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Toxicological assessment of citral and geraniol: Efflux pump inhibition in *Staphylococcus aureus* and invertebrate toxicity

Gildênia Alves de Araújo [a], Cícera Datiane de Morais Oliveira Tintino [a], Raimundo Luíz Silva Pereira [a], Isaac Moura Araújo [a], Cícera Laura Roque Paulo [a], João Arthur de Oliveira Borges [a], Ewerton Yago de Sousa Rodrigues [a], Ângella Eduarda da Silva [b], Francisco Assis Bezerra da Cunha [b], Zildene de Sousa Silveira [b], Nair Silva Macedo [b], Henrique Douglas Melo Coutinho [a,*], José Maria Barbosa Filho [c], Daniela Maria do Amaral Ferraz Navarro [e], Francisco Roberto de Azevedo [d], Saulo Relison Tintino [b]

[a] *Laboratory of Microbiology and Molecular Biology, Department of Chemical Biology, Regional University of Cariri, Crato, Brazil*
[b] *Laboratory of Semi-Arid Bioprospecting (LABSEMA), Department of Biological Chemistry - URCA, Crato, CE, Brazil*
[c] *Laboratory of Pharmaceutical Technology, Federal University of Paraíba — UFPB, João Pessoa 58033-455, Brazil*
[d] *Laboratory of Entomology, Federal University of Cariri - UFCA, Crato, CE CEP: 63048-080, Brazil*
[e] *Departamento de Química Fundamental, Universidade Federal de Pernambuco, Recife, PE 50740-560, Brazil*

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ABSTRACT

This study aimed to evaluate the antibacterial activity against multi-drug-resistant strains carrying efflux pumps and assess their toxicity on *Drosophila melanogaster* and *Aedes aegypti* models. Microdilution tests in broth were performed to determine the Minimum Inhibitory Concentration (MIC). The efflux pump inhibition was evaluated by analyzing the reduction in antibiotic MIC and Ethidium Bromide (EtBr) MIC when combined with the products. Mortality assay and negative geotaxis were conducted on *D. melanogaster* specimens, and insecticidal activity assays were performed on *A. aegypti* larvae. Only geraniol reduced the antibiotic MIC when combined, reducing from 64 μg/mL to 16 μg/mL in the 1199B strain of *S. aureus*. When combined with EtBr, both geraniol and citral reduced EtBr MIC, with geraniol decreasing from 64 μg/mL to 16 μg/mL and citral decreasing from 64 μg/mL to 32 μg/mL. Regarding the *S. aureus* K2068 strain, geraniol reduced the antibiotic MIC from 16 μg/mL to 8 μg/mL, and citral reduced it from 16 μg/mL to 4 μg/mL. In combination with EtBr, all monoterpenes reduced MIC from 64 μg/mL to 32 μg/mL. Both products exhibited toxicity in *D. melanogaster*; however, citral showed higher toxicity with a precisely determined LC50 of 2.478 μL. As for the insecticidal action on *A. aegypti*, both products demonstrated toxicity with cumulative effects and dose-dependent mortality.

1. Introduction

In October 2017, the World Health Organization [WHO] stated that bacterial resistance to antibiotics is one of the major global health issues as it prolongs hospitalization, increases treatment costs, and, even more critically, significantly raises mortality related to infectious diseases [18]. Among the classes of bacteria showing a resistance profile is the strain of *Staphylococcus aureus*. This bacterium is found in the normal skin microbiota of animals and humans, with a carriage rate of 20 30 % in the healthy human population [17–58]. Abscesses, lung infections, bacteremia, endocarditis, and osteomyelitis are all caused by *S. aureus* infections in humans [56]. One of the resistance mechanisms of *S. aureus* is efflux pumps.

Multidrug efflux pumps operate at the frontline to protect bacteria against antimicrobials by reducing the intracellular concentration of drugs. This protective barrier consists of a series of transporter proteins located in the bacterial cell membrane and periplasm, which remove various foreign substrates, including antimicrobials, organic solvents, toxic heavy metals, and other compounds, from bacterial cells [1]

Thus, the use of natural products has proven to be a promising

* Corresponding author.
E-mail address: hdmcoutinho@urca.br (H.D.M. Coutinho).

