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Influence of *Lippia thymoides* Mart. & Schauer and *Lippia origanoides* Kunth essential oils on planktonic cell and *Streptococcus mutans* biofilm growth

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ABSTRACT

Biofilms comprise of a complex structure of accumulated microorganisms, especially *Streptococcus mutans*, and are a major factor in the development of caries. This study aimed to evaluate the *in vitro* antimicrobial action of *Lippia thymoides* and *Lippia origanoides* essential oils against *S. mutans* UA159 by measuring minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) in biofilms. Cell viability was observed using scanning electron microscopy and confocal laser scanning microscopy. The MIC values were 35 µg/ml for *L. thymoides* and 40 µg/ml for *L. origanoides*, suggesting that the oils had antimicrobial effects on *S. mutans* strains and indicated a bactericidal action. These antimicrobial activity tests showed that *S. mutans* in the biofilm was significantly eliminated, with results better than the action of 0.2% chlorhexidine. These results confirm the antimicrobial activity of these essential oils and provide a promising means for the effective reduction of *S. mutans* biofilms.

Keywords: Antimicrobial susceptibility, biofilm, essential oils; *Lippia* sp., planktonic cells, *Streptococcus mutans*.

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INTRODUCTION

Dental caries are the most common infectious pathology of oral cavities and present as biofilms formed by microorganisms on the surface of teeth (Garcia et al., 2017; Marsh and Zaura, 2017). The main etiological factor of this disease is Streptococcus mutans (Garcia et al., 2017; Marsh and Zaura, 2017; de Matos et al., 2017). *S. mutans* is a gram-positive, anaerobic, facultative, and cariogenic bacterium that synthesizes sucrose and acidifies the buccal environment, which promotes tooth demineralization and participates in the pathogenesis of dental caries (Huang et al., 2013; Nomura et al., 2013; Falsetta et al., 2014).

The virulence of the species promotes adhesion and rapid colonization through the process of permanent cell division (Zijnge et al., 2010; Xiao et al., 2012; Wang et al., 2012). Microorganisms multiply and produce insoluble extracellular polymers that form a matrix of proteins, polysaccharides, lipids, and nucleic acids (Zijnge et al., 2010; Xiao et al., 2012; Wang et al., 2012). In addition to promoting fixation of new microorganisms and inorganic elements to the teeth, the polymer matrix becomes a protective physical and chemical barrier that allows

microorganisms to form three-dimensional structures that help them become more tolerant to antimicrobial agents (Zijnge et al., 2010; Xiao et al., 2012; Wang et al., 2012).

Increased bacterial resistance has warranted the development of new strategies to combat microorganisms. Chlorhexidine is a treatment that has effective antimicrobial activity (Yousefimanesh et al., 2015; Lee et al., 2016); however, when continuously used, it leads to side effects such as loss of taste, oral tissue aggravation, and tooth discoloration (Varoni et al., 2012; Yousefimanesh et al., 2015). *Lippia* genus of the *Verbenaceae* family are shrubs found in Central and South America and in Tropical Africa (Ombito et al., 2014; Pérez Zamora et al., 2018). These species present great pharmacological potential due to the presence of triterpenoids, steroids, iridoid glycosides, and phenols that include flavonoids and phenylpropanoids (Ombito et al., 2014; Pérez Zamora et al., 2018). Moreover, Lippia species have biological properties that make it a natural source for herbal products (de Vasconcelos et al., 2014; Porfírio et al., 2017) but there is limited information on its potential antimicrobial efficacy (de Vasconcelos et al., 2014; Costa et al., 2017; Porfírio et al., 2017). Lippia thymoides Mart. & Schauer (Verbenaceae) is commonly known as “broom rosemary” and found in Brazilian states with typical caatinga biomes (Silva et al., 2016). This plant presents small and aromatic leaves, thin vertical branches and grows to about 2 m high (Silva et al., 2016). It is popularly used in the treatment of headaches, hyperthyroidism, bronchitis, and rheumatism (Silva et al., 2016). *Lippia origanoides* Kunth *(Verbenaceae*) is found in semiarid regions of northeastern Brazil and is used culturally for treatment of respiratory, gastrointestinal, and throat inflammation (Botelho et al., 2007; Ribeiro et al., 2014; Vásquez et al., 2014; Soares et al., 2016; Menezes et al., 2018). Studies show that the *L. origanoides* essential oil has antimicrobial activity against microorganisms such as *Staphylococcus aureus*, *S. mutans, Lactobacillus casei*, and *Cryptococcus neoformans* (Ribeiro et al., 2014; Vásquez et al., 2014; Sarrazin et al., 2015; Soares et al., 2016; Veras et al., 2017; Menezes et al., 2018). In some of these reports, this species is also known by the scientific name *Lippia sidoides* Cham.

The objective of this study was to evaluate and characterize the antimicrobial action of *L. thymoides* and *L. origanoides* on planktonic and biofilm cultures that include *S. mutans*, as well as to analyze the levels and composition of the oils.

MATERIALS AND METHODS

Plant material and essential oils

L. thymoides leaves were collected in the winter of 2017 on a rainy day in the morning at a property located along the BA-265 highway in the municipality of Barra do Choça, Bahia, Brazil (Latitude: 14° 51.716’ South; Longitude: 40° 37.034’ West). The control material was been deposited under no. 2313 in the Mongóis, of Multidisciplinary Institute of Health of the Federal University of Bahia (UFBA) herbarium under the curatorship of Dr. Andrea Karla Almeida dos Santos.

Extraction of *L. thymoides* essential oil was conducted with steam distillation using artisanal extractor equipment developed by Adolfo Eustáquio dos Santos and José Wilson Rodrigues Soares Junior, in Montes Claros, Minas Gerais, Brazil.

Essential oil from *L. origanoides* was supplied by the Laboratory of Chemistry of Natural and Bioactive Products of the State University of Feira de Santana (UEFS). Its main constituents included carvacrol (53.89%), (*E*)-caryophyllene (5.86%), linalool (5.84%), bicyclogermacrene (4.16%), *p*-cymene (4.03%), and γ-terpinene (3.50%) (Menezes et al., 2018). This material was deposited under HUEFS 83373 in the UEFS Herbarium. The specimen was identified by Dr. Tânia Regina dos Santos Silva.

