



Antimicrobial and antibiofilm activity of *Cymbopogon flexuosus* essential oil microemulsions¹

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ABSTRACT

The objective of the study was to determine the antimicrobial activity antibiofilm and to identify the main components of the essential oil (EO) obtained from the leaves of *Cymbopogon flexuosus*. The antibacterial and antibiofilm activity was determined against *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella* Typhimurium ATCC 14028 and *Listeria monocytogenes* ATCC 19117. The effect of EO on biofilm was evaluated by quantifying viable cell number (CFU) and biomass by crystal violet (CV) analysis. The composition of the essential oils was determined by GC / FID and GC / MS. The results showed action against *L. monocytogenes*, *S. aureus* and *S. Typhimurium* with MIC and MBC values of 3.9 $\mu\text{L mL}^{-1}$, thus showing satisfactory antimicrobial activity, given this was the lowest concentration tested. For the antibiofilm activity, a significant reduction ($P < 0.05$) was observed for *S. typhimurium* and *S. aureus*. Biofilm biomass significantly reduced only for *S. aureus* and *P. aeruginosa*. EO presented the geranial and neral isomers as major components.

Keywords: biofilm; plants antibacterial; disinfectant; secondary plant metabolism; bacterial pathogens.

INTRODUCTION

In recent decades, studies on bacterial biofilms have been growing. These dynamic communities called biofilms consist of aggregates of microorganisms encased in a polysaccharide extrapolymer matrix called an exopolysaccharide (Donlan, 2002). In both natural and artificial environments, biofilms that are embedded in an aqueous fluid are subject to hydrodynamic forces. (Hall-Stoodley *et al.*, 2004).

The association of bacteria in biofilms differs significantly from those that are planktonic, as they demonstrate high resistance to antimicrobials, antibiotics and sanitizers. Parallel to this, they can also deceive the system of living hosts when they attack them. (Donlan, 2002; Donlan & Costerton, 2002). The adherence of bacterial species to surfaces used in the food industry

(stainless steel and polypropylene) can have the prospect of causing problems in these environments, especially the bacteria *Pseudomonas aeruginosa* (Donlan 2002; Oliveira *et al.*, 2012; Dalla Costa *et al.*, 2016).

In view of the cellular properties of sessile bacteria, it is of great interest that new substances be developed to inhibit from the initial process of biofilm formation, thus preventing reaching the mature and most resistant stage (Junker & Clardy, 2007; Nguyen *et al.*, 2012). Due to the emergence of bacterial strains resistant to most of the available antimicrobials, there is a renewed interest in the search for alternative antimicrobial agents (Nascimento *et al.*, 2000; Xiaofeng *et al.*, 2018). Moreover, the use of synthetic preservatives, many of them with carcinogenic and teratogenic activity, has diminished their acceptance by consumers (Nascimento *et al.*, 2000, Moreira *et al.*, 2005).

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It is important to highlight the consumer's search for the availability of natural foods that may bring some health benefits. These factors have contributed to increasing interest in the research of natural products that present biological activity antimicrobial. Plants rich in essential oils (EOs) can be used in foods to confer flavor, aroma, and pungency, in addition to having antioxidant activity, antimicrobial and nutritional properties. Besides these properties, antibacterial activity on biofilms has been observed, aiming at the possible use of these secondary metabolites or their constituents as sanitizers (Burt, 2004; Moreira *et al.*, 2005; Millezi *et al.*, 2012, Xiaofeng, *et al.*, 2018).

Among the EOs that have been in great demand throughout the world is the EO of the leaves of *Cymbopogon flexuosus* (lemongrass of East India or lemongrass). This fact, due to its varied range of applications in different types of industries, stands out for its lemon flavor of food and beverages, where it is used as a medicinal, condimental and/or preservative agent (Kumar *et al.*, 2009). The objective of this study was to characterize the chemical constitution, antimicrobial and antibiofilm activity of EO obtained from leaves of *Cymbopogon flexuosus*.

