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Antimicrobial and antibiofilm activity of the essential oil from dried leaves of *Eucalyptus staigeriana*

Atividade antimicrobiana e antibiofilme do óleo essencial de folhas secas de **Eucalyptus staigeriana**

Marcos Saldanha Correa[1], Joseli Schwambach[2], Michele Bertoni Mann[1], Jeverson Frazzon[1], Ana Paula Guedes Frazzon[1]*

ABSTRACT: In recent years, compounds with biological properties produced by plants have received attention as an alternative to control microorganisms. Essential oils extracted from green leaves of *Eucalyptus* sp. have been demonstrated to have antimicrobial activities, but so far there are no reports of antimicrobial activity of essential oils extracted from dried leaves of *Eucalyptus staigeriana*. So, the objectives of this study were to determine the chemical composition of the essential oils obtained from dried leaves of *E. staigeriana* ($EO^{dl}ES$) and to evaluate *in vitro* antimicrobial and antibiofilm activities of $EO^{dl}ES$ against gram-positive and gram-negative, resistance and multiresistant *Enterococcus faecalis* isolated from food and clinical samples. The characterization of $EO^{dl}ES$ was performed by gas chromatography–mass spectrometry (GC/MS). For this study, 26 bacterial strains were used, which included 11 reference strains and 15 antibiotic resistant and multiresistant *E. faecalis* strains. Antimicrobial activities of $EO^{dl}ES$ against gram-positive and gram-negative were determined using the disc diffusion method. The minimum inhibitory concentration (MIC) value was evaluated by a microbroth dilution technique. The antibiofilm effects were assessed by microtiter plate method. As a result, 21 compounds were identified, being oxygenated monoterpenes (69.58%) the major chemical family. $EO^{dl}ES$ showed only antimicrobial activity against gram-positive strains. *E. faecalis* resistant and multiresistant strains show the lowest MIC (3.12 to 6.25%), when compared with reference *E. faecalis* strain. $EO^{dl}ES$ has the ability to inhibit the biofilm formation, but little or none ability to inhibit the preformed biofilm. This study demonstrates that $EO^{dl}ES$ is a promising alternative to control important foodborne and clinic gram-positive resistant bacteria.

KEYWORDS: antimicrobial activity; antibiofilm activity; essential oils; dried leaves; antibiotic-resistant strains; *Eucalyptus staigeriana*.

RESUMO: Nos últimos anos, compostos com propriedades biológicas produzidas por plantas têm recebido atenção como alternativa de controle de micro-organismos. Óleos essenciais extraídos de folhas verdes de *Eucalyptus* sp. têm demonstrado atividades antimicrobianas. No entanto, até o momento não há nenhum relato de atividade antimicrobiana de óleos essenciais extraídos de folhas secas de *Eucalyptus staigeriana*. O objetivo deste estudo foi determinar a composição química dos óleos essenciais obtidos de folhas secas de *E. staigeriana* e avaliar *in vitro* a sua atividade antimicrobiana e de antibiofilme contra gram-positivas e gram-negativas e também resistentes e multirresistentes de *Enterococcus faecalis* isolados de amostras de alimentos e clínicas. A caracterização de *E. staigeriana* foi realizada por CG-EM. Para este estudo foram utilizadas 26 cepas bacterianas, que incluíram 11 cepas referência e 15 cepas de *E. faecalis* resistentes a antibióticos. A atividade antimicrobiana de *E. staigeriana* contra gram-positivas e gram-negativas foi determinada utilizando o método de disco-difusão. Os valores da concentração inibitória mínima foram avaliados pela técnica de microdiluição. Os efeitos de antibiofilme foram avaliados pelo método de placa de microtitulação. Como resultado, 21 compostos foram identificados, sendo monoterpenos oxigenados (69,58%) a grande família química. *E. staigeriana* mostrou apenas atividade antimicrobiana contra cepas gram-positivas. Cepas de *E. faecalis* resistentes e multirresistentes mostraram a menor concentração inibitória mínima (3,12 para 6,25%) quando comparado com a cepa referência de *E. faecalis*. *E. staigeriana* apresentou a capacidade de inibir a formação de biofilme, mas pouca ou nenhuma capacidade de inibir o biofilme pré-formado. Este estudo demonstra que o óleo essencial obtido de folhas secas de *E. staigeriana* é uma alternativa promissora para controle importante de bactérias gram-positivas resistentes de origem alimentar e clínicas.

