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ORIGINAL ARTICLE

Melaleuca alternifolia ESSENTIAL OIL COMBAT ANTIBIOTIC-RESISTANT *Streptococcus mutans* SUPPRESSING QUORUM-SENSING-DEPENDENT VIRULENCE AND BIOFILM FORMATION

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ABSTRACT

The practical application of *Melaleuca alternifolia* essential oil, commonly referred to as tea tree oil, as a topical antiseptic is notable. Its chemical composition has been extensively characterized, comprising primarily cyclic monoterpenes. Approximately half of these compounds are oxygenated, while the other half are hydrocarbons. *Streptococcus mutans*, a Gram-positive bacterium commonly inhabiting the human oral cavity, notably contributes to tooth decay. To ascertain the minimum inhibitory concentration (MIC) of *M. alternifolia* essential oil, the broth microdilution method was employed. Subsequent to these determinations, antibiofilm investigations, quantification of extracellular polymeric substances (EPS), and growth curve analysis were carried out. The findings indicated that *M. alternifolia* essential oil inhibited *S. mutans* at a concentration of 10 mg/mL. Statistical examination of the biofilm assay demonstrated a 60.5% inhibition of biofilm formation at a subinhibitory concentration of 5 mg/mL, with no discernible impact on the growth of *S. mutans*. Furthermore, at this concentration, *M. alternifolia* essential oil reduced the production of EPS by *S. mutans* by 30.3%. In conclusion, *M. alternifolia* essential oil exhibits notable potential as a topical antiseptic, characterized by its predominantly terpene composition. This study underscores its efficacy in inhibiting *S. mutans* biofilm formation and reducing EPS production, suggesting promising avenues for dental care applications.

KEY WORDS: Antibiotic, natural products, biofilm inhibitor, terpinen-4-ol.

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INTRODUCTION

Researchers have uncovered the antibacterial properties and skin permeability attributes of essential oils, catalyzing the emergence and evolution of aromatherapy (Ali et al., 2015). Essential oils represent a rich reservoir of biologically active compounds. The exploration of antibacterial properties within extracts from aromatic plants, particularly essential oils, has garnered increased interest. As a result, it is reasonable to expect a range of plant constituents to exhibit both specific and broad-spectrum antibacterial effects, along with potential antibiotic properties, within these oils (Vaou et al., 2021).

Numerous essential oils extracted from plants have been shown to have antibacterial properties against various bacteria and fungi. Although they typically exist as complicated mixes, their main monoterpenoid components may usually be used to explain their function (Chouhan et al., 2017). The use of *Melaleuca alternifolia* essential oil as a topical antiseptic goes back centuries. It has recently gained recognition as a safe, organic, and effective antiseptic. As a result, it has regained popularity, with many pharmaceutical and cosmetic products designed for external use incorporating it as a primary antibacterial agent or a natural preservative (Ali et al., 2015). Terpinen-4-ol, extracted through steam distillation from tea tree leaves and twigs, is the primary chemical constituent responsible for its antibacterial properties, yielding 1.8% of the plant's essential oil. Tea tree oil's chemical composition has been meticulously characterized, and it consists primarily of cyclic monoterpenes with an equal distribution of oxygenated and hydrocarbon compounds. Terpinen-4-ol contributes significantly to tea tree oil's broad-spectrum antibacterial efficacy (Cordeiro et al., 2020).

Tea tree oil has been scientifically proven to have a variety of medical benefits, including antiseptic, antifungal, and antibacterial properties (Kairey et al., 2023). Traditionally, Indigenous Australians have used it to treat colds, sore throats, and a variety of skin conditions (Nascimento et al., 2023). The theory that oil from a specific *M. alternifolia* clone has superior microbicidal activity has been made (May et al., 2000), but the evidence is not compelling. The international standard's components were chosen for various reasons, including verifying provenance and ensuring biological activity. For example, in terms of provenance, including minor components such as sabinene, globulol, and viridiflorol can be beneficial because it may make the formulation of synthetic oil from individual components difficult or economically impractical. In terms of biological activity, *M. alternifolia* essential oil antimicrobial properties are primarily attributed to terpinen-4-ol, the oil's major component (Lam et al., 2020).

