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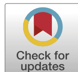

Lippia origanoides derivatives *in vitro* evaluation on polymicrobial biofilms: *Streptococcus mutans, Lactobacillus rhamnosus* and *Candida albicans*

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ABSTRACT

Objective: This work evaluated the *Lippia origanoides* derivatives *in vitro* effect on polymicrobial biofilms of *Streptococcus mutans, Lactobacillus rhamnosus* and *Candida albicans*. Additionally, the cytotoxic effect of the oils on human skin keratinocytes (HaCaT) and fibroblasts of the periodontal ligament (FLP) cell lines was evaluated.
Design: The minimum inhibitory concentration, the inhibitory activity on monomicrobial (*S. mutans*) and polymicrobial biofilm (*S. mutans, L. rhamnosus* and *C. albicans*) of *L. origanoides* four essential oils and terpenes (thymol and carvacrol) were evaluated. The cytotoxic effect of each one of the compounds was measured, and all the tests were compared against chlorhexidine.
Results: All the evaluated compounds reached an inhibition percentage of *S. mutans* monomicrobial biofilms formation of 100 % at 600 μg/mL ($p < 0.0001$). The highest concentration (2 MIC) eradicated 100 % of *S. mutans*-preformed biofilms after 5 min *L. origanoides* carvacrol + thymol and thymol chemotypes showed marked reductions in topography, the number of microbial cells and extracellular matrix on polymicrobial biofilm. The cytotoxic effect of the compounds was very similar to chlorhexidine.
Conclusions: L. origanoides essential oils have an inhibitory effect on mono and polymicrobial biofilms. The oils present a similar cytotoxic effect to chlorhexidine on HaCaT and FLP cell lines. However, including these compounds in formulations for clinical use is an exciting proposal yet to be investigated.

1. Introduction

A biofilm is an organized structure of microbial communities covered by an extracellular matrix that favors the fermentation of carbohydrates available from a diet, increasing acid production (Bertilsson et al., 2022; Wei et al., 2020) and it is associated with caries formation. Caries is a widespread disease described as a dynamic process that alternates phases of demineralization and remineralization of tooth structure (Peres et al., 2019). The main factors for biofilm formation include lifestyle, socioeconomic status, smoking, xerostomia, reduced exposure to fluoride, and poor oral hygiene (Al-Zahrani et al., 2022), but free sugar -defined as monosaccharides and disaccharides added to food- is the most critical factor (Al-Zahrani et al., 2022; Ferizi et al., 2022; Peres et al., 2019). These organized microbial communities can cause pathological alterations, thus their early disaggregation has been suggested (Bersan et al., 2014).

Although many studies have focused on studying the formation of monomicrobial biofilms (Covarrubias et al., 2018; Jordão et al., 2020; Ortega-Cuadros et al., 2018; Wall et al., 2019), only a few have concentrated their efforts on studying the association of at least two microbial species (Cieplik et al., 2019; O'Donnell et al., 2015). This becomes particularly relevant considering that biofilms are described as complex ecosystems that include the presence and interaction of multiple microorganisms and are affected by different environmental conditions, such as pH, oxygen and nutrient availability (Filoche et al., 2010).

Adequate oral hygiene (tooth brushing and flossing) is considered the gold standard for controlling oral problems; even a balanced diet has been described as a protective factor (Al-Zahrani et al., 2022). Other complementary measures include chemical control, which is significant in elderly patients with physical or mental limitations, orthodontic appliances users, post-surgery or patients with hyposalivation (Takenaka et al., 2019). The chemical control of bacterial plaque can be done through mouthwashes divided into products for cosmetic or therapeutic

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use based on different compounds, including delmopinol, hexetidine, povidone-iodide, cetylpyridinium chloride, essential oil, and chlorhexidine (Cieplik et al., 2019). Chlorhexidine is considered the gold standard antimicrobial agent for oral biofilm control because of its fungicidal, bactericidal, and bacteriostatic effects (Chaves-Quirós et al., 2020; Heliawati et al., 2022). Prolonged use of chlorhexidine causes undesirable side effects like yellow-brown pigmentations of enamel surfaces and disturbances in taste (Cieplik et al., 2019). To counteract the adverse effects of controlling oral plaque with chlorhexidine, natural compounds have been tested, and some of them have been found to have an inhibitory effect on plaque formation with little or no adverse effects. With increasing antimicrobial resistance and the need for more cost-effective treatments with fewer adverse effects (Kumari et al., 2019), the natural plants emerge as an interesting alternative for use in the clinical context (Khan et al., 2017).

On the other hand, Colombia has a great diversity of flora, which can be explored to develop therapeutic alternatives to commercial products. Among the plant resources, the medicinal and aromatic plants of the genus *Lippia* have traditionally been used in skin, gastrointestinal and liver diseases. Various studies demonstrate the antimicrobial, antioxidant and anti-inflammatory properties of the essential oils of species of this genus (Freires et al., 2015; Ribeiro et al., 2021). Essential oils are secondary plant metabolites that have been attributed a wide variety of benefits in alternative medicine and are widely accepted for use. Essential oils have even been tested for their effectiveness in eradicating oral bacterial biofilms in search of an alternative to using chlorhexidine with different plants (Ebani & Mancianti, 2020; Khan et al., 2020; Mouta et al., 2022). Another aspect that adds relevance to this type of research is the interest in developing non-antibiotic alternatives to treat infections by multiresistant microorganisms (Lu et al., 2018). Essential oils have also shown greater effectiveness in the control of antibiotic-resistant microorganisms (Ebani & Mancianti, 2020). The development of *C. albicans* biofilms is a highly regulated and coordinated process that protects the yeast from the action of the immune system and favors its dissemination and invasion (Wall et al., 2019). Besides, the prolonged use of antifungals can cause antimicrobial resistance, so it is important to find alternatives to their use (Jordão et al., 2020). This study aims to evaluate the antimicrobial effect of essential oils from native Colombian plant *Lippia origanoides* and two terpenes (thymol and carvacrol) on the polymicrobial biofilms of *Streptococcus mutans, Lactobacillus rhamnosus* and *Candida albicans.*

2. Materials and methods

2.1. Strains

Streptococcus mutans UA159, *Lactobacillus rhamnosus* ATCC 7469 and *Candida albicans* ATCC 10231 strains were purchased from the ATCC (American Type Culture Collection, Manassas, Virginia, USA). The bacteria and *C. albicans* were recovered and maintained on Brain Heart Infusion (BHI) broth and Sabouraud Dextrose Agar (SDA) (Merck, Darmstadt, Germany) respectively, in microaerophilic atmospheric conditions at 37 °C. BHI broth, SDA with Chloramphenicol, Mitis Salivarius Agar (AMS) (Thermo Scientific, Waltham, Massachusetts, USA) and Rogosa Agar (LBS) (Merck, Darmstadt, Germany) were used as the culture medium for biofilm assays.

