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Antibiofilm activity of the essential oil of citronella (*Cymbopogon nardus*) and its major component, geraniol, on the bacterial biofilms of *Staphylococcus aureus*

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Abstract Medicinal plants with antimicrobial action have been investigated for uses against biofilms, among which, *Cymbopogon nardus*, citronella, stands out as a promising species. The present study aims to evaluate the antimicrobial and antibiofilm action of the essential oil of *C. nardus* (EOCN) and geraniol on Gram-negative and positive bacteria from the determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration and inhibition of biofilms. In the results, the EOCN produced a 41 mm halo on *S. aureus*, which was susceptible with MIC values of 0.5 and 0.25 mg/mL for the EOCN and geraniol respectively, both with bactericidal effect. The antibiofilm action was confirmed, the EOCN and geraniol reduced the biofilm biomass of *S. aureus* up to 100% between 0.5 and 4 mg/mL concentrations. The reduction of cell viability was 0.25 and 1 mg/mL, of EOCN and geraniol, respectively. EOCN and geraniol were shown to be promising antibiotic against *S. aureus.*

Keywords Antibiofilm · *Cymbopogon nardus* · Essential oil · Food · Geraniol

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Introduction

Among the main causes of public health problems in the world are foodborne diseases (FDs). Foodborne diseases that are spread through the ingestion of contaminated water and/or food (Barreto et al., 2012). FDs are responsible for high rates of morbidity and mortality and for high costs of medical care (Fratamico et al., 2005).

The incidence of foodborne diseases has been underestimated. The World Health Organization estimates that 31 foodborne hazards have resulted in more than 600 million illnesses and 420,000 deaths worldwide in 2010. In Brazil, according to the Ministry of Health, there were 118 thousand registered cases of FDs from 2007 to 2016, and in 109 of these cases, the individuals died (Portal Saúde, 2016). Faced with this reality, it is necessary to find out the routes of exposure for key hazards for the prevention of FDs (Havelaar et al., 2015).

Contamination factors that contribute to the transmission of FDs are the following: direct transmission from animals to humans, vectors (insects), microbial adaptation, food handlers and food contaminated by processing surfaces (Hoffmann et al., 2017). Many pathogenic or corrosive microorganisms may be attached to food surfaces or may be present in the environments where food is prepared. One of the factors that exacerbates this issue is the lack of appropriate handling conditions and hygiene, which leads to surface and food contamination. *S. aureus* and *E. coli* are the predominant bacteria that form surface biofilms in the food industry and are responsible for numerous cases of food poisoning (Baptista, 2013, Di Conza et al., 2014; Freeman et al., 2014; Guimarães et al., 2017).

Biofilms are complex bacterial communities formed by the adhesion of microorganisms to a surface (biotic or abiotic); biofilms are embedded in an exopolymeric matrix

composed of polysaccharides, nucleic acids, and lipids, among other compounds (Bersan et al., 2014). One way to combat biofilms is to use sanitizers. Countless studies are being carried out to develop new sanitizing products that are more effective and are less toxic to humans.

Essential oils and their major components stand out as new strategies to combat biofilms, with potential applicability in the food industry (Bordi and Bentzmann, 2011). Essential oils are volatile aromatic products composed of secondary metabolites of plants. Essential oils are found in a concentrated form in various parts of the plants, such as the leaves, fruits or bark, and their therapeutic properties are being studied in the search for new antimicrobial drugs and antibiofilm strategies. In this context, the use of medicinal plants with antimicrobial activities constitutes an important strategy to combat this problem (Aguiar et al., 2014; Oliveira et al., 2011).

The literature highlights the essential oils from the leaves of *Cymbopogon nardus* for its biological activity against *Rhipicephalus microplus* (Agnolin et al., 2010); antibacterial activity against *Staphylococcus aureus*, *Listeria monocytogenes*, *E. coli*, *Salmonella choleraesuis* and *Pseudomonas aeruginosa* (Andrade et al., 2012); and antifungal activity against *Aspergillus* spp. (Aguiar et al., 2014). Geraniol, a major component of this essential oil, has shown antibacterial activity against *S. aureus* strain ATCC 6538 (Henrique et al., 2015).

