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BIOLOGY

Relaxing effect of eugenol and essential oils in *Pomacea canaliculata*

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ABSTRACT: *This study evaluated the potential relaxing and/or molluscicidal effects of eugenol and essential oils of Origanum majorana, Ocimum americanum, Hesperozygis ringens, and Piper gaudichaudianum in the gastropod Pomacea canaliculata. Compounds were tested at concentrations of 100, 250, 500, and 750μL L^{-1} to evaluate the relaxing effects. In the second experiment, animals were exposed to 10, 25, and 50μL L^{-1} of essential oils of H. ringens and P. gaudichaudianum for a period of 24h for the evaluation of molluscicidal effects. Eugenol and essential oils of O. majorana and O. americanum showed relaxing effects at ≥250μL L^{-1}, but the essential oils of H. ringens and P. gaudichaudianum did not promote relaxing or molluscicidal effects within the times and concentrations studied. Therefore, only eugenol and the essential oils of O. majorana and O. americanum can be used for relaxation purposes in P. canaliculata.*
Key words: *anesthesia, golden apple snail, mollusk, Ocimum americanum, Origanum majorana.*

Efeito relaxante de eugenol e óleos essenciais em *Pomacea canaliculata*

RESUMO: *Este estudo teve como objetivo avaliar os possíveis efeitos relaxantes e/ou moluscicidas do eugenol e óleos essenciais de Origanum majorana, Ocimum americanum, Hesperozygis ringens e Piper gaudichaudianum no gastrópode Pomacea canaliculata. Os compostos foram testados nas concentrações de 100, 250, 500 e 750μL L^{-1} para avaliar os efeitos relaxantes. Em um segundo experimento, os animais foram expostos a 10, 25 e 50μL L^{-1} por 24 horas aos óleos essenciais de H. ringens e P. gaudichaudianum para avaliação dos efeitos moluscicida. O eugenol e os óleos essenciais de O. majorana e O. americanum apresentaram efeito relaxante nas concentrações ≥250μL L^{-1}, mas os óleos essenciais de H. ringens e P. gaudichaudianum não promoveram efeito relaxante ou moluscicida no tempo e concentrações estudadas. Portanto, apenas o eugenol e os óleos essenciais de O. majorana e O. americanum podem serem usados para fins de relaxamento em P. canaliculata.*
Palavras-chave: *caracol maçã-dourada, moluscos, anestesia, Origanum majorana, Ocimum americanum.*

INTRODUCTION

Many mollusk species have economic importance, such in the production of pearls or in research (AQUILINA & ROBERTS, 2000; WYETH et al., 2009). Anesthetic substances have been used to improve their breeding in captivity (GARR et al., 2012), minimizing unpleasant sensations that these animals are likely to experience in handling and surgeries (COOPER, 2011), facilitating pearl withdrawal in bivalves (AQUILINA & ROBERTS, 2000). Almost all anesthetic substances used in shellfish are synthetic products such as benzocaine, MS-222 and 2-phenoxyethanol, and the minority are plant extractives (GARR et al., 2012). Essential oils (EOs) of *Lippia alba* and *Aloysia triphylla* (PARODI et al., 2012), for example, and isolated substances such as eugenol (GOMES et al., 2011; PARODI et al., 2012) and linalool (HELDWEIN et al., 2014) are effective to anesthetize fish and shrimps. Thus, we can assume that EOs may also be effective in mollusks.

Although some shellfish species have economic importance, others, such as *Pomacea canaliculata*, have become pests in agriculture and aquaculture because they are highly invasive and difficult to eliminate or control biologically (HAYES et al., 2015). In addition, they serve as intermediate hosts of disease-causing human parasites (SONG et al., 2016). *Pomacea canaliculata* is a gastropod

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from the family *Ampullariidae*, popularly known as golden apple snail. The uncontrolled spread of these gastropods has increased the demand for preventive and control measures through products that are non-toxic to other non-target organisms and the environment, as synthetic products used for this purpose have high residual effects (CALUMPANG et al., 1995). Products extracted from plants are promising alternatives because they are biodegradable and from renewable sources; they also have fewer adverse effects on the ecosystem. There are studies reporting molluscicidal activity of some plant extracts against *P. canaliculata* (DAI et al., 2011; KIJPRAYOON et al., 2014) and other mollusk species (RODRIGUES et al., 2013; HAMED et al., 2015). In this context, the aim of this study was to evaluate possible molluscicidal and/or relaxing effects of eugenol and the EOs of *Origanum majorana, Ocimum americanum, Hesperozygis ringens*, and *Piper gaudichaudianum* on *P. canaliculata*.

