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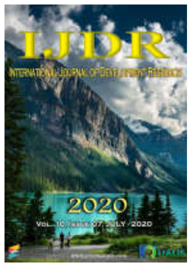

RESEARCH ARTICLE OPEN ACCESS

BACTERICIDE ACTION OF BIOACTIVE BASED ON ESSENTIAL OILS OF *PLANTS LIPPIA MENOSIDES CHAM*. AND *CYMBOPOGON WINTERIANUS JOWITT ON ESCHERICHIA COLI*

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ABSTRACT

This research aimed to verify the bactericidal action of a bioactive product, the base of the essential oils of the plants Rosemary-pepper (Lippia menosides), and Citronella (Cymbopogon winterianus) on the bacterium Escherichia coli. The work consisted of two stages, the first characterizes the bioactivated and the second of determining its bactericidal concentration. Chromatographic analysis of each oil revealed that the main components are thymol in pepper rosemary (71.5%) and citronellar in citronella grass (44.2%). The second stage consisted of testing the bioactive on E. coli, using 24 concentrations of the product, ranging from 1: 2 to 1: 8,388,608, obtained by microdilution, using a completely randomized design with 3 replications per treatment. The results revealed a percentage of bactericidal efficiency of 100% up to a concentration of 1: 1024. It is concluded that the bioactive studied is an effective product as a bactericide against E. coli, promising further research in the control of this bacterium in water reservoirs as a possible substitute for chlorine.

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INTRODUCTION

The need for research on natural products motivates the substitution of synthetic chemical substances with naturally low-cost and accessible materials since the benefits of research can contribute to the conservation of natural vegetation and the sustainability of semi-natural production systems (Candido; Lira, 2013). In this context the Essential oils from medicinal plants have been widely used in the perfumery, cosmetics, pharmaceutical industry, dentistry, medicine, and organic agriculture. Nowadays, large laboratories and research centers have been carrying out several studies with essential oils to understand the chemical components and identify potential antimicrobial activity. There are several reports on the biological activity of products of plant origin cited in the literature, with antifungal action (Nakahara *et al*. 2003, Medice *et al*. 2010), antibacterial action (Nogueira *et al*. 2007), anti-inflammatory and analytical action (Bose *et al*. 2007, Dlìaz-Viciedo *et al*. 2008), antioxidant (Scherer *et al*. 2009), allelopathic (Ootani *et al*. 2011), tick activity (Martin 2006, Vendrame *et al*. 2007, Olivo *et al*. 2008), insecticide (Lima *et al*. 2010), repellent (Raja *et al*. 2001), among others. The plant *Lippia menosides Cham* (Verbenaceae) is a shrub from Northeastern Brazilian, found mainly in the states of Ceará and the Rio Grande do Norte, popularly known as pepper rosemary in its contains composition essential oil rich in Timol and Carvacrol, which presents bactericidal, fungicidal, molluscicidal, and larvicidal properties (Andrade, 2015). In folk medicine, some uses of preparations obtained from L. menosides Cham are reported. Among them is its use in the form of tea or tincture of leaves, roots, or stems orally or topically as an antiseptic and antimicrobial (Almeida, 2010; Gomes, 2012). Citronella (*Cymbopogon winterianus*) is a perennial plant of the family Gramineae / Poaceae, it has between 0.6 to 1.0% of essential oil in its leaves. Oil extracted from fresh or partially dried leaves is traditionally used as a mosquito repellent. The oils extracted from plants, although insoluble in water, manage to impart an odor to it, constituting hydrolases and becoming an important source of flavorings in perfumery and spices. This medicinal and aromatic plant has grown in importance in Brazil due to the great demand for its essential oil, both in the domestic market and for export (Rocha *et al*., 2016). The development of bacterial infections in humans includes a variety of bacteria, among them Escherichia coli is the most common species of the Escherichia genus, associated with severe urinary tract infections, meningitis, and gastroenteritis (Murray; Rosenthal; Pfaller, 2010; Tortora; Funke; Case, 2008).