Gas chromatography

Identification of major compounds of *L. thymoides* was performed by comparing mass spectra obtained with gas chromatography separation (model GC-MS QP 2010 SE, Shimadzu, Kyoto, Japan), using data from Flavor and Fragrances of Natural and Synthetic Compounds (FFNSC). We considered only the spectra that resulted in compatibility greater than 95%. The relative percentage of each compound was then quantified. Helium gas was used as the mobile phase at a 1.0 ml min^{-1} flow rate. A DB-5HT column capillary from Restek (30 m, 0.25 mm id, 0.25 μm of film thickness) was used by programming the initial furnace temperature to 60°C, which was then increased to 240°C at a ratio of 3°C min^{-1}, for 60 min. Injector and detector temperatures were 250°C and the injection volume was 1.0 µl (Silva et al. 2019).

Bacterial culture

The reference strain *S. mutans* UA159 (ATCC® 700610™) was used as a test microorganism for antimicrobial assays and was acquired from the Collection of Microorganisms of Faculty of Dentistry in Piracicaba FOP-UNICAMP, Piracicaba, São Paulo. Bacterial cells were maintained in cryogenic systems and then activated on MSA culture medium supplemented with 1% PTS, 200 U/L bacitracin, and 15% sucrose (Mitis Salivarius Agar, Cat. #01337 Millipore; potassium tellurite solution, Cat. #17774 Millipore; Bacitracin, Cat. #B0125 Sigma-Aldrich; Sucrose, Cat. #84100 Millipore, Merck KGaA, Darmstadt, Germany). Planktonic cells and biofilms were tested in BHI medium (Brain Heart Infusion Broth, Cat. #53286 Millipore, Merck KGaA, Darmstadt, Germany) and BHI medium supplemented with 1% sucrose (Sucrose, Cat. #84100 Millipore Merck KGaA, Darmstadt, Germany), respectively (Stipp et al., 2013).

Measurement of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of essential oils

The MIC of *L. origanoides* and *L. thymoides* essential oils against *S. mutans* was determined by the broth microdilution method following the guidelines established by the Clinical and Laboratory Standards Institute (CLSI 2012a; CLSI 2012b), with some adaptation. For susceptibility tests, essential oil solutions were prepared in BHI medium supplemented with a final concentration of 1–4% dimethyl sulfoxide (DMSO) for 100–400 μl/ml essential oils/culture medium, and <1% DMSO for 15–91.85 μl/ml essential oils/culture medium (DMSO, Cat. #D1435 Sigma-Aldrich, Millipore, Merck KGaA, Darmstadt, Germany). A chlorhexidine solution was used as an experimental control for growth inhibition and

comparative parameters (chlorhexidine digluconate solution, Cat. #C9394 Sigma-Aldrich, Millipore, Merck KGaA, Darmstadt, Germany). These tests were conducted in four replicate test systems using sterile, disposable microdilution multi-well plates (96-well cell culture microplates, flat-bottom, polystyrene, Corning Inc., N.Y.) containing 20 μl of each bacterial inoculum at a final concentration equal to 1 × 10^8 CFU/ml of BHI and 80 µl BHI broth culture medium supplemented with final essential oil concentrations between 15 - 400 µl/ml, and final concentrations of chlorhexidine between 0.0053 – 0.0424 µg/ml (0.53–4.24%). These cultures were incubated at 37°C for 24 h with 10% CO_2 (Cole-Parmer, Vernon Hills, EUA).

Resazurin staining was used to confirm viable cells in the culture systems. A 35 µl aliquot of resazurin solution (0.01% aqueous solution; Resazurin sodium salt, Cat. #R7017 Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) was added in each well and incubated for 1 h to indicate the presentence of viable microorganisms with a colorimetric change from blue to pink-violet (Andrews, 2001). When there was no resazurin color change, the MIC was considered the lowest concentration of the test substance capable of inhibiting microorganism growth (Andrews 2001).

The MBC of *L. origanoides* and *L. thymoides* essential oils against *S. mutans* was analyzed as previously described (Moore et al. 2001). The total 20-µl volume of the well corresponding to the MIC without resazurin was homogenized with a pipette and used to inoculate MSA culture medium. Each aliquot was placed gently at a given point on the growth medium and kept at room temperature until the solution completely absorbed into the solid medium. Then, the plate was streaked to separate microorganisms and remove them from drug sources (e.g. oils and chlorhexidine) (Moore et al., 2001) and incubated at 37°C with 10% CO_2 for 24 h. The MBC was defined as the lowest concentration of the drug (i.e. oils or chlorhexidine) that eliminated ≥99.9% of the inoculum (0.05-0.25 colony); whereas, bacteriostatic activity was defined from the ≤99.9% reduction of the final inoculum (Cantón et al. 2003).

Susceptibility test of *S. mutans* biofilms

The *S. mutans* strain was grown on MSA medium at 37°C for 24 h in an atmospheric condition containing 10% CO_2. Five colonies were transferred to BHI broth and incubated at 37°C with 10% CO_2 for 18 h. The inoculum was set to a transmittance of 89.1% using a spectrophotometer (Thermo Scientific Multiskan GO UV/Vis, Microplate and Cuvette Spectrophotometer, ref. #51119200, Thermo Fisher Scientific Inc., Waltham, MA, USA) using a wavelength of 550 nm ($T = 89.1\% \rightarrow A_{550\ nm} = 2 - \log_{10} \%T \rightarrow A_{550\ nm} = 0.05$), in 40 ml BHI with 1% sucrose. Aliquots of 5 ml inoculum were transferred to a 6-well polystyrene plate (Corning® Costar® TC-Treated Multiple Well Plates, Cat. # CLS3516 Sigma, Merck KGaA, Darmstadt, Germany) and incubated at 37°C for 24 h in 10% CO_2 for biofilm formation. The culture media was then removed and the biofilms were washed three times with 1 ml 145 mM NaCl for 30 s under constant agitation at 180 rpm (Therno-Shaker Dry Block, model 1364, Nova, Brazil) in order to remove planktonic bacteria (Stipp et al., 2013).

