



Original Article

Antibacterial Activity of *Melaleuca alternifolia* (tea tree essential oil) on Bacteria of the Dental Biofilm

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Abstract

Objective: To evaluate the antibacterial activity of tea tree EO on *Streptococcus mutans* (ATCC 25175), *S. salivarius* (ATCC 7073) and *Lactobacillus rhaminosus* (ATCC 9595). **Material and Methods:** The antibacterial activity of *M. alternifolia* EO was evaluated by the broth dilution method, by which minimum inhibitory and bactericidal concentrations (MIC and MBC) were determined. Serial dilutions range from 70243.90 µg/mL to 26.14 µg/mL. The MIC evaluation was performed in 96-well microplates, in which 100 µL of Brain Heart Infusion (BHI), 100 µL of the EO dilution and 5 µL of the inoculum (final concentration = 5x10⁵ CFU/mL) were inserted. After 24 h of incubation, MIC was determined as the lowest concentration capable of inhibiting microbial growth, identified by the resazurin reaction (100 µg/mL). CBM was identified by the absence of subculture growths (50 µL) of dilutions equal to or greater than MIC. Tests were performed in triplicate and at three different times (n = 9). Pharmacological controls (0.05% and 0.12% Chlorhexidine), growth and sterility were used to validate the results. **Results:** The MIC of *M. alternifolia* compared to *S. mutans*, *S. salivarius* and *L. rhaminosus* was 1940.16 µg/mL, 3977.34 µg/mL and 3977.34 µg/mL, respectively. The MBC values were 70243.90 µg/mL, 3977.34 µg/mL and 34265.31 µg/mL, respectively. **Conclusion:** The essential oil of *M. alternifolia* presented antibacterial activity against the microorganisms evaluated when in high concentration.

Keywords: Tea Tree Oil; Phytotherapy; Streptococcus; Anti-Bacterial Agents.

Introduction

The control of the dental biofilm allows the maintenance of oral health and prevents the development of diseases such as caries and periodontal disease, which are strongly related to tooth loss [1]. Individuals who are motor deficient or are under development of this ability have difficulty in mechanical brushing [2]. This factor, associated with diet and the presence of cariogenic microbiota, alters the buccal balance [3].

For biofilm control in patients at risk of caries, educational and therapeutic actions such as supervised brushing and topical application of fluoride are particularly encouraged in communities with limited access to health services, but there is little scientific evidence that these interventions may reduce dental caries in children [4,5]. However, biologically active products are being incorporated into toothpastes and mouthwash solutions as supplements to traditional methods, this process may contribute to the cheapness and clinical efficiency of these formulations [6].

Natural products have demonstrated therapeutic potentials in the treatment of oral diseases [7]. Moreover, in relation to commercial products, they present low cost and minimize the side effects of conventional treatment [8].

The antimicrobial properties of *Melaleuca essential oil alternifolia* to oral biofilms have been reported in several studies [9-12]. In order for the product to have satisfactory antimicrobial activity, the Minimum Inhibitory Concentration (MIC) should be equal to or less than 100 µg/mL [13], however, the different commercial presentations of the natural products and the variety of methods to verify the antimicrobial activity make it difficult to compare of the studies [14].

It is important to note that in the early stages of development, the dental biofilm consists predominantly of oral streptococci and actinomyces, which colonize the buccal cavity in relation to commensalism. However, subsequent population changes lead to a higher proliferation of acidophilic microorganisms, such as *Lactobacillus* species, or Gram-negative anaerobes in the subgingival biofilm, which contribute to the onset and progression of dental caries and periodontal disease [15].

Thus, the antimicrobial control must interrupt or prevent the transition from the commensal to the pathogenic microbiota, allowing the microbial balance to be preserved. Therefore, the objective of the present study was to evaluate, *in vitro*, the antibacterial activity of *M. alternifolia* essential oil against strains of *Streptococcus mutans*, *Streptococcus salivarius* and *Lactobacillus rhaminosus*.

Material and Methods

General Outline of the Study

Quantitative study with inductive approach, descriptive procedure and direct laboratory documentation technique [16].

Products Tested

The essential oil of *M. alternifolia* was obtained from Laszlo Aromatherapy & Aromatology® (Belo Horizonte, Minas Gerais, Brazil), which produces and markets on an industrial scale. The physico-chemical properties were described by the supplier through a technical report (Table 1).

Table 1. Technical specifications by gas chromatography of the essential oil used in the study, supplied by the manufacturer.

