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Evaluation of antibacterial and toxicological activities of essential oil of *Ocimum gratissimum* L. and its major constituent Eugenol

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Abstract

The aim of this study was to evaluate the direct antibacterial activity, antibiotic modulation and toxicity of the essential oil of *Ocimum gratissimum* L. and its major compound Eugenol. The phytochemical analysis of the essential oil from leaves was investigated by Gas Chromatography coupled to Mass Spectrometry (CG-MS) and Eugenol was the major compound (63.25%). The Minimum Inhibitory Concentration and clinical antibiotics modulatory activities were performed using the 96-well plate microdilution methodology. *Drosophila melanogaster* was used as a model to assess the toxicity of the two substances. In conclusion, the eugenol and essential oil of *Ocimum gratissimum* L. showed direct antibacterial activity and associates with the tested antibiotics, which place them as promising agents against bacterial resistance. The

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essential oil showed less toxicity to fruit flies when compared to eugenol alone, although the results were numerically similar.

Keywords: Antibacterial agents. *Drosophila melanogaster*. *Ocimum gratissimum* L.. Essential oils.

Introduction

As ancient as the history of civilizations, medicinal plants have always been seen for their properties in offering relevant medicinal resources to ancient people (BADKE *et al.*, 2011). This perception collaborated with the dissemination of the therapeutic use of plant resources, which was established as a traditional secular practice and common to a diversity of cultures (BARRETO *et al.*, 2010).

With the development of research, it is now known that the usefulness offered by medicinal plants in the treatment of diseases is associated with the fact that they are considerable sources of secondary metabolites, which protect plants, in addition to being the target of clinical and therapeutic interests (RIMAWI *et al.*, 2020).

The discovery of new drugs for the treatment of bacterial infections has ensured the improvement of human health and the prolongation of its life (HE *et al.*, 2020). The need to discover new treatments for diseases occurs in theory due to the involvement of resistance, low responsiveness to drugs, such as those that treat problems such as cancer, and to the many adverse effects offered by the drugs currently commercialized (ZUHAIRI *et al.*, 2020).

In particular, bacterial resistance stands out in this context. The incidence of cases of antibiotic resistant bacterial have been observed whit increasing frequency over the past several decades, due to the prescription and incorrect use of antibiotics (OBEIDAT *et al.*, 2012). The worsening of this scenario has an impact on the inefficiency of the drugs used, leading to the discovery of new drugs, through the screening of possible phytochemicals and the manipulation of the apparatus made available by plant products (ACHMIT *et al.*, 2021). As a result, research that evaluates the antibacterial activity of essential oils has increased considerably (KUHN *et al.*, 2019).

In general, essential oils are volatile compounds of different combinations, obtained from flowers, leaves and barks, which may involve the presence of monoterpenes, sesquiterpenes, phenylpropanoids and others (BIZZO *et al.*, 2009).

Investigations have been carried out to determine the chemical profile of these oils and how they can be used in different areas, such as industry, pharmacy and food (KUCUKBAY *et al.*, 2014). Essential oils are abundant in different plant species, constituting most of the chemical substances of medicinal plants (SWAMY *et al.*, 2020).

In this context, the Lamiaceae family has gained evidence, for its usefulness to the human being in offering medicinal properties and cosmetic. It is estimated that this family has a total of 200 genera and more than 6900 species distributed around the world (MOHAMMADHOSSEINI, 2017). Among these is *Ocimum gratissimum* L., herbaceous, perennial and with a woody base, originally from the African continent and spontaneous in Brazil, mainly in the Northeast region (SHARMA *et al.*, 2011).

Borges *et al.* (2012) distinguished Eugenol as the most expressive chemical constituent of *Ocimum gratissimum* L. (alfavaca). This phenolic, aromatic and lipophilic phyto-compound has shown substantial biological activities, including antibacterial (ZHANG *et al.*, 2018).

Although encouraged, the widespread use of medicinal plants, such as their chemical components, can generate toxic responses (CUNHA *et al.*, 2015). In view of this problem, the *Drosophila melanogaster* (fruit fly) model has been consecrated in evaluating the pharmacological and toxicological effects offered by metabolites or inputs derived from medicinal plants (ZEMOLIN *et al.*, 2014).