PALAVRAS-CHAVE: atividade antimicrobiana; atividade antibiofilme; óleos essenciais; folhas secas; cepas resistentes a antibióticos; *Eucalyptus staigeriana*.

[1]Universidade Federal do Rio Grande do Sul – Porto Alegre (RS), Brazil
[2]Universidade de Caxias do Sul – Caxias do Sul (RS), Brazil
*Corresponding author: ana.frazzon@ufrgs.br
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INTRODUCTION

According to the World Health Organization (WHO, 2000), about 80% of the world's population uses medicinal plants to supply the primary medical care. In Brazil, plants in healing rituals come from the early days, with the indigenous culture, that uses plants in healing rituals (FERRO, 2008). As reported by MAZZARI; PIETRO (2014), approximately 66% of the Brazilian population without access to the modern medicine make use of folk medicines. It is estimated that 10,000 to 53,000 plants are used for medicinal purposes, but only a small part of them has been investigated, representing almost 1% of the flora (MAZZARI; PIETRO, 2014).

Essential oils are secondary metabolites produced by plants, being synthesized in different plant parts, such as buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood or bark. They play an important ecological role in protecting the plants against microorganisms and herbivores, but also to attract insect pollinators and seed dispersers. Among the plants producers of secondary metabolites highlighted, it is the Myrtaceae. This family includes 100 genera with more than 3,000 species on the planet. One of the best-known genera in this family is *Eucalyptus*. This genus covers more than 700 species found in various parts of the world, from which 300 species are extracted essential oils, which are used for their antiseptic and healing properties, fragrance and for food preservation (VUONG et al., 2015). Previous studies performed with essential oils of green leaves of *Eucalyptus* sp. showed antimicrobial activity (GILLES et al., 2010; SELIM et al., 2014).

In the last notification of the American Centers for Disease Control and Prevention (CDC), it is estimated that more than two million new cases and over 23,000 deaths were caused by antimicrobial-resistant microorganisms in the United States (USA) in 2013. It is estimated that the impact of antimicrobial resistance in 2050 will cause 10 million deaths per year if strategies to reduce this problem of antimicrobial resistance are not found (KRAKER et al., 2016). Resistant microorganisms like *Enterococcus* sp., *Staphylococcus* sp., *Pseudomonas* sp., *Escherichia coli*, and others (ANDRADE et al., 2003; SHERLEY et al., 2004; RIBOLDI et al., 2009; RIVERA; BOUCHER, 2011) have been isolated from clinical, food and environmental samples from different parts of the world, including Brazil.

In recent years, compounds with biological properties extracted from fresh leaves have received attention as an alternative to control microorganisms (NEGREIROS et al., 2016, SELIM et al., 2014). However, according to data, there are few reports antimicrobial activity of essential oils extracted from dried leaves (RADAELLI et al., 2016; SEMENIUC et al., 2017).

So, the objectives of this study were to determine the chemical composition of the essential oils obtained from dried leaves of *E. staigeriana* ($EO^{dl}ES$) and to evaluate *in vitro* antimicrobial and antibiofilm activities of $EO^{dl}ES$ against gram-positive and gram-negative resistance and multiresistant *Enterococcus faecalis* isolated from food and clinical samples.

MATERIAL AND METHODS

Plant material and chemical characterization of the essential oils from dried leaves of *Eucalyptus staigeriana*

Leaves of *E. staigeriana* were collected in Caxias do Sul (29º10'05"S; 51º10'46"W), Rio Grande do Sul, Brazil, in September 2014. The leaves were dried in a circulating air oven at the temperature of 30ºC for further extraction of the essential oil. The specimen was identified by the Universidade de Caxias do Sul (UCS) Herbarium team, and deposited in the Instituto de Biociências (ICN) Herbarium at UCS, under the voucher number 37937.

