The antimicrobial effects of *Citrus limonum* and *Citrus aurantium* essential oils on multi-species biofilms

**Abstract:** The aim of this study was to evaluate the effects of *Citrus limonum* and *Citrus aurantium* essential oils (EOs) compared to 0.2% chlorhexidine (CHX) and 1% sodium hypochlorite (NaOCl) on multi-species biofilms formed by *Candida albicans*, *Enterococcus faecalis* and *Escherichia coli*. The biofilms were grown in acrylic disks immersed in broth, inoculated with microbial suspension (10^6 cells/mL) and incubated at 37°C/48 h. After the biofilms were formed, they were exposed for 5 minutes to the solutions (n = 10): *C. aurantium* EO, *C. limonum* EO, 0.2% CHX, 1% NaOCl or sterile saline solution [0.9% sodium chloride (NaCl)]. Next, the discs were placed in sterile 0.9% NaCl and sonicated to disperse the biofilms. Tenfold serial dilutions were performed and the aliquots were seeded onto selective agar and incubated at 37°C/48 h. Next, the number of colony-forming units per milliliter was counted and analyzed statistically (Tukey test, *p* ≤ 0.05). *C. aurantium* EO and NaOCl inhibited the growth of all microorganisms in multi-species biofilms. *C. limonum* EO promoted a 100% reduction of *C. albicans* and *E. coli*, and 49.3% of *E. faecalis*. CHX was less effective against *C. albicans* and *E. coli*, yielding a reduction of 68.8% and 86.7%, respectively. However, the reduction of *E. faecalis* using CHX (81.7%) was greater than that obtained using *C. limonum* EO. Both *Citrus limonum* and *Citrus aurantium* EOs are effective in controlling multi-species biofilms; the microbial reductions achieved by EOs were not only similar to those of NaOCl, but even higher than those achieved by CHX, in some cases.

**Descriptors:** Biofilms; *Citrus limonum*; *Citrus aurantium*.

**Introduction**

The oral cavity is heavily colonized by a complex, relatively specific and highly interrelated group of microorganisms that are organized in biofilms. Microbial biofilms are composed of microorganisms that adhere to each other and/or to surfaces or interfaces and are embedded in an extracellular polymeric matrix, which includes water and nutrient channels.1 *Candida albicans* is a commensal yeast from the oral cavity and is the most virulent species of the genus. It is the main cause of oral candidiasis.2 *Escherichia coli* is a transient colonizer of the oral cavity, and the endotoxin produced by this bacterium plays an important role in the onset and perpetuation of periapical lesions and inflammatory bone resorption.3 *Enterococcus faecalis* is a normal inhabitant of the oral cavity.
and is associated with different forms of periodontal disease, including primary endodontic infections and persistent infections.\(^5\)

Mouth rinses are used as adjuncts to mechanical oral hygiene. The use of mechanical control alone to reduce recalcitrant biofilms in the oral cavity has been challenged, because it is considered time-consuming and, most importantly, insufficient for promoting effective oral hygiene.\(^1,6\) An increasing number of innovative formulation technologies have called for more predictive laboratory models to assess the preclinical biocidal efficacy of mouth rinses. Because biofilm microorganisms may be 10–1000 times more resistant to antimicrobial agents than planktonic cells of the same species,\(^7\) biofilm tests may be a more predictive assessment of mouth rinse efficacy than tests with free-living cells.

Medicinal plants may be explored to produce valuable herbal products, which are frequently used as natural alternatives to treat several diseases.\(^8\) The antimicrobial components of these plants can inhibit bacterial and fungal growth through mechanisms that are distinct from those used by ordinary antibiotics, underscoring their clinical significance.\(^9\)

Citric and acidic fruits contain healthy and nutritious contents. The peels of *Citrus* fruits are rich in flavonoids, especially many polymethoxylated flavones, which are very rare in other plants.\(^10\) The antimicrobial activities of several *Citrus* species, namely, *C. aurantium*, *C. bergamia*, *C. limonum*, *C. maxima*, *C. paradisi*, and *C. reticulata*, have been investigated, but the related tests have typically involved microorganisms in planktonic cultures,\(^11-14\) and few studies have been conducted on microbial biofilms.\(^15,16\)

The aim of this study was to evaluate the effects of *C. limonum* (lemon) and *C. aurantium* (bergamot) essential oils (EOs) compared to 0.2% chlorhexidine (CHX) and 1% sodium hypochlorite (NaOCl) (Byoformula, São Paulo, Brazil). The negative control used was 0.9% NaCl.