MATERIALS AND METHODS

The animals were obtained from earth ponds in a fish culture in São João do Polêsine city (Rio Grande do Sul, Brazil) and housed in the Fish Physiology Laboratory at the Universidade Federal de Santa Maria (UFSM) under the following conditions: temperature 19.02±0.02°C, pH 7.85±0.01, and dissolved oxygen levels 8.59±0.25mg L^{-1}. Identification of specimens was carried out by Dr. Carla Bender Kotzian (Department of Biology, UFSM).

Essential oils and isolated substance

Eugenol and the EO of leaves of *O. majorana* were purchased from Sigma-Aldrich, Brazil, and Vimontti® (Agroindústria São Caetano Ltda, Brazil), respectively. The other plant species (*O. americanum*, *H. ringens*, and *P. gaudichaudianum)* were collected in São João do Polêsine, São Francisco de Assis, and Santa Maria cities, Rio Grande do Sul State, respectively. The EOs were obtained from the aerial parts of the plants by hydrodistillation using a Clevenger apparatus for 2h and subsequently stored in amber glasses at -4°C in the dark prior to phytochemical analysis (EUROPEAN PHARMACOPOEIA, 2007).

Phytochemical analysis

Characterization of EOs was performed by chromatographic analysis using an Agilent 7890A gas chromatograph coupled to an Agilent 5975C mass selective detector (GC-MS). The identification of constituents was achieved by the comparison of retention indices, obtained by the use of a calibration curve of n-alkanes injected at the conditions mentioned for the samples, and the mass fragmentation patterns with the data of ADAMS (2009), NIST (2010), VIEIRA et al. (2014), and PINHEIRO et al. (2016).

Induction of relaxation and recovery

Animals (11.87±0.76g) were placed in continuously aerated 1-L aquaria (n=five animals/aquarium) in duplicate. Compounds and EOs previously dissolved in 95% ethanol (1:10) were tested at concentrations of 100, 250, 500, and 750µL L^{-1}. Control experiments were performed using aquaria containing only ethanol at a concentration equivalent to the highest dilution used for samples. The evaluation of relaxation was based on the experimental protocol described by GARR et al. (2012), with some adjustments. The maximum evaluation time was 40min, with the percentage of relaxed animals registered every 10min. Animals were considered relaxed when they did not show any resistance to the pulling of the operculum with the aid of a forceps. After relaxation, animals were transferred to anesthetic-free aquaria. Recovery time was evaluated at 10min intervals and the animal was considered recovered when it presented resistance to pulling of the operculum. Integrity and mortality were evaluated 24 hours after the experiment.

Molluscicidal effect

An additional experiment was performed with gastropods exposed to 10, 25, or 50µL L^{-1} of the EOs of *H. ringens* and *P. gaudichaudianum*, which revealed toxicity in the first experiment. The animals were placed in 1-L aquaria (n = five animals $aquarium^{-1}$, in duplicate) with each EO previously solubilized in 95% ethanol (1:10). A control group (water only) and another exposed to ethanol (450µL L^{-1}) were also observed. Mortality and integrity of animals were evaluated after 24 hours.

Statistical analysis

Data were submitted to Levene's test to determine homogeneity of variances, after which one-way ANOVA was performed, followed by Tukey's test or Kruskal-Wallis test, when appropriate. The tests were performed using the software package Statistica (version 11.0). The effective concentration that relaxes 50% of the animals (EC_{50}) was calculated with the SigmaPlot software. The minimum significance level was 95% ($P<0.05$). Data are reported as means ± SEM.

RESULTS

Chemical composition of essential oils

The EOs presented as major constituents the following compounds: terpinen-4-ol (29.13%), γ-terpinene (17.99%), and α-terpinene (9.72%) in the EO of *O. majorana*; dillapiole (84.32%) in the EO of *P. gaudichaudianum*; eucalyptol (24.31%), linalool (23.44%), and eugenol (14.86%) in the EO of *O. americanum*; and pulegone (74.27%) in the EO of *H. ringens*.

Induction of relaxation and recovery

Ethanol did not show any relaxing effect on animals within the time frame of the analysis. Essential oils, as well as eugenol, did not promote relaxation at 100µL L^{-1}, but the EOs of *O. americanum* and *O. majorana* and eugenol promoted relaxation at concentrations above 100µL L^{-1}. Use of 250µL L^{-1} eugenol induced relaxation of only 40% of the animals, but most animals relaxed at higher concentrations. The EO of *O. americanum* had a higher percentage of relaxed animals (70%) at 250µL L^{-1}, with the effect decreasing to 50% with increasing concentrations. In contrast, the two lowest concentrations (250 and 500µL L^{-1}) of EO of *O. majorana* relaxed only 10% of the animals, while at 750µL L^{-1}, 80% of the animals relaxed (Figure 1A). Other EOs tested (*P. gaudichaudianum* and *H. ringens*) did not promote relaxation, but caused a loss of mucus in the animals at all concentrations tested. There was no significant difference between the relaxation time in the concentrations of eugenol and EO of *O. americanum*. It was not possible to perform statistical analysis of the relaxation time between the different concentrations of the EO of *O. majorana* because of the small number of animals that relaxed (Figure 1B). The EC_{50} of eugenol and the EO of *O. majorana* were 269.5 and 662.1µL L^{-1}, respectively. The EC_{50} of the EO of *O. americanum* could not be calculated because no significant relationship concentration-response was reported.