2.2. Access to genetic resources and derived products for bioprospecting purposes

In accordance with Colombian legislation, this project was developed under the contract for access to genetic resources and derived products for bioprospecting purposes No. 270, signed between Ministerio de Ambiente y Desarrollo Sostenible and the Universidad Industrial de Santander. The plant material and its derived products (essential oils from *Lippia origanoides*) were provided by the Colombian State, through Ministerio de Ambiente y Desarrollo Sostenible.

2.3. Compounds

Two commercial terpenes (thymol and carvacrol) obtained from Sigma-Aldrich with at least 94 % purity and five essential oils of different chemotypes *of L. origanoides* were used in this study (Table 1). The oils were extracted and characterized by Centro Nacional de Investigaciones para la Agroindustrialización de Especies Vegetales Aromáticas Medicinales Tropicales (CENIVAM). All the information about obtaining and analyzing essential oils is previously described by Zapata-Zapata et al. (2022).

The essential oils were coded to carry out a blind evaluation and their identification was known only after performing the analysis. Stock solutions of essential oils and terpenes were solubilized in Dimethyl sulfoxide (DMSO) with 99 % purity (Sigma-Aldrich, Saint Louis, Missouri, USA).

2.4. Evaluation of the minimum inhibitory concentration (MIC)

The inhibitory activity of essential oils of *L. origanoides* and terpenes was determined through the broth microdilution technique in planktonic cultures of *S. mutans*, *L. rhamnosus* and *C. albicans*. The working solutions of derivatives of *L. origanoides* were prepared in BHI broth. Serial dilutions 1:2 were made at the concentrations required in each assay, with a final concentration of DMSO $\leq$ 2 %. Chlorhexidine (Sigma-Aldrich) was used at 2.34–300 μg/mL concentrations as a positive control of microbial growth inhibition.

2.5. S. mutans and L. rhamnosus planktonic cultures

The determination of the antibacterial activity of the compounds was carried out following the recommendations of Clinical & Laboratory Standards Institute (CLSI) in the document CLSI M07 (Clinical and Laboratory Standards Institute, 2018), with minimal modifications according to the technical needs of the assay. Briefly, 96 flat-bottomed polypropylene wells (Corning, New York, USA) contained essential oils and terpenes at 18.75–2400 μg/mL concentrations. The bacterial inoculum was prepared from pure cultures. The bacteria were suspended in BHI broth, and the turbidity was adjusted in a Multiskan Sky High spectrophotometer (Thermo Scientific, Waltham, Massachusetts, United States) to the equivalent of 1 on the McFarland scale (OD~0.257) for a subsequent 1:30 dilution in BHI broth with a final concentration of 1–5 $\times$ 10^5 CFU/mL. A sterility control was added, and the plates were incubated for 24 h at 37 °C with 5 % $C0_2$. Subsequently, 30 μL of 0.015 % (w/v) resazurin was added to each well and incubated for 3 h under the same conditions described previously. Resazurin was employed as an inhibitor of bacterial metabolism to inhibit turbidity caused by essential oils and avoid errors in the visual interpretation of results (Elshikh et al., 2016). The MIC was defined as the minimum concentration of the compound that prevents the color change from blue to pink-orange. Each assay was performed three times in triplicate.

2.6. C. albicans planktonic cultures

The broth microdilution technique for *C. albicans* was evaluated according to CLSI in guide M27 (Clinical and Laboratory Standards Institute, 2017) with minor modifications. Each well contained microbial inoculum of 1.5–3.5 $\times$ 10^3 CFU/mL. The final range of concentrations of the compounds was from 4 to 512 μg/mL. The plates were incubated at 37 °C at 5 % CO_2 for 24 h. The MIC was defined as the lowest concentration of the compounds that inhibited $\geq$ 90 % of the visible growth of *C. albicans* relative to the growth control (BHI broth with *C. albicans*). Chlorhexidine, fluconazole and amphotericin (Sigma-Aldrich) were used as a positive control for microbial inhibition. Each assay was performed at three different times in triplicate.

Table 1
Lippia origanoides essential oils and their principal constituents.

CODE	Plant species and chemotype	Collection site	*Voucher* number	Principal compounds (relative amount, %)
2206	*Lippia origanoides* (Carvacrol + Thymol Chemotype)	Barbosa – Santander, Colombia	COL 587104	Carvacrol (34.9), thymol (23.3), γ-terpinene (11.1), p-cymene (9.0), trans-β-caryophyllene (5.0), α-humulene (2.5), α-terpinene (1.8), β-myrcene (1.7), thymyl methyl ether (1.6), and carvacryl acetate (0.8).
0008	*Lippia origanoides* (Carvacrol Chemotype)	Bucaramanga – Santander, Colombia	Industrial University of Santander Herbarium 22034	Carvacrol (35.0), p-cymene (14.4), thymol (8.0), γ-terpinene (5.3), trans-β-caryophyllene (4.4), β-myrcene (2.4), carvacryl acetate (2.0), thymyl methyl ether (1.9), α-terpinene (1.7 %) and α-thujene (1.6).
0010	*Lippia origanoides* (Thymol Chemotype)	Bucaramanga – Santander, Colombia	Industrial University of Santander Herbarium 22035	Thymol (75.3), trans-β-caryophyllene (5.4), carvacrol (4.9), α-humulene (3.2), p-cymene (2.3), thymyl acetate (1.6), thymyl methyl ether (1.3), caryophyllene oxide (1.3), and trans-β-bergamotene (1.0).
0018	*Lippia origanoides* (Thymol + *p*-Cymene Chemotype)	Bucaramanga – Santander, Colombia	UIS herbarium 22039	Thymol (49.4), p-cymene (19.1), γ-terpinene (9.2), β-myrcene (5.2), α-terpinene (2.9), carvacrol (2.7), thymyl methyl ether (1.8), trans-β-caryophyllene (1.6), cis-β-ocimene (1.2), and limonene (0.9).
0019	*Lippia origanoides* (Thymol Chemotype)	Bucaramanga – Santander, Colombia	Industrial University of Santander Herbarium 22036	Thymol (71.7), p-cymene (10.5), carvacrol (4.4), β-myrcene (2.1), γ-terpinene (2.0), caryophyllene oxide (1.6), thymyl methyl ether (0.9), trans-β-caryophyllene (0.9), humulene epoxide II (0.7), and terpinen-4-ol (0.7).

