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In vitro larvicidal activity of geraniol and citronellal against *Contracaecum* sp (Nematoda: Anisakidae)

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Abstract

Human infection with fish parasites can result from the ingestion of incompletely cooked or raw fish, giving origin to parasitic diseases such as anisakiasis, caused by parasites of the Anisakidae family. The present study assessed the *in vitro* larvicidal effect of two monoterpene compounds, geraniol and citronellal, against *Contracaecum* sp (Nematoda: Anisakidae). Four hundred live larvae of *Contracaecum* sp obtained from "traíra" fish (*Hoplias malabaricus*, Bloch, 1974) were analyzed on 40 Petri dishes (10 larvae each) with the compounds to be tested. The final concentrations tested for each compound were 250, 125, 62.5, and 31.2 µg/mL and the evaluation was carried out at five different times (2, 4, 8, 24, and 48 h). The larvicidal action of geraniol and citronellal was statistically superior ($P < 0.005$) to the control (1% ethanol) at concentrations of 250 and 31.2 µg/mL (geraniol) and 250, 125, and 62.5 µg/mL (citronellal). However, no larvicidal activity was observed at concentrations of 125 and 62.5 µg/mL for geraniol and 31.2 µg/mL for citronellal. When the larvicidal action of geraniol was compared to that of citronellal, the former was found to be statistically superior ($P < 0.05$) to the latter at concentrations of 250 and 31.2 µg/mL. On the other hand, citronellal was statistically superior ($P < 0.005$) to geraniol at concentrations of 125 and 62.5 µg/mL. The larval mortality rate in terms of time (hours) was higher for geraniol with the passing of time at the 250 µg/mL concentration. At this concentration (in 48 h) the best larvicidal effect was observed with 90% lethality. The larvae were considered to be dead using no motility and loss of structural integrity as parameters. The data indicate that natural terpene compounds should be more explored for antihelminthic activity and can be useful for other studies about anisakiasis treatment.

Key words: Geraniol; Citronellal; Larvicide; *Contracaecum* sp

Introduction

Human infection with fish parasites can occur when raw or incompletely cooked fish is ingested. Anisakiasis is a disease caused by parasites of the Anisakidae family, among them *Contracaecum* sp. Many infections by nematodes of the Anisakidae family have been reported in several parts of the world (1-4). Their symptoms range from allergic reactions (5-7) to epigastric pain crises (8) and intestinal obstruction (9,10). The diagnosis and treatment are based mainly on the visualization and extirpation of the larvae from the gastroenteric mucosa by endoscopy (8). Pharmacological treatments have been suggested (11) but are not commonly used in clinical practice. The use of monoterpene compounds *in vitro* and *in vivo* has shown a significant anti-helminthic activity (12-15), presenting also phytotoxic (16) and acaricidal activities (17). The objective of the present study was to evaluate the anti-helminthic activity of the terpenic compounds geraniol and citronellal against *Contracaecum* sp larvae.

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Material and Methods

Third-stage *Contracaecum* sp larvae were obtained from the "traíra" fish (*Hoplias malabaricus*, Bloch, 1794) taken from the Cuiabá River, Brazil. The larvae were identified taxonomically and staged according to morphological characteristics (18,19). Representative specimens were deposited in the Helminthologic Collection of the Oswaldo Cruz Institute, under the number CHIOC 35505. Geraniol (Acros®, USA) and citronellal (Spectrum®, USA) were used at 96.6 and 94% purity, respectively, using 1% ethanol as control. Four hundred larvae were selected using motility and structural integrity as parameters. The larvae were distributed among 40 Petri dishes (10 larvae each), 20 of them containing an aqueous solution of geraniol and 20 containing an aqueous solution of citronellal (5 mL distilled water and 50 µL of the substance pre-dissolved in 96% ethanol, using the concentrations of 25, 12.5, 6.25, and 3.12 mg/mL). Ten control larvae were kept on a dish containing 1% ethanol. The final concentrations tested were: 250, 125, 62.5, and 31.2 µg/mL. The dishes were kept in a BOD incubator (FANEM, Brazil) at 37°C, with 90% humidity. The observations were carried out using a stereomicroscope 2, 4, 8, 24, and 48 h after the beginning of the experiment. The larvae were considered to be dead using no motility and loss of structural integrity as parameters.

The Fisher exact test was used to determine if there was a larvicidal activity of the product compared to control and to compare the two products. A regression equation was performed to evaluate the mortality rate of the larvae related to time. For analysis of the non-linear dose-reponse, the BioStat 2008 statistical program was used.