MATERIAL AND METHODS

Local and period of the experiment

Essential oil antimicrobial activity and antibiofilm were carried out in the Laboratory of Food Microbiology and Bromatology at the Federal Institute Catarinense, Concórdia, Santa Catarina, Brazil. The chemical composition characterization was carried at the Laboratory of Physical Chemistry of Embrapa Suínos e Aves, Concórdia, Santa Catarina, Brazil.

Essential oil (EO)

The essential oil extracted by hydro-distillation from leaves of *Cymbopogon flexuosus* were purchased from Ferquima Indústria e Comércio Ltda. (Vargem Grande, São Paulo, Brazil). For CG analysis, a solution was prepared by dissolving 100 mg of EO in 10 ml of CH₂Cl₂. These products had their quality parameters (purity, color, odor, density and refractive index at 20 °C) described in a report sent by the company.

Gas chromatography with flame ionization detection (GC-FID)

The EOs were analyzed using an Agilent 7820A gas chromatograph system (Agilent Technologies, Inc., Shanghai, China) equipped with a split/splitless injector, a flame ionization detector (FID), and an Agilent 7693A auto-injector. The sample solution (1 µl) was injected in the split

mode at a ratio of 1:10. Analyses were performed with an Agilent J&W HP-5 capillary column (30 m x 0.25 mm i.d., 1 µm film thickness, and stationary phase consisting of 5% diphenyl/95% dimethylpolysiloxane). The oven temperature program was as follows: held at 50 °C for 2 minutes, increased from 50 to 220 °C at a rate of 2 °C/min, held at 220 °C for 3 minutes. Nitrogen was used as carrier gas at a flow rate of 1.2 ml/min. The injector and FID temperatures were fixed 240 °C and 280 °C, respectively. The flow rates of air, H₂, and N₂ in the FID were 300, 30, and 30 ml/min, respectively. The OpenLAB CDS software was used for equipment management and data processing. The compositions of the essential oils were expressed as a percentage of normalized area.

Gas chromatography coupled to mass spectrometry (GC-MS)

The essential oils were analyzed using a Shimadzu GCMS-QP2010 gas chromatograph mass spectrometer (Shimadzu Corporation Technologies, Inc., Tokyo, Japan) equipped with a split/splitless injector. The sample solution (1 µl) was injected in the split mode at a ratio of 1:10. Analyses were performed with a Supelco Equity-5 capillary column (30 m x 0.2 mm i.d., 0.2 µm film thickness, and stationary phase consisting of 5% diphenyl/95% dimethylpolysiloxane). The oven temperature program was as follows: held at 50 °C for 2 minutes, increased from 50 to 220 °C at a rate of 2 °C/min, and held at 220 °C for 3 minutes. Helium was used as carrier gas at a flow rate of 1.2 ml/min (constant linear velocity of 39.2 cm/sec). The injector temperature was set at 240 °C. The interface and ion source temperatures were fixed at 220 and 200 °C, respectively. Quadrupole mass spectrometer was operated in electron impact mode at 70 eV, scanning the range m/z 35-350 in cycles of 0.5 s. The GCMS solution software was used for equipment management and data processing. Compounds were identified by searching the NIST 05 mass spectral library and by comparison of their retention indices relative to C7-C30 n-alkane series with those values found in an online database (El-Sayed, 2018).

Bacterial strains and standardization

The species *Staphylococcus aureus* ATCC 29213, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella* Typhimurium ATCC 14028 and *Listeria monocytogenes* ATCC 19117 were obtained from the Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, Brazil. Bacteria were incubated in Tryptic Soy Broth (TSB) (Sigma, India) medium overnight at 37 °C after calculations for the inoculation determined. The standardization of the number of cells was determined by a growth curve absorbance at 630 nm and CFU mL⁻¹ were measured until approximately 10⁸ CFU mL⁻¹.