In this way, this study characterized the chemical profile of the essential oil of *Ocimum gratissimum* L., presenting the possibility of its antibacterial effect and of its activity associated with antibiotics. Similar analysis was performed for Eugenol alone, comparing its effect with the results presented for essential oil. Finally, essential oil and Eugenol were analyzed through their effects on the induction of mortality or interference in the negative geotaxis mechanism of fruit flies.

Materials and methods

Botanical material

Two hundred and ten grams (210 g) of *Ocimum gratissimum* L. leaves were collected in July 2020 at the Crato Municipal Nursery, located at the Chico Mendes Institute for Biodiversity Conservation – ICMBio, according to coordinates 7°14'27.7"S 39°25'01.4"W. The leaves were collected at 12:00 PM, Eugenol peak time, as stipulated by Borges and collaborators (2012). An excicata of the specimen was deposited at the

Herbarium Dardano de Andrade de Lima – HCDAL of the Regional University of Cariri - URCA, Crato/CE, under number 14.389.

Essential Oil Extraction And Obtaining The Eugenol

The extraction of the essential oil of *Ocimum gratissimum* L. was carried out by the hydrodistillation method, using the Clevenger-type apparatus. The leaves were crushed, placed in a 5.0 L glass flask and placed in contact with 2.5 L of distilled water for subsequent boiling for 2 hours. The oil was separated by adding sodium sulfate (Na_2SO_4) anhydrous and preserved in refrigeration (-4°C) until its use. Eugenol (Sigma-Aldrich) was kindly provided by Dr. Francisco Assis Bezerra da Cunha from the Semi-Arid Bioprospecting and Alternative Methods Laboratory - LABSEMA, Regional University of Cariri (URCA), Crato/CE.

Chemical analysis of the essential oil of *Ocimum gratissimum* L.

Analyzes in CG/EM were performed on the chromatograph (GCMS-QP2010 SE, AOC-5000 auto injector from SHIMADZU), following the following analysis conditions: Injector 250°C ; initial oven temperature of 50°C , with a first heating ramp of $5^\circ\text{C}/\text{min}$ to 180°C , remaining for 4 minutes; a second ramp of $10^\circ\text{C}/\text{min}$ to 260°C , remaining for 10 minutes; Split ratio of 1:100; interface temperature: 250°C and 250°C source; mass range from 50 to 400 Daltons; ionization by electron impacts, 70 eV. For component chromatography, a DB5-MS (Agilent) column, $30\text{ m} \times 0.25\text{ mm}$ with an inner film thickness of $0.25\text{ }\mu\text{m}$ and a stationary phase of diphenyl dimethylpolysiloxane and helium as carrier gas was used. The identification was carried out by comparing the mass spectra with those of the Nist08 libraries and comparison with the base records of the literature.

Preparation of the essential oil of *Ocimum gratissimum* L. and Eugenol

In a test tube 10 mg of essential oil and 500 μL of DMSO were added. This solution was transferred to another tube and diluted in 9265 mL of sterile distilled water, resulting in a solution with a final concentration of 1,024 $\mu\text{g}/\text{mL}$ that was used in all tests. The same process was repeated for Eugenol.

Determination of the minimum inhibitory concentration (CIM)

The bacterial strains *Escherichia coli* ATCC 25922, *Escherichia coli* 06, *Klebsiella pneumoniae* ATCC 4352, *Pseudomonas aeruginosa* ATCC 9027, *Pseudomonas aeruginosa* 24, *Staphylococcus aureus* ATCC 25923, *Staphylococcus aureus* 10 and *Streptococcus mutans* ATCC 0446 were seeded in Petri dishes containing HIA culture medium and incubated at 37 °C for 24 h.

Then, a sample of each culture was dragged and diluted in test tubes containing sterile saline, in triplicate. After this procedure, the turbidity was adjusted according to the McFarland 0.5 scale. A 100 µL aliquot of each bacterial inoculum (referring to 10% of the total solution) was transferred to a tube containing 900 µL of a 10% Brain and Heart Infusion Broth (BHI) solution.