2.7. L. origanoides essential oils and terpenes in vitro inhibition effect on S. mutans biofilms

The inhibitory activity of *L. origanoides* essential oils and terpenes on the formation of monomicrobial biofilms of *S. mutans* was determined by viable counts of the microbial population (CFU/mL) recovered from each biofilm after treatment. In 96-well plates, essential oils and terpenes that showed inhibitory activity on planktonic cultures at four different concentrations diluted in BHI broth were added. The evaluated concentrations of the compounds ranged between 75 and 600 μg/mL. As an inhibition control, chlorhexidine was added at a concentration of 1200 μg/mL (0.12 % p/v). An 3–5 $\times$ 10^6 CFU/mL of *S. mutans* inoculum was added. Growth controls (bacteria inoculum in BHI broth) and sterility controls (BHI broth without microorganisms) were included. The 96-well plate was covered with the MBEC Assay® device (Innovatech, Edmonton, Canada) (Ceri et al., 2001) and incubated for 24 h at 37 °C and 5 % CO_2 under continuous agitation at 110 RPM. Subsequently, the BHI broth was changed after 24 h incubation, and the plate was incubated for 48 h for *S. mutans*. After the final incubation, the MBEC device was washed in a 96-well plate with 200 μL of 0.9 % NaCl for two minutes to remove non-adherent cells. Finally, the MBEC device was transferred to a 96-well plate with 180 μL of BHI broth to perform biofilm recovery through sonication in an m150 soniclean ultrasound bath (Midmark Corporation, Dayton, Ohio, United States) for 30 min for *S. mutans*. With the material resulting from sonication, serial dilutions were made from 1:10–10^{-6} for subsequent inoculation using the spot technique (10 μL) in BHI. After 48 h of incubation, a microbial count was performed, and the percentage of biofilm inhibition was calculated between the microorganisms treated with essential oils, terpenes and CHX compared to the growth control according to the following formula of biofilm formation:

$$LOG = LOG10(CFU)$$

$$\%inhibitionoreradication = \left(\frac{LOGcc - LOGbpt}{LOGcc}\right) * 100$$

LOGcc is log10 CFU/mL of the biofilm growth control, and LOGbpt is the biofilm formation exposed to the compounds and activity controls.

2.8. L. origanoides essential oils and terpenes in vitro effect on S. mutans preformed biofilms

To evaluate *L. origanoides* essential oils and terpenes effect on preformed *in vitro S. mutans* films, the methodology described by Ortega-Cuadros et al. (2018) was followed with some modifications. Monomicrobial *S. mutans* biofilms were formed for 48 h, using the commercial MBEC device (Ceri et al., 2001). A 1 McFarland scale suspension was prepared from pure cultures, and a 1:30 dilution of this inoculum was subsequently made. Of each inoculum, 160 μL were added to the MBEC device. A sterility control consisting of BHI broth without microorganisms was added. The MBEC device was incubated at 37 °C, 5 % CO_2 with continuous shaking at 110 RPM. After the incubation time for biofilm formation, the MBEC device was adjusted to a 96-well plate with a 0.9 % NaCl solution for 2 min to remove non-adherent cells. The MBEC device was set in a 96-well plate containing 180 μL of the essential oils and terpenes with concentrations from 0.25X MIC to 2X MIC for *S. mutans.* As growth inhibition control, chlorhexidine was used at concentrations of 1200 μg/mL (0.12 % p/v) and 2000 μg/mL (2 % p/v) and 2.4 % DMSO. The MBEC device was incubated at 37 °C, 5 % CO_2 with continuous shaking at 110 RPM. Subsequently, the MBEC device was transferred to a 96-well plate with a neutralizing solution and allowed to stand for 30 min to limit the action time of the compounds under evaluation. The neutralizing solution was prepared from a 1:41 dilution of a universal and a surfactant solution. The universal solution consisted of 1 g of L-histidine, 1 g of L-cysteine and 2 g of reduced glutathione in 20 mL of sterile distilled water. The surfactant

solution was composed of 1 g of Saponin and 471 μL of Tween 80 dissolved in 50 mL of BHI broth. The MBEC Assay® device was subjected to sonication at 42 kHz through an m150 soniclean ultrasound bath for 30 min for *S. mutans* biofilms. With the material obtained from the sonication, serial dilutions were made from 1:10–10^6 in BHI broth, and 10 μL have inoculated in BHI. The microbial count was performed after 48 h of incubation, and the percentage of logarithmic reduction of biofilms was calculated using the previously described formula of biofilm formation.

2.9. *L. origanoides* essential oils and terpenes inhibitory activity on a preformed polymicrobial biofilm

The effect of *L. origanoides* four essential oils and two commercial terpenes on a polymicrobial biofilm was evaluated according to Guggenheim et al. (2001) with some modifications. Biofilm formation was carried out by incubating glass disks with sterile human saliva at 37 °C under continuous agitation for one hour. Saliva was collected from one of the researchers without a history of caries and active lesions, without gingivitis or periodontitis, with the normal salivary flow (>1 mL/min) and who had not taken antibiotics three months before obtaining the sample. The donor did not brush, eat, or drink for 2 h before sampling. The collection was performed through saliva stimulated by chewing a piece of paraffin (0.7 ± 0.1 g) for 15 min or until collected 50 mL. With the collected samples, a poll was made and treated with 2.5 mM dithiothreitol to reduce the aggregation of salivary proteins. Subsequently, it was centrifuged at 4000 g for 10 min at 4 °C; the supernatant was filtered using a 0.45 μm filter and stored at −20 °C until use (maximum one week). Sterility control was performed by seeding 100 μL of sterile saliva on blood agar and BHI at 37 °C in aerobiosis and microaerophilia for 72 h. After incubation with saliva, the glass disks were incubated in 24-well plates, and 1080 μL of a suspension of each bacterium at a final concentration of 1–5 × 10^8 CFU/mL (*S. mutans* and *L. rhamnosus*) and 240 μL of *C. albicans* at a final concentration of 0.5–5 × 10^5 CFU/mL. Parafilm-sealed dishes were incubated at 37 °C, 5 % CO_2 for 24 h. After incubation, the glass disks were incubated for 5 min in a 24-well plate with 0.9 % NaCl and transferred to a new 24-well plate in which essential oils were previously added at concentrations of 512, 1024, 1536 and 2048 μg/mL. Chlorhexidine at 2 % (2000 μg/mL) and 0.12 % (1200 μg/mL) was added as antimicrobial activity control, and BHI broth with and without DMSO was added as a control for the effect of the solvent and growth, respectively. The biofilm was incubated with the different test solutions and controls for 5 min at 37 °C under continuous agitation to simulate hygiene oral product conditions in clinical use. Subsequently, the glass disks were transferred to a new 24-well plate with 2500 μL of neutralizing solution for 30 min and sonicated at 42 kHz. Finally, serial dilutions from 1:10–10^6 were made with the sonication product, and 10 μL of each dilution was inoculated into the selective agars for each microorganism: *S. mutans* in mitis salivarius agar, *L. rhamnosus* in rogosa agar, and *C. albicans* in sabouraud dextrose agar with chloramphenicol. After incubation of each agar at 37 °C, 5 % CO_2 for 48 h, a microbial count was performed, and the percentage of logarithmic reduction was calculated with the previously described formula of biofilm formation.

2.10. Scanning electron microscopy

Each biofilm exposed to the highest concentration of the compounds in which there was a decrease in microbial viability was selected to observe the large-scale morphological changes. The polymicrobial biofilms were fixed with 2.5 % glutaraldehyde for 12–24 h; three washes were performed with PBS and then increasing concentrations of 50 %, 75 % and 95 % ethanol. Final dehydration with 100 % ethanol for 45 min was made. Samples were fixed with graphite tape and plated with gold on a cathode sputter (DENTON VACUUM Desk IV Beijing, China) to a thickness of ~20 mn. Each sample was analyzed in a High Vacuum Scanning Electron Microscope (JEOL JSM 6490 LV Tokyo, Japon). The secondary electron detector (SEI) was used to evaluate the morphology and topography of the sample.