The essential oil from dried leaves of *E. staigeriana* ($EO^{dl}ES$) was extracted through steam distillation according to CASSEL et al. (2009), with modification on the extraction time to 1 h. The characterization of the compounds was performed by gas chromatography–mass spectrometry (GC-MS), with gas chromatography (Hewlett Packard 6890) coupled to a mass selective detector (Hewlett Packard MSD5973), equipped with the software Hewlett Packard ChemStation and Wiley 275 spectrum.

The analyses were carried out using a fused silica capillary column INNOWax (30 m × 0.25 mm id, 0.25 µm film thickness) (Hewlett Packard, Palo Alto, USA) with the following conditions: column temperature, 40ºC (8 minutes) and 180 to 3ºC/minute, 180–230 to 20ºC/minute, 230ºC (20 minutes); 280ºC interface; Reason 1:100 division; carrier gas He (56 KPa); speed: 1.0 mL/minute; ionization energy 70 e V; and 40–350 mass range. The injected volume was 0.4 µL (diluted in hexane 1:10). Analytical gas chromatography was performed on a Hewlett Packard 6890 gas chromatograph with a flame ionization detector (FID) equipped with the Hewlett Packard ChemStation software. Using a capillary column bonded phase INNOWax (30 m × 0.32 mm id, 0.50 µm film thickness) (Hewlett Packard, Palo Alto, USA) with the following conditions: temperature of the column, 40ºC (8 minutes) and 180 to 3ºC/minute, 180–230 to 20ºC/minute, 230ºC (20 minutes); gun temperature 250ºC, temperature of 250ºC detector; reason of 1:50 division; carrier gas H_2 (34 KPa). The injection volume was 1 µL (diluted in hexane 1:10). The individual components were identified by a combination

of spectrum Wiley library mass and comparison with data from the literature (ADAMS, 2005).

Strains and cultivation

The total of 26 bacterial strains were tested, being 11 reference strains (*Bacillus cereus* ATCC 14579, *Bacillus pumilus* IA/ICBS, *Listeria monocytogenes* ATCC 7644, *Enterococcus faecalis* ATCC 29212, *Streptococcus gallolyticus* ATCC 9809, *Streptococcus agalactiae* ATCC 13813, *Staphylococcus aureus* ATCC 4163, *Staphylococcus epidermidis* ATCC 35984, *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 27853 and *Salmonella choleraesuis* ATCC 14028); and 15 *E. faecalis* resistant and multiresistant strains isolated from food and clinical samples. All strains belonged to bacterioteca from laboratory 220 at the Department of Microbiology, Immunology and Parasitology from Universidade Federal do Rio Grande do Sul.

The microorganisms were distributed in four experiments:

- evaluation of antimicrobial activity against gram-positive and gram-negative ATCC strains;
- minimum inhibitory concentration (MIC) was evaluated against seven gram-positive ATCC and 15 resistant and multidrug-resistant *E. faecalis* strains;
- inhibition of biofilm formation against 21 strains;
- inhibition of preformed biofilm against five strains.

Before each experiment, bacterial cells were inoculated in Brain Heart Infusion agar (BHIA, Oxoid) and incubated at 37ºC for 24 hours. For experimental procedures, a loopful of the BHIA cultured of each isolate was resuspended in sterile saline solution 0.9% (w/v) until it reached the turbidity standards of 0.5 McFarland (approximately 1×10^8 CFU/mL).

Determination of in vitro antimicrobial activity of Eucalyptus staigeriana

In vitro antimicrobial activity of EO^{dl}ES was investigated using the disk diffusion method, as described by PONCE et al. (2003). The inoculums adjusted to a 0.5 MacFarland standard was uniformly spread on the surface of Müeller-Hinton agar (MH, HiMedia Laboratories). Sterile filter papers discs of 6 mm impregnated with 10 µL of pure EO^{dl}ES were placed on the surface of the culture medium at the center of the dish. Plates were incubated for 24 hours at 37ºC. All trials were conducted in triplicate. After this period, the antimicrobial activity was evaluated by measuring the inhibition zone. The sensitivity to the oils was classified by the diameter of inhibition using the patterns described by PONCE et al. (2003):

- not sensitive (-) for diameters less than 8 mm;
- sensitive (+) for diameters 9–14 mm;
- very sensitive (++) for diameters 15–19 mm;
- extremely sensitive (+++) for diameters larges than 20 mm.