Minimal Inhibitory Concentration (MIC)

Minimal Inhibitory Concentration (MIC) was detected by the microdilution method with adaptations (NCCLS/CLSI, 2003). The results were expressed in $\mu\text{L mL}^{-1}$. The essential oil was diluted at concentrations $50 \mu\text{L mL}^{-1}$ to $3.9 \mu\text{L mL}^{-1}$ in Brain Heart Infusion – BHI (Himedia, China) broth medium with 0.5% tween 80 in the wells of the microdilution plates. Subsequently, $90 \mu\text{L}$ of the BHI culture medium and finally $10 \mu\text{L}$ of the adjusted bacterial suspension were added at a concentration of approximately 10^8CFU mL^{-1} .

In each line, sterility controls (BHI broth with 0.5% tween 80 added with BHI and without the addition of inoculum) and growth controls (BHI broth with 0.5% tween 80 added with BHI and inoculum) were maintained. The plates were incubated at 36°C for 18 h – 24 h and microbial growth was visually detected and confirmed by the addition of $20 \mu\text{L}$ of 0.5% (m/v) aqueous triphenyl-tetrazolium chloride (TTC) solution, with additional incubation of 1 h, at the same temperature. The MIC was defined as the lowest concentration of each extract that totally inhibited microbial growth.

Minimum bactericidal concentration (MBC)

The minimum bactericidal concentration of the EO was determined from the plates used for the determination of the MIC, through a methodology adapted from Celiktas *et al.*, 2007. From each well where no microbial growth occurred, $10 \mu\text{L}$ was transferred to TSA plates. Plates were incubated at 36°C for 24 h and colony growth was checked. The MBC was defined as the lowest concentration of EO lemongrass, which totally prevented the microbial growth in the plaques.

Antibiofilm activity

For biofilm formation, inoculation was performed at approximately 10^8CFU mL^{-1} (in the calibration curve) in 96-well polypropylene microplates. The EO and the bacterial inoculum in a ratio of 1:1 (v/v) were added in triplicate. To prepare the EO dilutions, 2% DMSO and 0.85% saline water plus 0.5% tween 80 were used as diluting solutions, a 2.5% stock dilution whose concentrations were: 0.195 %, 0.39% and 0.78% in TSB medium supplemented with 0.5% tween 80 (Millezi *et al.*, 2016 with modifications).

In addition, the treatments with oils inoculated also in triplicate the positive control (0,0% of EO) containing the bacterial inoculum and the TSB broth (1:1 v/v), the negative control only EO and TSB medium (1:1 v/v) and white (only one TSB medium). Afterward, the microplate for the orbital was sent to the agitator (Tecnal, Brazil) and shaken at 37°C at 80 rpm for 24 h (Millezi *et al.*, 2016). This procedure was also performed for analyses of Biomass Quantification and Quantification of Viable Cells after 24 h.

Crystal Violet Analysis (CV)

Biomass biofilms were quantified by adapting the crystal violet (CV) staining method by Stepanovic *et al.* (2000) and after 24 hours of incubation, the untreated control, containing only culture medium and the bacterial inoculum, were used to classify the bacteria as biofilm formation capacity. For fixing, we added $200 \mu\text{L}$ of 99 % methanol (Vaz Pereira, Portugal) to each well containing adhered cells or biofilms treated with essential oils, as previously described. After the time of 15 minutes, the methanol was removed and the polystyrene microplates were allowed to dry at room temperature. Then $200 \mu\text{L}$ of CV stain (1 % v/v) (Merck, Portugal) was added to all wells. After 5 minutes, CV excess was removed and plates were washed in distilled water. Finally, $230 \mu\text{L}$ of acetic acid (33% v/v) (Pronalab, Portugal) were added to the wells to dissolve CV stain, and the absorbance was measured at 630 nm in spectrophotometer Elisa (Biotek, USA).

The design of the analysis followed in three replications and triplicate.