2.11. Cytotoxicity assessment

To evaluate the cytotoxic effect of each of the compounds in this study, the cell lines, HaCaT (Human adult low Calcium high Temperature) of adult human skin keratinocytes and FLP (fibroblasts of the periodontal ligament) were used. The HaCaT cell line was gently donated by Professor Belfran Carvonel of the Genetic, Regeneration and Cancer Group (GRC) at the University of Antioquia. The FLP cell line originated from a primary crop of a patient's periodontal ligament fibroblasts. To obtain the periodontal fibroblasts, approval of the research and ethics committee of the Faculty of Dentistry of the Pontificia Unviersidad Javeriana through act #005 of February 12, 2021 was obtained. Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco), supplemented with 10 % Fetal Bovine Serum (SBF, GIBCO®, USA) and 1 % Penicillin (100 U/mL)-Streptomycin (100 ug/mL) (P/S) (GIBCO®, USA) and gentamicin (100 U/mL, GENFAR®, Colombia) at 37 °C and 5 % CO_2. The evaluation of cytotoxicity was performed by the MTS technique, using a tetrazolium compound [3-(4,5-dimethylthiazol-2-yl) -5- (3-phenyl carboxymethyl) -2- (4-sulfophenyl) - 2H-tetrazolium, inner salt; MTS]. This colorimetric assay determines the number of viable cells based on the bioreduction of a tetrazolium compound into a colored formazan (Cell Titer 96® AQueous Non-Radioactive Cell Proliferation Assay, Promega Corporation, Madison, Wisconsin, USA). In flat-bottom 96-well plates, 16 × 10^3 cells/well of each cell line were added in DMEM medium and the previous protocol, described by Sadeghi-Aliabadi et al. (2009), was followed. After 12 h of incubation, the medium was removed, and serial 1:2 dilutions of the compounds were added, obtaining concentrations between 1.95 and 2000 μg/mL for 5 min at 37 °C, 5 % CO_2. Once the incubations were finished, the stimuli were removed, and 20 μL of MTS and 100 μL of DMEM were added to each well, followed by a new incubation for 3 h. The absorbance was determined in a spectrophotometer microplate reader (BioRad, Hercules, California, USA) at 493 nm. CC_{50} values will be obtained by linear regression analysis from 3 independent assays in triplicate.

2.12. Statistical analysis

The data was tabulated and stored in the Microsoft Excel program. Statistical analysis was performed using the Prism 6.0 statistical program (GraphPad, San Diego, California, USA), and a $p < 0.05$ was considered significant. To evaluate the effect of the oils on microbial viability, Kruskal Wallis and the Bonferroni post-test were used. The data are expressed as the median and range.

3. Results

3.1. Minimum inhibitory concentration (MIC)

Compounds extracted from *L. origanoides* were coded and their composition is described in Table 1. The main components of the oils extracted from *L. origanoides* are thymol and carvacrol, for which both commercial compounds were evaluated and their chemical structure is presented in Fig. 1 (PubChem database, National Institutes of Health, Bethesda, MD, USA). *L. origanoides* five essential oils and two commercial terpenes (thymol and carvacrol) showed inhibition in the 3 microorganisms evaluated (*S. mutans* UA159, *L. rhamnosus* ATCC 7469 and *C. albicans* ATCC 10231) for which they were selected to evaluate their

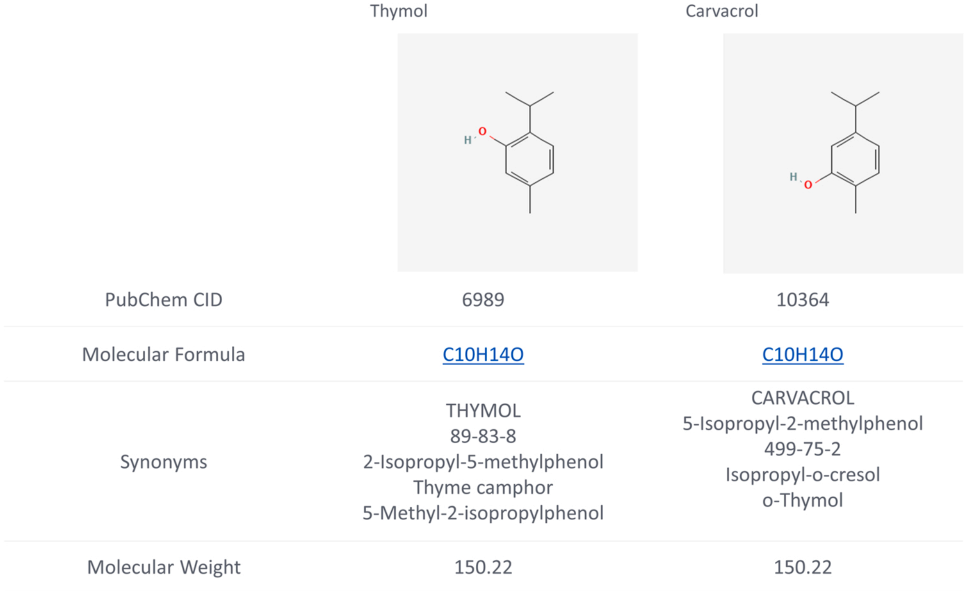


Fig. 1. Thymol and carvacrol chemical structures. Chemical structures of thymol and carvacrol, molecular formula, synonyms and molecular weight. Images used belong to the PubChem library. https://pubchem.ncbi.nlm.nih.gov/compound/6989, https://pubchem.ncbi.nlm.nih.gov/compound/10364.

activity in inhibiting the formation of monomicrobial biofilms. Compounds were used at a maximum of 2400 and 512 μg/mL concentrations for bacteria and yeast, respectively (Table 2). Essential oils 0018, 0019 and thymol showed more significant activity against *S. mutans* (600 μg/mL). *L. rhamnosus* and *C. albicans* MICs were 1200 μg/mL and 512 μg/mL respectively for all compounds with inhibitory activity. Chlorhexidine showed a MIC of 4.69 and 9.37 μg/mL for bacteria and yeast, respectively. The MIC of amphotericin and fluconazole as reference antifungals against *C. albicans* was also evaluated. Limonene and perillyl alcohol presented a higher MIC than the other terpenes evaluated.

3.2. L. origanoides derivatives inhibitory activity determination on S. mutans monomicrobial biofilms formation

The inhibitory activity of five *L. origanoides* essential oils, thymol and carvacrol on *S. mutans* UA159 formation biofilm was performed. An exposure time of 24 h was evaluated, with 4 concentrations per compound (75–600 μg/mL), according to the values obtained in the MIC on planktonic cultures (Fig. 2). All the evaluated compounds reached an inhibition percentage of 100 % at a concentration of 600 μg/mL ($p > 0.0001$). Thymol, carvacrol and essential oil 0010 reached an inhibition of 7.9 % ($p = 0.0056$), 8.0 % ($p = 0.0393$) and 8.4 % ($p = 0.0242$), respectively at 300 μg/mL.