According to research, monoterpenes can infiltrate and damage cell membrane structures, contributing to their antimicrobial effects. Due to their

lipophilic nature, monoterpenes tend to migrate from an aqueous environment into membrane structures (Zengin & Baysal, 2014). Tea tree oil ingestion can be highly toxic, with serious health consequences. Symptoms may include drowsiness, confusion, hallucinations, coma, unsteadiness, weakness, nausea, vomiting, diarrhea, abnormal blood cell counts, and severe rashes. To avoid accidental ingestion, tea tree oil should be stored away from children and pets (Hammer et al., 2006). Tea tree oil should not be applied near or inside the mouth because it can have negative consequences. Additionally, applying tea tree oil to the skin may cause allergic reactions. As the oil matures and its chemical composition changes, it increases the likelihood of causing an allergic reaction. Skin irritation, allergic contact dermatitis, systemic contact dermatitis, linear immunoglobulin A illness, erythema multiforme-like reactions, and systemic hypersensitivity reactions are all possible negative consequences (de Groot & Schmidt, 2016). *M. alternifolia* thrives in its natural habitat and contributes to local ecosystems. However, its introduction into non-native areas should be carefully controlled to avoid ecological imbalances. Sustainable cultivation practices are critical to ensuring the long-term availability of tea tree oil while minimizing environmental impact (Chouhan et al., 2017).

Streptococcus mutans, a gram-positive, facultatively anaerobic coccus, is prevalent in the human oral cavity and significantly contributes to tooth decay (Lemos et al., 2019). Belonging to the group termed *streptococci*, which encompasses all species within the genus *Streptococcus*, it plays a pivotal role in dental health (Szafrański et al., 2021). *S. mutans* comprises a group of seven closely related species collectively known as *mutans streptococci*. These bacteria primarily inhabit the mouth, throat, and intestine. Dental caries, or tooth decay, is attributed to various mechanisms, including adhesion to enamel surfaces, production of acidic metabolites, accumulation of glycogen reserves, and synthesis of extracellular polysaccharides (EPS) (Liu et al., 2020). Within the oral cavity of humans, particularly within dental plaque, a complex multispecies biofilm that forms on tooth surfaces, *S. mutans* finds its natural habitat. A recent study observed that *M. alternifolia* exhibits greater efficacy than *S. costus* against *Porphyromonas gingivalis*, *Enterococcus faecalis*, and *S. mutans*. This suggests that oral dentifrices containing *M. alternifolia* extracts could potentially contribute to maintaining oral health and treating conditions like periodontitis (BinShabaib et al., 2022). Additionally, *M. alternifolia* holds potential for use in mucosal areas due to its ability to combat microorganisms and reduce inflammation. For mucosal applications, such as in oral or vaginal health, its effectiveness can be leveraged in carefully formulated products that ensure proper dilution and safety to avoid irritation or toxicity. Despite its established use on skin, exploring *M. alternifolia* oil's benefits in mucosal contexts could provide new therapeutic options, provided rigorous research and formulation standards are met to ensure efficacy and safety in these sensitive areas (Chouhan et al., 2017; Lam et al., 2020).

Despite the abundance of reviews on the medicinal properties of *M. alternifolia* (tea tree oil), none offer comprehensive details regarding its antimicrobial, antibiofilm activities, and pharmaceutical applications. If our trial results show that tea tree oil is an effective antibacterial agent, it could be ideal for use in various solutions without adverse effects on human health or the environment (Nascimento et al., 2023). This study aimed to assess the antibiofilm and antivirulence capabilities of *M. alternifolia* essential oil against *S. mutans*.

MATERIAL AND METHODS

Oil Acquisition

Melaleuca alternifolia essential oil was obtained from Aramacks Pvt. Ltd. in Delhi, India, for this study. A qualified botanist independently verified the oil's authenticity. The oil was stored in an amber glass bottle at room temperature to protect it from light and oxidation.