Results

When the action of citronellal and geraniol was compared to that of the 1% ethanol control, both compounds were found to have larvicidal activity against *Contracaecum* sp ($P < 0.005$), at concentrations of 250 and 31.2 µg/mL for geraniol and at concentrations of 250, 125, and 62.5 µg/mL for citronellal. When the actions of geraniol and citronellal were compared, geraniol was found to have a higher larvicidal action than citronellal ($P < 0.05$) at concentrations of 250 and 31.2 µg/mL and citronellal was found to have a higher larvicidal activity than geraniol ($P < 0.005$) at concentrations of 125 and 62.5 µg/mL. The estimated equation for the mortality rate of *Contracaecum* sp larvae in relation to time (hours) obtained with the geraniol concentration of 250 µg/mL was: $y = 7.33297 + 1.899925x$ ($R^2 = 0.8926$), showing a higher larvicidal activity of the compound the longer it stayed at this concentration (Table 1). We concluded that both products had larvicidal activity against *Contracaecum* sp.

Table 1. The observed and calculated mortality rates of *Contracaecum* sp larvae in response to 250 µg/mL geraniol *in vitro*.

Time	Observed mortality N (%)	Calculated mortality
2 h	1 (10%)	11.1%
4 h	0 (0%)	14.9%
8 h	3 (30%)	22.5%
24 h	7 (70%)	52.9%
48 h	9 (90%)	98.5%

To identify the best regression model, the F-test was used. The equation $y = 7.33297 + 1.899925x$, where y = mortality (%) and x = time (h), was obtained by the minimum-squares method ($R^2 = 0.8926$).

Discussion

Geraniol had a larvicidal effect after 2 h at the maximum concentration tested (250 µg/mL), killing 10% of the larvae. After 48 h, 90% lethality was observed, also at the concentration of 250 µg/mL. At the 31.2 µg/mL concentration, the larvicidal effect was achieved within 48 h, with death of 10% of the larvae. Other investigators who used geraniol against *Anisakis simplex* larvae (13) obtained a maximum lethal effect between 8 and 24 h at the concentration of 12.5 µg/mL. Thus, the lethality effect against *Anisakis simplex* was obtained using much smaller concentrations than necessary to obtain the same effect against *Contracaecum* sp. The larvae of *Anisakis* sp showed higher sensitivity than *Contracaecum* sp, a fact that must be analyzed as an acceptable difference between the species of parasites used. However, in the present study, when the sensitivity of *Contracaecum* sp larvae to the different concentrations of geraniol and citronellal was analyzed, absence of lethality was observed at the concentrations of 62.5 and 125 µg/mL geraniol and of 31.2 µg/mL citronellal.

This result should be examined considering also variations in sensitivity between the larvae used, although all were from the same species, same stage and the same population. The lack of lethality observed at geraniol concentrations of 62.5 and 125 µg/mL and at the citronellal concentration of 31.2 µg/mL can be explained by an interspecific variation of larval resistance. Another hypothesis for explaining the changes in larval sensitivity to the compounds tested was the age of the larvae collected. Even though the larvae were in the same stage, they might have originated from infections that had occurred at different times and thus during different periods of life. The effect of larval age on their sensitivity to terpene compounds deserves investigation.

Geraniol was initially used in tests against *Caenorhabiditis elegans* helminths, showing an ED_{50} of 66.7 µg/mL (12) and the study led to the discovery of an antihelminthic effect on this species of nematodes. This compound was later used for the *in vitro* evaluation of its effect against *Anisakis simplex* (13), showing a significant larvicidal ef-

fect. The result of the present *in vitro* evaluation of geraniol against *Contracaecum* sp confirms the anthelminthic action of this compound, emphasizing the need for future *in vivo* tests for the use of this compound in the treatment of human and animal anisakiasis. The use of other monoterpene compounds has been investigated, such as eugenol against *Caenorhabiditis elegans* (20), showing a larvicidal effect with an ED_{50} of 62.1 µg/mL. Other investigators evaluated the effect of eugenol against *Anisakis simplex* larvae but did not obtain a larvicidal effect at the maximum concentration tested of 12.5 µg/mL (13). Although the effect of eugenol against *Contracaecum* sp larvae has not been tested, with these results we can observe that, even though monoterpene compounds have been described as anthelminthics, the dose of the product tested and the sensitivity of the helminth species directly influenced the results.

The non-linear dose-reponse relationship of geraniol against larvae of *Contracaecum* sp showed an LD_{50} = 182.38 and LD_{100} = 470.93 in 48 h. These data suggest therapeutic potential of this compound. However, more studies are necessary to clarify the toxicity, phamacokinetics and pharmacodynamic effects for tests *in vivo.* The citronellal LD_{50} and LD_{100} were too high to indicate therapeutic potential under the conditions tested.

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