Quantification of colony forming units (CFU) in biofilm

For the quantification of colony forming units (CFUs) in biofilm, microwells where the crops were washed in sterile water to remove planktonic bacteria $200 \mu\text{L}$ sterile distilled water was added to the wells and viable biofilm cells were removed by sonication (6 minutes) and then serial dilution of samples. The plating of the sample aliquots was then plated on TSA (Trypan Soy Agar, Acumedia) medium, and plates were incubated at 37°C in an aerobic incubator for 18h prior to enumeration. The number of cultivable bacterial cells was determined and expressed as Log CFU cm^{-2} and three replications.

Statistical analysis

Statistical analysis was performed using Prism version 3.0 (GraphPad Software, Inc., La Jolla, USA). The assumptions for the parametric test were checked before the analysis. Data was analyzed One-way ANOVA Bonferroni test was performed and $P < 0.05$ was considered significant. The analyses were performed in triplicate and assays were performed on three separate occasions.

RESULTS AND DISCUSSION

Identification and quantification of EO constituents

A total of 19 different components were identified in *C. flexuosus* EO (Table 1). The results obtained showed that geranial was the predominant component, representing 41.80%, followed by neral 33.25%.

The *C. flexuosus* plant, popularly known as lemongrass is widely used as a medicinal and condimentar plant. This use is not random, this plant has compounds that confer biological properties, antioxidant and antimicrobial, as it is the case of EOs, secondary plant metabolites that are addressed in some studies (Rota *et al.*, 2008; Sharma *et al.*, 2009; Xiaofeng *et al.*, 2018) and especially in this research, which describes results on its constitution and antibacterial activity.

Several factors such as temperature, light intensity, soil moisture, fertilizers, and developmental stages of the plant/parts greatly influence the EO content and the composition of lemongrass (Ganjewala *et al.*, 2008). EO production is dependent on the early growth stages of leaf and inflorescence, respectively (Singh *et al.*, 1989). In general, EO production is directly proportional to the yield of the

biomass of the plant/plant parts. In lemongrass, younger leaves produce EO of higher quality with very high citral content (75%), where as older leaves have a low level of citral when harvested at a given point. Lemongrass EO consists mainly of citral, a mixture of geranial and neral isomers (40% and 32% respectively) (Ganjewala & Gupta, 2013). In this study, the proportions were 41.8% geranial and 33% general, corroborating with the literature.

Antimicrobial activity

The results showed action against *S. aureus*, *S. Typhimurium* and *L. monocytogenes*, with MIC and MBC values $3.9 \mu\text{L mL}^{-1}$ showing satisfactory antimicrobial activity (Table 2) because this was the lowest concentration tested. None of the concentrations were efficient to inhibit *P. aeruginosa*, so the cells with the highest concentration

Table 1: Chemical composition (%) of essential oils from *Cymbopogon flexuosus*

Compound ^a	RI ^b	RIE ^c	<i>C. flexuosus</i> (%)
α -Pinene	939	920	0.15
Camphene	954	935	0.79
6-Methyl-5-hepten-2-one	985	985	1.66
β -Myrcene	990	989	0.37
D-Limonene	1030	1025	0.27
(Z)- β -Ocimene	1040	1036	0.19
(E)- β -Ocimene	1050	1046	0.13
4-Nonanone	1053	1072	0.59
β -Linalool	1100	1101	1.54
Isopulegol	1145	1143	-
Citronellal	1153	1152	0.23
4,5-Epoxy- <i>t</i> -carene	-	1182	1.04
α -Terpeniol	1185	1189	0.61
β -Citronellol	1228	1231	-
Neral	1238	1240	33.25
Geraniol	1253	1259	5.37
Geranial	1267	1270	41.80
Citronellyl acetate	1353	1352	-
Geranyl acetate	1383	1382	4.23
β -Elemene	1391	1386	-
β -Caryophyllene	1419	1410	2.01
γ -Muuroleone	1477	1505	1.07
α -Muuroleone	1499	1494	-
δ -Cadinene	1524	1516	0.42
Elemol	1547	1544	-
Geranyl butyrate	1562	1570	-
Nerolidol	1564	1576	-
Monoterpenes hydrocarbons			1.89
Oxygenated monoterpenes			83.85
Sesquiterpenes hydrocarbons			3.50
Oxygenated sesquiterpenes			-
Others			6.49
Total identified			95.72