3.3. L. origanoides essential oils and terpenes effect on S. mutans preformed biofilms

The eradication effect on preformed monomicrobial biofilms was identified after 5 min of treatment with the compounds (Fig. 3). All compounds evaluated at the highest concentration (2 MIC) eradicated 100 % of the biofilm, similar to 0.12 % chlorhexidine ($p < 0.0001$). The compounds thymol, carvacrol, 0008, 0010, and 0019 also achieved this eradication percentage at a concentration of 1 MIC ($p < 0.0001$). Essential oils 2206 and 0018 at 1 MIC achieved 21.3 % ($p = 0.0043$) and 37.9 % ($p < 0.001$), respectively. Essential oil 0019 at 0.5 MIC achieved 29.4 % eradication on *S. mutans* biofilm ($p < 0.0001$).

3.4. Polymicrobial biofilm eradication by four L. origanoides essential oils, thymol and carvacrol

The eradication effect of four *L. origanoides* essential oils, thymol and carvacrol on *S. mutans, L. rhamnosus* and *C. albicans* formation biofilm was performed and all data were compared against microbial growth control (Fig. 4). Essential oil 0019 reached a maximum eradication of the *S. mutans* biofilm at a concentration of 4 MIC (39.24 %, $p < 0.0001$) and chlorhexidine achieved an eradication on this same bacteria of 17.39 % and 34.68 % ($p < 0.0001$), at 0.12 % and 2 % respectively. Essential oil 0019 concentration 3 MIC achieved an eradication percentage of 35.83 % ($p < 0.0001$) compared to the growth control of *L. rhamnosus*. Chlorhexidine (0.12 % and 2 %) reached an eradication of 17.24 % ($p = 0.0001$) and 26.81 % ($p = <0.0001$) respectively. As for *C. albicans*, thymol at concentrations of 3 and 4 MIC reached eradication percentages of 35.05 % and 38.88 % ($p = <0.0001$). Essential oil 0019 concentrations 3 and 4 MIC eradicated 38.70 % and 38.88 % ($p = <0.0001$), respectively. But the most significant eradication power was obtained by essential oil 2206 at the 4 MIC concentration with 42.36 % ($p < 0.0001$), even higher than the treatment with chlorhexidine at 0.12 % and 2 % (24.19 % and 36.62 % $p = <0.0001$).

3.5. L. origanoides essential oils and terpenes morphological effect on a preformed polymicrobial biofilm

S. mutans, L. rhamnosus and *C. albicans* polymicrobial biofilm was formed for 24 h in microaerophilia at 37 °C to evaluate, through scanning electron microscopy, the structure of the biofilm and its extracellular matrix on glass discs previously treated with sterile human saliva. The biofilm was exposed to *L. origanoides* essential oils and two terpenes at a concentration of 4MIC (2048 μg/mL) for 5 min. A culture medium with 2 % DMSO was used as a negative control, and 2 % (p/v) chlorhexidine was used as a control for eradication activity. Compared to the growth control (Fig. 5G), a decrease in the microscopic forms of *C. albicans* is observed in all treatments. The essential oils coded as 0019 (Fig. 5F) and 2206, (Fig. 5C) showed marked reductions in topography, number of microbial cells and extracellular matrix. The activity control

Table 2
Minimum Inhibitory Concentration (MIC) of compounds on Streptococcus mutans UA159, Lactobacillus rhamnosus ATCC 7469 and Candida albicans ATCC 10231.

Code	MIC (μg/mL)		
	Streptococcus mutans	*Lactobacillus rhamnosus*	*Candida albicans*
2206	1200	1200	512
0008	1200	1200	512
0010	1200	1200	512->512
0018	600	1200	256–512
0019	600	1200	512
Mixture	1200	>2400	>512
Thymol	600	1200	512
Carvacrol	1200	1200	512
Limonene	>2400	>2400	>512
Perilyl alcohol	>2400	NE	>512
Chlorhexidine	4.68	4.68	9.37
Amphotericin B	NA	NA	0.0625–0.125
Fluconazole	NA	NA	0.5

NE: Not evaluated, NA: Not apply

(2 % chlorhexidine, Fig. 5H) shows a reduction in the extracellular matrix and forms of *C. albicans*. This effect on cell morphology seems to be greater in decreasing order with the compounds 2206, 0019, thymol, 0018, 0010 and carvacrol.

3.6. *L. origanoides* essential oils and terpenes in vitro cytotoxic effect on HaCaT and periodontal fibroblast cell line

The cytotoxicity of the oils and terpenes was determined using the MTS colorimetric assay by determining the cytotoxic concentration 50 (CC_{50}) on HACAT and primary culture periodontal fibroblasts (FLP) cell lines at 5 min of incubation (Table 3), to simulate conditions of use in clinical practice. Although all the compounds evaluated in this study have a cytotoxic effect on each cell line used, 0010 requires 2.5 % more concentration to reach the CC_{50} compared to the chlorhexidine control on HaCaT cells. Furthermore, 0019 requires a 7.4 % higher concentration of the compound to reach the CC_{50} compared to the use of chlorhexidine on the primary culture of periodontal fibroblasts.

4. Discussion

Bacteria adhesion of teeth surface is de most prevalent problem associated with poor oral hygiene (Wei et al., 2020; Filoche et al., 2010). During biofilm formation, several microbial species adhere, including *Lactobacillus casei, Streptococcus sanguis, S. mutans, S. mitis,* and *S. sobrinus* (Bersan et al., 2014). Despite the significant evidence in this regard on biofilms, few studies evaluated the effect of essential oils on the formation of polymicrobial biofilms that include yeasts such as *C. albicans,* an aspect of great importance if one considers that a diverse ecosystem exists within the oral cavity where bacteria and yeast coexist (O'Donnell et al., 2015). In this study, the use of essential oils based on *L. origanoides* was evaluated, and their effect was compared with chlorhexidine in a short application time (5 min), simulating their use in

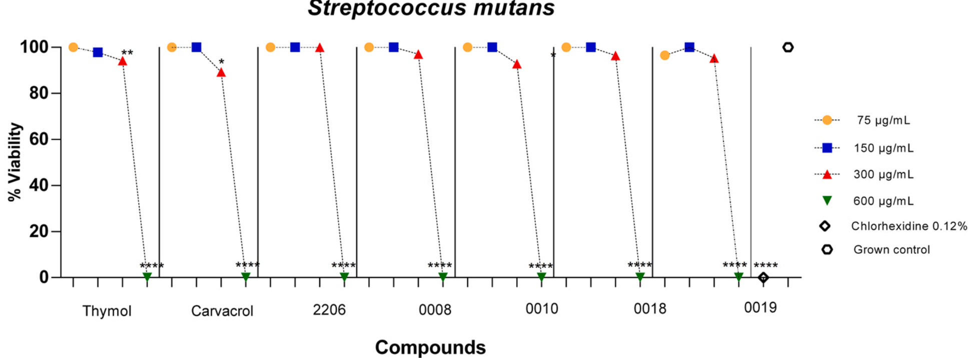


Fig. 2. ***S. mutans*** **biofilm inhibition by** ***L. origanoides*** **essential oils thymol and carvacrol.** The graph shows the percentage of *S. mutans* biofilm viability after exposure of five *L. origanoides* essential oils, thymol and carvacrol at four different concentrations (75–600 μg/mL). A 0.12 % chlorhexidine control was also included. All the results were compared with grown control (****p = < 0.0001; **p = <0.01, *p = <0.05).