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alternative in antibacterial activity and the reversal of bacterial resistance. In this regard, Brazil, due to its vast biodiversity, holds high potential for research in this area [2–15].

Terpenic natural products are responsible for over 30,000 different secondary metabolite compounds that play distinct roles defense compounds in plants against viral, bacterial, and parasitic infections in various organisms where they are found [49]. They are also abundant in fruits, vegetables, and flowers [9]. On the other hand, high concentrations of terpenes can be toxic, making them an effective defense mechanism against herbivores and pathogens.

Monoterpenes belong to a large and diverse group of naturally occurring compounds. The basic structure of monoterpenes, or monoterpenoids, consists of two linked isoprene units [62]. Terpenes have exhibited various biological activities, including antimicrobial actions [30]. Among the classes of monoterpenes, geraniol and citral stand out. Geraniol is a well-known terpenoid compound and is the primary component of many essential oils [59]. Geraniol is mainly found in aromatic herbaceous plants and is economical and easy to produce [40–59]. In recent years, it has been demonstrated that geraniol has significant antibacterial activity against gram-negative bacteria such as *Escherichia coli* and Salmonella Typhimurium [16] and *Streptococcus pneumoniae* and *S. aureus* [22].

Citral [3,7-dimethyl-2,6-octadienal] is a monoterpene aldehyde formed by a natural blend of geranial [trans-citral] and neral [cis-citral]. It is found in various plants such as myrtle, bergamot, lemon balm, lemongrass, and verbena [11–52]. Studies have shown that citral possesses various pharmacological activities, including anti-inflammatory, anticancer, analgesic, antispasmodic, antiparasitic, and immunomodulatory actions [4–33,35]. Furthermore, several studies have reported antimicrobial effects of citral against various pathogenic bacteria [37–44]. The use of plant secondary metabolites has been employed for control purposes. Various substances derived from the intermediate or final products of plant secondary metabolism, such as rotenoids, pyrethroids, alkaloids, and terpenoids, can significantly interfere with the metabolism of other organisms [27].

In the process of discovering new antibacterial agents, assessing the toxicity of natural products is a crucial step to determine the toxicological profile and safety in eukaryotic cells [29]. Therefore, *Drosophila melanogaster* is recommended as an alternative model by the European Centre for the Validation of Alternative Methods [ECVAM] [55]. Using this model to assess the selectivity and risk of toxicity to organisms [bioindicators] like *Aedes aegypti* affected by chemical agents is carried out through ecotoxicity [21].

In the context, the objective of this study is to evaluate the action of the monoterpenes citral and geraniol against bacterial strains carrying efflux pumps, as well as to assess their toxicity in *Drosophila melanogaster* and *Aedes aegypti* models.

2. Materials and methods

2.1. Substances

All substances, including citral and geraniol used in the tests, were acquired from Sigma Aldrich Brasil. The antibiotics Norfloxacin and Ciprofloxacin were diluted in dimethyl sulfoxide (DMSO) and sterile water. Chlorpromazine (CPZ) and Ethidium Bromide (EtBr) were dissolved in sterile distilled water, while 4-chlorophenyl-m-chlorocarbonyl cyanide hydrazone (CCCP) was dissolved in methanol/water (1:3, v/v). All substances were diluted to a standard concentration of 1024 μg/mL.

2.2. Microbiological assays

The strains of *S. aureus* 1199B, which express the NorA efflux pump protein and expel antibiotics and other drugs, such as DNA intercalating dyes; and the strain *S. aureus* K2068, carrying the MepA efflux pump, were used. These strains were provided by Prof. S. Gibbons (University of London). The strains were stored in Brain Heart Infusion (BHI) liquid culture medium with glycerol at −80°C. For the experiments, the strains were cultured for 24 hours at 37°C on solid Heart Infusion Agar (HIA) medium (Difco Laboratories Ltd., Brazil). The direct antibacterial activity was evaluated by determining the minimum inhibitory concentration (MIC) for the compounds citral and geraniol. The broth microdilution method proposed by CLSI (2015) [7] was used with adaptations. To verify whether citral and geraniol act as potential inhibitors of the NorA and MepA efflux pump, their ability to decrease the MIC of EtBr and the antibiotics Norfloxacin and Ciprofloxacin, when combined, was compared to the standard efflux pump inhibitors, CCCP and CPZ.