**Evaluation of EOs and other chemical solutions**

EOs were collected from air-dried peels of *C. aurantium* (bergamot) and *C. limonum* (lemon) fruits. The peels were placed in a round-bottom distillation flask to which distilled water was added. The EOs were obtained by hydrodistillation for 3 h with the Clevenger apparatus. The oils were separated, dried over anhydrous sodium sulfate, and stored in an amber bottle at 4°C until use.\(^17\)

The antimicrobial effects of the EOs were also compared to those of 0.2% CHX and 1% NaOCl (Byoformula, São Paulo, Brazil). The negative control used was 0.9% NaCl.

**Biofilm formation**

The biofilm formation was developed as proposed by Pereira *et al.*,\(^18\) with some modifications. The biofilms were grown on 50 sterilized acrylic resin (AC) discs (Clássico, São Paulo, Brazil), with a diameter of 6 mm each. The AC discs were placed in the first rows of 24-well plates (Costar Corning, New York, USA) containing 2 mL of sterile brain heart infusion (BHI) broth (Difco, Detroit, USA) supplemented with 5% sucrose and inoculated with 0.1 mL of each bacterial suspension. The AC discs were then incubated at 37°C for 48 h. The media was not changed during the incubation period.

After 48 h of incubation, the AC discs contain-
Susceptibility testing of biofilms

The 50 AC discs with the biofilms formed after 48 h were placed in the fourth row of the plate (24 wells) and exposed for 5 minutes to 2 mL of the following solutions:
- C. aurantium EO (n = 10),
- C. limonum EO (n = 10),
- 0.2% CHX (n = 10),
- 1% NaOCl (n = 10) and
- 0.9% NaCl (n = 10).

Following the experimental periods, each biofilm was washed with 2 mL of 0.9% NaCl to remove the substances tested. The discs were placed in tubes containing 10 mL of 0.9% NaCl and sonicated (Sonoplus HD 2200, 50 W. Bandelin Eletronic, Berlin, Germany) for 30 s to disperse the biofilms. Biofilm suspensions were serially diluted in 0.9% NaCl to produce dilutions of 10⁻¹ to 10⁻⁵ times the original concentration. One hundred microliter aliquots of each dilution were seeded on agar (in duplicate):
- Sabouraud dextrose agar with 50 mg/L chloramphenicol (União Química, São Paulo, Brazil) for C. albicans,
- MacConkey agar for E. coli (HiMedia, Mumbai, India) and
- m-Enterococcus agar (Difco, Detroit, USA) for E. faecalis.

The number of colony-forming units per milliliter (CFU/mL) was determined after 48 hours of incubation. The results were log-transformed (log₁₀) and analyzed by analysis of variance (ANOVA), and the Tukey test. A p value of < 0.05 indicated a statistically significant difference. The percentage of reduction was calculated using the 0.9% NaCl results as a reference.

Results

This study aimed at evaluating the effects of C. limonum EO and C. aurantium EO, compared to other solutions of known antimicrobial activity, such as 0.2% CHX and 1% NaOCl, on multi-species biofilms formed on acrylic resin by C. albicans, E. faecalis and E. coli reference strains. All tested solutions significantly decreased (p < 0.05) the viability of microorganism growth in multi-species biofilms formed on acrylic resin discs, as compared to the control group, which was treated only with 0.9% NaCl. Figures 1, 2 and 3 show these results.

C. aurantium EO and NaOCl were the most effective solutions and inhibited the growth of all microorganisms in multi-species biofilms. C. limonum EO promoted a 100% reduction of C. albicans and E. coli, and 49.3% of E. faecalis biofilms.

We did not achieve the complete elimination of any microorganism with 0.2% CHX, which was less effective against C. albicans and E. coli, with a reduction of 68.8% and 86.7%, respectively. However the reduction of E. faecalis with CHX (81.7%) was greater than that using C. limonum EO.

Discussion

Microorganisms form biofilms as a means of defense and of facilitating physiologic processes. The layered organization of the biofilm protects the microorganisms from changes in pH and other antimicrobial insults. Microbiota within the biofilms were aseptically transferred to the second and third rows of the plate (24 wells) and washed twice with 2 mL of 0.9% NaCl to remove loosely bound material.
The antimicrobial effects of \textit{Citrus limonum} and \textit{Citrus aurantium} essential oils on multi-species biofilms

Enhanced by coaggregation, which is the adhesion of two or more microorganism species. Biofilms can be up to 1000 times more resistant to antimicrobials than planktonic cells from the same species. These issues have called for alternative strategies to control biofilms, particularly in the context of treating oral disease.