In view of the above observations, the present work aims to verify if the essential oil from the species *C. nardus* and its major component, geraniol, have antimicrobial activity and the potential to inhibit the formation of in vitro biofilms of strains of *S. aureus* and *E. coli*. The results of this study may guide future tests to validate the use of this essential oil as an antimicrobial.

Materials and methods

Collection and identification of botanical material

The leaves of *C. nardus* were collected in April 2014 in the municipality of Cariré, Ceará, Brazil. The plant material was identified by Dr. Elnatan Bezerra de Souza and deposited under accession number 20807 to the Dr. Francisco José de Abreu Matos Herbarium of the Vale do Acaraú State University, Sobral, CE, Brazil.

Extraction and chemical characterization of the essential oil of *Cymbopogon nardus*

The essential oil of *C. nardus* was obtained by extraction using the Cleavenger hydrodistillation method. For the extraction, 320 g of fresh leaves was used; the leaves were crushed, placed in a 5-L round-bottom flask with 2 L of distilled water, and boiled for 2 h. During this time, the water/oil mixture present in the dispenser was separated based on the density difference between the two. The obtained essential oil was weighed and stored under refrigeration in a labelled amber bottle. The yield of the essential oil was expressed as the percentage obtained from the ratio of the mass (g) of the oil extracted to the mass (g) of the leaves in the flask multiplied by 100.

The chemical constituents of the essential oil of *C. nardus* were analyzed in laboratory of Chemistry Natural Products at Federal University Rural of Rio de Janeiro, Seropédica, Rio de Janeiro. The major component of the essential oil, namely, geraniol, was commercially obtained from Sigma Aldrich®, Sigma, St. Louis, MO, USA.

Analyses of constituents of the essential oil

The qualitative analysis of the chemical composition of the essential oil was performed by GC-MS in a Shimadzu QP-2010 instrument (Shimadzu, Kyoto, Japan). The compounds were identified by analysis of the fragments patterns displayed in the mass spectra, and their identities were confirmed by comparing their mass spectra with those present in the database (NIST, 2006) and comparing their retention rates (Kovat) to of known compounds which were obtained by injection of a mixture of standards containing a homologous series of C8–C30 alkanes as described by Van Den Dol and Kratz (1963).

$$KI = 100n + 100\left(\frac{\log t'_{R(i)} - \log t'_{R(n)}}{\log t'_{R(n+1)} - \log t'_{R(n)}}\right)$$

Substances and preparation of solutions

The analyzed substances were the essential oil of the *C. nardus* and the major component of this essential oil, geraniol; chlorhexidine and imipenem were used as positive controls.

Bacterial strains and culture conditions

The following strains were used in this study: *S. aureus* ATCC 6538, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 4083, *Streptococcus pyogenes* ATCC 19615, *E. coli* ATCC 11303 and *Pseudomonas aeruginosa* ATCC 115442. The strains were purchased from the Institute Oswaldo Cruz-Fiocruz and are part of the American Type Culture Collection (ATCC). All strains were stored under refrigeration at − 18 °C in skim milk medium enriched with glycerol.

In the experiments, a 100 µL aliquot of the stock solution was inoculated in TSA medium (trypticase soy agar) and grown in a greenhouse at 37 °C for 24 h. After this initial activation, the culture was revived with the addition of 100 µL of inoculum to 10 mL of TSB (trypticase soy broth) followed by incubation for 18 h under the same conditions described above. In the antimicrobial activity assays, the cultures were washed with Mili-Rios water, and their concentrations were adjusted to 10^7–10^8 CFU/mL with the use of a microplate reader (SpectraMax i3 Multi-Mode Microplate Reader) at 620 nm.

Antimicrobial activity

The antimicrobial activities of the compounds were tested by performing antimicrobial susceptibility tests on the bacteria by the agar-diffusion method and by microdilution tests in broth; these tests were used to determine the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC).