Biofilms were exposed to 200 μl *L. origanoides* or *L. thymoides* essential oils at concentrations of 100% and 0.06% (diluted in 0.6 μl/ml sterile distilled water) for 5 min. Then, biofilms were removed from the plates by sonication in 1 ml 145 mM NaCl sterile solution, diluted serially in NaCl solution (from 10^{-1} to 10^{-8}), plated using the spread-plate method on BHI agar and incubated at 37°C for 24 h in an atmosphere of 10% CO_2. The assays were performed four times and the dates were reported as colony forming units per milliliter (CFU/ml). A 0.2% (2 μg/ml) chlorhexidine solution and sterilized mineral oil (Mineral oil, Cat. #M5904 Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) were used as controls for non-viable (i.e. anti-biofilm action) and viable cells, respectively (Stipp et al., 2013).

Scanning electron microscopy

The *S. mutans* inoculum was prepared for scanning electron microscopy (SEM) according to a protocol previously described (Stipp et al., 2013). Biofilms were grown on sterilized circular glass coverslips (Knittel Glass, Brunsvique, Germany) in 12-well polystyrene plates (Corning® Costar® TC-Treated Multiple Well Plates, Cat. #CLS3512 Sigma, Merck KGaA, Darmstadt, Germany) lined with sterilized absorbent paper. Aliquots of 3 ml bacterial inoculum were added to plate wells and incubated at 37°C for 24 h at 10% CO_2. Newly grown biofilms were gently washed three times with 1 ml 145 mM NaCl solution and treated with 200 μl *L. origanoides* or *L. thymoides* essential oils (100% and 0.06%) for 5 min at room temperature. Biofilms were then washed again with NaCl solution and treated with 200 µl 2% glutaraldehyde (Glutaraldehyde solution, Cat. #G5882 Sigma-Aldrich, Merck KGaA, Darmstadt, Germany) for 30 min. The resulting products were washed three times with sterile distilled water and dried at room temperature.

Biofilm specimens grown on circular glass coverslips were dehydrated with increasing concentrations of ethanol (50, 70, 90 and 100%) for 10 min each, dried at room temperature, fixed on metal stubs with carbon strips (Double Sided Sticky Carbon Tape, Cat. # C261, TAAB Laboratories Equipment Ltd., Glasgow, England) and metallized with gold by an SCD 050 metallizer (Bal-Tec AG, Balzers, Liechtenstein, Germany). Metallized samples were analyzed by scanning electron microscopy (JSM-5600 Lv JEOL, Tokyo, Japan) using a voltage of 15 kV and 7.000× magnification.

Confocal laser scanning microscopy

Streptococcus mutans biofilms exposed to essential oils and chlorhexidine were analyzed by confocal laser scanning microscopy (LEICA TCS SP5 Microscope, Leica Microsystems, Wetzlar, Germany). The inoculum was prepared following a previously described method (Stipp et al. 2013). Bacterial biofilms were grown on glass slides (Lab-Tek Chamber Slide™, Cat. #177399; Nalgene Nunc International, Naperville, IL, USA) in BHI medium supplemented with 1% sucrose, and incubated at 37°C for 24 h in 10% CO_2. The biofilms were washed three times with 1 ml 145 mM NaCl for 30 s with constant agitation at 180 rpm (Thermo-Shaker Dry Block, model 1364, Nova, Brazil), before 200 μl essential oils (100% and 0.06%) or chlorhexidine (0.2%) was added and incubated for an additional 5 min. Biofilms were washed twice with NaCl solution and stained with 200 μl fluorescent solution (LIVE/DEAD™ BacLight™ Bacterial Viability Kit, Invitrogen™, Cat. #L7012, Eugene, OR, USA) for 15 min. This method monitored cell viability as a function of cell membrane integrity; therefore, cells with compromised membranes (i.e. non-viable cells) were visualized with propidium iodide staining (false colored in red), while cells with an intact membrane (i.e. viable cells) were visualized with SYTO 9 staining (false colored green). Depending on the intensity of both dyes in overlapping images, non-viable cells were visualized as having a yellowish tone.

The cell viability (i.e. percentage of green pixels: viable cells; percentage of red/yellow pixels: non-viable cells), relative biomass of the biofilm ($\sum PX \leftrightarrow \sum \mu m \rightarrow \sqrt[3]{\sum n_{\mu m}} = n_{\mu m^3}$, where PX and μm are units were measured in pixels and micrometers, respectively; 1 μm = 1.2933 pixels or 1 μm^3 = 1.089515 pixels) and thickness of the biofilm (µm) were evaluated using images captured at 1 μm resolution intervals (512 × 512 pixels) using Leica LAS AF Lite software (LEICA). The 3D reconstructions of biofilms were acquired with Fiji version 2.0.0-rc- 67/1.52c (National Institutes of Health de Wayne Rasband, Bethesda, USA).

RESULTS

Determination of *L. thymoides* essential oil composition

The chemical analysis of *L. thymoides* essential oil showed that it was composed of 22.8% p-cymene followed 19.14% γ-terpinene, 16.20% thymol methyl ether, and 5.48% thymol (Table 1).

Antimicrobial effects of essential oils

The MIC values of *L. thymoides* and *L. origanoides* essential oils on *S. mutans* planktonic cells are presented in Table 2. According to MBC values, all the compounds tested had bactericidal activity.

Anti-biofilm effect of essential oils

The average number of *S. mutans* UA159 colony forming units from biofilms grown on polystyrene microplate surfaces and those exposed to mineral oil, chlorhexidine (0.2% w/vol.), *Lippia thymoides* essential oils (0.06% and 100%) and *Lippia origanoides* essential oils (0.06% and 100%) were $8.34 \times 10^9 \pm 2.97 \times 10^8$, $2.47 \times 10^9 \pm 6.00 \times 10^7$, $1.81 \times 10^8 \pm 5.64 \times 10^7$ (0.06%), $2.35 \times 10^5 \pm 4.63 \times 10^5$ (100%), $2.36 \times 10^8 \pm 1.09 \times 10^8$ (0.06%) and $6.36 \times 10^4 \pm 1.60 \times 10^4$ (100%), respectively (Table 3). Chlorhexidine and *Lippia* species had $SMIC_{70}$ and $SMIC_{>97}$, respectively, indicating that based on the average CFU/ml values of *S. mutans* biofilms, *L. origanoides* and *L. thymoides* were capable of reducing bacterial growth by >97% compared to the growth control (mineral oil), whereas chlorhexidine had about 70% of this capacity (Table 4 and Figure 1).