As controls, antimicrobial discs specific to each of the evaluated strains were employed.

Determination of minimum inhibitory concentration of *Eucalyptus staigeriana*

Broth microdilution assay was carried out in a sterile U-bottomed 96-well polystyrene microtitre plate, as described by NEGREIROS et al. (2016). In each well of a polystyrene microtitre plate, 100 µL of Müeller-Hinton broth (MHB, HiMedia Laboratories) was dispensed, followed by 100 µL of the EO^{dl}ES into the first well and serial dilutions — to achieve the final oil concentrations of 50 to 0.09% — of the EO^{dl}ES. Subsequently, 10 µL of the bacterial suspension (10^8 CFU mL^{-1}) were added to each well, and the microtitre plates were incubated for 24 hours at 37ºC. The control of the growth promotion was composed of 100 µL of MHB and 10 µL of the inocula; the control of sterility was composed of 100 µL of MHB; and the control of extract was composed of 100 µL of MHB plus 100 µL of extract. The lowest concentration that completely inhibits visible growth was established as the MIC.

Inhibitory effects of *Eucalyptus staigeriana* on biofilm

The ability of the EO^{dl}ES to inhibit *in vitro* biofilm formation and the preformed biofilm was evaluated according to SLAVERS et al. (2016), with some modifications. Biofilm formation was quantified by crystal violet. Controls were prepared by replacing the inoculum volume by tryptic soy broth (TSB), and essential oil by sterile water. Positive control was carried out with the *S. epidermidis* strain ATCC 35984 and as negative control culture medium only, without inoculum. The tests were carried out in quadruplicate and the optical density measured in absorbance at 450 nm. It was considered a producer of biofilm samples, whose reading of the average for each strain was increased to the value of the cutting point (ODc), defined by the formula expressed on Equation 1:

$$ODc = [CN + 3\ (SD)] \quad (1)$$

In which:
CN: the average reading of negative control;
SD: the standard deviation of the reading the CN.

The strains were classified as strong (4 ODc ≤), moderate (2 ODc ≤ of ≤ 4 ODc), weak (ODc ≤ of ≤ 2 ODc) and biofilm-producing (≤ ODc).

The percentage of inhibition of biofilm followed the formula of Equation 2:

$$\%\ \text{inhibition of biofilm} = 100\text{-}(\text{OD of treated sample}/\text{OD of positive control untreated}) \times 100 \quad (2)$$

(JADHAV et al., 2013)

In which:
OD: optical density.

The assays were conducted in quadruplicate, on two separate occasions. The microplates were incubated at 37ºC for 24 hours under sterile conditions to allow cell adherence. Biofilm formation was quantified by crystal violet and metabolic activity of cell assays. Controls were prepared by replacing the inoculum volume by TSB, and essential oil by sterile water.

Inhibition of the formation of biofilm

In each well of sterile flat-bottomed 96-well polystyrene microtitre plate, 180 µL of TSB (HiMedia Laboratories) and 20 µL of the bacterial suspension (10^8 CFU mL^{-1}) in 0.5 McFarland were added, and the final concentrations of the essential oil were equivalent to of ½ MIC, MIC and 2 × MIC of EO^{dl}ES. The microplates were incubated at 37ºC for 24 h to allow cell adhesion. EO^{dl}ES activity on microbial biofilm formation was tested on strains classified as non-forming, weak, moderate and strong biofilm former. Controls were prepared by replacing the inoculum volume by TSB, and essential oil by sterile water.

Inhibition to preformed biofilm

For this test, 20 µL of the bacterial suspension (10^8 CFU mL^{-1}) in 0.5 McFarland was added to each well containing 180 µL of TSB, being the plate incubated at 37ºC for 6 h to allow the preformation of biofilm. Next, EO^{dl}ES equivalent to ½ MIC, MIC and 2 × MIC were added to each well for the period of 18 h at 37ºC. In this experiment, the EO^{dl}ES activity has been tested against strains classified as weak, moderate and strong biofilm-forming. Controls were prepared by replacing the inoculum volume by TSB, and essential oil by sterile water.