Each well of a 96-well microdilution plate was filled with 100 µL of the solution formed, and then the essential oil was microdiluted in a 1:1 ratio, the concentrations ranged from 512 µg/mL to 8 µg/mL. A well with no essential oil added was used as a positive control of bacterial growth. The same process performed for the essential oil was repeated with Eugenol. The reading of the Minimum Inhibitory Concentration (MIC) was performed by colorimetric method after 24 hours of incubation at 37 °C in a bacteriological oven. Bacterial growth was analyzed by adding 20 µL of resazurin (0.4 mg / ml) to each well. After the addition of resazurin, the plates were incubated for 1 h at room temperature and after this period the colorimetric variation was observed, since there was no bacterial growth in the wells that remained blue, and there was bacterial growth in the wells that changed from blue to pink coloring (GALUCCI *et al.*, 2009.). The tests were performed in triplicate.

Evaluation of modulating antibiotic activity

To evaluate the activity associated with antibiotics, the method described by Coutinho and collaborators *et al.* (2010). The substances (Chlorpromazine, Eugenol and essential oil) were tested in their sub-inhibitory concentrations (MIC/8). Each substance was diluted in tubes containing 900 µL of BHI 10%, where 100 µL of the bacterial inoculum of the strains *Escherichia coli* 06, *Pseudomonas aeruginosa* 24 and *Staphylococcus aureus* 10 were added separately. 100 µL of the obtained concentration were placed in each well of the microdilution plate. Microdilutions were performed with

antibiotics Amikacin, Ampicillin, Norfloxacin and Penicillin and the enzymatic inhibitor Sulbactam (associated with Ampicillin), all of them with an initial concentration of 1,024 $\mu\text{L/mL}$. The last well of each plate was used as a growth control. The plates were incubated in an oven at 37 °C for 24 hours and the Minimum Inhibitory Concentrations of each tested substance were determined by colorimetric method, through the addition of resazurin (GALUCCI *et al.*, 2009). The tests were performed in triplicate.

Toxicity assay

Breeding and stocking of *Drosophila melanogaster*

Drosophila melanogaster (Harwich strain) was obtained from the National Species Stock Center, Bowling Green, OH. The flies were grown according to methodology described by Cunha *et al.* (2015) in 340 mL glass containers and grown in medium containing: 83% corn mass, 4% sugar, 4% lyophilized milk, 4% soybean, 4% wheat or oat bran and 1% salt). When cooking the mixture, 1 g of Nipagin (Methylparaben) was added. After the mixture cooled in the growth flasks, 1 mL of a solution containing *Saccharomyces cerevisiae* was added.

The flies were grown in BOD type incubator at a temperature of 25 °C $\pm$ 1 °C, with light-dark cycles 12:12 h and a relative humidity of 60%.

Mortality assay

Mortality tests were performed in accordance with the methodology of Cunha *et al.* (2015). Adult flies, male and female, were placed in 130 mL containers (6 cm high and 6.5 cm in diameter). The pots were divided into control and test groups. One milliliter (1mL) of 20% sucrose solution in distilled water was placed at the bottom of all control pots. The test groups consisted of pots that contained eugenol or essential oil of *Ocimum gratissimum* L. The fumigation methodology was adopted, where the compounds are added on a filter paper in the tube cap and thus are volatilized and absorbed. The concentrations of 1, 5 and 10 $\mu\text{L} / \text{mL}$ of Eugenol and essential oil of *O. gratissimum* were used, with a counting time of 1, 2, 3, 6, 9, 12, 24 and 48h. The temperature of 25 °C $\pm$ 1 °C was maintained throughout the test, as well as the relative humidity of 60% in a BOD type incubator with a light-dark cycles 12: 12h. The tests were performed in sextuplicate, with each pot containing a total of 20 flies.

Negative geotaxis assay

Damage to the locomotor system was determined by the negative geotaxis test, as described by *Cunha et al.* (2015). Live flies, after exposure to Eugenol or essential oil of *O. gratissimum* at predetermined times, were taken to the bottom of the pots. After one minute, the flies that reached 4 cm in height in the containers were counted and this procedure was repeated after the 1-minute interval.