^a Compounds are listed in order of their elution from a Supelco Equity-5 column (30 m, 0.2 mm, 0.2 μm)

^b Retention index obtained at <http://www.pherobase.com/>

^c Retention index experimentally determined on a Supelco Equity-5 column, using a homologous series of C7-C30 alkanes

(500 $\mu\text{L mL}^{-1}$) were counted in comparison with the positive control, in which a viable cell reduction of 1.6 CFU Log 10 mL.

The bioactive potential of lemongrass oil and constituents are rapidly increasing, which is reflected in the growing number of reports being published. Since lemongrass oil is easily available, non-toxic safe and has a pleasant aroma, it is becoming increasingly popular among pharmaceuticals, cosmetic and medicines. The chemical properties of the lemongrass oil have been promising in the integrated pest management program, as this property may lead to the development of alternatives promising, being more natural and desirable than synthetic chemical pesticides (Kumar *et al.*, 2007). Most of the bioactivities of lemongrass oil has been attributed to its one or more major chemical constituents, namely neral and geraniol (Santoro *et al.*, 2007; Kumar *et al.*, 2007; Sharma *et al.*, 2009).

Crystal Violet Analysis (CV)

The studies to verify the adhesion capacity of the bacteria in the polyethylene plates showed that and *P. aeruginosa* adhered strongly, proving the biofilm formation in 24 hours of culture. *Salmonella* Typhimurium and *Listeria monocytogenes* were classified as weakly biofilm (Table 3).

Table 2: Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *Cymbopogon flexuosus* EO ($\mu\text{L mL}^{-1}$)^a

	MIC	MBC
Gram-positive bacteria		
<i>Staphylococcus aureus</i> ATCC 29213	3,90	3,90
<i>Listeria monocytogenes</i> ATCC 19117	3,90	3,90
Gram-negative bacteria		
<i>Salmonella</i> Typhimurium ATCC 14028	3,90	3,90
<i>Pseudomonas aeruginosa</i> ATCC 27853	> 50	> 50

^a The tests were performed in triplicate and the modal values are presented

Table 3: Adhesion ability of bacterial species strains to microplates polystyrene according to the classification proposed by Stepanovic *et al.* (2000)

Bacterial species	Adhesion after 24 h
<i>Staphylococcus aureus</i>	+++
<i>Pseudomonas aeruginosa</i>	+++
<i>Salmonella</i> Typhimurium	+
<i>Listeria monocytogenes</i>	+

+, OD_c < OD_d 2 OD_c, weakly adherent/weak biofilm producer; ++, 2xOD_c < OD_d 4xOD_c, moderately adherent/moderate biofilm producer; +++, 4xOD_c < OD_d, strongly adherent/strong biofilm producer; where OD is the optical density of the negative control and OD_c is the cut-off OD value defined as three standard deviation values above the mean OD of the negative control.

The EO effect on *S. aureus* biomass was efficient, with a significant reduction ($P < 0.05$) for all concentrations (figure 1A). *Listeria monocytogenes* and *S. Typhimurium* showed to be weakly adherent (figure 1B e 1D), however, the EO demonstrated significant activity against adherence ($P < 0.05$) of *S. Typhimurium*. *Pseudomonas* biomass, presented a significant reduction ($P < 0.05$) 67.13% in the concentration 0.39% and 0.195% (57.8%) (Figure 1C).