This study aimed at evaluating the antimicrobial effects of \textit{C. limonum} EO and \textit{C. aurantium} EO, compared to other solutions of known antimicrobial effects, such as 0.2\% CHX and 1\% NaOCl, on multi-species biofilms formed on acrylic resin by \textit{C. albicans}, \textit{E. faecalis} and \textit{E. coli} reference strains. When we compared the EOs with these solutions, we found that the antimicrobial effects of the EOs were similar to that of 1\% NaOCl, and that these effects promoted a 100\% reduction of \textit{C. albicans} and \textit{E. coli}. \textit{C. aurantium} EO and 1\% NaOCl also produced the complete elimination of \textit{E. faecalis} in biofilms. These results were more favorable than those using 0.2\% CHX. Currently, CHX is the safest and most efficient antimicrobial agent used to reduce microorganisms in the oral cavity. However, it is cytotoxic when injected into the peri-apical tissues, where it may cause severe inflammatory reactions. These factors have prompted the search for other antimicrobial agents.

In this study, the viability of \textit{C. albicans} in multi-species biofilms was directly affected by \textit{C. limonum} EO and \textit{C. aurantium} EO, which eliminated the yeast in the concentrations that were used. Other EOs from various \textit{Citrus} species (\textit{C. limonum}, \textit{C. paradisi}, \textit{C. bergamia}, \textit{C. sinensis} and \textit{C. reticulata}) showed antimicrobial activity on planktonic cultures of gram-positive and gram-negative bacteria, including \textit{E. coli} and yeasts such as \textit{C. albicans}. Oral candidiasis is caused by \textit{Candida} yeasts, which are present in the oral cavities of approximately half of all healthy individuals.\textit{ C. albicans} is the most virulent and prevalent species, isolated in up to 50\% of cases of oral candidiasis; it colonizes the oral surface, proliferates and causes damage through the expression of its virulence factors, such as adherence to host cells, morphological transition, biofilm formation, hydrophobicity and secretion of hydrolytic enzymes.

\textit{C. limonum} EO and \textit{C. aurantium} EO promoted...
a 100% reduction of E. coli in multi-species biofilms in this study. A study conducted by Soković et al. analyzed the activities of various EOs, including C. aurantium and C. limonum, against pathogenic microorganisms, and observed growth inhibition of E. coli planktonic cultures in disc diffusion and microdilution tests. Ashok-Kumar et al. evaluated the antibacterial activities of five different solvent extracts (ethyl acetate, acetone, ethanol, petroleum ether and water) prepared from the peels of two citrus fruits (C. sinensis and C. limonum) against five pathogenic bacteria: Staphylococcus aureus, Bacillus subtilis, E. coli, Klebsiella pneumoniae and Salmonella typhi. These authors observed high antibacterial activity against these bacteria in planktonic cultures, and concluded that extracts of C. sinensis and C. limonum can be as potent as methicillin and penicillin. In the oral cavity, E. coli is considered a transient colonizer, and is more prevalent in the mouth as a successional community during antibiotic therapy, and also in immunocompromised and hospitalized patients. Among the virulence factors of E. coli, the endotoxin produced by this bacterium plays a role in early and perpetuation of periapical inflammatory lesions and bone resorption.

In this study, C. limonum EO reduced the viability of E. faecalis in multi-species biofilms by 49.3%, whereas C. aurantium EO eliminated this microorganism. Laird et al. also achieved the complete elimination of E. faecalis biofilms established on stainless steel surfaces with a vaporized blend of citrus EO (Citri-V™, orange:bergamot, 1:1 v/v). The results of previous studies investigating the effects of citrus vapor on Enterococcus sp. suggested that it acts on the cell membrane, insofar as treated cells show a loss of membrane integrity and an increase in cell permeability compared to untreated cells, with a loss of membrane potential and a reduction in intracellular ATP. In the oral cavity, E. faecalis is commonly isolated in root canal systems as an outcome of failed endodontic treatment, on account of its ability to adhere to dentin, invade dentinal tubules and form biofilms; this may contribute to bacterial resistance and persistence after intracanal antimicrobial procedures.

The mechanisms by which EOs can inhibit microorganisms involve different modes of action and may in part be an outcome of microorganism hydrophobicity. As a result, the oils are partitioned into the lipid bilayer of the cell membrane, affecting the respiratory chain and leading to the leakage of vital cell contents. The impairment of bacterial enzyme systems may also be a potential mechanism of action. Various components of EOs can permeabilize the cell membrane, increasing the penetration of antibiotics. Interference with bacterial enzyme systems may be another potential mechanism of action. Chemical analyses have shown that limonene is the most abundant chemical component of C. aurantium and C. limonum EOs, at 90% and 59.7%, respectively. Limonene was tested separately for antimicrobial activity and was confirmed to have bactericidal activity against gram-positive and gram-negative bacteria, including E. coli.

Conclusion
The current results indicate that EOs are effective in controlling multi-species biofilms, and that the microbial reductions achieved by EOs are not only similar to those of NaOCl, but even higher than those achieved by CHX, in some cases.

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The antimicrobial effects of *Citrus limonum* and *Citrus aurantium* essential oils on multi-species biofilms

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