Identification of chemical composition by Gas Chromatography-Mass Spectrometry (GC-MS)

The Gas Chromatography-Mass Spectrometry (GC-MS) analysis procedure followed the methodology described by Sankar Ganesh and Ravishankar Rai (2018). In brief, oil analysis was carried out on a Shimadzu QP-2010 gas chromatograph equipped with a Shimadzu GCMSQP-2010 plus detector and an SGE BPX-5 fused silica capillary column. The oil was diluted and injected into the device at a volume of 0.1 mL. The oven temperature program began at 60 °C and was held for 1 min before increasing to 230 °C at a rate of 3 °C/min and remaining for 10 min. Helium was used as the carrier gas, with a 1.0 mL/min flow rate. The detector and injector temperatures were set to 250 °C and 230 °C, respectively. The retention index (RI) was calculated using a mixture of n-alkanes and compared to the National Institute of Standards and Technology (NIST) to identify analytes. The electronic library and literature data rely on RI. The area under peaks was used to identify essential oil components.

Bacterial strain and growth environment

The strains of *S. mutans* employed in this research were acquired from Saveetha Dental College and Hospital in Tamil Nadu, India. These bacterial strains were cultured aerobically in Brain Heart Infusion (BHI) broth at 37 °C with agitation at 100 rpm for a duration of 24 h. Previous studies have outlined standard microbiological procedures for identifying genera and species (Holt & Krieg, 2001). Distinctive growth patterns were observed on both BHI agar

and blood agar plates. Various phenotypic assays were conducted and recorded, including gram staining, catalase, oxidase, motility, and citrate utilization. Regular sub-culturing of bacterial cultures was performed for experimental purposes, with consistent biochemical confirmation.

Antimicrobial activity of M. alternifolia *essential oil*

The antibacterial effectiveness of tea tree oil was assessed using the standard agar well-diffusion method (Manzanelli et al., 2023). Cultures of *S. mutans* were evenly spread onto Mueller Hinton agar (MHA) plates using a sterile swab saturated with the bacterial suspension. A sterile cork borer was used to create an 8 mm-diameter well in MHA plates, which were then filled with 30 µL (range of 20 mg/mL) *M. alternifolia* essential oil. Afterward, the plates were positioned upright in an incubator set at 37 °C for one day. Following this incubation period, the diameter of the inhibition zones surrounding the wells was measured using a Vernier caliper to assess the antibacterial efficacy.

Antibiogram of S. mutans

Streptococcus mutans antibiotic susceptibility was determined using the Kirby-Bauer disk diffusion method. The *S. mutans* bacterial culture was initially evenly distributed onto MHA plates using a sterile swab saturated with the bacterial suspension. These plates were then tested against a selection of conventional antibiotics. The criteria for selecting these antibiotics were based on their established efficacy in clinical settings for treating *S. mutans* infections, their spectrum of activity, and their relevance to our study objectives. The antibiotics tested included metronidazole (30 µg), ampicillin (10 µg), rifampicin (5 µg), cefotaxime (4 µg), and ciprofloxacin (15 µg) (HiMedia, Mumbai, India).

Evaluation of minimum inhibitory concentration

The minimum inhibitory concentration (MIC) of *M. alternifolia* essential oil against *S. mutans* was determined using the broth microdilution method across a range of concentrations from 10 mg/mL - 0.019 mg/mL. The MIC assessment followed standard protocols (Packiavathy et al., 2014). Briefly, 20 µL of BHI broth culture with the cell mass equivalent of 0.5 McFarland turbidity standard (equivalent to 1.5×10^8 CFU/mL) was added to tubes containing BHI broth. The bacterial culture tubes were subjected to serial dilution with *M. alternifolia* essential oil and subsequently incubated at 37 °C for 24 h. Bacterial growth was monitored, and 2,3,5-triphenyl tetrazolium chloride (TTC) was added to indicate cell viability. After incubating for 20 min, color changes were checked to confirm the results. The MIC was determined as the lowest concentration of *M. alternifolia* essential oil, which significantly

inhibited *S. mutans* growth. Sub-MIC antibiofilm studies were then carried out using these MIC values.