Scanning electron microscopy

The ultrastructure of *S. mutans* biofilms exposed to *L. thymoides* and *L. origanoides* essential oils (0.06% and 100%), chlorhexidine (0.2%), and mineral oil (growth control) for 5 min were analyzed by scanning electron microscopy (SEM). Untreated controls revealed uniform and densely organized cell communities, while biofilms treated with both concentrations of *L. thymoides* essential oils showed changes in the single plane of bacterial division (i.e. streptococcal chains breakdown) and cell deformity, while those treated with *L. origanoides* showed only slight changes in the single plane of bacterial division. Chlorhexidine treatment had no obvious effect on structural and morphological development of *S. mutans* biofilms (Figure 2).

Biological and structural effects of essential oils on biofilms

Confocal laser scanning microscopy (CLSM) was conducted to determine the biological (i.e. percentage of viable cells and non-viable cells) and greatness parameters (i.e. relative biomass and thickness) of the *S. mutans* biofilms exposed to *Lippia* sp. essential oils compared with mineral oil and chlorhexidine. In *in vitro* experimental conditions, the biological and greatness parameters of biofilms exposed to chlorhexidine and all concentrations of *Lippia* sp. essential oils were not significantly different from each other ($p < 0.05$). However, the antimicrobials were significantly different from mineral oil only in the biological parameters measured ($p < 0.05$; Table 5; Figures 3 and 4). These results indicate that essential oils affect cell viability more than biofilm development.

Table 1. Chemical composition of essential oil of leaves of *L. thymoides*.

Formula	Component	%
$C_{10}H_{14}$	m-cymene	22.8
$C_{10}H_{16}$	γ-terpinene	19.14
$C_{10}H_{14}O$	thymol methyl ether	16.20
$C_{10}H_{14}O$	thymol	5.48

Table 2. Minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of essential oils of *L. thymoides*, *L. origanoides* and chlorhexidine on *S. mutans*.

Potential antimicrobial agents	MIC (µg/ml)	MBC (CFU/ml)
L. thymoides	35	Absent
L. origanoides	40	Absent
Chlorhexidine	0.0053	Absent

CFU correspond to colony formation unit.

DISCUSSION

Chromatography of *L. thymoides* essential oils has revealed a predominance of monoterpene, especially p-cymene, which can enhance the action of other antimicrobial agents contained in the oil through synergy,

Table 3. Average numbers of colony forming units of *S. mutans* UA159 coming from biofilms grown on polystyrene microplate surfaces and exposed to the mineral oil, chlorhexidine (0.2% w/vol.), *Lippia thymoides* essential oils (0.06% and 100% vol/vol) and *Lippia origanoides* essential oils (0.06% and 100% vol/vol).

Serial dilutions	Controls (CFU/ml)	
	Mineral oil	**Chlorhexidine (0.2%)**
10^{-1}	$1.26\times10^{4} \pm 7.39\times10^{1}$	$7.49\times10^{3} \pm 6.29\times10^{1}$
10^{-2}	$1.13\times10^{5} \pm 1.76\times10^{3}$	$6.43\times10^{4} \pm 3.41\times10^{2}$
10^{-3}	$1.05\times10^{6} \pm 2.38\times10^{4}$	$5.24\times10^{5} \pm 1.61\times10^{4}$
10^{-4}	$9.43\times10^{6} \pm 1.12\times10^{5}$	$4.26\times10^{6} \pm 4.69\times10^{4}$
10^{-5}	$8.35\times10^{7} \pm 8.52\times10^{5}$	$3.68\times10^{7} \pm 1.99\times10^{6}$
10^{-6}	$7.72\times10^{8} \pm 9.34\times10^{6}$	$2.65\times10^{8} \pm 8.72\times10^{6}$
10^{-7}	$6.60\times10^{9} \pm 2.07\times10^{8}$	$1.89\times10^{9} \pm 4.65\times10^{7}$
10^{-8}	$5.92\times10^{10} \pm 2.16\times10^{9}$	$1.76\times10^{10} \pm 4.23\times10^{8}$
M ± SD	$8.34\times10^{9} \pm 2.97\times10^{8}$	$2.47\times10^{9} \pm 6.00\times10^{7}$
Serial dilutions	***Lippia thymoides*** **(CFU/ml)**	
	0.06% (vol/vol)	**100% (vol/vol)**
10^{-1}	$3.81\times10^{3} \pm 6.32\times10^{1}$	$5.28\times10^{2} \pm 6.57\times10^{2}$
10^{-2}	$2.27\times10^{4} \pm 2.26\times10^{3}$	$2.89\times10^{3} \pm 3.95\times10^{3}$
10^{-3}	$1.85\times10^{5} \pm 3.04\times10^{3}$	$1.77\times10^{4} \pm 3.09\times10^{4}$
10^{-4}	$1.50\times10^{6} \pm 1.29\times10^{5}$	$2.25\times10^{5} \pm 4.01\times10^{5}$
10^{-5}	$8.46\times10^{6} \pm 1.26\times10^{6}$	$1.63\times10^{6} \pm 3.27\times10^{6}$
10^{-6}	$4.75\times10^{7} \pm 9.95\times10^{6}$	0
10^{-7}	$2.38\times10^{8} \pm 5.28\times10^{7}$	0
10^{-8}	$1.15\times10^{9} \pm 3.87\times10^{8}$	0
M ± SD	$1.81\times10^{8} \pm 5.64\times10^{7}$	$2.35\times10^{5} \pm 4.63\times10^{5}$
Serial dilutions	***Lippia origanoides*** **(CFU/ml)**	
	0.06% (vol/vol)	**100% (vol/vol)**
10^{-1}	$4.37\times10^{3} \pm 2.73\times10^{1}$	$7.59\times10^{2} \pm 2.41\times10^{2}$
10^{-2}	$3.44\times10^{4} \pm 1.57\times10^{3}$	$5.48\times10^{3} \pm 1.30\times10^{3}$
10^{-3}	$2.30\times10^{5} \pm 4.39\times10^{3}$	$4.74\times10^{4} \pm 2.20\times10^{4}$
10^{-4}	$1.46\times10^{6} \pm 1.15\times10^{5}$	$4.55\times10^{5} \pm 1.04\times10^{5}$
10^{-5}	$8.98\times10^{6} \pm 1.16\times10^{6}$	0
10^{-6}	$7.08\times10^{7} \pm 9.51\times10^{6}$	0
10^{-7}	$2.99\times10^{8} \pm 2.83\times10^{7}$	0
10^{-8}	$1.51\times10^{9} \pm 8.31\times10^{8}$	0
M ± SD	$2.36\times10^{8} \pm 1.09\times10^{8}$	$6.36\times10^{4} \pm 1.60\times10^{4}$

