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Original Article

Properties of Compounds Citral and Geraniol on *Trichomonas gallinae*: Activity in Vitro and Cytotoxicity

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Received 21 Jul 2024
Accepted 18 Sep 2024

Keywords:
Trichomoniasis;
Citral;
Geraniol;
Monoterpene;
Cytotoxicity

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Abstract

Background: Avian trichomoniasis is an important disease that causes bird mortality, both wild and captive, around the world. This study evaluated the in vitro cytotoxicity, antioxidant, and antiparasitic activity of citral (3.7-Dimetil-2.6-octadienal) and geraniol (trans-3.7-Dimetil-2.6-octadien-1-ol) against *Trichomonas gallinae* trophozoites.

Methods: In vitro assays were conducted at the Laboratory of Protozoology and Entomology (LAPEN) at the Federal University of Pelotas (UFPel), Brazil in 2019 using tests with 10^6 parasites and citral and geraniol at concentrations ranging from 10 to 100 µM and four controls: NC (culture medium and trophozoites), MTZ (trophozoites plus 100 µM of metronidazole), and TW (trophozoites plus vehicles used for solubilizing derivatives (0.01% Tween).

Results: The citral (60 µM) and geraniol (50 µM) concentrations reduced the trophozoites's viability by 100%. The molecular docking experiment demonstrated that citral and geraniol might inhibit a hydrogen enzyme for *T. gallinae* survival.

Conclusion: The major compounds of lemongrass have potential antitrichomonal activity against *T. gallinae* in vitro.

Introduction

Avian trichomoniasis is an important disease-causing bird mortality, both wild and captive, around the world (1,2). Its transmission occurs directly during courtship, feeding of chicks, and predation by birds of prey (3), being transmitted mainly by pigeons (*Columba livia*) to other bird species (*Columbiformes, Falconiformes, Passeriformes*) (4). There are yellowish caseous lesions in the upper digestive tract, particularly in the mouth, esophagus, and harvest of infected birds, leading to anorexia, weight loss, and asphyxia. Birds are characterized mainly by caseous masses and ulceration in the oral cavity and other parts of the upper digestive tract, which may affect feeding, causing hunger or suffocation (2). It presents in an acute or chronic subclinical form, depending on the host species, the pathogenicity of the strain, and immunological status (5-7).

Lemongrass oil and citral have several biological activities, such as antineoplastic, antimicrobial, antifungal, antiprotozoal, anthelmintic, and anti-arthropod activities (8-11). Lemongrass oil contains mainly citral (12), a natural combination of two isomeric aldehydes, that is, the geraniol isomers (α-citral) and neral (β-citral) (13). Geraniol is an acyclic alcohol with a floral odor, which appears as a yellowish oily liquid (14). In addition to its use in fragrances, it has several biological activities, such as antiseptic, anthelmintic, antioxidant, anti-inflammatory, and antifungal (15-18). In this context, identifying new, safer, and more efficient therapeutic agents with antiparasitic action is desirable so that the considered plants are viable alternatives.

We aimed to evaluate the cytotoxicity and antiparasitic activity of in vitro citral (3.7-Dimethyl-2.6-octadienal) and geraniol (trans-3.7-Dimethyl-2.6-octadien-1-ol) and against trophozoites from *Trichomonas gallinae*.

Materials and Methods

Ethics approval

This research was approved by the SISBIO on 08/02/2018, under number 61235-1.

Reagents

Citral, geraniol, DMSO, MTZ, and TYM (Tripticase Yeast Extract Maltose) were commercially acquired from Sigma-Aldrich®.

Trichomonas gallinae

Samples of *T. gallinae* were recovered by the wet mount method of naturally infected pigeons. Twelve native pigeons (*C. livia*) (2 to 8 weeks old) were captured in urban areas using swabs; samples were taken from the oral cavity and from membranous lesions of the oropharyngeal region. The culture of the parasite was prepared by immersion of oral swabs in medium TYM supplemented with 10% adult serum, antibiotic meropenem), antifungal amphotericin B) (Sigma-Aldrich®) and incubated at 37 °C (19). The cultures were then examined under an optical microscope at (100x and 400x) for mobile trophozoites observation. Cultures were observed over seven consecutive days to verify the growth of trophozoites. Within 48 h, trophozoites that had more than 95% mobility and normal morphology were replicated (20).