Biofilm inhibition assay

The effect of *M. alternifolia* essential oil on biofilm formation was evaluated using the crystal violet (CV) staining assay method (Sankar Ganesh & Ravishankar Rai, 2018). Overnight culture of *S. mutans* (20 µL) was inoculated into a microtiter plate with 180 µL of fresh BHI medium and varying concentrations ranging from 10 mg/mL – 0.019 mg/mL. The plate was then incubated for two days at 37 °C. Following the incubation period, the biofilm on the surface was stained with 0.1% CV solution. Washing the plate with sterile distilled water removed any planktonic cells that were present. After a 10-minute incubation, the CV-bound biofilm was dissolved in 200 µL of 70% ethanol. The concentration was measured using a UV-Vis spectrophotometer (Biobase BK-D 590 Double beam scanning UV/Vis China) by measuring crystal violet's optical density (OD) at 520 nm for each well, using the control for comparison. Additionally, the OD at 600 nm was measured for the overnight *S. mutans* culture alone to determine if growth had any influence.

The Inhibition Percentage was calculated using the following equation:

$$\text{Inhibition Percentage} = [(\text{Control OD 520nm} - \text{Treated OD 520nm}) / (\text{Control OD 520nm})] \times 10$$

Analysis of bacterial growth curve

The growth of *S. mutans* was monitored in the presence and absence of *M. alternifolia* essential oil at a concentration of 5 mg/mL. Cultures were incubated at 37 °C for a day, with cell density measurements taken at OD 600 nm every h throughout the incubation period.

Estimation of Exopolysaccharide (EPS) in S. mutans

The exopolysaccharide (EPS) extraction procedure followed a pre-established protocol (Venkatramanan et al., 2020). Initially, cultures of *S. mutans* were grown aerobically in BHI broth containing 1% glucose, and *M. alternifolia* essential oil was added at concentrations ranging from 10 mg/mL – 0.019 mg/mL, along with a control group (*S. mutans* without *M. alternifolia* essential oil) and incubated at 37 °C in a shaking incubator (100 rpm) for a day. After incubation, the cultures were centrifuged at 10,000 rpm for 15 min. The resulting bacterial pellets were reconstituted in 50 mL of high-salt

buffer (HSB) containing 10 mM KPO_4, 7.5 mM NaCl, and 2.5 mM $MgSO_4$, and centrifuged again at 10,000 rpm for 30 min. Ethanol was added to the supernatant (triple the volume of 3-HSB) and then centrifuged at 10,000 rpm for 30 min. The isolated EPS was subsequently dissolved in Milli-Q water. For analysis, 1 mL of precipitated EPS was mixed with 1 mL of cold 5% phenol and 5 mL of concentrated H_2SO_4, resulting in a red coloration. The intensity of this colour in both the control and treated EPS samples was measured at OD 490 nm using a UV-Vis spectrophotometer (Biobase BK-D 590 Double beam scanning UV/Vis China).

Statistical evaluation

Each experiment was conducted in triplicate, and statistical significance was thoroughly assessed for the biofilm assay, growth curve analysis, and EPS quantification. The statistical analysis was performed using Microsoft Excel 2018, a software developed by Microsoft Corporation. A *p*-value of less than 0.05 indicated a statistically significant difference.

RESULTS

GC-MS analysis

The compounds present in *M. alternifolia* essential oil were characterized by comparing each peak's retention times and mass spectra with information from the NIST library. The specified results and the percentage range for *M. alternifolia* essential oil recommended by ISO 4730:2017 are delineated in Table 1. The primary constituents include terpinen-4-ol, present at 35.1%, significantly higher, and within the recommended concentration of 35% to 48% as per ISO standards. Other significant components are γ-terpinene at 20.31%, α-terpinene at 10.13%, and 1,8-cineole (Eucalyptol) at 4.1%. These compounds contribute to the oil's therapeutic properties and are within the recommended ranges: γ-terpinene (14.0 to 28.0%), α-terpinene (6.0 to 12.0%), and 1,8-cineole (≤10%).

Minor constituents include terpinolene (3.45%), p-cymene (3.12%), α-terpineol (2.65%), aromadendrene (1.3%), α-thujene (0.82%), and viridifloral (0.15%). These components also fall within or near the recommended ranges ISO sets, ensuring the oil's quality and efficacy. For instance, α-terpineol falls within the 2.0 to 5.0% range, aromadendrene is within the 0.2 to 3.0% range, and viridifloral is within the ≤1.0% limit. Overall, the analysis validates the consistency and quality of the *M. alternifolia* essential oil.