Antimicrobial susceptibility test by disc-diffusion in agar

For the antimicrobial susceptibility test by agar diffusion, modifications were made to the standard M2-A8 method (Clinical and Laboratory Standards Institute-CLSI) translated and distributed in Brazil by the National Agency of Sanitary Surveillance. The strains *S. aureus*, *S. epidermidis*, *E. faecalis*, *S. pyogenes*, *E. coli* and *P. aeruginosa* were activated in TSB, and two biological replicates were tested for each strain.

The cell densities of the bacterial cultures were adjusted with 0.85% saline solution to 10^6–10^8 CFU/mL or the equivalent of 0.5 on the McFarland scale and then inoculated on Müeller-Hinton agar medium. Sterile, 6-mm diameter white disks were soaked with 20 µL of essential oil *Cymbopogon nardus* (EOCN) (18 mg/mL), geraniol (19 mg/mL), imipenem positive control (10 mg/mL) or chlorhexidine positive control (10 mg/mL); after drying, the discs were placed in triplicate on the plates in which the bacteria were inoculated.

Minimum inhibitory concentration (MIC)

To determine the MICs by means of the microdilution test in broth, the compounds EOCN, geraniol and chlorhexidine were tested against the strains *S. aureus* and *E. coli*. The MIC test was standardized according to the Methods for Dilution Antimicrobial Susceptibility Test for Bacteria That Grow Aerobically; Approved Standard-Tenth Edition, CSLI document M07-A10 (2015) with modifications, as described below.

The minimum inhibitory concentration was determined by the microdilution technique in 96-well polystyrene plates. The calculations for the preparation of the solutions containing EOCN and geraniol were performed based on the densities of the substances. To facilitate the solubilization, 2% Tween 20 was used. For the assembly of the plates, EOCN, geraniol and chlorhexidine were prepared at an initial concentration of 16 mg/mL and serially diluted in culture medium so that the concentrations varied ranged from 0.125 to 8 mg/mL. Then 100 µl of culture medium containing the microorganisms were adjusted to the concentration of 10^7–10^8 CFU / ml and added. The negative control consisted of growing the microorganisms in the TSB culture medium with 2% Tween 20. The lowest concentration of each substance at which no microbial growth was detected was considered the MIC, through a visual reading the lower attention.

Minimum bactericidal concentration (MBC)

The minimum bactericidal concentration was determined by removing a 10-µL aliquot from the solutions considered to contain each substance at its MIC. The aliquot, in triplicate, was inoculated in Petri dishes containing TSA, and the dishes were placed in an incubator at 37 °C. Bacterial growth was observed after 24 h (Sá et al., 2012).

Antibiofilm activity

The ability to inhibit biofilm formation was determined by the indirect biomass-quantification technique using crystal violet (CV) and by the colony-forming unit (CFU)—counting technique, which provides data on the viability of the cells contained in the biofilm. The plaques were assembled in a process similar to the MIC test, and after 24 h, the antibiofilm activity was verified; the bacterium used was *S. aureus*.

Quantification of biofilm biomass

After the 24-hour incubation, the plates were washed with sterile water (200 µL/well) to remove the loosely adhered and air-dried cells. The adhered cells of the biofilm were fixed in the wells of the plate by the addition of 200 µL of methanol for 15 min. After 15 min, the methanol was removed, and 200 µL of 1% crystal violet was added for 15 min. Then, the plates were washed again and air dried. Next, 200 µL of ethanol (96%) was added to the wells, and the plates were left shaking for 5 min and then read on a microplate reader at 595 nm (Sá et al., 2012).

Counting of colony-forming units (CFU)

After the incubation period, the culture medium was removed, and the plates were subjected to three washes with distilled water. Then, 200 µL of 0.9% saline was added to the wells, and the plate was sonicated in an ultrasonic bath (LK-D32 Ultrasonic Bath) operating at 50 kHz for 10 min. The liquid from the wells was pooled to a total volume of 1 mL, from which a 20 µL aliquot was withdrawn and serially diluted in a volume of 180 µL of saline (10^{-1}–10^{-6}). In a Petri dish containing TSA, three aliquots of 10 µL were cultured, constituting a triplicate for each concentration. These plates were placed in a greenhouse for 24 h at 37 °C, the counting of the colony forming units was performed by manually counting the visible wells after incubation (Sá et al., 2012).