Constituent	%	Constituent	%
α -pinene	2.45	γ -terpinene	6.75
cis-calamene	1.16	Terpinolene	1.25
β -pinene	0.75	Terpinen-4-ol	50.98
α -terpinene	11.16	α -terpineol	5.16
Limonene	3.10	β -gurgunene	1.20
1.8- cineol	0.70	Viridiflorene	0.84

For the microdilution assay, an emulsion of the essential oil was prepared using an emulsifying agent (Tween 80) (Sigma-Aldrich, St. Louis, Missouri, USA). To this end, 0.8 ml of the essential oil, 0.05 ml of Tween 80 and 4.2 ml of sterile distilled water were added in sterile glass tubes. The assembly was shaken for 5 minutes in a Vortex type shaker apparatus (Mod. AP56, Phoenix) and the final concentration obtained was 14.4000 $\mu\text{g}/\text{mL}$ (144 mg/mL) considered in this study the initial standard solution for the evaluated product. Dilutions were obtained according to the supplier's information, where the substance density was considered as 0.9 g/mL [17].

In order to validate the technique used in this study, pharmacological, growth and sterility controls, represented respectively by: chlorhexidine digluconate were reactivated in Petri dishes (Alamar Tecno Científica Ltda., Diadema, SP, Brazil) and incubated in an oven at 37° C for 48 hours. After this time, a bacterial suspension was prepared in Brain Heart Infusion (BHI) broth (HiMedia Laboratories®, Mumbai, India). The inoculum at the concentration of 2×10^7 colony forming units per milliliter (CFU/mL) was standardized using a spectrophotometer Orion AQ3010 (0.05% and 0.12% CHX) (FGM Produtos Odontológicos, Joinville, SC, Brazil), developing bacterial suspension, and sterile culture medium.

The reference strains used in the study were *S. mutans* (ATCC 25175), *S. salivarius* (ATCC 7073) and *L. rhaminosus* (ATCC 9595). The microorganisms, maintained in culture medium with glycerol, Turbidity Meter (Termo Scientific) at the optical density of 0.2 to 625 nm¹⁸.

Determination of Antibacterial Activity

The antibacterial activity was determined by obtaining the Minimum Inhibitory Concentration (MIC) from the microdilution assay in a 96-well microtiter plate (Alamar Tecno Científica Ltda, Diadema, SP, Brazil) according to the M07A10 Standard of Clinical and Laboratory Standards Institute [18].

The essential oil and pharmacological controls (0.05% and 0.12% CHX) were distributed along columns (1 to 12) in the wells of the microtiter plate. In each of the wells, 100 μL of broth

(BHI) was inserted for bacterial culture. Then, 100 µl of the essential oil emulsion was added to obtain the initial concentration of 7.2% (70243.90 µg/mL) in the first column of the microtiter plate. Subsequent concentrations of the essential oil were obtained after serial dilution of the natural product on the microtiter plate, starting from the initial concentration of 7.2% of the marketed product (70243.90 µg/mL) to 0.003% (26.14 µg/mL) by transferring 100 µL of the contents to the subsequent well (Table 2). In the wells containing the pharmacological control, antimicrobial solutions were also serially diluted and, therefore, a comparative parameter for the test solution (Table 2).

Table 2. Concentrations of tea tree essential oil (EO) and pharmacological controls (µg/mL) used in the antimicrobial activity assay against *S. mutans*, *S. salivarius* and *L. rhaminosus* [18].

Well	Tea tree EO (µg/mL)	0.05 % Chlorhexidine (µg/mL)	0.12% Chlorhexidine (µg/mL)
1	70243.90	243.90	585.36
2	34265.31	118.97	285.54
3	16714.78	58.03	139.28
4	8153.55	28.31	67.94
5	3977.34	-	-
6	1940.16	-	-
7	946.42	-	-
8	461.66	-	-
9	225.2	-	-
10	109.85	-	-
11	53.58	-	-
12	26.14	-	-

After the dilution of the evaluated substances, 5 µL of the suspension of the microorganisms (2×10^7 CFU / mL) were inserted in all wells, except in the column corresponding to the sterility control. The final concentration of microorganisms per well was 5×10^5 CFU/mL, as established by Standard M07A10 [18].

After the incubation period (24 h at 37° C), the MIC corresponded to the lowest concentration of the essential oil in which bacterial growth did not occur, identified by the formation of microbial precipitate or turbidity in the culture medium after the incubation period.

The MIC determination was confirmed by the addition of 10 µL resazurin (Sigma-Aldrich, St. Louis, Missouri, USA) (100 µg/mL). This colorimetric indicator is blue in its oxidation state and becomes pink when reduced by viable cells. Thus, the detection of microbial growth was confirmed by the reduction of resazurin salt [19].

After incubation for 4 h, the results were read, in which the dye was pink (viable) or blue (non-viable) after oxidation by cellular metabolism [20]. The wells with the highest concentration of tea tree essential oil and visible growth after incubation were analyzed for bacterial viability from subculture on BHI Agar (HiMedia Laboratories, Mumbai, India) [21].

The Minimum Bactericidal Concentration (MBC) was obtained by sowing 50 µL aliquots of dilutions equal to or greater than MIC in Petri dishes containing BHI Agar. After sowing, petri

dishes were incubated in a bacteriological oven at 37° C for 48h. The CBM was considered to be the lowest concentration of the substance in which visible growth inhibition of the subculture was observed or the formation of up to three Colony Forming Units (CFU) [22]. The tests for determination of MIC and MBC were performed in three independent experiments, each in triplicate.