Table 1. Identification of the major compounds of *Melaleuca alternifolia* essential oil compared with the recommended standard composition.

Compound	%	Retention time (min)	% recommended (ISO 4730:2017)
α-terpineol	2.6	12.62	2.0 to 5.0
terpinen-4-ol	35.1	14.36	35 to 48
1,8-cineole (Eucalyptol)	4.1	9.65	≤ 10
Terpinolene	3.4	9.44	1.5 to 5
γ--terpinene	20.3	8.7	14.0 to 28.0
p-cymene	3.1	6.59	0.5 to 8.0
Aromadendrene	1.3	23.56	0.2 - 3.0
α-thujene	0.8	4.25	------
Viridifloral	0.2	28.65	≤ 1.0
α-terpinene	10.1	7.25	6.0 to 12.0

Biochemical Characterization of S. mutans

Various morphological types were observed, and the morphological attributes of the bacterial isolate were evaluated on a conventional culture medium. In Gram staining, *S. mutans* exhibited characteristics indicative of Gram-positive cocci. Additionally, they tested negative for catalase activity, were non-motile, and exhibited no oxidase activity.

Antimicrobial susceptibility

The observed diameter of the inhibition zone attributed to *M. alternifolia* essential oil was 19 mm, indicating its effectiveness against *S. mutans* in terms of antimicrobial activity. This inhibition zone was observed at a concentration of 20 mg/mL.

Antibiotic sensitivity testing (ABST)

Antibiotic susceptibility testing was carried out following the protocols outlined by the Clinical and Laboratory Standards Institute (CLSI) in 2022. It was found that metronidazole, ciprofloxacin, and rifampicin had no zone of inhibition, indicating that *S. mutans* is resistant to these drugs (Table 2).

Table 2. Antibiogram of *Streptococcus mutans* against different antibiotics.

S1 nº	Antibiotic	Inhibition zone diameter (mm)
1	Metronidazole	R*
2	Ampicillin	11 ± 1.6
3	Rifampicin	R
4	Cefotaxime	14 ± 1.5
5	Ciprofloxacin	R

*R indicates resistance to the antibiotic. Values represent the mean inhibition zone diameter (mm) ± standard deviation for *S. mutans.*

M. alternifolia essential oil inhibited *S. mutans*

A serial dilution technique was utilized to assess the antibacterial effectiveness of *M. alternifolia* essential oil over a spectrum of concentrations ranging from 10 mg/mL to 0.019 mg/mL (Figure 1A). The results revealed growth inhibition of *S. mutans* at the 10 mg/mL concentration endpoint. Consequently, sub-MIC concentrations of *M. alternifolia* essential oil were used to investigate its anti-biofilm and anti-virulence properties.