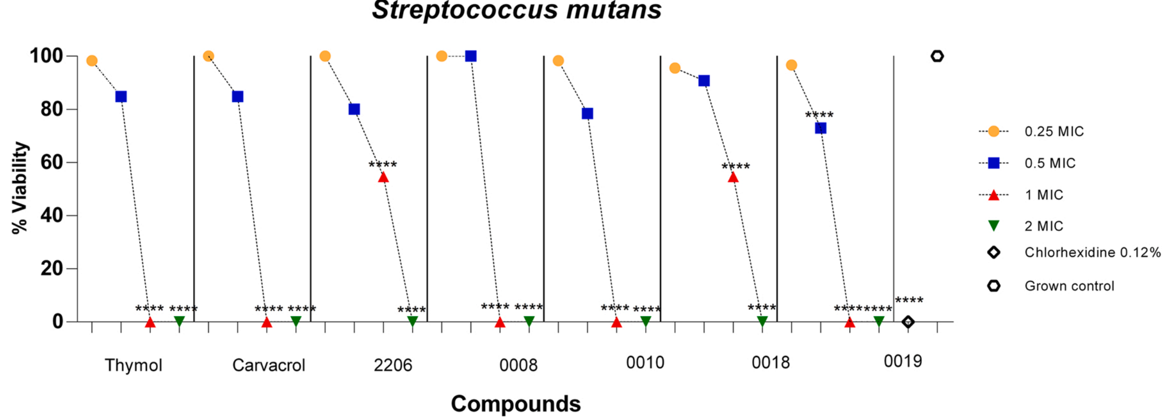
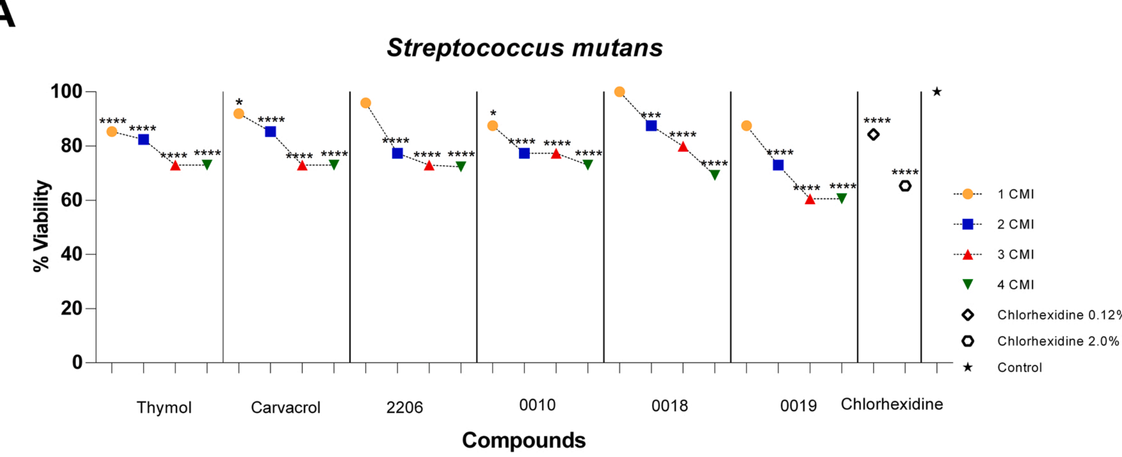
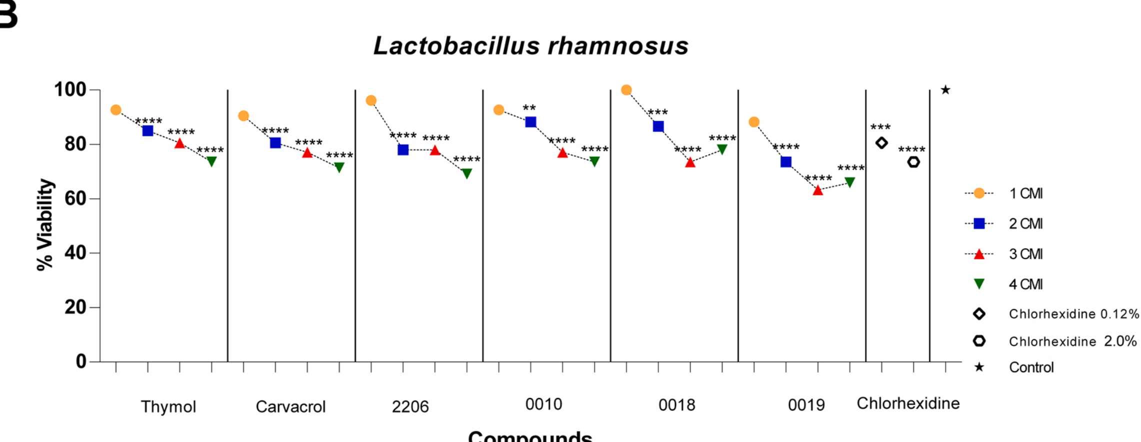


Fig. 3. ***S. mutans*** **biofilm eradication by** ***L. origanoides*** **essential oils, thymol and carvacrol.** The graph shows the percentage of *S. mutans* biofilm viability after using five *L. origanoides* essential oils, thymol and carvacrol at four different concentrations (0.25–2 MIC). A 0.12 % chlorhexidine control was also included. All the results were compared with grown control (****p = < 0,0001).





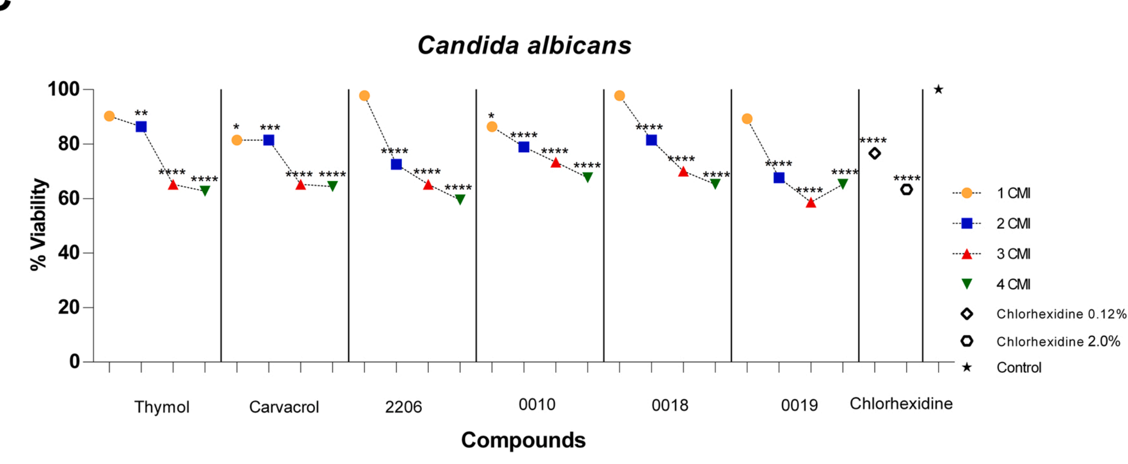


Fig. 4. ***S. mutans, L. rhamnosus* and *C. albicans* biofilm eradication by four *L. origanoides* essential oils, thymol and carvacrol.** The graph shows biofilm of *S. mutans* (A), *L. rhamnosus* (B) and *C. albicans* (C) viability after the action of four *L. origanoides* essential oils and two commercial terpenes: thymol and carvacrol at four different concentrations (1–4 MIC), 0.12 % and 2 % chlorhexidine. All the results were compared with the grown control (*p = 0.05; **p = 0.01; ***p = 0.001; ****p < 0.0001).

oral hygiene clinical practice. Our results show that *L. origanoides* oils have a microbicidal and cytotoxic effect similar to chlorhexidine.