Statistical analysis

The statistical evaluation of the data was performed with the program Prism, GraphPad®, San Diego, California, USA, version 5.0. The statistical test used for multiple comparisons was ANOVA, followed by the Bonferroni test. Values of $p < 0.01$ were considered statistically significant and are indicated by an asterisk. The MBC and CFU tests were performed with three replicates. All tests were performed in three independent experiments.

Results and discussion

Yield and chemical composition of the essential oil of *Cymbopogon nardus* (Table 1)

The yield of the essential oil of *C. nardus* obtained by hydrodistillation was 1.33%. The extraction of the essential oils of aromatic plants depends on several factors, such as the extraction method, cultivation method, environmental conditions, period of collection, and altitude (Simões et al., 2010).

In the chemical analysis of the *C. nardus* essential oil, seventeen constituents were found (Table 1). The components found in higher amounts were the monoterpenes geraniol (33.88%), citronellal (27.55%) and citronellol (14.40%). The results found are in agreement with those of Hazwan et al. (2014), who found that geraniol constitutes 42.38% of the essential oil extracted from the leaves of the same plant.

The activity of geraniol has been described in other studies and seems to play a primary role in the antimicrobial activity of EOCN (Aguiar et al., 2014; Hazwan et al., 2014; Millezi et al., 2014).

Table 1 Chemical composition, retention index of the literature (RI_{Lit}), percentage of the identified components (%) from the essential oil of *Cymbopogon nardus* (EOCN) aerial parts

No.	Compounds	RI_{Lit}	%
1	Limonene	1029	1.20
2	γ-Terpinene	1060	0.42
3	Linalool	1097	0.82
4	Citronellal	1153	27.55
5	Citronellol	1226	14.40
6	Neral	1238	0.90
7	Carvone	1243	10.06
8	Geraniol	1253	33.88
9	Geranial	1267	1.26
10	Citronellyl acetate	1352	0.51
11	Neril acetate	1361	0.66
12	E-Muurola-3,5-diene	1453	0.54
13	δ-Cadinene	1523	0.58
14	Elemol	1550	3.38
15	Germacrene D-4-ol	1575	1.75
16	Z-Bisabolol-11-ol	1619	0.80
17	Eremoligenol	1631	1.09
	Total		99.80

Antimicrobial activity

Disc-diffusion in agar

The data for inhibition by EOCN and geraniol are shown in Table 2.

Treatment with EOCN and geraniol led to the formation of inhibition halos 41 and 19 mm in diameter, respectively, when tested with *S. aureus*. Comparing these data with the values presented by commercial antibiotics, it can be seen that the inhibition halo formed in the EOCN treatment is 16 mm larger than the inhibition halo that formed in the chlorhexidine treatment.

The inhibition halos when *S. epidermidis* bacteria were tested with EOCN and geraniol were 16 and 19 mm in size, respectively. The sizes of the inhibition haloes of geraniol and chlorhexidine were close, i.e., 19 and 18 mm, and *S. epidermidis* were similar in the presence of geraniol and chlorhexidine.

Inhibition halos were observed for the bacteria *E. faecalis* and *S. pyogenes* when using EOCN, and the results were similar to the inhibition halos induced by chlorhexidine. EOCN did not induce the formation of inhibition halos for *P. aeruginosa* and *E. coli* bacteria. When treated with geraniol, *E. coli* presented a halo of 15 mm, and this value was close to the size of the halo formed upon chlorhexidine treatment, i.e., 18 mm.