In vitro assay

In vitro assays were conducted at the Laboratory of Protozoology and Entomology (LAPEN) at the Federal University of Pelotas (UFPel), Brazil in 2019. To examine the susceptibility of *T. gallinae* to citral and geraniol, sterile 96-well plates were used for incubation with different concentrations. The parasites were seeded at an initial density of 10^6 trophozoites/mL of medium TYM and incubated with citral and geraniol. Three controls were considered: A - trophozoites only, B - trophozo-

ites plus the vehicle used for solubilization of the derivatives (DMSO), C - trophozoites plus 100 µM metronidazole (MTZ) as positive control. Citral and geraniol were added to the wells to obtain final concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 µM, respectively. Subsequently, the microculture plates were incubated at 37°C with 5% CO2 for 24 h to generate anaerobic conditions. After this period, a preparation with trophozoites containing trypan blue 0.4% (1:1) was evaluated in the Neubauer chamber. Cultures with viability equal to or greater than 95% were used for assays, having considered motility, morphology, and exclusion by dye. Only the concentrations of citral and geraniol that showed a reduction in the viability of the trophozoites to 100% were used to determine the MIC (minimum inhibitory concentration). The trophozoites used to establish MIC and below and above concentrations, as well as inoculated controls in fresh TYM medium at 37°C, were counted in the Neubauer chamber with trypan blue every 24 hr for 96 hr to confirm MIC. The best concentration, presented after MIC citral analysis and geraniol, performed the kinetic growth curve at the following times: 1, 6, 12, 24, 48, 72, and 96 hr by the dye exclusion method. A death time curve was constructed to obtain an activity profile of the efficiency of the citral and geraniol against *T. gallinae* flagellate trophozoites. All assays were performed independently in triplicate (21).

Cytotoxicity

The cytotoxic effect of citral and geraniol was assessed on Vero cells (1.8×10^6 cells/plate) by MTT assay. Briefly, 100 µl of cells were seeded in 96-well polystyrene plates and 24h later were added of different citral and geraniol concentrations (100 µM, 80 µM, 60 µM, 40 µM, 20 µM, 10 µM). After 14 h of incubation, an MTT (Sigma-Aldrich, St. Louis, MO) solution (5mg/mL) was added in all wells and the plates were incubated for 4 h until violet color development. The supernatant was replaced by 200 µl of dimethyl sulfoxide (DMSO). The optical density was determined by spectrophotometry (550 nm), and the results were expressed in the percentage of viable cells relative to cell control, untreated.

Statistical analysis

Statistical analysis was carried out by one-way ANOVA of variance followed by the Newman-Keuls post hoc test. Descriptive statistics data were expressed as mean ± S.E.M. Probability values less than 0.05 ($P < 0.05$) were considered statically significant. The statistical analysis was accomplished using GraphPad Prism version 8.0 for Windows, Graph Pad Software (San Diego, CA, USA).

Results

In vitro assay

The results of the in vitro study on the inhibiting effect of citral and geraniol compounds in *T. gallinae* and the comparison of this activity with the drug metronidazole are showed in Fig. 1. The analysis of anti-*T. gallinae* from citral showed that at concentrations of 60, 70, 80, 90 and 100 µM, there was no statistically significant difference from the metronidazole. The compound was 100% effective after 12h in the kinetic growth curve of incubation at a concentration of 60 µM (MIC) (Fig. 1). Geraniol was effective from the concentration of 50, 60, 70, 80, 90, and 100 µM, showed no statistically significant difference from the metronidazole and the MIC was 50 µM (Fig. 1). The growth of trophozoites was completely inhibited by the compound within 24 hours of incubation in the kinetic growth curve at 50 µM (Fig. 2).

Cytotoxic assay

The cytotoxic assay of Geraniol, showed that it did not cause effects and damage to VERO cells in 24 hours of time-dependent dose treatment, compared to the control proliferation (100%). Although citral showed antiprotozoal activity, a decrease in VERO cell

proliferation was detected after 24 hours. As noted, the number of viable cells for concentrations was 10 to 100 µM, respectively.