In this study, the effect of four *L. origanoides* essential oils on the formation of mono and poly microbial films was evaluated, in addition to comparing their effect with that presented by the commercial compounds (thymol and carvacrol), and chlorhexidine. It was observed that the MICs of the compounds were higher than 600 ug/mL for S. mutans, higher than 1200 ug/mL for L. rhamnosus and higher than 512 ug/mL for C. albicans. Chlorhexidine presented a MIC of 4.69 and 9.37 μg/mL for bacteria and yeasts, respectively. Limonene and perillyl alcohol showed MIC > 2400 and > 512 μg/mL for bacteria and yeast. All the evaluated compounds caused *S. mutans* biofilms inhibition at 600 μg/mL concentrations. Regarding eradicating *S. mutans-preformed* biofilm, all compounds behave similarly to chlorhexidine at high concentrations (2 MIC). However, essential oils 0008, 0010 and 0019 also exhibited this eradication effect at 1 MIC. Essential oil 0019 and 2206 presented the

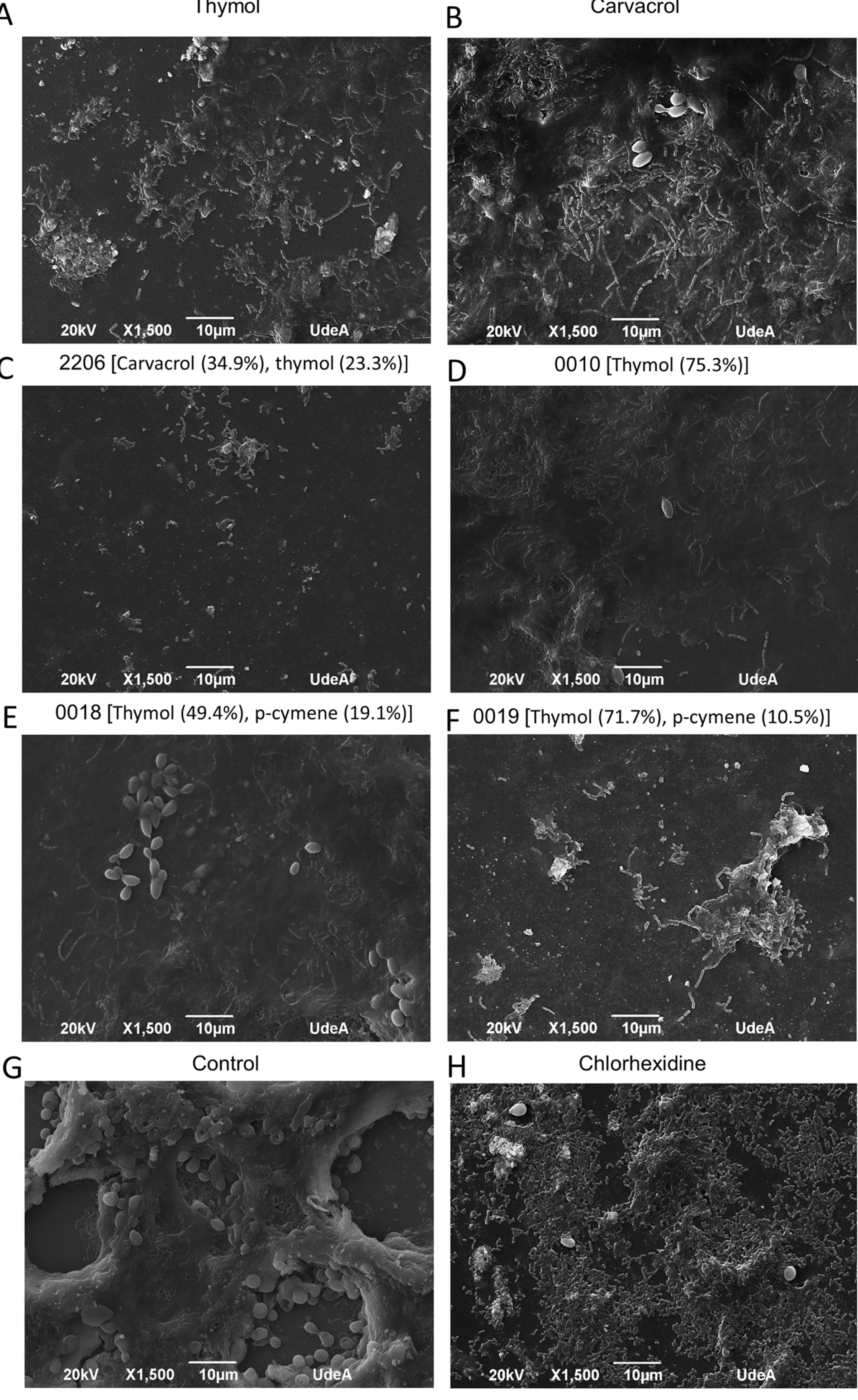


Fig. 5. *L. origanoides* essential oils and terpenes morphological effect on *S. mutans, L. rhamnosus* and *C. albicans* a preformed polymicrobial biofilm. Essential oils effect on cell morphology of *S. mutans, L. rhamnosus* and *C. albicans*. Scanning electron microscopy was employed to evaluate the eradication effect of the compounds A. thymol, B. carvacrol, C. 2206, D. 0010, E. 0018, F. 0019 and compared to G. control and H. Chlorhexidine 2 %. Photograph with 1500X magnification.

best eradication effect of the polymicrobial biofilm. Compound 0019 achieved a polymicrobial eradication percentage similar to chlorhexidine at concentrations of 4 MIC for *S. mutans*, 3 MIC for *L. rhamnosus*, and 3 and 4 MIC for *C. albicans*. Essential oil 2206 achieved a similar effect to chlorhexidine at MIC of 4 for all microorganisms. It was also possible to observe that the effectiveness of the compounds was reduced to a higher number of microorganisms; that is, the essential oils evaluated had more effects when applied to monomicrobial films than when they were tested on polymicrobial films. This eradication effect could be verified using electron microscopy, observing that 0019 and 2206 cause a decrease in the biofilm topography, in the number of microorganisms and in the extracellular matrix. This matrix of extracellular polysaccharides favors the firm adhesion of microorganisms to a solid surface (Bersan et al., 2014; Filoche et al., 2010). A possible mechanism of action of essential oils on microorganisms includes the rupture of the cell membrane (Ebani & Mancianti, 2020), which could explain the

Table 3
Lippia origanoides essential oils, thymol and carvacrol cytotoxic concentration 50 on adult human skin keratinocytes (HACAT) and fibroblasts of the periodontal ligament (FLP) cell lines.