2.3. Assays in Drosophila melanogaster

D. melanogaster (Harwich strain) was obtained from the National Species Stock Center, Bowling Green, OH. The flies were cultivated in flasks 15 cm high and 6.5 cm in diameter, in a medium containing: 83 % corn mass, 4 % sugar, 4 % freeze-dried milk, 4 % soybean, 4 % soybean bran, wheat or oats and 1 % salt. When cooking the mixture, 1 g of Nipagin (methylparaben) was added. After cooling the mixture in the growth flasks, 1 mL of solution containing *Saccharomyces cerevisiae* was added. Flies were cultured in photoperiod BOD incubators at a temperature of 25 °C ± 1 °C and a relative humidity of 60 %. The fumigation bioassay methodology proposed by Cunha et al. [8] was used to evaluate the toxicity of citral and geraniol. Damage to the locomotor system was determined by the geotaxis test, as described by Bao et al. [4].

2.4. Insecticidal activity against Aedes aegypti larvae

Eggs donated from the Diptera farm of the Chemical Ecology Laboratory of the Department of Fundamental Chemistry of the Federal University of Pernambuco (UFPE) were used. Before the experiments, the eggs were placed to incubate with distilled water and fish food to obtain larvae in L3, in a B.O.D-type air-conditioned chamber for three days, with temperature (25 ± 2ºC) and relative humidity (70 ± 10 %). The insecticidal and the enhanced insecticidal activities were performed according to Chantraine et al. [6].

2.5. Statistical analysis

Antibacterial assays were performed in triplicate replications and the results were expressed as the geometric mean of the replications. Hypothesis testing was performed for the antibacterial assays using two-way ANOVA followed by the Bonferroni post hoc test, utilizing GraphPad Prism 7.0 software. For toxicity data analysis, a two-way ANOVA followed by the Tukey test was conducted.

3. Results and discussion

3.1. Antibacterial activity on efflux pump-carrying strains

Direct antibacterial activity of the monoterpene geraniol was observed with a Minimum Inhibitory Concentration (MIC) of 512 μg/mL against K2068. In contrast, no direct antibacterial activity was observed for citral, with an MIC ≥ 1024 μg/mL, a value considered clinically irrelevant, against the K2068 strain. Against 1199B, no antibacterial activity was observed. However, the absence of antibacterial activity or low activity observed in citral makes it advantageous to be used as an efflux pump inhibitor. This is because one of the ideal advantages of inhibitors is the absence of direct antibacterial activity.

Volatile compounds found in essential oils have demonstrated significant antibacterial potential. The activity of monoterpenes, in particular, stands out, as they can target various bacterial components [46]. Many monoterpenes can disrupt the lipid fraction of the bacterial

plasma membrane, leading to changes in membrane permeability and leakage of intracellular materials [57]. They can also cause morphological alterations, cytoplasmic changes, disruptions in cell division, and modifications in the cell wall [29].

The antibacterial activity of geraniol against *S. aureus* has been previously mentioned in the literature [31]. It has been shown that the MIC values of geraniol are < 600 μg/mL against *S. aureus*, *E. coli*, and other bacteria, indicating excellent antibacterial activity. This is consistent with the data obtained in the present study [31–50]. Therefore, the antibacterial activity of geraniol may be related to the mechanisms mentioned earlier.

3.2. Activity of reducing minimum inhibitory concentration of antibiotics and ethidium bromide

When associated with the antibiotic, among the analyzed monoterpenes, only geraniol was able to reduce the MIC of the antibiotic in the 1199B strain of *S. aureus*, decreasing it from 64 μg/mL to 16 μg/mL. However, when associated with ethidium bromide, both geraniol and citral were able to reduce the MIC of ethidium bromide, with geraniol decreasing it from 64 μg/mL to 16 μg/mL, and citral decreasing it from 64 μg/mL to 32 μg/mL (Fig. 1).

Terpenes are already known for their biological activities. Terpenes such as Nerolidol [63], estragole [64] and isoeugenol [65] have shown the ability to reduce the MIC of norfloxacin and EtBr as well as geraniol and citral in *S. aureus* 1199B strains.

