli* and *S. aureus* cultures showed a significant decrease in the total DNA content in comparison to their untreated control cultures respectively (fig. 4) and hence the EO treatment could have inhibited the DNA synthesis in the bacterial cells.

Determination of bacterial cell death and morphological changes by confocal laser scanning microscopy

The standard concentration (75%) of EOIPL treated *S. aureus* culture was subjected to confocal laser scanning microscopic analysis in order to study the changes in the cell morphology. The confocal laser scanning microscopic images showed the absence of viable cells and disrupted cell structures in essential oil treated *S. aureus* culture (fig. 5) which was similar to the result obtained with standard antibiotic drug, tetracycline (30µg/ml) treatment.

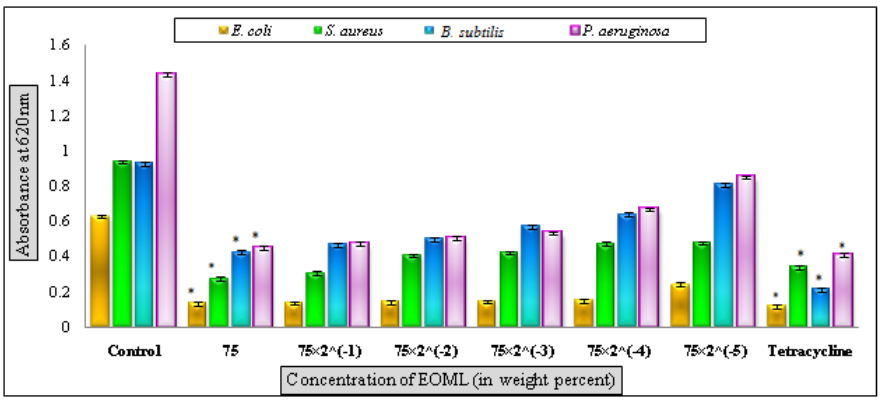

Fig. 3: Minimum inhibitory concentration for the EOIPL of *M. alternifolia* against bacterial pathogens. Each value is mean ± SEM of three independent observations. '*' represents statistical significance between control versus essential oil-treated organisms at $p < 0.05$

Molecular docking

Docking studies were performed by accelrys discovery studio 2.1 to find the possible binding site of Autolysin. From the binding site analysis, the binding pockets were identified and the largest binding pocket was selected as active site for the docking studies. The protein was docked with terpinen-4-ol (fig. 6), an important component present in the EO mixture and its corresponding Libdock score was calculated. The docking result proved that terpinen-4-ol showed the conventional hydrogen bond interactions (table 4) within active site at the amino acid residues ASN 286 and HIS 260 (fig. 7).

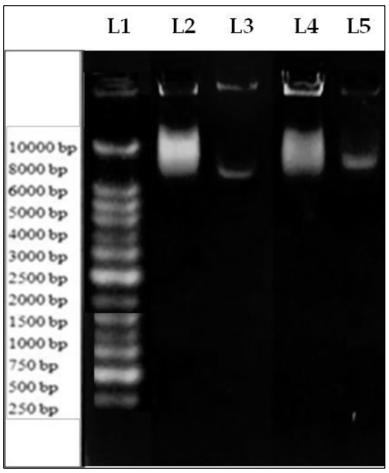

Fig. 4: EOIPL induced-DNA fragmentation in *E. coli* and *S. aureus*. Lane 1- Molecular weight marker; Lane 2- *E. coli* Control; Lane 3- *E. coli* treated with EOIPL; Lane 4- *S. aureus* Control; Lane 5- *S. aureus* treated with EOIPL

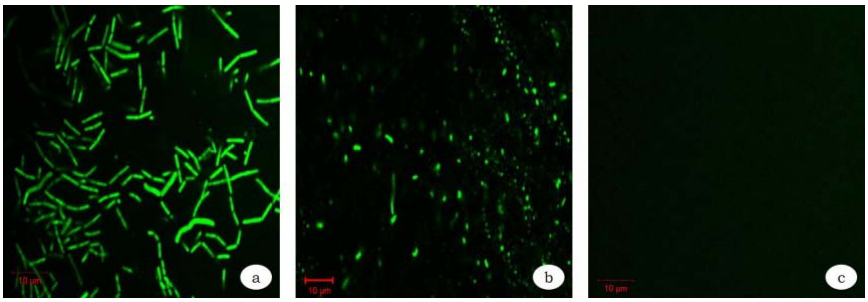

Fig. 5: EOIPL induced-cell death in *S. aureus* a-*S. aureus* Control, b- *S. aureus* treated with EOIPL, c- *S. aureus* treated with tetracycline (30µg/ml)

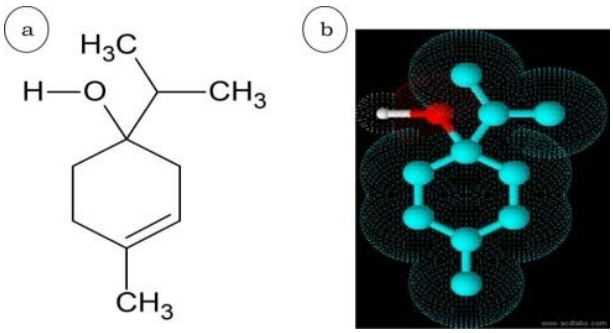

Fig. 6: 2a-Two-dimensional structure of terpinen-4-ol, b-Three-dimensional structure of terpinen-4-ol