Statistical Analysis

Data from the serial dilutions in which the MIC was observed were tabulated in the Microsoft Office Excel 2007® Program and analyzed descriptively, obtaining the fashion values.

Results

S. mutans strains (ATCC 25175), *S. salivarius* (ATCC 7073) and *L. rhaminosus* (ATCC 9595) were found to be susceptible to the action of the tea tree essential oil, as they showed values of MICs below 70243.90 µg/mL (Table 3). The tea tree essential oil had lower values of MIC on *S. mutans* (1940.16 µg/mL) and lower values for CBM on *S. salivarius* (3977.34 µg/mL).

Table 3. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) obtained for the tea tree essential oil (melaleuca) against test strains, in three independent experimental events. Values expressed in (µg/mL).

Events	1°		2°		3°		Mode	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Strains								
<i>S. mutans</i>	1940.1	>70243.9	1940.1	70243.9	1940.1	70243.9	1940.1	70243.9
<i>S. salivarius</i>	8153.5	8153.5	3977.3	3977.3	3977.3	3977.3	3977.3	3977.3
<i>L. rhaminosus</i>	3977.3	34265.3	3977.3	16714.7	3977.3	34265.3	3977.3	34265.3

The CIM and CBM of the pharmacological controls (0.05% and 0.12% CHX) demonstrated absence of bacterial growth at both concentrations. Sterility control and growth control showed, respectively, the absence of contamination of the culture medium and the viability of the strains tested.

Discussion

The present study used the broth dilution method, considered the most sensitive method to determine antimicrobial activity [23], presenting a value of 1940.16 µg/mL against *S. mutans* strain (ATCC 25175).

Studies comparing agar and dilution techniques in broth presented statistical differences between these methods [23]. When evaluating the antimicrobial activity on planktonic cultures, the values for the MIC of the tea tree oil, using the diffusion method in agar, *S. mutans* (ATCC 25175) and *S. mutans* (JC-2) were found to be 1000 µg/mL [24,25]. The difference between the results observed in the literature [24,25] and the data from the present study are probably related to the method determination of antimicrobial activity.

Previous study have identified MIC values lower than *L. rhaminosus* (300 µg/mL) and higher for *S. mutans* (2500 µg/mL) [9]. Differences in MIC values can be justified by the different strains tested, as well as by the method of obtaining the essential oil, its seasonality, or place of cultivation.

Several studies have evaluated the effect of the tea tree essential oil on bacteria of the dental biofilm [9,24-28], but no study included *S. salivarius*, making it difficult to compare the results found in this investigation, in which it presented 3977.34 µg/mL for MIC and MBC, and this value is considered high [14].

Although the incorporation of substances into the natural products is observed as an artifice of potentializing their action [29], the present study did not do this evaluation. However, the combination of the tea tree essential oil and chlorhexidine against *S. mutans* did not obtain good results, because when compared to the oils of cinnamon (620 µg/mL) and manuka (*Leptospermum scoparium*) (620 µg/mL), the tea tree EO (2500 µg/mL) presented the worst performance [25], configuring the low additive potential of this natural product.

In previous studies, the use of tea tree essential oil as an alternative antibacterial was considered unfavorable. When evaluating its effect on the salivary levels of *S. mutans* in the formulation of 2% solution for mouthwash, there was no statistically significant difference between the groups that used Listerine and 0.12% chlorhexidine [11]. As observed in the present study, tea tree EO seems to demonstrate effect at high concentrations. Under the conditions of the present study, the MIC of tea tree EO against *S. mutans* equals a formulation of 0.2% (2 mg/mL). Given clinical use, a concentration of 10×MIC would be acceptable to demonstrate effectiveness. However, the same clinical study mentioned previously reported unpleasant taste (66.6%) and burning sensation (77.8%) [30], highlighting inconveniences of clinical use.

Natural products must have minimum inhibitory concentrations of 100 µg/mL or less to be considered satisfactory [14]. Although the tea tree EO presented antimicrobial action against the strains evaluated, the MICs probably did not make the investigations more thorough, since the yield to make this product therapeutically would exceed limits of economic viability.

Clinical studies previously conducted with this product [12,30] demonstrate some antimicrobial efficacy [12], but inefficiency of clinical use [30]. The MICs found in the present study would also exceed the limit of cytotoxicity, since the 0.2% concentration has been shown to have a deleterious effect on human endothelial cells [24]. The antimicrobial and therapeutic potential of the tea tree essential oil should be explored from the isolation of bioactive molecules from bioprospecting. The capacity for therapeutic use in the form of essential oil appears to be limited.

Conclusion

The tea tree essential oil has antibacterial activity against microorganisms evaluated in high concentration. The use of this product in therapeutic strategies should be considered for its

antimicrobial efficacy and suitability for use. Bioactive molecules appear to be part of the composition of this natural product.

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