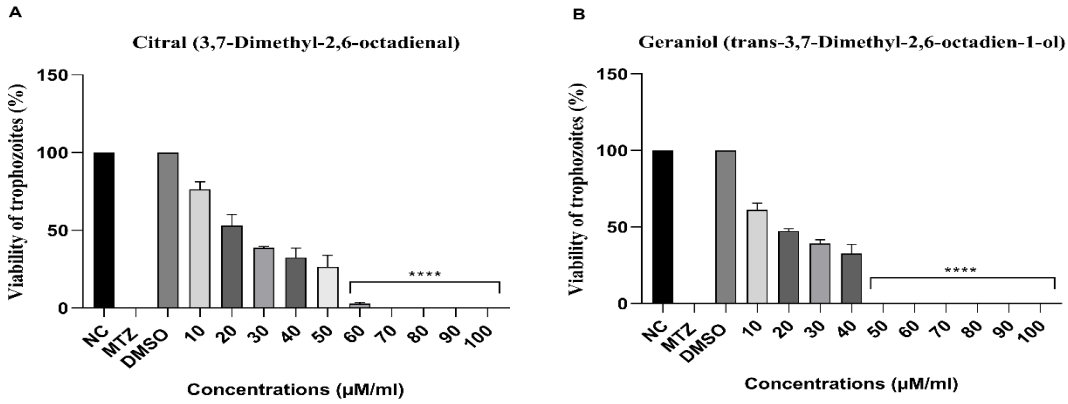

Fig. 1: Anti-*Trichomonas* activity in the culture medium of the formulation of the major compounds (citral and geraniol) at concentrations from 10 to 100 µM, as controls: NC (negative control), MTZ (metronidazole 100 µM), DMSO (diluent 0.6%). The analyses were evaluated 24 hours after treatment. The columns indicate the groups and * indicates the statistical difference when compared to the MTZ control by the Tukey test (P <0.05)

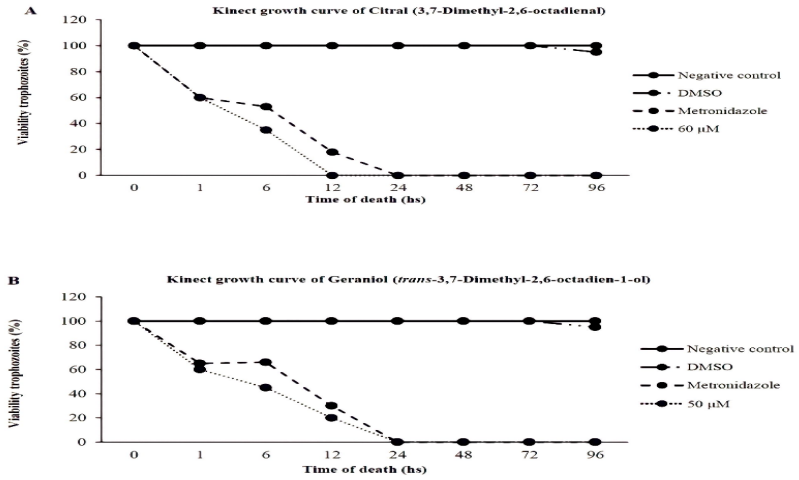

Fig. 2: Time of death of *Trichomonas gallinae* after treatment with the control groups, treatment groups with the standard drug, as well as citral (A) and geraniol (B) in the period of 1, 6, 12, 24, 48, 72 and 96 h

Discussion

Several medications have been tested against trichomoniasis in the buttocks (22, 23). Therefore, there is no specific medicine for the treatment of this disease in birds, because metronidazole has not been used, a medicine for human use for the treatment, which was adapted for use in birds due to the lack of development of a new antitrichomonal medicine

(24). In this context, several synthetic composts demonstrate activity against *T. gallinae*, as well as low toxicity to the host, and may be alternative treatment potencies compared to nitroimide-based medications (25).