The antibiofilm activity investigated EOs and extracts of pepper mint (*Mentha x Piperita* L.), coriander (*Coriandrum sativum* L.) and anise (*Pimpinella anisum* L.) against Gram-positive bacteria (*S. aureus*) and Gram-negative (*E. coli*) (Bazargani & Rohloff, 2016). The authors performed the minimum inhibitory concentration (MIC) using the double-series dilution method. The antimicrobial activity was observed for almost all plant extracts and EOs against both bacterial strains with stronger activity against *S. aureus*, again observing the greater resistance to Gram-negative species. All EOs had MIC values of 0.8 to 0.63 $\mu\text{L mL}^{-1}$ and 8 of 14 plant extracts of 2 and 4 $\mu\text{L mL}^{-1}$ for both bacteria. The CV and XTT reduction assays demonstrated inhibition of bacterial fixation by at least 50% for coriander EO, indicating further investigations due to the high activity potential of this oil.

In our study, there was a satisfactory reduction of biofilm biomass, suggesting rupture of the structure, however, the viable cells were not significantly reduced, thus allowing the cells to produce the exopolysaccharides again and reconstitute the biofilm structure. Only *Salmonella* cells were significantly reduced at all concentrations. The 0.78% concentration was efficient in the reduction of viable cells in *S. aureus* biofilm, the treatment time parameter and the temperature factor is variables to be tested in order to reduce the viable cells.

Quantification of colony forming units (CFU) in biofilm

Quantification of viable cells in biofilm demonstrated that only *S. Typhimurium* was significantly reduced in the OE treatments at the three concentrations ($P > 0.05$). *Staphylococcus aureus* was significantly reduced only at the concentration of 0.78% ($P < 0.05$). *Listeria monocytogenes* and *Pseudomonas aeruginosa* were resistant at all concentrations ($P > 0.05$) (Figure 2).

In some cases, the activity of the compound alone is not always more efficient than that of EO. In a study by Millezi *et al.* (2016), EOs treatments of palmarosa, cinnamon, and green tea, as well as their respective major compounds, respectively, geraniol, eugenol, and terpine-4-ol, reduced planktonic and biofilm populations of *S. aureus* and *E. coli*, however, EOs proved to be more efficient. The activity of antibacterial substances varies according to some types of bacteria and arrangements. Bacteria in the planktonic

state are more sensitive than strongly adhered bacteria forming biofilms. Satisfactory MIC and MBC results for planktonic bacteria may not have the same effect for bacteria of the same species in biofilms. After bacteria establish biofilms, they initiate different gene expression than free or planktonic, causing phenotype changes (Prigent-Combaret & Lejeune, 1999). Importantly, among these changes is the greater capacity of resistance to antimicrobials. Sessile bacteria in biofilms are more resistant to physical and chemical forms of eradication, and thus cause persistent infections. (Costerton, 2005; Rota *et al.*, 2008, Simões *et al.*, 2018). In this way, bacterial cells in biofilms can survive and persist after cleaning and disinfection if performed inefficiently. This fact may

represent a possible source of food poisoning in humans and animals, as well as problems with economic losses (Chavant *et al.*, 2007).

The activity of EOs may be related to the capacity of current in the plasma membrane and cell wall, causing through diffusion process because they are lipophilic compounds (Bakkali *et al.*, 2008). The complexity of the mechanism EOs action in the bacterial cell is described: cell wall destruction, plasmatic membrane damage such as protein and enzyme inactivation, interference with proton motive force, coagulation of cytoplasmic fluid (Burt 2004; Ayala-Zavala *et al.*, 2009).