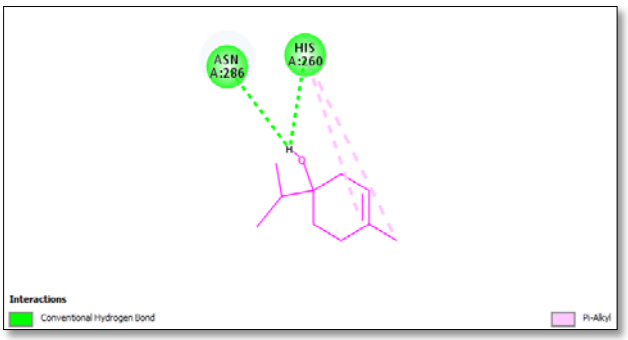

Fig. 7: Receptor- ligand interaction analysis through molecular docking studies, terpinen-4-ol interacted with autolysin with hydrogen bond interactions (green dotted lines)

Table 4: Interaction analysis of receptor-ligand complex

Compound	Target protein	PDB ID	No. of poses	Absolute energy	Libdock score	No. of H-bond	Residues	Bond length (Å)
Terpine-4-ol	Autolysin	2B0P	99	18.318	59.44	2	Asn286 His260	2.3686 2.2266

DISCUSSION

In order to treat drug-resistant bacteria, there must be new arrival of alternative strategies in drug to deal with infectious disease caused by antibiotic-resistant bacteria. Nowadays many authors have been found that various phytochemicals present in the herbal medicinal plants that could be able to treat such infections with good bactericidal effects. Plant derived essential oils have the ability to penetrate the skin and pass into the blood that showed a good synergistic effect of EOs. Most of the essential oils available today are extracted by steam distillation process as it causes minimum changes to the essential oil composition during extraction. Lee *et al.* (2013) has reported that TTO that comes from *M. alternifolia* leaves contains over 100 components (mostly monoterpenes, sesquiterpenes and terpene alcohols [9]. Studies demonstrated that terpinen-4-ol, a monoterpene is the most abundant (minimum 30%) component, besides this TTO also contains various amounts of 1, 8-cineole that causes skin irritant [10]. In this study the EOs from *M. alternifolia* showed best results in the treatment of skin infections (*E. coli* and *S. aureus)* as well as respiratory disease (*B. subtilis* and *P. aeruginosa*) causing bacteria. When compared with other bacteria, *S. aureus* was highly susceptible to EOIPL of *M. alternifolia* with larger inhibition zone whereas in *E. coli* showed less inhibition zone due to the presence of lipoproteins and lipopolysaccharides in their cellular walls that form a barrier to hydrophobic compounds. The EOIPL exhibited maximum inhibitory activity against the bacteria due to higher content of secondary metabolites in it. In MIC, the standard concentration (75%) of EOIPL disrupts the permeability barrier of cell membrane structures at the exponential growth stage of the cell and [11] the accompanying loss of chemiosmotic control is the most likely source of its lethal action at minimum inhibitory levels. Genomic DNA fragmentation is the indicator of cell death achieved by the activation endonucleases causing nicks in the DNA strands. This activity of the antibacterial reduces the replication of DNA. In the present study, the EOIPL induced DNA fragmentation in both *E. coli* and *S. aureus* cells, which might be due to the activation of endonucleases and disturbance in the hydrogen bonding involved in the stacking of nucleic acid bases and inhibition of DNA gyrase activity involved in unwinding of super coiled DNA through ATPase inhibition. The presence of glycosides, flavonoids and also phenolic and non-phenolic compounds present in the essential oil might induce the DNA cleavage in whole cells [12]. The standard concentration (75%) of EOIPL treated *S. aureus* culture was subjected to CLSM analysis that affected the cell viability by losing the ability to regulate potassium transfer across the membrane that leads to an outpouring of potassium interaction with from the cell [13]. Generally in bacteria the stress signals are sensed by the membrane-bound signal transduction system and hence disturbance caused in the membrane integrity is the measure of cytotoxicity of an antibacterial drug [14]. In accordance with these reports, our results from CLSM images showed that the EO derived from the *in-vitro* propagated leaves of *M. alternifolia* inhibited the growth *S. aureus* and also damaged the cell structural morphology, wherein decreased viable cells were observed. It has been suggested that terpenes promote membrane disruption binding to polysaccharides or enzymes promoting inactivation [15, 16]. Docking pattern was reliable with stability of the complex evaluated from its absolute energy [17] and also showed a firm interaction of hydrogen bond length being less than 3Å RMSD [18]. The mechanism of action of terpinen-4-ol causes the release of membrane bound cell wall autolytic enzymes will induce lysis eventually. The activation of autolytic enzymes may have been due to weakening of the cellwall and the subsequent rupture of the cytoplasmic membrane due to osmotic pressure [19].

CONCLUSION

Thus the study revealed the significance of the EOIPL derived from *M. alternifolia* leaves as a potential antibacterial agent and further investigation in this aspect could help in exploring their mode of action as well as the pathway in which it interacts with the target (receptor) proteins it in order to bring about bacteriostatic and bactericidal effect.

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AUTHORS CONTRIBUTIONS

Ms. Jeyakani did all the experimental work and manuscript writing. Ms. Kumari Niirmala did molecular docking studies and manuscript editing. Dr. Rajalakshmi did research guidance and critical revision of manuscript.

CONFLICT OF INTERESTS

The authors declare that they have no conflict of interest.

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