Statistical analysis

The results of the EO^{dl}ES into inhibit the biofilm formation were analysed through ANOVA, and means were compared with Tukey's test using the Statistical Package for the Social Sciences (SPSS). Differences with $p < 0.05$ were considered statistically significant.

RESULTS

Essential oil characterization

The constituents identified for EO^{dl}ES are given in Table 1. The GC-MS and gas chromatography-flame ionization detector (GC-FID)analysis showed the presence of 21 compounds, and major chemical families were oxygenated monoterpenes (69.58%) and hydrocarbons (monoterpenes 28.84%), being the major constituents the geranial (28.67%), neral (19.68%) and limonene (17.29%).

In vitro antimicrobial activity of *Eucalyptus staigeriana* against gram-positive and gram-negative strains

Table 2 presents the antimicrobial activity of EO^{dl}ES against the microorganisms tested. The results obtained in this study indicated that essential oils showed in *vitro* activity against gram-positive bacteria, in which the larger diameter halos were detected to *S. aureus* ATCC 4163 (> 45 mm) and *B. cereus* (44 mm). The EO^{dl}ES had no active against gram-negative strains.

Table 1. Chemical composition of the essential oil dried leaves of *Eucalyptus staigeriana* (EO^{dl}ES) by gas chromatography-mass spectrometry (GC-MS) and gas chromatography-flame ionization detector (GC-FID).

Components	LTPRI	Area (%)
α-Pinene	8.171	0.83
α-Phellandrene	16.268	0.28
Myrcene	16.494	0.47
Limonene	18.288	17.29
1.8-Cineol	18.743	6.16
b-Terpinene	20.901	0.62
cis-β-Ocimene	21.397	0.3
o-cimene	22.305	0.44
δ-Careno	22.944	5.61
Linalool	35.967	1.3
Cariofilene	37.84	0.26
Terpinen-4-ol	38.362	0.85
Neral	41.837	19.68
Methyl geranate	42.226	3.78
Geranial	43.921	28.67
Geranul acetate	44.658	2.16
Citronellol	45.092	1.31
Nerol	46.462	1.72
Geraniol	48.243	3.77
Espatulenol	56.906	0.14
Eugenol	57.636	0.18
Monoterpenes hydrocarbons		25.84
Oxygenated monoterpenes		69.58
Hydrocarbon sesquiterpenes		0.26
Oxygenated sesquiterpenes		0.14
Total components		95.82
Extraction yield		0.77%

LTPRI: linear temperature programmed retention indices tabulated (ADAMS, 2005); Area: relative percentage of each component was obtained directly from chromatographic peak areas, considering the sum of all eluted peaks as a hundred percent.

Table 2. Results of the *in vitro* antimicrobial activity test of essential oil of dried leaves of *Eucaplyptus staigeriana* ($EO^{dl}ES$) against gram-negative and gram-positive strains.

Strains	Halo diameter (mm) (sensitivity criterion)
Escherichia coli ATCC 10536	8 (-)
Pseudomonas aeruginosa ATC 27853	6 (-)
Salmonella choleraesuis ATCC 14028	8 (-)
Staphylococcus aureus ATCC 4163	>45 (+++)
Staphylococcus epidermidis ATCC 35984	42 (+++)
Enterococcus faecalis ATCC 29212	23 (+++)
Streptococcus gallolyticus ATCC 9809	12 (+)
Streptococcus agalactiae ATCC 13813	30 (+++)
Listeria monocytogenes ATCC 7644	31 (+++)
Bacillus pumilus IA/ICBS	28 (+++)
Bacillus cereus ATCC 14579	44 (+++)

-: not sensitive for diameters less than 8 mm; +: sensitive for diameters 9–14 mm; ++: very sensitive for diameters 15–19 mm; +++: extremely sensitive for diameters larges than 20 mm.