E. coli is often transmitted by eating or contaminated food that has not been pasteurized, washed or properly cooked. It is found in the intestines of certain types of cattle and can be lodged in the meat of the slaughtered animal (most often in the ground meat). It can also be found in unpasteurized milk (raw milk), raw apple cider, and made cheeses with unpasteurized milk. Vegetables and greens (including lettuce, spinach, and sprouts) can transmit E. coli if contaminated by animal feces during its growth and are not washed properly (Oliveira, 2018). *Escherichia coli(E.coli)* is an important microbiological indicator used in studies of water quality. It is characterized by being a bacterium quite abundant in the feces of warm-blooded animals, being found in natural water, sewage, and soils that have received recent fecal contamination. When the individual is exposed to contaminated water, in the presence of some pathogenic strains of E. coli, he may suffer from moderate to severe diarrhea, severe hemorrhagic colitis and uremic hemolytic syndrome (HUS), and in more extreme cases he may die (ORTEGA *et al*., 2009; Lima, 2015). Also, it is possible to establish a relationship between the existence of E. coli and the presence of other microbiological agents such as viruses and bacteria that cause waterborne diseases such as pneumonia, hepatitis, amebiasis, giardiasis, gastroenteritis, typhoid fever, infectious hepatitis, and cholera, among others (Roveri, 2013). Based on the reports above, the present study sought to demonstrate the possible antimicrobial action of the bioactive based on the essential oils of the plants *Lippia sidoides (rosemary pepper*) and *Cymbopogon winterianus Jowit* (citronella) against Escherichia coli, which is an important microbiological indicator used in studies water quality.

METHODOLOGY

Botanical Material: The leaves of pepper rosemary (*L. sidoides*) and citronella (*C. winterianus*), raw materials used to obtain the essential oils that make up the bioactive, were obtained from the Horto de Medicinal and Aromatic Plants of the Agricultural Sciences Center of the Federal University of Ceará (UFC), located at Fazenda Experimental Vale do Curu, in the municipality of Pentecoste - CE, about 100 km from Fortaleza. The plants were grown according to appropriate technology (Plantas Medicinais, 2006; Mattos, 2000), and their exsiccates deposited at Herbário Prisco Bezerra of UFC, being identified as EAC 54139 and EAC 54141 respectively for pepper rosemary and citronella.

Determination of the Antimicrobial Activity of Lippia Sidoides Cham and Cymbopogon Winterianus Extracts: For the test of the bactericidal effect of Bioactive the base of the essential oils of the plants Lippia menosides Cham. and Cymbopogon winterianus, the was completely randomized design with 3 replications and 24 treatments was used. The following procedure was adopted to obtain 23 different concentrations of the bioactive: 100 μL of the Bioactive plus 100 μL of HCM to obtain the stock solution (1: 1) where it was subjected to successive dilutions obtaining the concentrations of 1: 2; 1: 4; 1: 8; 1:16; 1:32; 1:64; 1: 128; 1: 256; 1: 512 to 1: 8,388 and then 20μL of the microorganism suspension was added to each well of the microplates. Two (02) Control Groups were used for this research, the first consisting of an antibiotic with effective and known action against E. coli in the concentration determined by the manufacturer, in this case, amoxicillin, and the second without substance no antimicrobial.

He tests were carried out in the microbiology laboratory of the Centro Universitário Católica de Quixadá (UNICATÓLICA) and they have a quantitative character. In this work, the following strain was used: Escherichia coli, standard strain *Escherichia Coli* NEWP 0022, each disc contains standard microorganisms in high concentration. (Above 100,000 CFU / mL) and a concentration <105 CFU / mL of non-pathogenic origin for the sole purpose of proving this study. The samples isolated in CMH culture medium were intended to favor the growth of these bacteria, at the Microbiology Laboratory of the Centro Universitário Catolica de Quixadá-UNICATÓLICA. The composition of the standard Cepa disk is *Escherichia Coli* NEWP 0022.Each disk contains standard microorganisms in high concentration. (Above 100,000 CFU / mL), Concentration> 105 CFU / mL. Using flaming forceps, a disk was removed aseptically from the flask and suspended in 4 mL of CMH broth. The material was incubated at 35º ± 2º C for a period of (18-24 hours). "Overnight" period. After the incubation period, tests started.