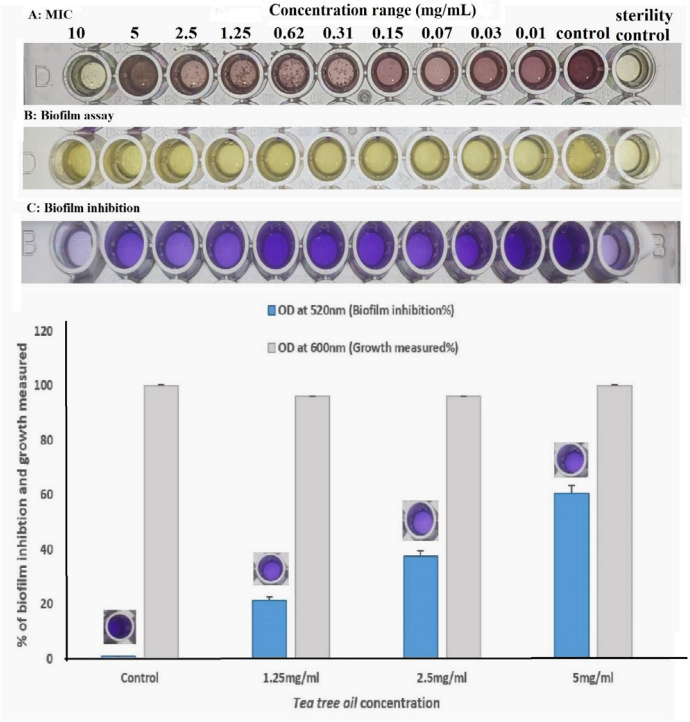

Figure 1. Minimum inhibitory concentration and biofilm inhibition assay of *Melaleuca alternifolia* essential oil against *Streptococcus mutans*. (A) Growth is indicated by the presence of a cherry red colour, observed at concentrations ranging from 5 mg/mL to 0.019 mg/mL. The absence of growth is indicated by no cherry red color (10 mg/mL). (B) Effect of *M. alternifolia* essential oil on *S. mutans* growth after 48 h incubation. The concentrations tested are 10 mg/mL, 5 mg/mL, 2.5 mg/mL, and 1.25 mg/mL. (C) Biofilm inhibition of *S. mutans* treated with sub-inhibitory concentrations of 5 mg/mL, 2.5 mg/mL, and 1.25 mg/mL of *M. alternifolia* essential oil. (D) Percentage of biofilm inhibition in *S. mutans* at sub-inhibitory concentrations of *M. alternifolia* essential oil. At 5 mg/mL, there is 60.5% inhibition; at 2.5 mg/mL, there is 37.6% inhibition; and at 1.25 mg/mL, there is 21.5% inhibition.

M. alternifolia essential oil inhibited biofilm in *S. mutans*

The capability of *M. alternifolia* essential oil to hinder biofilm formation by *S. mutans* was assessed using 0.1% crystal violet dye in a static microtiter plate. Spectrophotometric analysis indicated that tea tree oil at concentrations of 1.25 mg/mL, 2.5 mg/mL, and 5 mg/mL significantly decreased biofilm formation by approximately 21.5%, 37.6%, and 60.5%, respectively (Figure 1, B, C, D).

Bacterial growth curve analysis

The growth curve analysis was performed on *S. mutans* cultures with and without *M. alternifolia* essential oil. The findings suggest that at a concentration of 5 mg/mL, *M. alternifolia* essential oil did not inhibit bacterial growth (as depicted in Figure 2). The spectrophotometric evaluation indicated no notable difference between the untreated and treated bacterial cells at 600 nm.

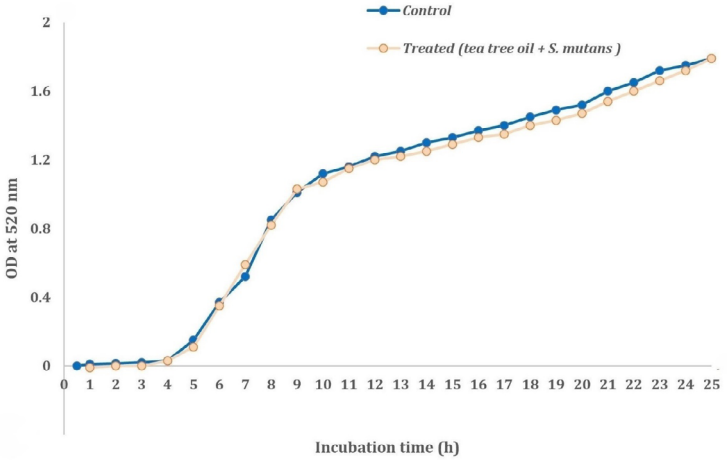

Figure 2. Displays the growth curve analysis of *Streptococcus mutans* under two conditions. Grown without any treatment (control) and in the presence of *Melaleuca alternifolia* essential oil (tree tea oil) at a 5 mg/mL concentration.

Effects of M. alternifolia essential oil on EPS synthesis in S. mutans

The study investigated the influence of *M. alternifolia* essential oil on the synthesis of EPS, which is crucial for maintaining the structural integrity of biofilms. The findings revealed that *M. alternifolia* essential oil, at concentrations of 1.25 mg/mL, 2.5 mg/mL, and 5 mg/mL, resulted in a reduction in EPS production in *S. mutans* by 10.75%, 18.8%, and 30.25%,

respectively (Figure 3). These findings highlight the effectiveness of *M. alternifolia* essential oil in reducing EPS production in *S. mutans*, thereby influencing the architecture of the biofilm.