Statistical analysis

The antibacterial assays were performed in triplicates and the results were expressed as the geometric mean. Statistical hypothesis analysis was performed for the antibacterial assays using a Two-Way ANOVA followed by Bonferroni's post hoc test, using the GraphPad Prism 7.0 software. To analyze the toxicity data, a two-way ANOVA followed by Tukey's multiple comparisons test was performed. No statistical differences using the same concentration as a function of time were observed.

Results

The yield of the essential oil of *Ocimum gratisimum* (EOOg) was 0.39%. Phytochemical characterization, performed by mass chromatography, identified the presence of 10 compounds, as can be seen in Figure 1.

When analyzing the constituents individually, it is observed that Eugenol (63.25%), Cineole (10.83%), Beta-silinene (8.43%) and Trans-caryophyllene (6.85%) are the major components (Table 1).

Nº	RT	Similarity (%)	Constituent	Kovats Index	Molecular Formula	Absolute Area	Relative Area
1	9.525	95	1,8-cineole-eucalytol	991	C ₁₀ H ₁₈ O	648767	10,83%
2	9.574	93	Cis-ocimene	1050	C ₁₀ H ₁₆	112333	1,88%

3	14.428	93	l-alpha-terpineol	1189	C ₁₀ H ₁₈ O	50805	0,85%
4	18.955	97	Eugenol	1356	C ₁₀ H ₁₂ O ₂	3787946	63,25%
5	20.077	91	Beta-elemene	1375	C ₁₅ H ₂₄	50833	0,85%
6	20.959	96	Trans-caryophyllene	1419	C ₁₅ H ₂₄	410342	6,85%
7	21.921	94	Alpha-humulene	1438	C ₁₅ H ₂₄	60036	1,00%
8	22.585	96	Germacrene-D	1480	C ₁₅ H ₂₄	182146	3,04%
9	22.810	95	Beta-selinene	1485	C ₁₅ H ₂₄	504974	8,43%
10	22.985	94	Alpha-selinene	1494	C ₁₅ H ₂₄	180493	3,01%

Table 1: Relative composition of *Ocimum gratissimum* L. essential oil.

Source: Elaborated by the author (2021).

The analysis of the Minimum Inhibitory Concentration (MIC) was performed using the microdilution method. The values obtained for the MICs of each substance were considered clinically significant when less than 1,000 µg/mL (HOUGHTON et al., 2007). The Minimum Inhibitory Concentration (MIC) obtained in the tests with Eugenol (EUG) demonstrated its action against the strains: *Klebsiella pneumoniae* ATCC 4352, *Pseudomonas aeruginosa* ATCC 9027, *Pseudomonas aeruginosa* 24, *Staphylococcus aureus* ATCC 25923 and *Streptococcus mutans* ATCC 0446 (Table 2a).

Following the analysis of antibacterial activity, the EOOg showed results similar to those obtained by the EUG, with clinically significant responses evidenced against the same strains tested (Table 2b).

Table 2: Minimum inhibitory concentration (MIC) of Eugenol and Essential Oil of *Ocimum gratissimum* L. against bacterial strains.

	STRAINS	MIC
(a) EUGENOL	<i>Escherichia coli</i> ATCC 25922	1,024.00 µg/mL
	<i>Escherichia coli</i> 06	1,024.00 µg/mL
	<i>Klebsiella pneumoniae</i> ATCC 4352	512.00 µg/mL
	<i>Pseudomonas aeruginosa</i> ATCC 9027	406.37 µg/mL
	<i>Pseudomonas aeruginosa</i> 24	322.54 µg/mL
	<i>Staphylococcus aureus</i> ATCC 25923	203.19 µg/mL
	<i>Staphylococcus aureus</i> 10	1,024.00 µg/mL
	<i>Streptococcus mutans</i> ATCC 0446	322.54 µg/mL
(b) ESSENTIAL OIL	<i>Escherichia coli</i> ATCC 25922	1,024.00 µg/mL
	<i>Escherichia coli</i> 06	1,024.00 µg/mL
	<i>Klebsiella pneumoniae</i> ATCC 4352	645.08 µg/mL
	<i>Pseudomonas aeruginosa</i> ATCC 9027	812.75 µg/mL
	<i>Pseudomonas aeruginosa</i> 24	512.00 µg/mL
	<i>Staphylococcus aureus</i> ATCC 25923	80.63 µg/mL
	<i>Staphylococcus aureus</i> 10	1,024.00 µg/mL
	<i>Streptococcus mutans</i> ATCC 0446	812.75 µg/mL

Source: Elaborated by the author (2021).