Determination of the Minimum Inhibitory Concentration (CIM): MIC was determined by the microplate dilution technique (96 holes) according to the methodology described according to the M7-A6 standard of the Manual Clinical and Laboratory Standards Institute (CLSI, 2006) for aerobic bacteria. The microplate holes (96 wells) were filled with 100μL of HCM and then 100μL of Bioactive was added to the base of the essential oils of the Lippia menosides Cham plants. and Cymbopogon winterianus and serial plate dilution were performed. Also, 20μL of the microorganism suspension was distributed in each well of the microplates As a positive control, amoxicillin and negative control with nothing, that is, without any antimicrobial substance, were used. The microplates were incubated in an oven at 37ºC for 24 hours. The tests were performed in triplicate. Readings were performed with the resazurin developer (100μg / mL) of which 30μL was added to each hole of the microplates in the tests with bacteria. In 2 hours, the presence of blue represents the absence of growth and pink, the presence of bacterial growth (Palomino *et al*., 2002).

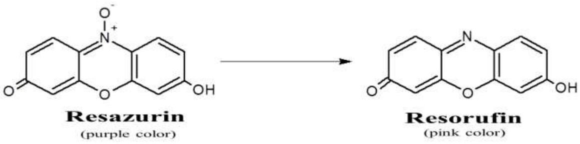

Figure 1. Reaction of redox oxidation of resazurin

Resazurin (7-hydroxy-3H-phenoxazin-3-one10-oxide) is considered the most used indicator in conditions of reduction in culture media (Fukushima *et al*., 2003). The mechanism is based on the reduction of resazurin (purple color) to resorufin (pink color), shown in Figure 2. Resazurin has a direct correlation with the quantity/proliferation of living organisms, which include bacterial cells and even mammalian cells (O'brien *et al*., 2000).

RESULTS AND DISCUSSION

The essential oils of *Lippia menosides Cham* and *Cymbopogon winterianus*, had their activity of access to genetic heritage, in the terms summarized below, was registered with SisGen, in compliance with the provisions of Law No. 13,123 / 2015 and its regulations.

Table 1. Constituents of the essential oil of Lippia

Constituent	%
Tymol	71,55
Para cymene	12,44
Caryophyllene	5,44
Myrcene	3,28
Gama-terpinene	1,95

Considering that essential oils have been extensively studied, with special attention to the use in the control of infectious processes, chemical analyzes were carried out through Mass Spectrum Gas Chromatography (CGL / EM) to characterize them, which are present in the Bioactive in the study. Through the analysis of the chromatograms of the essential oils of *Lippia menosides Cham* and *Cymbopogon winterianus*, it was possible to identify the main phytochemicals existing in the essential oil, where he presented Thymol as a major constituent in a concentration of 71.5% and the Citronella 44.20%, respectively . These data corroborate with Botelho *et al*. (2007) who analyzed the antimicrobial activity of *Lippia menosides* oil against pathogens of the oral mucosa and observed Timol with a concentration of 56.9% and 16.7%, respectively. The other constituents of the essential oil of Lippia menosides found in this study can be seen in Table 2. From the data described in Table 2, it is observed that 5 constituents present in the essential oil of Cymbopogon winterianus were identified, with the major components being the acyclic monoterpenes, citronellal (44.20%), geraniol (32.31%), citronellol (12.56%), elemol (6.43%) and limoneno (4,49%).