Minimum inhibitory concentration of the *Eucalyptus staigeriana*

The results of the MIC of $EO^{dl}ES$ against gram-positive strains is listed in Table 3. The MICs ranged from 0.39 to 6.25%, being the lowest observed for the followed standard strains, *S. aureus* ATCC 4163, *B. pumilus* IA, *S. agalactiae* ATCC 13813 and *L. monocytogenes* ATCC 7644, and for the clinical *E. faecalis* strains, 1220, 606 and 1240. Notably, the MIC for antibiotic multiresistant *E. faecalis* strains (1.56 to 6.25%) was higher than the standard strain of *E. faecalis* ATCC 29212 (0.78%).

In vitro antibiofilm activity of *Eucalyptus staigeriana*

In vitro *inhibition of biofilm formation*

$EO^{dl}ES$ was able to inhibit the biofilm formation in all concentrations tested (Table 4). The Crystal Violet assay showed that the biofilm formation was significantly inhibited ($p < 0.05$) by MIC values.

Table 3. Minimum inhibitory concentration values of essential oil of dried leaves of *Eucalyptus staigeriana* ($EO^{dl}ES$) against gram-positive strains.

Strains	Source	Resistance profile*	MIC (%)
Staphylococcus aureus ATCC 4163	Standard Strain	_	0.39
Bacillus pumilus IA/ICBS	Standard Strain	_	0.39
Streptococcus agalactiae ATCC 13813	Standard Strain	_	0.39
Listeria monocytogenes ATCC 7644	Standard Strain	_	0.39
Enterococcus faecalis ATCC 29212	Standard Strain	_	0.78
Staphylococcus epidermidis ATCC 35984	Standard Strain	_	0.78
Streptococcus gallolyticus ATCC 9809	Standard Strain	_	0.78
Enterococcus faecalis 2389	Clinic/ICBS	V, E, G	0.78
Enterococcus faecalis 1950	Clinic/ICBS	V, G, T	0.78
Enterococcus faecalis 1953	Clinic/ICBS	V, E, G, T	0.78
Enterococcus faecalis 151	Clinic/ICBS	T	0.78
Enterococcus faecalis 612	Clinic/ICBS	C, Ch, E, Ni	1.56
Enterococcus faecalis C13	Food/ICBS	E, T	1.56
Enterococcus faecalis 1854	Clinic/ICBS	V, E, G, A	1.56
Enterococcus faecalis E2	Food/ICBS	E	3.12
Enterococcus faecalis C2	Food/ICBS	E, G, T	3.12
Enterococcus faecalis G9	Food/ICBS	V, G, T	3.12
Enterococcus faecalis 603	Clinic/ICBS	T, C, Ch, E, ST	3.12
Enterococcus faecalis E18	Food/ICBS	E, T	3.12
Enterococcus faecalis 1240	Clinic/ICBS	T, C, Ch, E, N	6.25
Enterococcus faecalis 1220	Clinic/ICBS	T	6.25
Enterococcus faecalis 606	Clinic/ICBS	T	6.25

MIC: minimum inhibitory concentration; ICBS: Instituto de Ciências Básicas da Saúde; *antibiotics; A: ampicillin; C: ciprofloxacin; Ch: chloramphenicol; E: erythromycin; ST: streptomycin; G: gentamicin; N: norfloxacin; Ni: nitrofurantoin; T: tetracycline; V: vancomycin.

In vitro inhibition of biofilm preformed

The essential oil showed little or no activity on the preformed biofilm for the majority of selected strains. The largest reductions were obtained at the concentration of ½ MIC in *E. faecalis* ATCC 29212 and clinical strain 2389, and at MIC and 2 × MIC in clinic strain 1240 ($p > 0.05$) (Table 5).

DISCUSSION

In this study, the major constituents of EO^{dl}ES were geranial (28.67%), neral (19.68%) and limonene (17.29%). GILLES et al. (2010) identified 29 compounds in oil extracted from fresh leaves of *E. staigeriana*, being the main components 1.8-cineole (34.8%), neral (10.8%), and geranial (10.8%). Similar results were found by MACEIL *et al.* (2010), who found out that the main compound extracted was limonene (28.82%), followed by citral (10.77%). There are many factors that affect the oil compounds, such as plant condition, time of harvest, and seasonal factors. In addition, the extraction methods also affect the composition of the oils (VUONG et al., 2015).