Regarding the *S. aureus* K2068 strain, a reduction in the antibiotic MIC was observed in all associations with ciprofloxacin, decreasing from 16 μg/mL to 8 μg/mL. However, the best result was achieved with citral, reducing from 16 μg/mL to 4 μg/mL. In the association with ethidium bromide, a reduction in CIM also occurred, all monoterpenes reduced it from 64 μg/mL to 32 μg/mL (Fig. 2).

corroborating this study, terpenes such as abietic acid [66] and limonene [67] were also able to reduce the MIC value of Ciprofloxacin and EtBr in strains of *S. aureus* K2068, demonstrating the capacity that this class of substance can present in combating antibiotic resistance.

It is known that the number of antibiotic-resistant strains is increasing [32]. However, the development of new antibiotics is slow in response to this rapid demand. Therefore, potentiating or restoring the activity of existing antibiotics is currently the main strategy for controlling multidrug-resistant bacterial strains. Essential oils and their volatile constituents have shown increasing promise in restoring antibiotic activity.

3.3. Toxicity in Drosophila melanogaster

According to Fig. 3, the highest mortality was observed after

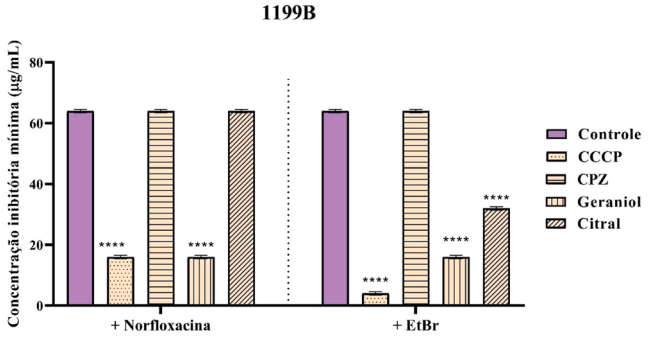

Fig. 1. Evaluation of the NorA efflux pump inhibitory activity by the sesquiterpenes geraniol and citral against the *S. aures* 1199B strain. Associated with norfloxacin and ethidium bromide. Two-way ANOVA followed by Bonferroni post hoc. CPZ = chlorpromazine; EtBr = ethidium bromide; **** = $p < 0.0001$ vs control.

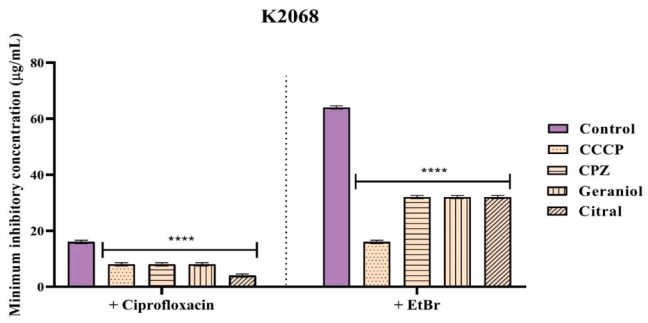

Fig. 2. Evaluation of the MepA efflux pump inhibitory activity by the sesquiterpenes geraniol and citral against the *S. aures* K2068 strain. Associated with norfloxacin and ethidium bromide. Two-way ANOVA followed by Bonferroni post hoc. CPZ = chlorpromazine; EtBr = ethidium bromide; **** = $p < 0.0001$ vs control.

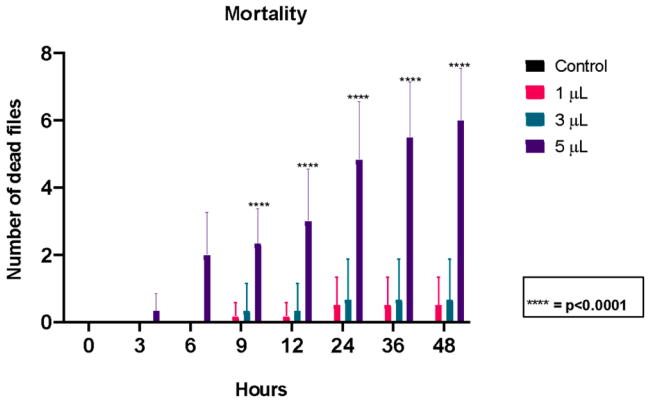

Fig. 3. Evaluation of toxicity through mortalityin exposure to geraniol against ***Drosophila melanogaster*** after 48 h of exposure, with an equivalence of 2 mg/mL = 2 μL, with $EC_{50} > 5$ μL.