M, SD and CFU correspond to average, standard deviation and colony formation unit, respectively. Dilution assays were performed in replicate system (four times).

antagonism, and additive effects (Marchese et al., 2017). The second most prevalent compound was γ-terpinene, which can reduce the amount of cell binding on the substrate surface, disrupting membrane structure and diffusion through the polysaccharide biofilm matrix, promoting destabilization of the biofilm (Miladi et al., 2017). Thymol was present in *L. thymoides* essential oil, which can cause deformities in cellular physical arrangements due to its potential effect on plasma membrane permeability (Soković et al., 2010; Ferreira et al., 2016; Miladi et al., 2017; Veras et al. 2017). Similarly, the compound carvacrol was predominantly detected in the essential oil of *L. origanoides*, and can affect the ionic gradient and permeability cell membranes, making them more fluid (Ultee et al. 2002; Miladi et al. 2017). Studies indicate that carvacrol decreases the capacity of the cytoplasmic membrane, attenuates intracellular pH, inhibits ATP synthesis and induces potassium (K^+) leaks, which contributes to cell death (Ultee et al. 2002). The compounds thymol, carvacrol, *y*-terpinene, and *p*-cymene synergistically interact with the antibiotic effect of tetracycline against oral pathogens, providing

Table 4. Average numbers of colony forming units of *S. mutans* UA159 corresponding to the $SMIC_n$ of biofilms grown on polystyrene microplate surfaces and exposed to the mineral oil, chlorhexidine (0.2% w/vol.), *Lippia thymoides* essential oils (0.06% and 100% vol/vol) and *Lippia origanoides* essential oils (0.06% and 100% vol/vol).

n **(CFU/ml)**	**Scientific** ***n*** **(CFU/ml)**	**SMIC**	**Test substance**
8336908681	8.34×10^9	$SMIC_0$	Mineral oil (growth control)
6252681511	6.25×10^9	$SMIC_{25}$	-
4168454340	4.17×10^9	$SMIC_{50}$	-
2473766864	2.47×10^9	$SMIC_{70.3}$	Chlorhexidine digluconate (0.2%)
2084227170	2.08×10^9	$SMIC_{75}$	-
1667381736	1.67×10^9	$SMIC_{80}$	-
833690867.9	0.83×10^9	$SMIC_{90}$	-
416845433.9	0.41×10^9	$SMIC_{95}$	-
236129971.6	0.23×10^9	$SMIC_{97.167}$	*Lippia origanoides* (0.06%)
180645553.8	0.18×10^9	$SMIC_{97.833}$	*Lippia thymoides* (0.06%)
234927.5	2.35×10^5	$SMIC_{99.997}$	*Lippia thymoides* (100%)
63581.3	6.36×10^4	$SMIC_{99.999}$	*Lippia origanoides* (100%)
0	0.00×10^0	$SMIC_{100}$	Sterility control

$SMIC_n$ correspond to biofilm sessile minimun inhibitory concentration capable of reducing bacterial growth at *n*%. Data obtained by regression analysis, using average numbers of CFU of the biofilm assays: *y* (CFU/ml) = –83,369,086.81 × *X* ($SMIC_n$) + 8,336,908,680.83 ($R^2 = 1$).