EO^{dl}ES showed *in vitro* antimicrobial activity only against gram-positive strains. Similar results were observed by GILLES et al. (2010) using *S. aureus* and *E. faecalis* strains, which reported high sensitivity for the essential oil of *E. staigeriana*. *E. coli*, *P. aeruginosa* and *S. choleraesuis* strains were no sensitive to EO^{dl}ES. GILLES et al. (2010) also observed that *P. aeruginosa* and *E. coli* were not sensitivity to the essential oils extracted from EO^{dl}ES. The antimicrobial activity of essential oils depends on the constitution and the number of compounds present.

Each compound may present a different mechanism to control the microorganisms, which involves a series of chemical reactions in the bacterial cell. The presence of the outer membrane in gram-negative bacteria — composed of polysaccharides and lipopolysaccharides — may prevent that essential

Table 4. Percentage of inhibition on the biofilm formation of gram-positive strains exposed to essential oil of dried leaves of *Eucalyptus staigeriana* (EO^{dl}ES).

Strains	Biofilm formation classification (% of inhibition)			
	C+	MIC	½ MIC	2X MIC
Enterococcus faecalis ATCC 29212	S^b	N (96^a)	N (98^a)	N (99^a)
Staphylococcus aureus ATCC 4163	S^b	N (97^a)	N (92^a)	N (97^a)
Streptococcus agalactiae ATCC 13813	M^b	N (79^a)	N (75^a)	N (84^a)
Listeria monocytogenes ATCC 7466	M^b	N (95^a)	N (87^a)	N (97^a)
Streptococcus gallolyticus ATCC 9809	S^b	N (96^a)	N (93^a)	N (96^a)
Staphylococcus epidermidis ATCC 35984	S^b	N (100^a)	N (98^a)	N (100^a)
Enterococcus faecalis E18. C13. E2. C2. G9.	S^b	N (97.8^a ± 1.36)	N (96.6^a ± 3.28)	N (97.8^a ± 1.84)
151. 1854. 603. 606. 1220.1953	S^b	N (99.8^a ± 0.32)	N (99.6^a ± 0.64)	N (99.6 ± 0.48)
1240	M^b	N (99^a)	N (96^a)	N (94^a)
2389. 1950	W^b	N (93^a ± 0.0)	N (85^a ± 14.0)	N (98^a ± 4.0)
612	N^b	N (94^a)	N (87^a)	N (98^a)

Each bacterium was evaluated individually, and the values are expressed as percentages. Same superscript letters do not differ statistically ($p > 0.05$). MIC: minimum inhibitory concentration; C+: positive control without the addition of EO^{dl}ES; S: strong; M: moderate; W: weak trainer; N: non-biofilm builder.

Table 5. Percentage of inhibition on the preformed biofilm of *Enterococcus faecalis*, *Staphylococcus aureus* and *Listeria monocytogenes* strains exposed to essential oil of dried leaves of *Eucalyptus staigeriana* (EO^{dl}ES).

Strains	Biofilm formation classification (% of inhibition)			
	C+	MIC	½ MIC	2x MIC
E. faecalis ATCC 29212	S^b	S (-4^b)	W (39^a)	S (-28^b)
S. aureus ATCC 4163	S^b	S (-23^a)	S (-16^a)	S (-24^a)
L. monocytogenes ATCC 7644	M^b	M (28^a)	M (36^a)	M (37^a)
E. faecalis 1240	M^b	W (48^a)	M (-6^b)	S (46^a)
2389	W^b	W (-25^b)	N (21^b)	W (9^b)

Each bacterium was evaluated individually, and the values are expressed in percentages. Same letters do not differ statistically ($p > 0.05$). Negative (-) indicates stimulus in biofilm formation. MIC: minimum inhibitory concentration; C+: positive control without the addition of EO^{dl}ES; S: strong; M: moderate; W: weak; N: non-biofilm builder. Same superscript letters do not differ statistically ($p > 0.05$).

oils active compounds reach the cytoplasmic membrane of gram-negative bacteria. In addition, this outer membrane also contains porins, which acts as a hydrophilic channel, making the passage of selective transmembrane of small hydrophilic molecules to the cell interior. On the other hand, the cell structure of gram-positive bacteria allows hydrophobic molecules to accumulate on the wall or the passage to the interior of the cell, allowing essential oils, generally hydrophobic, to act against this microorganism (NAZZARO et al., 2013).