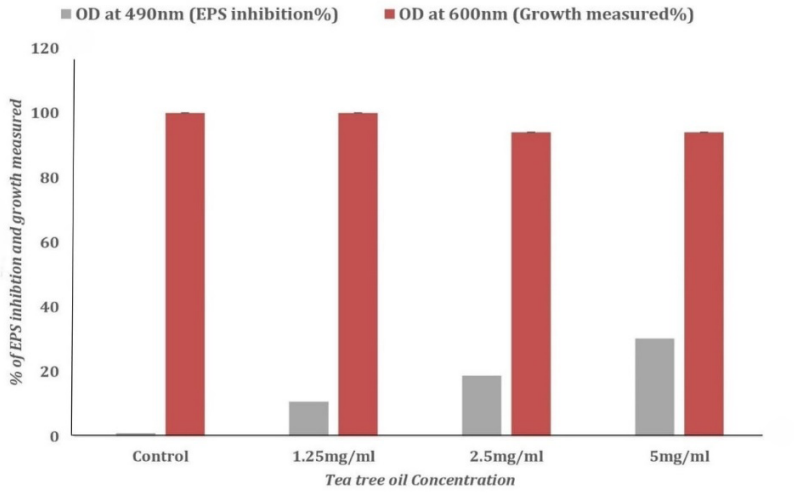

Figure 3. Represents the inhibition of exopolysaccharide (EPS) production and growth. *Melaleuca alternifolia* essential oil (tree tea oil) suppressed the EPS pigment of *Streptococcus mutans*, decreasing it to levels of 10.75%, 18.8%, and 30.25% at sub-inhibitory concentrations of 1.25 mg/mL, 2.5 mg/mL, and 5 mg/mL, respectively.

DISCUSSION

The *Melaleuca alternifolia* essential oil has been shown to inhibit a wide variety of bacteria, making it a potential replacement for topical antibiotic use (Song et al., 2020). The correlation between bacterial strains' resistance to multiple drugs (MDR) and their vulnerability to *M. alternifolia* essential oil is notably inverse. This suggests that as a bacterium becomes resistant to more antibiotics, it's also more likely to resist tea tree oil. However, it's essential to consider the potential influence of the extract on specific respiratory enzymes or metabolic processes, which cannot be entirely discounted (Hammer et al., 2003). Research findings have shown that *M. alternifolia* essential oil disrupts respiration and induces leakage of cellular potassium in *Escherichia coli*. These observations and the data provided indicate that the extract compromised the cell membrane integrity of *E. coli*, *S. aureus*, and *Candida albicans*. Small ions like H+, K+, Na+, and Ca2+ cannot pass through the cytoplasmic membranes of bacteria or the plasma and mitochondrial membranes of yeast,

which enables cells and organelles to regulate the entry and exit of various compounds (Carson et al., 2006).

The extract's antibacterial properties come from its capacity to compromise the permeability barrier of microbial membrane structures. Similar to other broad-spectrum, membrane-active disinfectants and preservatives such as phenol derivatives, chlorhexidine, and p benzoic acid derivatives, this mode of action is the same against *E. coli*, *S. aureus*, and *C. albicans* (Geremias et al., 2021).

Melaleuca alternifolia essential oil is a complex mixture of more than 100 different terpenes and alcohols. The oil contains terpinen-4-ol, 1,8-cineole, α-terpineol, terpinolene, α- and β-terpinene, accounting for up to 90% of its total content (de Groot & Schmidt, 2016). According to recent research, the ingredient 1,8-cineole is no longer considered the primary cause of hypersensitivity reactions, previously linked to adverse side effects like mucus and skin irritation. While high concentrations of the extract have been linked to cytotoxicity, the current evidence calls for additional *in vivo* testing to confirm this association (Pazyar et al., 2013). This investigation used a 5% oil concentration in broth to match the typical concentration found in several foods.