Cell line	Compound Composition (%)	CC_{50} μg/mL	R^2	p-value
HaCaT	Thymol	1774	0.7510	0.0025
	Carvacrol	1792	0.7538	0.0024
	2206 Carvacrol (34.9), thymol (23.3)	1877	0.7415	0.0029
	0010 Thymol (75.3)	1950	0.6181	0.0120
	0018 Thymol (49.4), p-cymene (19.1)	1864	0.7847	0.0015
	0019 Thymol (71.7), p-cymene (10.5), carvacrol (4.4)	1890	0.7797	0.0016
	Chlorhexidine	1901	0.5494	0.0223
FLP	Thymol	1630	0.4817	0.0381
	Carvacrol	1621	0.4984	0.0335
	2206 Carvacrol (34.9), thymol (23.3)	1646	0.4920	0.0352
	0010 Thymol (75.3)	1622	0.4929	0.0350
	0018 Thymol (49.4), p-cymene (19.1)	1636	0.4488	0.0483
	0019 Thymol (71.7), p-cymene (10.5), carvacrol (4.4)	1671	0.4987	0.0335
	Chlorhexidine	1556	0.4269	0.0563

CC 50: cytotoxic concentration 50

observed decrease of microorganisms, especially yeasts, in the polymicrobial biofilm. This anti-biofilm effect has also been described for oregano oil (Lu et al., 2018). Finally, the cytotoxic effect of the compounds on HaCaT and FLP cell lines was evaluated and it was observed that essential oil 0019 has a CC_{50} greater than that observed by chlorhexidine. It is also described that chlorhexidine is a toxic compound, which has been shown to induce apoptosis and/or necrosis of cultured fibroblasts (Chaves-Quirós et al., 2020; Coelho et al., 2020) and odontoblast-like cells (Lessa et al., 2010).

Essential oils 0019 (thymol chemotype) and 2206 (thymol and carvacrol chemotype) derived from *L. origanoides* presented similar effects to that chlorhexidine reported regarding the inhibition and eradication of mono and polymicrobial films by microorganisms such as *S. mutans, L. rhamnosus* and *C. albicans.* However, these thymol and carvacrol-rich chemotypes also exhibit a cytotoxic effect similar to that of chlorhexidine. This effect may be associated with the higher concentration of terpenes in both chemotypes of *L. origanoides*, which have demonstrated an antimicrobial effect against biofilm formation (Bersan et al., 2014). Despite their cytotoxicity, the subsequent exploration of these two compounds in other models and tests is an incentive to the problem of antimicrobial resistance to the use of chlorhexidine (Huang et al., 2022), and also its adverse effects with prolonged use such as affectation in taste and change in dental color (Cieplik et al., 2019). Even with its side effects, the use of chlorhexidine has demonstrated a prolonged effect against a broad spectrum of microorganisms, even in the presence of body fluids such as blood (Bersan et al., 2014). Chlorhexidine has two chloroguanide chains linked by a central hexamethylene chain, its main application is surface disinfectant and as a topical antiseptic (Heliawati et al., 2022).

The results found in this study agree with what was reported by Khan et al. (2017) who used two chemotype oils of thymol and carvacrol from the plant *Origanum vulgare* L. They observed that 100 μg/mL of the compounds reduced the bioavailability of *S. mutans* by more than 50 % through cell lysis. Thymol and carvacrol are terpenes that have been shown to be effective as antimicrobials and have antiviral and antitumor effects (Elshikh et al., 2016).

It should be noted that this *in vitro* study evaluated the effect of different essential oil chemotypes on a polymicrobial biofilm composed of three microorganisms. However, it has been described that different microenvironments present diverse microbial compositions, ranging from 50 to 1000 species (Lamont et al., 2018). This work can be considered the basis for future research in which the effect of these compounds on other microorganisms associated with oral health is evaluated. For example, in a study by Pardo-Castaño et al. (2020), the antimicrobial effect of collinin and isocollinin, chlorhexidine and sodium hypochlorite (NaClO) was evaluated on periodontal bacteria (*Aggregatibacter actinomycetemcomitans, Porphyromonas gingivalis, Fusobacterium nucleatum, Prevotella intermedia, Escherichia coli, Dialister pneumosintes, Staphylococcus aureus*, and *Pseudomonas aeruginosa*) finding a microbicidal effect at MICs of 2.1 μg/mL and 4.2 μg/mL, for collinin and isocollinin, respectively. This may be especially important for microorganisms such as *P. gingivalis* whose prevalence in the Colombian population is 72 % (Lafaurie et al., 2007) and in Latin America, it can reach up to 89 % (Contreras et al., 2015).

Evaluating the effect of these oils on bacteria resistant to antibiotics may also be an exciting option. A study that evaluated oregano oil showed significant anti-bacterial activity against 11 multi-antibiotic resistant bacteria, including *Acinetobacter baumannii, Pseudomonas aeruginosa*, and methicillin-resistant *Staphylococcus aureus*, in addition to eradicating biofilms formed by these bacteria (Lu et al., 2018).

The use of natural compounds rich in thymol and carvacrol not only have antimicrobial activity but beneficial effects for health, in general, have also been described with the use of plant oils, so their research will always be an attractive topic (Elshikh et al., 2016). The acceptance by users of healthcare products of natural origin is associated with the fact that they are cheaper, less toxic and less likely to induce microbial resistance compared to medicines (Bersan et al., 2014; Gad & Fouda, 2020). All these characteristics make the derivatives of *L. origanoides* an interesting and potential product for oral health.

Finally, the results of this study suggest that essential oils derived from the *L. origanoides* plant, especially those with a predominance of terpenes in their composition such as thymol or carvacrol, have an antimicrobial effect similar to that of chlorhexidine. Both thymol and carvacrol affect the formation of polymicrobial biofilms, reduce the extracellular matrix and affect the viability of microorganisms without a more significant cytotoxic effect than that presented by the reference compound. The antimicrobial properties of essential oils against various bacteria and fungi, including oral pathogens, have been widely demonstrated (Bersan et al., 2014; Ebani & Mancianti, 2020; Heliawati et al., 2022).

5. Conclusion

L. origanoides thymol chemotype and thymol/carvacrol chemotype show an inhibitory and eradication effect on mono and polymicrobial biofilms, similar to those reported by chlorhexidine. Both compounds had a cytotoxic effect similar to chlorhexidine on periodontal fibroblasts and human keratinocytes.

Declaration of Competing Interest

The authors present no conflicts of interest to declare. The funding source for this work had no intervention in the design and execution of this research.

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