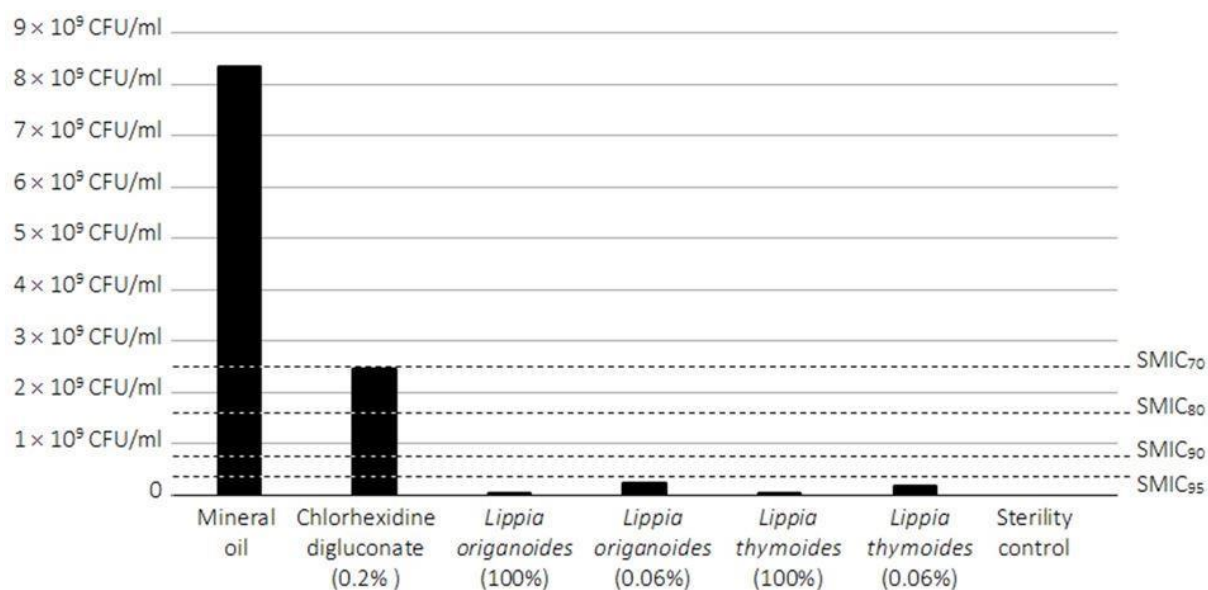

Figure 1. Profile of average numbers of colony forming units of *S. mutans* UA159 coming from biofilms grown on polystyrene microplate surfaces and exposed to the mineral oil, chlorhexidine (0.2% w/vol.), *Lippia thymoides* essential oils (0.06% and 100% vol/vol) and *Lippia origanoides* essential oils (0.06% and 100% vol/vol).

antimicrobial, and anti-biofilm activities (Miladi et al., 2017).

External factors such as climate, soil conditions, and ambient temperature in plant cultivation could interfere with the anti-biofilm efficacy of essential oils and generate possible variation in their composition and biological potential. However the studies show that the seasonal variation of external factors have little influence on *L. origanoides* essential oil composition or its antimicrobial activity (Sarrazin et al., 2015). The *L. thymoides* essential oil had satisfactory antimicrobial activity against strains of *S. mutans* (Table 2). Previous studies identified the efficacy of *L. thymoides* essential oil isolated from plants harvested during the winter in combating the gram-positive *S. aureus* and *Micrococcus luteus* bacteria (Silva et al., 2016), and results presented in this report argue

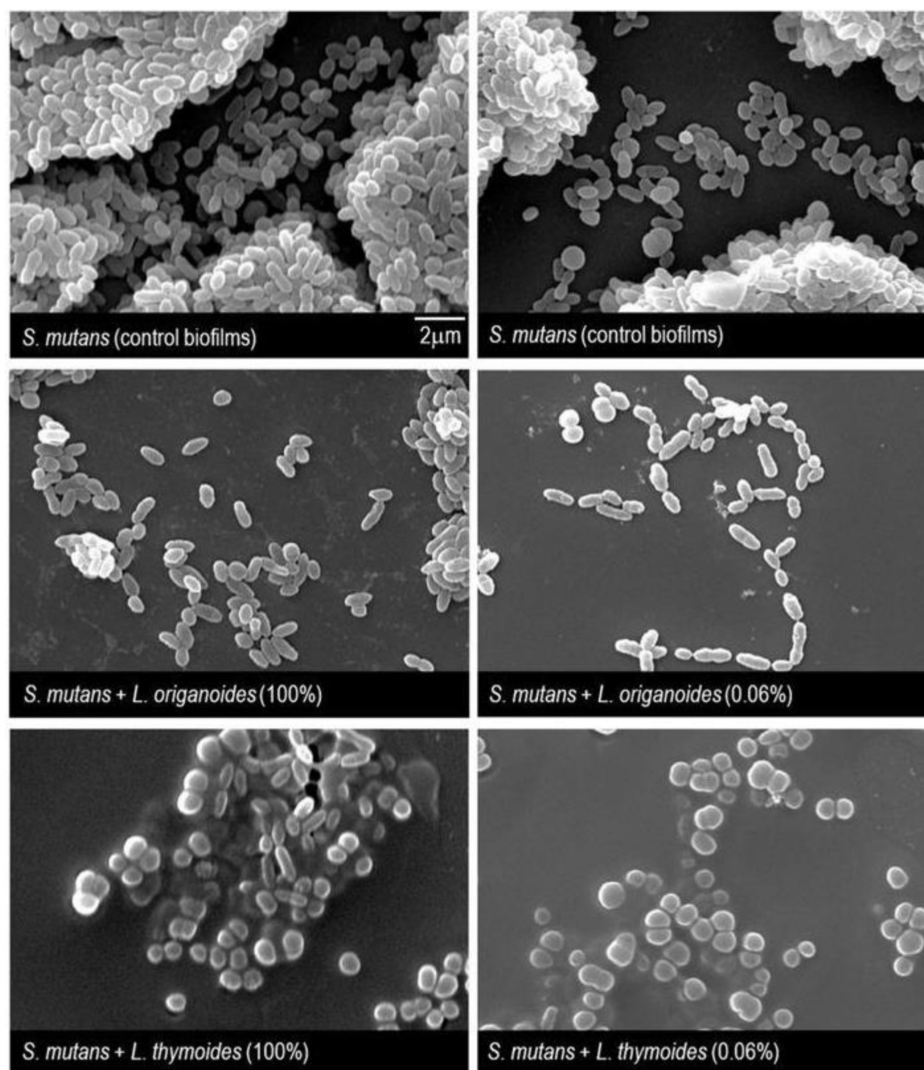

Figure 2. SEM images (magnification of 7,000× at 15kV) of *S. mutans* UA159 biofilms grown on polystyrene microplate surfaces and exposured to the mineral oil, chlorhexidine (0.2% w/vol.), *Lippia thymoides* essential oils (0.06% and 100% vol/vol) and *Lippia origanoides* essential oils (0.06% and 100% vol/vol).

Table 5. Biological (non-viable cells) and (relative biomass and thickness) greatness parameters of *S. mutans* UA159 biofilms grown on polystyrene microplate surfaces and exposed to the mineral oil, chlorhexidine (0.2% w/vol.), *Lippia thymoides* essential oils (0.06% and 100% vol/vol) and *Lippia origanoides* essential oils (0.06% and 100% vol/vol), obtained by CLSM. Data generated by Leica LAS AF Lite software (LEICA, Wetzlar, Germany).