Our study identified the compounds present in *M. alternifolia* essential oil. The results reveal that terpinen-4-ol is the most prevalent compound, making up 35.11% of the oil at a retention time of 14.36 min (Table 1). In comparison, recent studies have shown that terpinen-4-ol is the most prevalent compound (Johansen et al., 2022). This highlights the significance of terpinen-4-ol in essential oil. Additionally, Puvača et al. (2021) observed the antimicrobial activity of *M. alternifolia* essential oil using the disk diffusion method and found it effective against *Citrobacter koseri*, *Salmonella typhi*, and *E. coli*. The inhibition zones ranged from 13 to 21 mm. This activity was generally more intense than that observed in our study. Similarly, in another study Kim et al. (2004) found that *M. alternifolia* oil consisted of terpinen-4-ol (43.2%), γ-terpinene (20.6%), and α-terpinene (9.6%). Elmi et al. (2019) analysed their *M. alternifolia* oil sample and discovered 41.49% terpinen-4-ol, 20.55% γ-terpinene, and 9.59% α-terpinene. Both studies found higher percentages of these main components than our *M. alternifolia* oil.

Melaleuca alternifolia essential oil can be used topically to kill germs in common carrying areas like the perineum, groin, armpits, nostrils, and hairline. It can also be used in hand-washing formulas to reduce the spread of various multi-resistant germs associated with hospital-acquired infections. The current research underscores the antimicrobial and antibiofilm capabilities of *M. alternifolia* essential oil against *S. mutans*. Initial evaluations demonstrated that the essential oil exerted antimicrobial effects at a minimum concentration of 10 mg/mL. However, a study found that the MIC of *M. alternifolia* against

S. aureus, *E. coli*, and *Pseudomonas aeruginosa* were 2 mg/mL, 8 mg/mL, and 12 mg/mL, respectively, indicating significant antimicrobial activity (Zhang et al., 2018). Similarly, another study found that *M. alternifolia* essential oil effectively killed all clinical strains of *S. aureus*, including planktonic and biofilm-forming strains. They found that a 0.5% concentration of *M. alternifolia* essential oil killed 99% of bacteria in just 60 min (Kwieciński et al., 2009). This concentration was more effective but also higher than the one used in our study.

Furthermore, our findings suggest that *M. alternifolia* essential oil effectively hindered *quorum sensing* (QS)-mediated biofilm formation by *S. mutans* in a dose-dependent manner at sub-MIC concentrations. At its highest concentration of 5 mg/mL, *M. alternifolia* essential oil notably suppressed biofilm formation while having no impact on the growth of planktonic cells (Figure 1D). The effect of tea tree oil on biofilm formation has been observed. A study found that *M. alternifolia* essential oil nanoparticles had a positive effect on inhibiting Gram-negative bacteria (Comin et al., 2016). Recently, a study found that *M. alternifolia* essential oil inhibited *C. albicans* adhesion and biofilm formation on both biotic and abiotic surfaces (Sudjana et al., 2012). Similarly, Al-Shuneigat et al., (2005) observed that the *M. alternifolia* essential oil product significantly reduced adherence to polystyrene in biofilm-forming *Staphylococcus* strains at a concentration of 625 ppm. Additionally, our investigation indicated that *M. alternifolia* essential oil, at concentrations of 1.25 mg/mL, 2.5 mg/mL, and 5 mg/mL, significantly reduced EPS production by *S. mutans* by 10.75%, 18.8%, and 30.25%, respectively (Figure 3).

Altogether, findings from our study highlight the potential of *M. alternifolia* essential oil to disrupt biofilm formation by targeting EPS synthesis in *S. mutans*. Moreover, its ability to combat microorganisms and reduce inflammation makes it suitable for mucosal applications, such as in oral or vaginal health. Carefully formulated products that ensure proper dilution and safety can leverage their effectiveness while avoiding irritation or toxicity. However, further studies are needed to isolate active compounds with anti-QS and anti-biofilm properties.

Our findings indicate that *M. alternifolia* essential oil possesses significant antibacterial properties and effectively reduces biofilm formation, as demonstrated through comprehensive *in vitro* studies. These results enhance our understanding of *S. mutans* behavior and offer potential avenues for targeted interventions in managing oral infections. This research contributes to the broader context of antimicrobial resistance (AMR) and oral health, emphasizing the importance of further investigation to develop effective strategies for controlling microbial populations in the oral cavity.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest to disclose.

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