The percentage of recovered animals exposed to eugenol was not related to concentration. Mollusks exposed to the highest concentration of the EO of *O. americanum* presented a higher percentage of recovery, and the few specimens exposed to all concentrations of the EO of *O. majorana* that relaxed showed recovery within the observation time (Figure 1C). Recovery time was not significantly affected by eugenol concentration. Low number of animals that recovered from the exposure to the EOs did not allow a statistical analysis of this parameter (Figure 1D).

Eugenol and the EOs of *O. americanum* and *O. majorana* did not cause mortality after 24 hours; however, the EOs of *H. ringens* and *P. gaudichaudianum* at 750µL L^{-1} provoked 20 and 30% mortality, respectively. Other animals exposed to *P. gaudichaudianum* and *H. ringens* and remained alive exhibited fragile and brittle shells.

Molluscicidal effect

No mortality was observed in any of the treatments after 24h, but gastropods exposed to 50µL L^{-1} of both EOs tested showed damages in their shells, as reported in the previous experiment.

DISCUSSION

Eugenol and the EOs of *O. americanum* and *O. majorana* promoted muscle relaxation in *P. canaliculata*. This effect was expected, since these compounds have an anesthetic effect in fish (GOMES et al., 2011, SILVA et al., 2015; CUNHA, et al., 2017). Eugenol also presented anesthetic activity in crustaceans (PARODI et al., 2012). In gastropods, clove oil at 350µL L^{-1} showed 100% efficacy in relaxing *Pomacea paludosa* within about 20min (GARR et al., 2012), while eugenol, the main constituent of clove oil, was 100% effective in the present study at relaxing *P. canaliculata* only at a higher concentration (750µL L^{-1}) and with a longer exposure time. Animals required less time to recover from eugenol exposure, but only 40% of the animals that relaxed with 750µL L^{-1} recovered within the observation period. The intermediate concentration of eugenol tested (500µL L^{-1}) showed the best result, with 100% animals recovered.

The EO of *O. americanum* presented as major constituents eucalyptol, linalool, and eugenol, corroborating the results reported by SILVA et al. (2015). The relaxing effect of this oil can be attributed to eugenol, which has been proven to have a relaxing effect in *P. canaliculata*, and linalool, which also has an anesthetic effect on silver catfish, *Rhamdia quelen* (HELDWEIN et al., 2014). In contrast, eucalyptol showed no anesthetic activity up to 17mg L^{-1} when tested in this species (HELDWEIN, 2011), but was effective in blocking the excitability of the superior cervical ganglion neurons of *in vitro* rats (FERREIRA-DA-SILVA et al., 2009). Most likely, the relaxing effect of the EO of *O. americanum* is due to the synergistic action of its compounds. Regarding efficacy, it was possible to relax 70% of the animals at 250µL L^{-1} within 30min, whereas higher concentrations had lower efficacy; although, there was no difference in relaxation time. Similar results were obtained for *Ostrea edulis* anesthetized with urethane and menthol; namely, lower concentrations were more effective (CULLOTY & MULCAHY, 1992).