Several factors interfere with qualitatively and quantitatively in the production of essential oil and the fidelity of the final product. Yield and composition are directly influenced by conditions such as climate, soil, time of planting and harvesting, fertilization, use of pesticides, irrigation, weather, and environmental conditions, state of use (green or dry), OE extraction technique, latitude, and longitude. Thus, essential oil of the same species can vary, changing the content of its compounds and, consequently, its medicinal properties (LEAL, 2003). Thymol corresponds to a phenolic compound derived from phenylpropane with little solubility in water, having a strong, pleasant, and characteristic aroma. The literature points out several properties of this substance, with antiseptic being the most significant of them, as it gives thymol the ability to act by inhibiting the proliferation of microorganisms (Morais, 2002). Brito *et al*. (2015), in phytochemical *analyzes of the essential oil of Lippia sidoides*, pointed out thymol as the major component, reaching concentrations of 83%. The same study compared the activity of OELS (essential oil of *Lppia sidoides*) with thymol compound isolated against strains of *Candida sp*. and noted that both inhibited their growth considerably. The presence of citronellal compounds in the oil of this plant was reported by Labinas and Crocomo (2002). Quintans-Júnior *et al*. (2008) found the presence of geraniol (40.06%), as the main constituent, followed by the compounds citronella (27.44%) and citronellol (10.45%) Leite *et al*. (2011) found as major components of the essential oil of C. winterianus the citronella (36.19%), geraniol (32.82%) and citronellol (11.37%) Oliveira *et al*. (2010) reported that the essential oil of C. winterianus presented as major components citronellal (34.60%), geraniol (23.17%) and citronellol (12.09%). According to Chen and Vijoen, (2010), geraniol has antiseptic activity, inhibiting the growth of fungi and bacteria. Although essential oils have a variable amount of substances, it is common for one or two compounds to predominate in greater quantities. Antimicrobial activity may be associated with the presence of one of these compounds or with the synergistic action of two or more compounds present (Silva; Bastos, 2007). It presents the composition of the evaluated essential oils, determined by gas chromatography (CG -DIC and CG / EM). In this study, it was possible to identify 05 compounds, which represented 71.55% to 44.20% of the composition of volatile oils.

Microdilution methods: The antimicrobial activity of the bioactive based on *Lippia menosides Cham*. and *Cymbopogon winterianus Jowitt* in *Escherichia coli* at different dilutions were evaluated as shown in Table 4 and Figure 3. With this result, the Minimum Inhibitory Concentration (MIC) against Escherichia coli was thus determined. In the experiment, the positive control of the microbial growth medium was adequate. The result of the antibacterial activity of the bioactive shows that the bioactive showed the ability to inhibit the growth of the microorganism in well B-2, which has a 1: 1024 dilution.

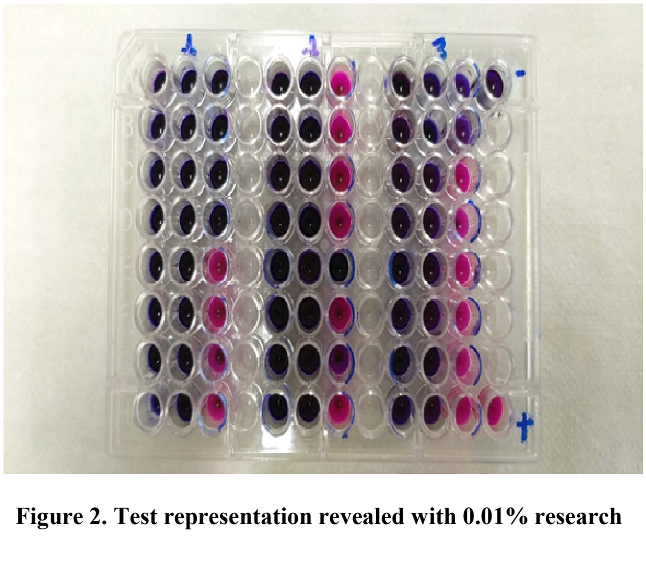

Figure 2. Test representation revealed with 0.01% research

Table 2. Minimum inhibitory concentration (MIC) of the bioactive based on *Lippia menosides Cham*. and *C ymbopogon winterianus Jowitt* on *Escherichia coli.*