48 hours of exposure at the highest concentration. At concentrations of 1 μL and 3 μL, there was no major difference in mortality during the 48 hours observed. According to Fig. 4, the most significant changes in fly motility were observed at concentrations of 5 μL, and after 12 hours the number of flies that managed to climb to the top fell by half, and greater action after 48 hours, with the reduction in motility above 50 %. According to Fig. 5, the highest mortality was observed at the highest concentration, showing a dose-dependent relationship. However, concerning the exposure time, little association with increased mortality was observed. Among the analyzed monoterpenes, citral showed the highest toxicity, with a precisely determined LC_{50} of 2.478 μL. As observed in Fig. 6, clear changes in geotaxis were observed depending

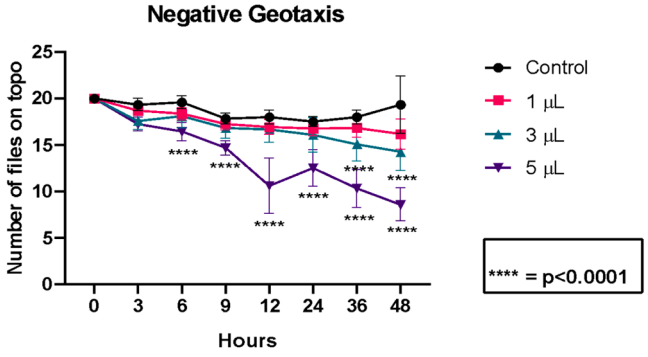

Fig. 4. Analysis of negative geotaxis in 48 h by checking the flies that manage to climb to the top, in different concentrations of exposure to geraniol.

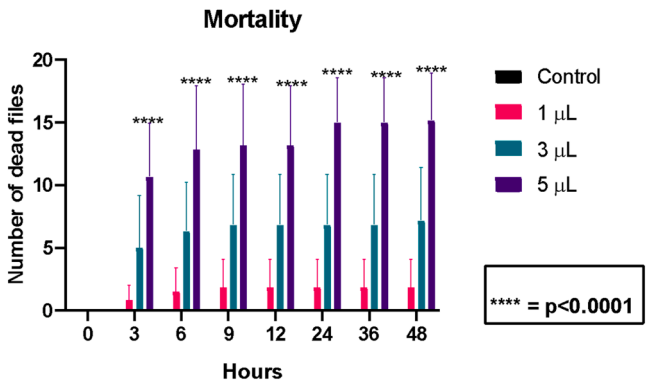

Fig. 5. Assessment of toxicity due to mortality in exposure to citral against ***Drosophila melanogaster*** after 48 hours of exposure, with an equivalence of 2.5 mg/mL = 2 µL, with EC_{50}: 2.478 µL in 3 hours of exposure to citral.

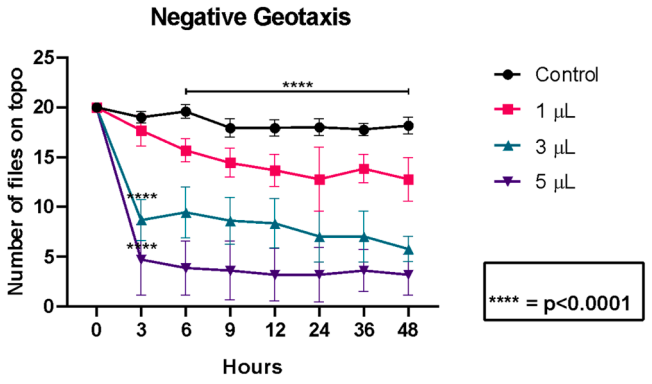

Fig. 6. Analysis of negative geotaxis in 48 hours by checking the flies that manage to climb to the top, in different concentrations of exposure to citral.

on the concentration, with the most intense changes occurring at a concentration of 5 µL. Regarding the exposure time, the changes were less pronounced, remaining almost constant over time with only slight variations.