The sensitivity of the gram-positive bacteria to EO^{dl}ES is an important finding of our study, especially to antibiotic resistant or multiresistant *E. faecalis* strains. Sensitivity of gram-positive bacteria tested to the EO^{dl}ES is also very important, since they are classified as opportunistic pathogens, highlighting the clinically important bacteria antibiotic resistant strains (RIVERA; BOUCHER, 2011). NEGREIROS *et al.* (2016) also observed that the essential oil from of *H. psiadioides* was able to inhibit enterococci vancomycin resistant strains.

The sensitivity of the gram-positive bacteria to the EO^{dl}ES is an important finding of our study, especially due to the inhibition of antibiotic resistant or multiresistant *E. faecalis* strains by the essential oils tested. The sensitivity of gram-positive bacteria tested to essential oils is very important, since the bacteria are classified as opportunistic pathogens (RIVERA; BOUCHER, 2011). NEGREIROS et al. (2016) observed the activity of the essential oil from *Baccharis psiadioides* inhibits enterococci resistant to vancomycin, a clinically important bacterium.

The MIC of EO^{dl}ES ranged from 0.39 to 6.25%, showing high values to resistant and multiresistant strains. These results are in agreement with other studies that evaluated the MIC of essential oils from different plants against enterococci. NEGREIROS *et al.* (2016), evaluating the MIC, from essential oils of *B. psiadioides* against the same strains tested here, obtained values of MIC > 1.25% in agar dilution and 4 to 16% in broth dilution test. SELIM et al. (2014) showed that the MIC of essential oils from leaves of *Eucalyptus globulus* against vancomycin-resistant enterococci (VRE) strains, using the agar dilution technique, was 0.5 to 1%.

The results found in this study showed that EO^{dl}ES has the ability to inhibit the biofilm formation in all strains tested. There are no reports in the literature of anti-biofilm activity involving the EO^{dl}ES. The essential oil applied before the formation of the biofilm could interact with proteins of bacterial surface compromising the phase of initial connection on surfaces, as well as interfere with *quorum sensing* systems. The success in inhibiting cellular link can be explained as the cellular connection, which is the initial stage in the formation of the biofilm with previous conditioning of the surface conditioning, provides a favorable environment for bacterial fixation (SCHILLACI et al., 2013; SELIM et al., 2014; KIFER et al., 2016).

Unlike the ability to inhibit the formation of biofilms in all concentrations tested, the EO^{dl}ES presented little or no activity removal preformed biofilm to the most bacteria tested. This result can occur due to overproduction of exopolysaccharide, which has the role to protect the metabolically active bacteria embedded in biofilm community, that the compounds used could eliminate only the cells closest to the interface biofilm (SELIM et al., 2014; KIFER et al., 2016). However, we observed reduction in preformed biofilm on three *E. faecalis* strains. MERGHNI et al. (2016), in studies with other essential oils, verified that the antibiofilm activity was observed in ½ MIC reaching percentages of inhibition of 50 to 70% of *S. aureus* of the biofilms. Nowadays, no target therapy in biofilm is available on the market, and still the best strategy against the biofilm is to avoid your training rather than trying to eliminate them after they graduate (SCHILLACI et al., 2013).

In conclusion, this study showed that the essential oil from dried leaves of *E. staigeriana* has potential for gram-positive pathogens control, highlighting clinical and food, and *Enterococcus* resistant strains. EO^{dl}ES can emerge as a promising alternative to control antimicrobial resistant bacteria, as well as for the prevention of contamination linked to the formation of biofilm.

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