Table 2 Values (mm) of the inhibition halos obtained by the use of E EOCN and geraniol against gram-positive and gram-negative bacteria

Microorganisms	EOCN	Geraniol	IMP	CLR
Staphylococcus aureus	41 ± 0.94	19 ± 0.47	59 ± 1.70	25 ± 0.47
Staphylococcus epidermidis	16 ± 2.05	19 ± 1.25	52 ± 2.05	18 ± 0.82
Enterococcus faecalis	13 ± 0.94	11 ± 0.94	35 ± 2.36	14 ± 0.47
Streptococcus pyogenes	12 ± 0.82	10 ± 1.25	38 ± 1.70	17 ± 3.09
Pseudomonas aeruginosa	0	10 ± 0.47	32 ± 0.94	17 ± 1.89
Escherichia coli	0	15 ± 1.63	19 ± 2.05	18 ± 2.16

EOCN Essential oil *C. nardus*, *IMP* Imipenem, *CLR* Chlorhexidine

Second, Carvalho (2012) attributed the antibacterial effect of *C. nardus* to the presence of the geraniol and citronellal constituents in its composition. Monoterpenes have the ability to cross the cell wall and the ability to permeabilize and depolarize the plasma membrane (Tong et al., 2013).

MIC and MBC values of EOCN and geraniol for S. aureus and E. coli

The results of the antimicrobial activities of EOCN and geraniol are shown in Table 3.

The MICs of EOCN and geraniol for *S. aureus* were 0.5 mg/mL and 0.25 mg/mL, respectively. The data show that both EOCN and geraniol exhibit bacteriostatic activities at the concentrations stated above. The EOCN MIC values for *S. aureus* found in this study are in agreement with a study by Silveira et al. (2012) that evaluated the antimicrobial activity of EOCN from leaves and found geraniol to be the major component; that study found the EOCN MIC values to be 0.600 mg/mL. With respect to geraniol, the results of our study corroborate those found by Coutinho et al. (2015), who determined the MIC to be 0.24 mg/mL for *S. aureus*.

The MBC values for *S. aureus* bacteria were as follows: 4 mg/mL for EOCN and 2 mg/mL for geraniol. In one study, the MBC for *S. aureus* was found to be 2.5 mg/mL for geraniol. On the other hand, the MBC of EOCN and geraniol for *E. coli* was not detected (Coutinho et al., 2015).

Inhibition of bacterial biofilm formation by Staphylococcus aureus

The biomass reduction of the *S. aureus* biofilm occurred in both EOCN and geraniol treatments, as shown in Table 4.

According to the table, it can be seen that EOCN reduced the biomass between 95.0 and 100.0% when used in a concentration range of 0.5–4 mg/mL. Geraniol showed data similar to chlorhexidine at all concentrations; it is worth noting that at a concentration of 0.125 mg/mL there was a reduction of 84.2% in the biofilm biomass of *S. aureus*. It is observed that from 0.5 to 4 mg/mL the reduction in biomass ranged from 93.3 to 100%, and there was no statistically significant difference between these data at different concentrations or when using different substances. Thus, it can be concluded that the reduction in biomass promoted by the substances in this study was equivalent to the positive control at the measured concentrations.

The use of essential oils and their byproducts has become a major strategy to combat the formation and development of biofilms. Second, Chaieb et al. (2011) reported the inhibition of bacterial biofilm formation based on the bactericidal effects and antiadhesion potentials of many oils or isolated compounds. The tested substances, namely, EOCN and geraniol, show promise as inhibitors of biofilm formation by *S. aureus*, a bacterium that has caused many outbreaks of food poisoning (Guimarães et al., 2017). The cellular viability data are shown in Fig. 1.