An evaluation of the associated activity of EUG and EOOg with antibiotics was also carried out. In addition, the effect of the combination of chlorpromazine (CPMZ), associated with the antibiotics of interest, and Sulbactam (SULB) in combination with Ampicillin was also tested. The results obtained in this analysis were distributed in sections, organized according to pairs of antibiotics, which act on the external and internal portion of the bacterial cell.

Figures 2a and 2b represent the results of the combination of the compounds mentioned above, against the *Escherichia coli* 06 strain. A possible synergistic effect was evidenced in the association between CPMZ + Norfloxacin, and EUG + Norfloxacin. The same could be seen with Amikacin, when associated with EUG and EOOg. On the other hand, CPMZ when associated with Amikacin, caused an increase in MIC, resulting in a possible antagonistic phenomenon (Figure 2a).

It was observed that the combination of Sulbactam and Ampicillin caused a reduction in MIC, from 1,024 µg/mL to 128 µg/mL, possibly potentiating the activity of ampicillin against the strain tested (Figure 2b).

Figures 2c and 2d show a combined activity of the antibiotics Norfloxacin, Amikacin, Penicillin and Ampicillin, for the bacteria *Pseudomonas aeruginosa* 24. Chlorpromazine continued to intensify the action of Norfloxacin, as well as with *Escherichia coli* 06. This possible synergistic effect was also evidenced in the association CPMZ + Amikacin and EUG + Amikacin. However, the EUG + Norfloxacin combination was possibly antagonistic, with a corresponding increase in the inhibitory concentration of the antibiotic against the strain tested. This information can be seen in Figure 2c.

Sulbactam continued to intensify the action of Ampicillin, by reducing the MIC from 512 $\mu\text{g/mL}$ to 4 $\mu\text{g/mL}$, on the other hand, there was a possible antagonistic process in the association of this antibiotic with the EUG, given the increase in MIC from 512 $\mu\text{g/mL}$ to 812.7 $\mu\text{g/mL}$ (Figure 2d).

Figure 2e shows a possible antagonism in the combination of Norfloxacin + CPMZ and Norfloxacin + EUG against *Staphylococcus aureus* 10. This antagonism was repeated for Amikacin + CPMZ and Amikacin + EUG. However, there was an intensification of Amikacin activity, when combined with essential oil, similarly to what happened in the *Escherichia coli* 06 strain.

Interestingly, EUG and EEOg caused a possible synergistic effect (reduction of MIC from 1,024 $\mu\text{g/mL}$ to 161.3 $\mu\text{g/mL}$ and 128 $\mu\text{g/mL}$, respectively) when combined with Penicillin against *Staphylococcus aureus* 10 (Figure 2f) which it did not happen with the strains *Escherichia coli* 06 and *Pseudomonas aeruginosa* 24. In view of the Gram-positivity of *Staphylococcus aureus*, it is assumed that this effect for Penicillin is not prominent in Gram-negatives. The combination of Sulbactam + Ampicillin and EEOg + Ampicillin also potentiated the control effect, the latter contrary to what was seen in the other strains.

The *Drosophila melanogaster* model was chosen to assess the toxicity of EUG and EEOg. For both, concentrations of 1, 5 and 10 $\mu\text{L/mL}$ were used. EEOg showed a EC_{50} equal to 2,677 $\mu\text{L/mL}$ after 6h of exposure. The concentration of 1 $\mu\text{L/mL}$ did not differ significantly when compared to the control. The concentration of 5 $\mu\text{L/mL}$ showed greater toxicity after 48 hours of contact. After 6 hours of exposure to a concentration of 10 $\mu\text{L/mL}$ of EEOg, it started to increase significantly, demonstrating the toxicity at this concentration. (Figure 3a).