Toxicity in *Drosophila melanogaster* is an important parameter for evaluating the toxicity of substances with pharmaceutical potential, especially essential oils and their aromatic derivatives. Many genes present in *D. melanogaster* are also found in humans, making it an important model organism for assessing biological activities [36]. Several studies indicate that essential oils and their aromatic derivatives can exhibit toxicity against *Drosophila melanogaster*, leading to mortality or alterations in the locomotor apparatus [8–61]. One advantage of using essential oils and their aromatic derivatives is that they can be evaluated through the fumigation method due to their volatile nature [8].

Studies indicate that citral exhibits high toxicity against *Drosophila melanogaster*, with an LC50 of 0.06 µL/L after 24 hours of exposure [61]. In the present study, the LC50 of 2.478 µL in a 130 mL container was evaluated after 3 days. The differences in the presented results are due to the specific methods employed, which are different. There is also a recent study indicating that citral negatively influences the development of *Drosophila melanogaster* larvae, causing alterations in larval development [51]. Regarding geraniol, a study presents the geraniol LC50 value as 10.42 µL/mL against *D. melanogaster* larvae after 24 hours of exposure, a result consistent with the obtained results here, showing an LC50 greater than 5 µL after 24 hours [20]. Geraniol has also demonstrated a neuroprotective effect in *Drosophila melanogaster*, indicating its low toxicity [43].

The toxicity of citral and geraniol may be associated with their action on the nervous system of *D. melanogaster*. Previous studies on the mechanism of action of some monoterpenes in insects indicate that they can target various proteins in the nervous system. These proteins include: A) octopamine receptors [13,14], B) acetylcholinesterase enzyme [33], C) nicotinic acetylcholine receptors [38,39], D) tyramine receptors [14–28], E) ionotropic γ-aminobutyric acid receptors [19–43].

3.4. Larvicidal activity against Aedes aegypti and enhancement of insecticide action

In the insecticidal activity of citral, dose-dependent mortality was observed with cumulative toxic effects over time, with the highest mortality occurring after 72 hours of exposure, with all larvae dead. In the association trial with pyriproxyfen, dose-dependent and cumulative toxicity effects were also observed. Even at a lower oil volume of 5 µL, insecticide enhancement was observed. The values were considered significant when compared with pyriproxyfen alone (Fig. 7).

In the insecticidal activity of geraniol, dose-dependent mortality and cumulative toxic effects over time were also observed. The only volume that did not change over time was the combination of pyriproxyfen with a volume of 6.2 µL of geraniol. It should be noted that potentiation of pyriproxyfen was also observed in this combination (Fig. 8).

It is known that essential oils have a remarkable insecticidal capacity, to the extent that it is believed that octopaminergic pathways are their main mode of action [13]. Regarding larvicidal action against *Aedes aegypti*, many studies have shown the potential of essential oils as possible bio-larvicides [6–12]. However, few studies demonstrate the direct action of isolated monoterpenes as presented in this study. There are also very few studies on the ability to enhance existing insecticides against *A. aegypti*. This latter activity has become increasingly relevant, considering studies indicating resistance of larvae to existing insecticides [3]. It is important to mention that pyriproxyfen acts on larval development, with a slower action, whereas essential oils and monoterpenes can act on the cholinergic system, GABA system, mitochondrial system, and other targets, with a faster action compared to pyriproxyfen [45–47]. This contributes to their potential use as combined agents with synergistic action on insects. The low mortality or absence of pyriproxyfen observed in the assays may be associated with both resistance to it and its slow action, the latter case not reaching the necessary time for mortality.

According to the results obtained, citral exhibited insecticidal action similar to that observed in *D. melanogaster*, confirming the larvicidal action observed in *A. aegypti*. Therefore, citral demonstrates strong toxic action against insects. Some studies have shown the toxic activity of citral against both adult and larval insects [40]. In the study by Lacher [27], citral exhibited an LC50 of 0.03 µl/cm2 against Housefly larvae (*Musca domestica* L.), a result consistent with the findings of this study, indicating its potent larvicidal potential.