In this study, the treatment with citral and geraniol compounds at the highest concentrations reduced the viable trophozoites in the culture medium by 100% after 24 hours, corroborating other studies who used essential oils in their studies, demonstrating the antitrichomonal effects against *T. gallinae* trophozoites in the in vitro assay (26-28). The mechanism of citral release is similar to that of other aldehydes, affecting the inhibition of the activity of thiol group enzymes in the cytoplasmic membrane (29). It can probably be explained by the ability of this compost to promote damage to the cell membrane and alter its integrity and permeability (30-33).

The inhibitory effects of citral have been observed in vitro against *T. cruzi*, reducing the epimastigote and trypomastigote forms and acting in the process of differentiation of protozoa. Essential oils are lipophilic and this contributes to greater penetration into the cell membrane of the protozoan, interacting with polysaccharides, fatty acids, and phospholipids, being the first mechanism of action, causing cell death due to loss of ions and cell content (34).

Antiprotozoal activity has been detected against promastigotes, axenic amastigotes, and intracellular amastigote forms of *Leishmania amazonensis*. This study may be responsible for changes in the morphology of the protozoan, causing the rupture of the plasma membrane, interfering with the cell division process, and promoting the disintegration of the parasite (35). The compounds induced a decrease in the total lipid content of G. *citri-aurantii* cells, indicating the destruction of cell membrane structures. These results suggest that the antifungal activity of citral may be assigned to disruption of cell membrane integrity and leakage of cell components (36). Geraniol has antioxidant, anti-inflammatory and antimicrobial properties and is active against the bacteria *Listeria monocytogenes* and *Salmonella enterica*. In the gaseous form, it has activity against *Haemophilus influenzae*, *Streptococcus pneumoniae*, *S. pyogenes* and *Staphylococcus aureus* (37). There are reports of activities against fungi, *Candida albicans*, Saccharomyces *cerevisiae*, and the nematode *Caenorhabditis elegans* (38-41).

The mechanism of action of geraniol is associated with its lipophilic properties. It can preferentially influence the membrane structures or the synthesis of these structures, changing the permeability and fluidity of the membrane (41). In addition to its lipophilic characteristic, it has a chemical structure very similar to geranyl pyrophosphate, a sterol found in cell membranes and functions as an important regulator of membrane fluidity (42). This similarity suggests that the action of geraniol is associated with the inhibition of enzymes involved in the ergosterol biosynthesis process, thus causing dynamic alterations in the cell membrane of microorganisms.

MTT cell viability showed that citral reduced VERO cell growth in a dose and time-dependent manner. Cytotoxic and genotoxic effects are reported by citral at a concentration of 0.5 µg/mL in human hematopoietic cultured cells and leukocytes, showing a significant decrease in cell viability, corroborating our study (43). For geraniol, the assay by the MTT method indicated that cell viability remained above 100%, demonstrating that it did not show cytotoxicity at the tested concentrations. Cytotoxic potential of six concentrations of geraniol (100-2000 µg / mL) in human lymphocytes; both of the tested concentrations were cytotoxic, which differs from our study. This difference is possibly related to the difference in time and concentration of exposure to geraniol; while we used 24 h, treated the cells for 3 h (44).

In a study to evaluate the antiparasitic potential of an essential oil compound, resveratrol, in *T. vaginalis* (45), hydrogen activity increased after resveratrol administration, while Fe-hydrogenase activity decreased, indicating

the same effect caused by the most commonly used drug for trichomoniasis (Metronidazole). Inhibition of this enzyme is a known effect of metronidazole. The authors concluded that resveratrol mainly inhibits the Fe-hydrogenase enzyme and iron transport.

Conclusion

Although the chemical characteristics of geraniol and citral are different compared to resveratrol, these compounds may not bind strongly to Fe-hydrogenase, but may act on iron transport and thus cause metabolic changes in *T. gallinae*.

Acknowledgements

This study was carried out with the support of the Coordination of Improvement of Higher Education Personnel-Brazil (CAPES) Financing Code 001. The authors are grateful for the financial support of FAPERGS for the ADR/2017 process.

Conflicts of Interest

The authors declare there are no conflicts of interest.

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