There was variation in the mortality pattern according to the EUG concentration used, which presented EC_{50} equivalent to 2,681 $\mu\text{L/mL}$, after 6 h of exposure. Concentrations of 1 and 5 $\mu\text{L/mL}$ showed a significant degree of mortality after 48 and

24 hours of exposure, respectively. On the other hand, the concentration of 10 μL differed from the control after 6 hours, being more toxic in relation to the others (Figure 3b). Thus, EOOg was less offensive when compared to EUG.

The concentration of 1 $\mu\text{L/mL}$ of EOOg was not significant in the impairment of *D. melanogaster* geotaxy. Fact that differs from that visualized for the concentration of 5 $\mu\text{L/mL}$, which caused an interference in the flight ability from 12 h of the beginning of the tests. The concentration of 10 $\mu\text{L/mL}$ of EOOg caused the negative geotaxis of the fruit flies to be compromised right after the first 3 hours of testing, with a substantial degree of impairment (Figure 4a).

The Eugenol also affected the negative geotaxis of *D. melanogaster*. Figure 4b shows how much each concentration varied compared to the control. In this case, it is verified that the exposure to 1 $\mu\text{L/mL}$ of the compound causes impairment of the mobility of the flies after 48 hours of exposure, while the concentration of 5 $\mu\text{L/mL}$ causes the same effect after 12h of contact. The previous exposure to 10 $\mu\text{L/mL}$ of EUG was even more toxic, in view of the negative geotaxis impairment even in the first counting time.

Discussion

Pessoa *et al.* (2015) chemically characterized the essential oil of *Ocimum gratissimum* L., distinguishing the following compounds: Eugenol (67.30 to 74.99%), β -selinene (8.18 to 8.27%), β - ilangene (4.97 to 5.09%), Viridiflorene (3.38 -3.47%), 1,8-cineol (3.07 to 3.11%) and γ -himachalene (2.00 - 2.62 %). The relative composition of the EOOg presented in this study was similar to that presented by Pessoa *et al.* (2015), in view of the one presented for Eugenol (63.25%) and β -selinene (8.43%).

Catherine, Deepika and Negi (2012) mentioned the effectiveness of Eugenol against the bacteria *Escherichia coli* and *Staphylococcus aureus*. The performance of Eugenol in the microtiter methodology was expressive, represented in the very low values of MIC for bacteria: *Bacillus cereus* (0,125 $\mu\text{g/mL}$), *Escherichia coli* (0,125 $\mu\text{g/mL}$), *Helicobacter pylori* (0,0312 $\mu\text{g/mL}$), *Staphylococcus aureus* (0,25 $\mu\text{g/mL}$) e *Streptococcus pyogenes* (0,5 $\mu\text{g/mL}$). A slightly higher MIC was obtained for *Pseudomonas aeruginosa* (8 $\mu\text{g} / \text{mL}$), although this is as significant as those of the strains mentioned (JEYAKUMAR; LAWRENCE, 2020).

Aguiar *et al.* (2015) examined the antibacterial action of EOOg against bacteria (standard and resistant) *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. In this analysis, only the standard *Staphylococcus aureus* MIC was significant.

These data corroborate with this study, and it is useful to reinforce that EOOg showed equal effectiveness in relation to the standard strains of *Klebsiella pneumoniae*, standard and resistant strains of *Pseudomonas aeruginosa*, standard strains of *Staphylococcus aureus* and *Streptococcus mutans*. In turn, Joshi (2013), concluded that the essential oil of *Ocimum gratissimum* L. also has considerable activity against *Escherichia coli* and *Klebsiella pneumoniae*, by dilution in tubes.

Jeyacumer and Lawrence (2020) suggest that the EUG causes a disturbance in the lipid fraction of the bacterial membrane, causing a change in permeability that facilitates the interaction of the compound with intracellular portions, thus justifying its effect. The mechanism of action of EOOg is related to its majority composition, with prevalence of Eugenol, as described by Joshi (2013).

The evaluation of the combined activity was performed for the antibiotics Amikacin, Ampicillin, Norfloxacin and Penicillin, aiming to evaluate the possibility of EUG and EOOg to intensify or not the action of these drugs. EUG is the major compound present in a chemotype of *Ocimum basilicum* L. Studies carried out with the latter have shown that the association of its essential oil with Neomycin and also with Amikacin generates a possible synergistic effect, evidenced by the reduction of MIC of these antibiotics, against *Staphylococcus aureus* (NUNES *et al.*, 2014). This information contrasts with what was verified in this study for EOOg and even for the EUG.