Regarding geraniol, there are also reports in the literature of its insecticidal action. In the study by Kaur [24] the larvicidal effects of

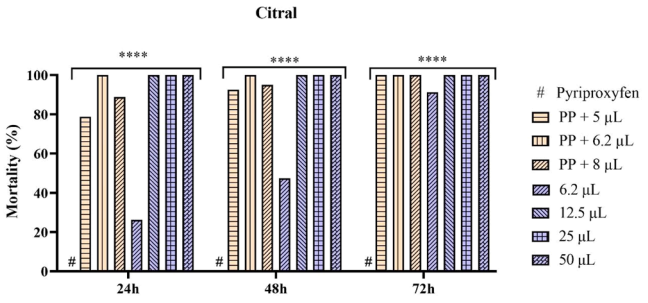

Fig. 7. Action of citral on the mortality of *Aedes aegypti* larvae in the time of 24, 48 and 72 hours. Results are expressed as percentage of mortality. Two-way ANOVA followed by Bonferroni post hoc. #: Pyriproxyfen; * ** * $p < 0.0001$ *vs* pyriproxyfen control; PP: Pyriproxyfen.

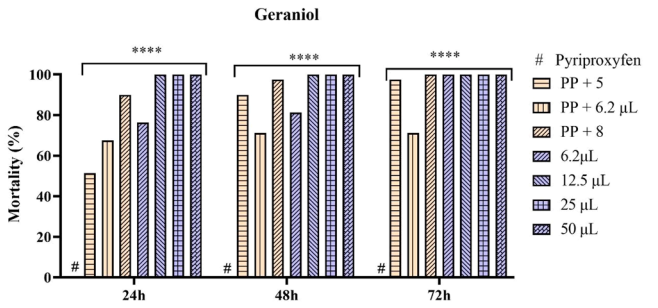

Fig. 8. Action of geraniol on the mortality of *Aedes aegypti* larvae in the time of 24, 48 and 72 hours. Results are expressed as percentage of mortality. Two-way ANOVA followed by Bonferroni post hoc. #: Pyriproxyfen; **** $p < 0.0001$ *vs* pyriproxyfen control; PP: Pyriproxyfen.

geraniol and citral nanoemulsions on *Spodoptera litura* and *Helicoverpa armigera* larvae were presented. There is a study indicating the repellent power of geraniol in nanoformulation against *A. aegypti*, reaffirming its toxicity against this invertebrate [10].

4. Conclusions

This study demonstrated that the monoterpenes geraniol and citral have promising properties in combating bacterial resistance and in controlling invertebrates. Both acted as inhibitors of efflux pumps (NorA and MepA), reducing the MIC of antibiotics and ethidium bromide in resistant strains of *Staphylococcus aureus*, with geraniol also showing direct antibacterial activity. In addition, citral and geraniol demonstrated significant toxicity in *Drosophila melanogaster*, with citral being more toxic (LC_{50} = 2.478 µL), and exhibited strong larvicidal action against *Aedes aegypti*, promoting dose-dependent mortality and a synergistic effect with pyriproxyfen. These results reinforce the potential of these natural compounds as effective tools in reversing antimicrobial resistance and in the management of disease vectors, highlighting the importance of exploring sustainable alternatives to global public health challenges.

Ethics approval and consent to participate

Not applicable.

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CRediT authorship contribution statement

Gildênia Alves de Araújo: Conceptualization. **Cícera Datiane de Morais Oliveira Tintino:** Investigation. **Raimundo Luíz Silva Pereira:** Investigation. **Isaac Moura Araújo:** Conceptualization. **Cícera Laura Roque Paulo:** Methodology. **João Arthur de Oliveira Borges:** Methodology. **Ewerton Yago de Sousa Rodrigues:** Methodology. **Ângella Eduarda da Silva:** Methodology. **Francisco Assis Bezerra da Cunha:** Writing – original draft. **Zildene de Sousa Silveira:** Software. **Nair Silva Macedo:** Methodology. **Henrique Douglas Melo Coutinho:** Supervision. **José Maria Barbosa Filho:** Supervision. **Daniela Maria do Amaral Ferraz Navarro:** Writing – original draft. **Francisco Roberto de Azevedo:** Resources. **Saulo Relison Tintino:** Writing – original draft.

Declaration of 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.

Data Availability

Data will be made available on request.

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