DILUTIONS	Repetitions			MIC
	I	II	III	(%)
1:1	BLUE	BLUE	BLUE	100
1:2	BLUE	BLUE	BLUE	100
1:4	BLUE	BLUE	BLUE	100
1:8	BLUE	BLUE	BLUE	100
1:16	BLUE	BLUE	BLUE	100
1:32	BLUE	BLUE	BLUE	100
1:64	BLUE	BLUE	BLUE	100
1:128	BLUE	BLUE	BLUE	100
1:256	BLUE	BLUE	BLUE	100
1:512	BLUE	BLUE	BLUE	100
1:1024	BLUE	BLUE	BLUE	100
1:2.048	PINK	BLUE	BLUE	66
1:4.096	BLUE	PINK	PINK	33
1:8.192	PINK	PINK	PINK	0
1:16.384	PINK	PINK	PINK	0
1:32.768	PINK	PINK	PINK	0
1:65.536	PINK	PINK	PINK	0
1:131.072	PINK	PINK	PINK	0
1:262.144	PINK	PINK	PINK	0
1:524.288	PINK	PINK	PINK	0
1:1.048.576	PINK	PINK	PINK	0
1:2.097.125	PINK	PINK	PINK	0
1:4.194.304	PINK	PINK	PINK	0
1:8.388.608	BLUE	BLUE	BLUE	0

* CIM -0,78 mg/mL* blue color represents absence of growth and pink color, presence of bacterial growth

Table 3. Summary of the analysis of variance of data on the percentage of efficiency of the bioactive based on Lippia menosides Cham. and Cymbopogon winterianus Jowitt on Escherichia coli in different dilutions.

Variation Sources	G. L.	Q. M.[1]
Treatments	23	58.71**
Residue	48	2.25
C.V. (%)	-	27.03

[1]Original data transformed to Y = (X + 1) 1/2.
** - Significant at 1% probability by the F test.
G.L. Degree of freedom
C.V Coefficient of variation

Table 4. Average values of the percentage of efficiency of the bioactive based on Lippia menosides Cham. And Cymbopogon winterianus Jowitt on Escherichia coli in different dilutions

DILUTION (ml / ml)	EFFICIENCY (%)
1:1	100 A
1:2	100 A
1:4	100 A
1:8	100 A
1:16	100 A
1:32	100 A
1:64	100 A
1:128	100 A
1:256	100 A
1:512	100 A
1:1024	100 A
1:2.048	66 AB
1:4.096	33 BC
1:8.192	0 C
1:16.384	0 C
1:32.768	0 C
1:65.536	0 C
1:131.072	0 C
1:262.144	0 C
1:524.288	0 C
1:1.048.576	0 C
1:2.097.125	0 C
1:4.194.304	0 C
1:8.388.608	0 C

- Means followed by the same letter in the column do not differ at the level of 1% by the Tukey test.

Statistical analysis: The data of the analysis of variance contained in TABLE 4 reveal a highly significant difference for treatments represented by the variable percentage of efficiency of the bioactive based on *Lippia sidoides*. and *Cymbopogon winterianus* in 24 different dilutions. The averages relative to the efficiency percentage (%) of the bioactive on Escherichia coli in different dilutions are contained in TABLE 5 It is verified by its examination that the averages of the efficiency percentage of the bioactive presented highly significant statistical variation with average values between 0 to 100%. It is also observed that the maximum dilution of the bioactive with 100% efficiency was 1: 1,024, although statistically, it does not differ from the average value reached when the dilution was 1: 2048 (66%). From 1: 8,192 all dilutions of the bioactive did not show more bactericidal efficiency (0%).

Conclusion

The results obtained in the chromatographic analysis identified as major chemical components thymol (75.4%) for pepper rosemary and Cymbopogon *winterianus*, the major components acyclic monoterpenes, citronellal (44.20%), geraniol (32.31%), and citronellol (12.56%), the percentages identified suggest the presence of antimicrobial activity. Although the antimicrobial activity cannot be attributed only to the major compound, this is because this antimicrobial action can also occur through the synergy between the various compounds of essential oils and due to the mixture and complexity of the chemical components existing in the plant. Therefore, in view of the richness both quanti and qualitatively of these oils, the biativo test was carried out, where the antimicrobial effect and its minimum inhibitory concentration (MIC) were evaluated.The bioactive was efficient in a significant dilution, showing that the use of this component on *Escherichia coli*. From these results, it is possible that future studies can test the bioactive in cisterns used by rural communities in northeastern Brazil so that the water treatment can be carried out with a natural product based on essential oils from the plants of *Lippia menosides Cham*. And *Cymbopogon winterianus Jowitt*.With the possibility of replacing chemicals.

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