Aguiar *et al.* (2015) studied the direct modulating effect of the essential oil of *Ocimum gratissimum* L. in association with the aminoglycosides Amikacin and Gentamicin. The significant results obtained in tests involving the bacteria *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were possibly antagonistic. In this study, we present the possibility of synergism for Amikacin (against *Escherichia coli* and *Staphylococcus aureus*), Norfloxacin (against *Pseudomonas aeruginosa*), Ampicillin and Penicillin (against *Staphylococcus aureus*).

The sequence inhibition of a common biochemical pathway, as well as enzymes that protect microorganisms, the combination of active agents in the cell wall or their association with components that act in other pathways, facilitating their entry into the cell, characterize the main reasons that trigger processes of potentiation of the action of drugs (SANTIESTEBAN-LOPEZ; PALOU; LÓPEZ-MALO, 2007).

The factors that cause antagonism in the drug combination are still poorly understood. However, it is known that the association of bacteriostatic and bactericidal agents, the combined use of compounds that compete for the same target in a

microorganism and the very molecular interactions between different active components, are the main reasons behind the development of antagonistic processes (GÖNI *et al.*, 2009).

The effect of Eugenol, when combined with antibiotics, can be understood by what was explained by Debaio *et al.* (2019). This phyto-compound acts by creating deformations in the bacterial membrane, such as that seen against *Escherichia coli*, damaging its integrity and morphology, thus causing an overflow of intracellular content and inactivation of the microorganism. Furthermore, there is also a predisposition of the EUG to bacterial genomic DNA, responsible for the aggregation of DNA molecules. Due to the lipophilic character of monoterpenes, such as EUG, the interaction with polysaccharides, fatty acids and lipids of the bacterial cell membrane is facilitated, thus causing a disturbance in the permeable barrier (LIMAVERDE *et al.*, 2017).

An analogous mechanism can be perceived in the performance of essential oils, capable of compromising the integrity of the bacterial membrane and the conformation of its cell wall, facilitating the influx of drugs (MATIAS *et al.*, 2011). The hydrophobic compounds of these oils, such as Eugenol, are able to adhere to both the plasma membrane and phospholipids and membrane proteins, thus promoting the assimilation of antibiotics (LUZ *et al.*, 2014;).

The conformation of the cellular envelope of microorganisms, induce variations in the effectiveness of chemical components against bacteria. The gram-positive peptideoglycan thick layers guarantee greater sensitivity to bioactive components, since they can interact with the bacterial envelope in different ways and degrees (REQUENA; VARGAS; CHIRALT, 2019). This phenomenon justifies the performance of EEOg by potentiating the action of Amikacin and Penicillin against *Staphylococcus aureus* 10 and EUG by intensifying the action of Penicillin against the same strain in this study.

Chlorpromazine, in non-antibacterial concentrations, has been used as an agent capable of inhibiting efflux pumps, Coutinho *et al.* (2010) discussed the effect of reducing the MIC of Chlorpromazine, concluding its activity for Amikacin, Kanamycin and Tobramycin, in addition to describing an antagonistic effect for Gentamicin and Neomycin, with data referring to tests with *Escherichia coli*.

Sulbactam is a semi-synthetic compound capable of inhibiting the beta-lactamase enzyme (KU; YU, 2021). The combination of SULB together with Ampicillin considerably expands the spectrum of action of the latter, against *Klebsiella pneumoniae* and *Staphylococcus aureus*, for example. The possible synergistic interactions, presented

in this study, involving CPMZ and SULB, may be suggestive of efflux pump and beta-lactamase enzyme inhibition processes, respectively. However, specific studies must be carried out to prove these mechanisms of action.