The EOCN induced reduction in cell viability in the *S. aureus* biofilm occurred at EOCN concentrations up to 0.5 mg/mL; however, at concentrations from 1 to 8 mg/mL, the inhibition was 9.62 log CFU/mL. Geraniol reduced

Table 3 MIC and MBC values (mm/ml) of EOCN and geraniol for *S. aureus* and *E. coli*

Microorganisms	MIC		MBC	
	EOCN (mg/ml)	Geraniol (mg/ml)	EOCN (mg/ml)	Geraniol (mg/ml)
S. aureus	0.5	0.25	4	2
E. coli	> 8	> 8	> 8	> 8

MIC Minimal inhibitory concentration, *MBC* Minimal bactericidal concentration, *EOCN* Essential oil *C. nardus*

Table 4 Percentage reduction of *S. aureus* biofilm biomass by EOCN, geraniol and chlorhexidine (mg/mL)

	4 mg/ml	2 mg/ml	1 mg/ml	0.5 mg/ml	0.25 mg/ml	0.125 mg/ml
EOCN	100.0 ± 0.0Aa*	98.9 ± 1.0Aa	98.3 ± 1.6Aa	95.0 ± 2.7Aa	72.7 ± 7.1Ab	61.0 ± 6.6Ab
GER	98.5 ± 0.6Aa	99.9 ± 0.0Aa	99.0 ± 0.3Aa	98.1 ± 0.6Aa	95.0 ± 2.5Ba	84.2 ± 3.5Ba
CLR	95.7 ± 1.4Aa	93.3 ± 2.2Aa	100.0 ± 0.0Aa*	99.7 ± 0.2Aa	97.6 ± 1.1Ba	99.8 ± 0.1Ba

EOCN Essential oil *Cymbopogon nardus*, *GER* Geraniol, *CLR* Chlorhexidine

*Averages ± standard error followed by the same letter do not differ statistically from one another, upper-case letters correspond to the columns, and lower-case letters correspond to the rows. One-way ANOVA test ($p < 0.01$) with the Bonferroni post-test

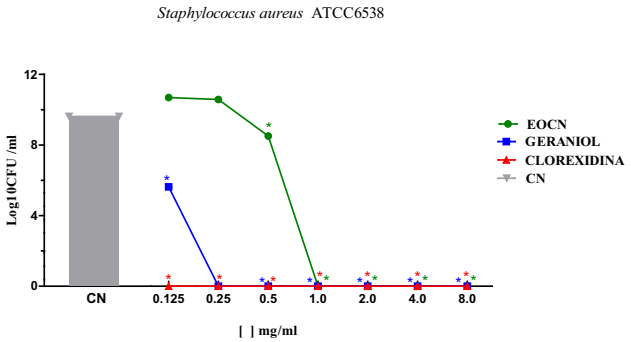

Fig. 1 Cell viability in *S. aureus* biofilms after the action of EOCN and geraniol. *$p < 0.01$ compared to the negative control (CN). EOCN—Essential Oil *C. nardus*

cell viability at the concentrations tested, with a reduction of 4 log CFU/mL compared to the negative control at 0.125 mg/mL. It is noteworthy that between 0.25 and 8 mg/mL of geraniol, the results are similar to those seen with chlorhexidine.

Geraniol is an acyclic monoterpene present in a large number of plant species. Second, Trombetta et al. (2005) showed that monoterpenes have antimicrobial activity against gram-positive bacteria. This antimicrobial activity occurs because the action of monoterpenes causes perturbation in the lipid fraction of the plasma membrane of the microorganism, which results in alterations of the permeability of the membrane and consequently in cellular death by plasmolysis.

For the food industry, the presence of biofilms entails serious economic losses. The use of essential oils is a new alternative for the disinfection of industrial surfaces. Oliveira et al. (2010) used the oil from the leaves of *C. nardus* alone or in combination and were able to reduce the number of cells in the biofilms of *L. monocytogenes* adherent to the surface by 100% (5.64 Log CFU/mL) after 60 min of contact.

The tested substances, namely, geraniol and the essential oil of *C. nardus*, presented antimicrobial activity; gram-positive bacteria were more susceptible in the planktonic form, and the data revealed values similar to those of the positive control. In addition, both EOCN and geraniol are bactericidal against *S. aureus*. The antibiofilm activity was evidenced by the inhibition of the formation of *S. aureus* biofilms and verified by the reduction in the formed biofilm biomass and the reduction in cell viability when compared to the negative control.

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