Oliveira *et al.*, (2012) studied the antibacterial effect EO *Cinnamomum cassia* bark, *Melaleuca alternifolia* and

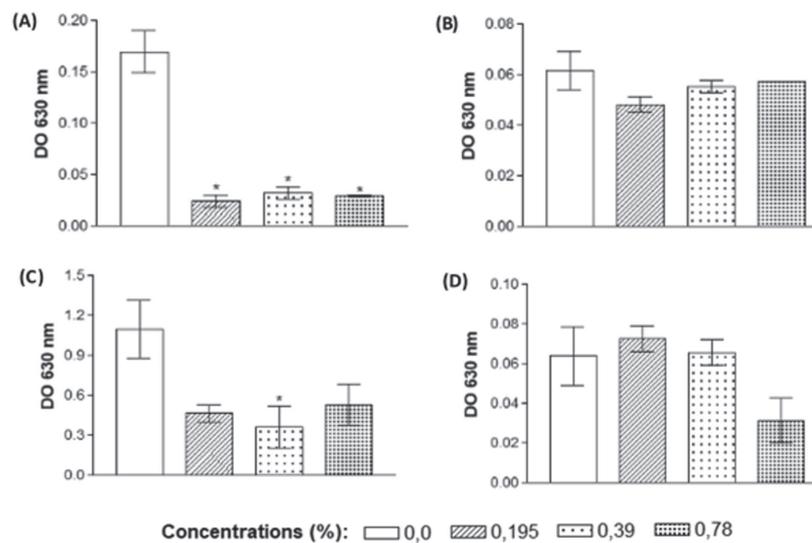


Figure 1: Quantification of biofilm biomass *Staphylococcus aureus* (A), *Salmonella Typhimurium* (B); *Pseudomonas aeruginosa* (C) e *Listeria monocytogenes* (D). The values are means of three assays, and the bars indicate standard deviation. *P < 0.05 in ANOVA test.

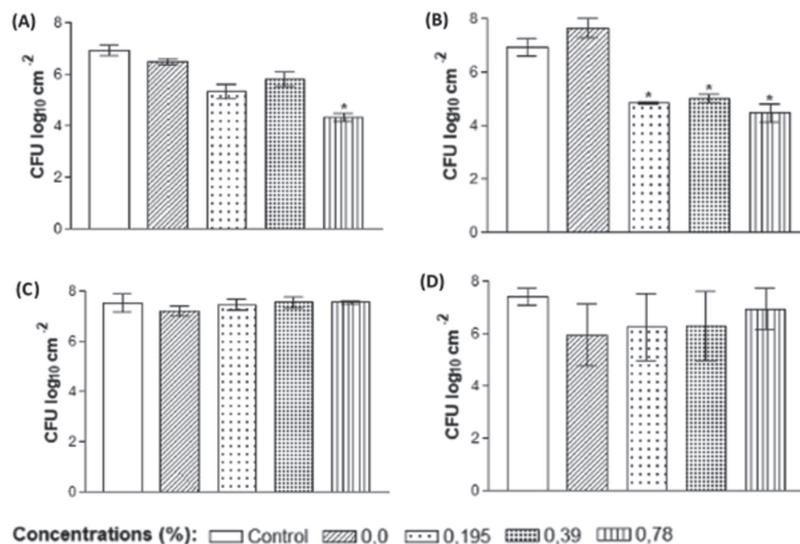


Figure 2: Quantification of viable cells in biofilm *Staphylococcus aureus* (A), *Salmonella Typhimurium* (B); *Pseudomonas aeruginosa* (C) e *Listeria monocytogenes* (D). The values are means of three separate assays, and the bars indicate standard deviation. *P < 0.05 in one-way analysis of variance test.

C. flexuosus against *E. coli* and *L. monocytogenes*. The tests result showed antibacterial activity; however, EO of *C. cassia* was shown to be the most effective for biofilm control. The combination *C. flexuosus* and *M. alternifolia* presented greater efficiency than the individual one. *L. monocytogenes* has been shown to be more suitable to the action of EOs than to EPEC, which demonstrates the greater resistance of Gram-negative bacteria.

CONCLUSION

This study demonstrated that the EOs have important biological properties, therefore they should be researched and tested. Further investigations, including dose-response and toxicological studies, are needed to establish these promising natural compounds as sanitizers in the real complex of food-processing ecosystems.

DECLARATION OF CONFLICTING INTERESTS

The Author(s) declare(s) that there is no conflict of interest.

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