According to Zhang *et al.* (2016), oxygenated monoterpenes are agents of considerable toxicity against *Drosophila melanogaster*, which can be represented by the variation of LC₅₀ of 0.015 and 0.02 µL/L. This data is consistent with that presented to the EUG in this study, taking into account the EC₅₀ value equivalent to 2,681 µL/mL. The main factor behind this is associated with the specific characteristics of the EUG, such as: the presence of the hydroxyl group and its position, as well as the characteristic positioning of its double bonds, which can vary in a different way in the degree of mortality of fruit flies, as seen for α-pinene and β-pinene (XIE *et al.*, 2014, ZHANG *et al.*, 2016).

The toxicological response of essential oils is related to the additive and synergistic effects of different compounds (ESSOUNG *et al.*, 2020). Tests carried out with *Ocimum tenuiflorum* showed their fumigant capacity against *S. oryzae*, by inhibiting the enzyme acetylcholinesterase (AChE). This action is represented by the LC₅₀ value of 479 µL/L (BHAVYA; CHANDU; DEVI, 2018).

A similar effect was evidenced for EEOg in this study, whose mortality was similar to that of the EUG, which is justified, since the literature describes the EUG as one of the main compounds of *Ocimum gratissimum* L. In particular, the compounds present in oils essential elements confer volatile characteristics, which facilitate their entry into the intracellular environment, the influx of terpenes, for example, can cause the formation of free radicals, leading to death (HUA *et al.* 2018).

The toxicological effect on the negative geotaxis of *Drosophila melanogaster* was prominent for EUG and EEOg. As a survival strategy in adverse situations, fruit flies increase the activity levels of the enzyme acetylcholinesterase (AChE), thus hydrolyzing acetylcholine and preventing it from accumulating in unfavorable environmental conditions or stress (HU *et al.*, 2019). Thus, it is suggested that the EUG and EEOg acted by inhibiting the action of AChE, allowing the stress of the flies with impairment of their locomotor system.

Conclusion

Eugenol is the most expressive component of the essential oil of *Ocimum gratissimum* L. The results obtained in the tests of antibacterial activity and combined with antibiotics were promising, demonstrating that both EUG and EOOg can be viable alternatives for reversing bacterial resistance. Even so, further studies should be carried out to better evaluate the performance of the tested substances, as well as to delimit the toxicological effect in relation to other concentrations and also by using other model organisms.

Authorship contribution declaration

Júlio César Silva: Methodology and validation. **Raimundo Luiz Silva Pereira:** Methodology. **Thiago Sampaio de Freitas:** Software. **Janaína Esmeraldo Rocha:** Validation. **Nair Silva Macedo:** Methodology. **Carla de Fatima Alves Nonato:** Methodology. **Marina Leite Linhares:** Formal analysis. **Daniely Sampaio Arruda Tavares:** Formal analysis. **Francisco Assis Bezerra da Cunha:** Resources, Supervision. **Henrique Douglas Melo Coutinho:** Conceptualization, Supervision. **Sidney Gonçalo de Lima:** Resources, Supervision. **Francisco Nascimento Pereira Júnior:** Writing-review and editing. **Fabiola Fernandes Galvão Rodrigues:** Conceptualization, Supervision. **George Joaquim Garcia Santos:** Conceptualization, Supervision.

Declaration

Competing interest: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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FIGURES

Fig. 1 Chromatogram CG-MS of the essential oil of *Ocimum gratissimum* L. 1 = 1,8-cineole-eucalytol; 2 = Cis-ocimene; 3 = 1-alpha-terpineol; 4 = Eugenol; 5 = Beta-element; 6 = Trans-caryophyllene; 7 = Alpha-humulene; 8 = Germacrene-D; 9 = Beta-selinene; 10 = Alpha-selinene

Fig. 2 Evaluation of antibiotics modifying activity (Amikacin, Ampicillin, Norfloxacin and Penicillin) by EUG and EEOg against bacterial strains (*Escherichia coli* 06, *Pseudomonas aeruginosa* 24 and *Staphylococcus aureus* 10)

Fig. 3 Effect of Essential oil of *Ocimum gratissimum* L. (a) and Eugenol (b) on the mortality test of *Drosophila melanogaster* at different concentrations and time

Fig. 4 Effect of the Essential oil of *Ocimum gratissimum* L. (a) and Eugenol (b) on the negative geotaxis behavior of *Drosophila melanogaster* in different concentrations and time