Test substance	**Biological parameters**		**Greatness parameters**	
	NVC (%)	**VC (%)**	**T (μm)**	**RB (μm^3)**
Mineral oil (growth control)	9.250	90.750	90	306.674
Chlorhexidine (0.2%)	67.018	32.982	107	288.025
Lippia origanoides (100%)	59.244	40.756	97	285.023
Lippia origanoides (0.06%)	70.885	29.115	118	249.611
Lippia thymoides (100%)	59.838	40.162	92	293.985
Lippia thymoides (0.06%)	79.771	20.229	103	312.358

*Cells with compromised membrane that are considered to be dead or dying. NVC, VC, T and RB correspond to non-viable cells, viable cells, thickness of the biofilm and relative biomass of biofilm, respectively. Values with the same letter (A, B or C) are not significantly different ($p < 0.05$).

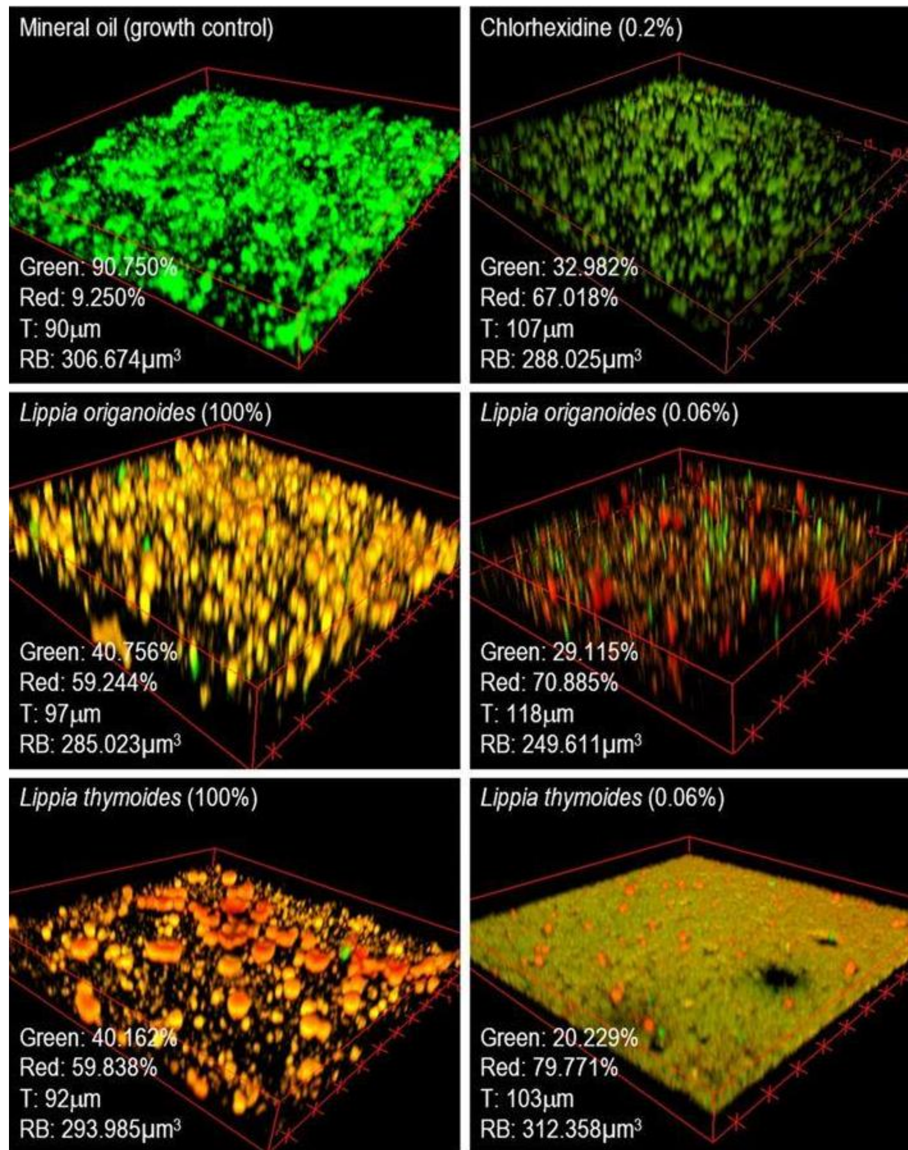

Figure 3. CLSM images of *S. mutans* UA159 biofilms grown on polystyrene microplate surfaces and exposed to the mineral oil, chlorhexidine (0.2% w/vol.), *Lippia thymoides* essential oils (0.06% and 100% vol/vol) and *Lippia origanoides* essential oils (0.06% and 100% vol/vol).

that its effects against *S. mutans* also help reduce biofilm development.

The essential oil from *L. origanoides* also had antimicrobial efficacy against *S. mutans* (Table 2). Previous studies have demonstrated that, among microorganisms related to dental caries, *S. mutans* has the highest sensitivity to this essential oil (Botelho et al. 2007). *L. origanoides* essential oil has a synergistic effect in combination with penicillin and is able to reduce the MIC for *S. mutans* (Veras et al., 2017). However, this essential oil has less effective antimicrobial action compared to carvacrol and thymol compounds, suggesting that the smaller scale compounds present in oil may have an antagonistic effect antimicrobial action (Botelho et al., 2007).

S. mutans pathogenicity includes extensive biofilm formation, which can increase antimicrobial tolerance, making these bacteria difficult to treat (Wang et al., 2012). To determine the action of essential oils on oral biofilms, essential oils were diluted in order to compare the range of their potential antimicrobial effects on oral biofilms. Essential oils can be cytotoxic at high concentrations and therefore, understanding minimum dosage can improve product marketability. *In vitro* studies with mouthwashes have demonstrated that some products were cytotoxic to gingival fibroblasts, confirming

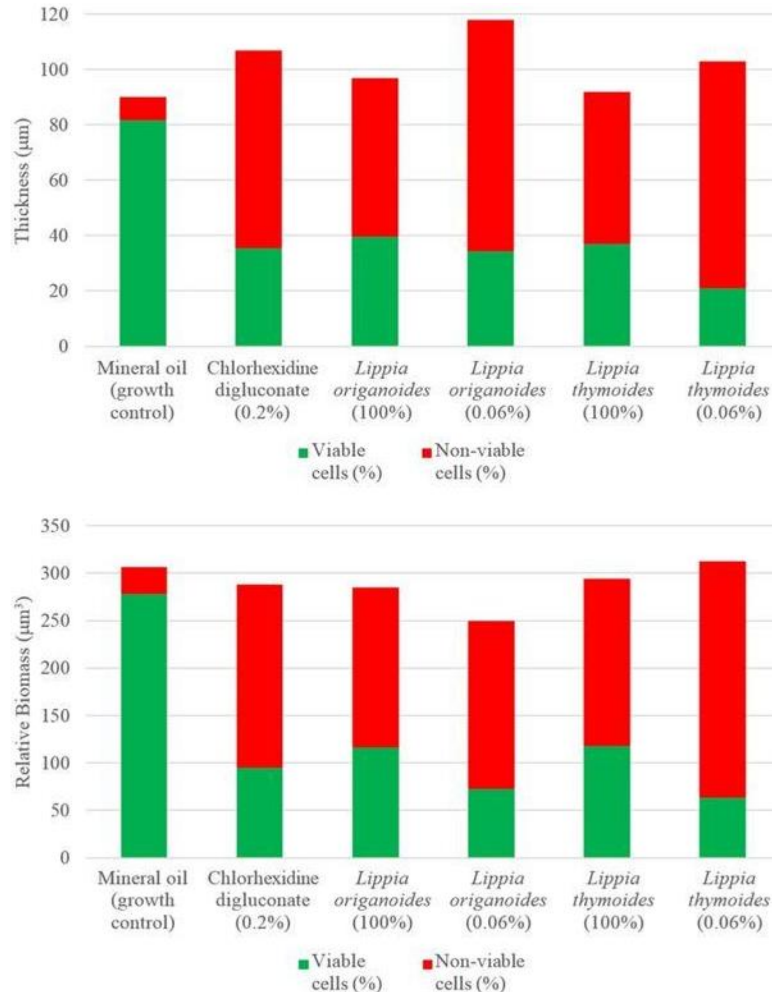

Figure 4. Profile of the biological (non-viable cells) and (relative biomass and thickness) greatness parameters of *S. mutans* UA159 biofilms grown on polystyrene microplate surfaces and exposed to the mineral oil, chlorhexidine (0.2% w/vol.), *Lippia thymoides* essential oils (0.06% and 100% vol/vol) and *Lippia origanoides* essential oils (0.06% and 100% vol/vol).

dose- and time-dependent aspects of the products' effects (de Oliveira et al., 2018). However, research with *L. origanoides* essential oil did not show acute or chronic toxicity, nor did it indicate significant alterations in physiological or organ function in treated rats (Andrade et al., 2014). We hypothesize that the essential oils from *L. origanoides* is a promising and marketable antimicrobial product or addition to current antimicrobial products.

In this study, 0.2% chlorhexidine was used as a positive control in our biofilm experiments, based on a previous report that the antimicrobial activity of 0.2% chlorhexidine is concentration dependent irrespective of the number of treatments, and that its maximum effective concentration decreases on *S. mutans* biofilms over time (Lee et al., 2016). Our quantitative biofilm cell viability assay suggested that *L. thymoides* and *L. origanoides* essential oils significantly eliminated *S. mutans* from a biofilm better than 0.2% chlorhexidine (Figure 1). Aside from the research presented here, there is a general lack of information on the antimicrobial action of *L. thymoides* and *L. origanoides* essential oils against biofilms. However, a crystal violet assay and qualitative analysis of biofilms showed that thymol, a compound present in *L. thymoides* essential oil, significantly reduced *S. mutans* biofilm formation (Khan et al., 2017). A study indicated that a single application of mouthwash containing essential oil had immediate antimicrobial potential and biofilm penetration capacity that was better than that observed with 0.2% chlorhexidine under the same conditions (Quintas et al., 2015).

Although the *L. origanoides* essential oil had less antimicrobial efficiency than that of *L. thymoides* on biofilms, both substantially reduced the number of colonies (Figure 1). Additionally, carvacrol, a predominant

compound in *L. origanoides* essential oil, decreased the expression of the *gtf B* gene, suggesting a mechanism for its *S. mutans* biofilm formation inhibition (Khan et al., 2017).

SEM analysis revealed no structural differences in cells treated with *L. origanoides* essential oil, unlike *L. thymoides* essential oil, which were lysed and deformed cells (Figure 2). These results suggest that *L. origanoides* essential oil does not affect the morphology of bacterial cells in biofilms, at least not after 5 min of treatment. However, a study of the individual *L. origanoides* essential oil components thymol and carvacrol indicated that overexpression of autolysin genes involved in reformulation of the *S. mutans* cell wall (Khan et al., 2017). As described in this work, some compound(s) present in essential oil may have an antagonistic effect on the action of other compounds in the oil (Botelho et al., 2007).

The results obtained by confocal microscopy show that *L. thymoides* and *L. origanoides* essential oils could penetrate into the biofilm volume, which increased cell death more than that of samples treated with 0.2% chlorhexidine (Figures 3 and 4). In another study on the penetration capacity of essential oils, the penetration capacity of a single application of essential oil was higher than that of 0.2% chlorhexidine (Quintas et al., 2015). Other research also suggests that thymol and carvacrol compounds found in *L. thymoides* and *L. origanoides* essential oils, respectively, exteriorize the overexpression of the *sodA* and *ymcA* genes, assuming that therapeutics containing these compounds elicit general and oxidative stresses in microbial cells (Khan et al., 2017). However, a single application of essential oils is not sufficient to decrease biofilm thickness (Quintas et al., 2015).

The *L. thymoides* and *L. origanoides* essential oils have an antimicrobial effect on *S. mutans* bacteria. Our results confirm that *L. thymoides* and *L. origanoides* essential oils are promising and effective treatments for reducing *S. mutans* biofilms. However, these essential oils were not evaluated for their toxicity and therefore, subsequent studies of the action of these oils on bacterial cells and biofilms, as well as on their degree of cytotoxicity, will be needed to develop new drug therapies and clinical studies.

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