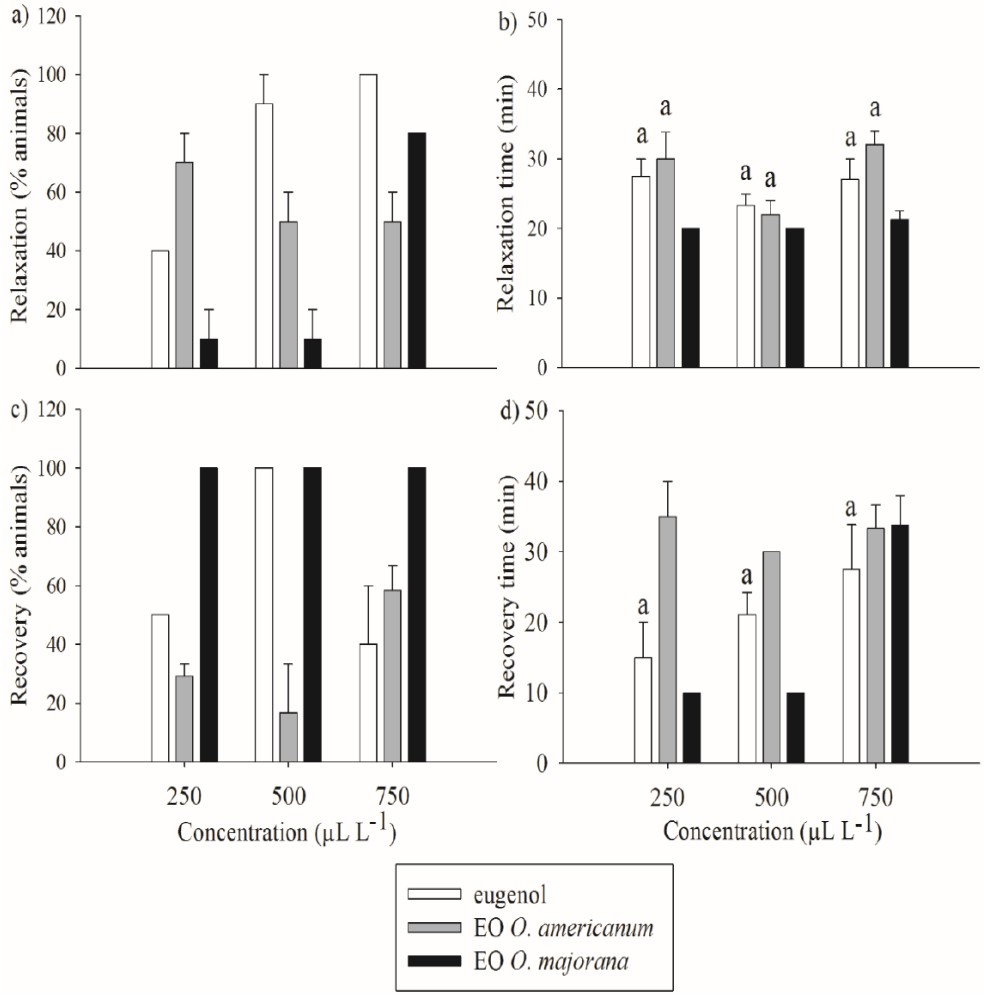

Figure 1 - Relaxation percentage (a), time to induction of relaxation (b), recovery percentage (c), and recovery time (d) of *Pomacea canaliculata* exposed to eugenol and essential oils of *Origanum majorana* (EO *O. majorana*) and *Ocimum americanum* (EO *O. americanum*). Different letters indicate a significant difference between concentrations in the same treatment by one-way ANOVA and Tukey´s test ($P < 0.05$). No statistical analysis was performed in "a" and "c" and for the EO of *O. majorana* (b) and the EOs of *O. americanum* and *O. majorana* (d).

Terpinen-4-ol, γ-terpinene, and α-terpinene were the main constituents identified in the EO of *O. majorana*. This same EO presented anesthetic activity in silver catfish at concentrations ≥100µL L^{-1} (CUNHA, et al., 2017). In *P. canaliculata,* the relaxing effect was obtained from 250µL L^{-1} (10% efficacy), but only produced a satisfactory effect at a higher concentration (750µL L^{-1}). However, all animals that relaxed with EO of *O. majorana* recovered up to 40min. High concentrations of nembutal (1,000µL L^{-1}) and 2-phenoxyethanol (3,000µL L^{-1}) were also necessary for the relaxation of *Haliotis iris* (AQUILINA & ROBERTS, 2000) and *Pomacea paludosa* (GARR et al., 2012), respectively.

The EOs of *H. ringens* and *P. gaudichaudianum* at concentrations of up to 750µL L^{-1} did not cause muscle relaxation in the golden apple snail. The EO of *H. ringens* promoted deep anesthesia (111 to 554µL L^{-1}) without mortality in silver catfish (SILVA et al., 2013), and its effect was attributed to the presence of pulegone (96.66%), an allosteric modulator of GABA (TONG & COATS, 2010). As GABA immunoreactive neurons are reported in the central nervous

system of gastropods (HATAKEYAMA & ITO, 2000; GUNARATNE et al., 2014; 2016), this result was unexpected.

Pomacea canaliculata can cause losses in agriculture (COWI, 2002) and public health problems because it is an intermediate host of the nematode *Angiostrongylus cantonensis*, responsible for causing meningitis in humans (SONG et al., 2016). For this reason, the molluscicidal activity of the EOs of *H. ringens* and *P. gaudichaudianum* were tested. Both EOs did not cause mortality within 24h exposure at the lower tested concentrations (10, 25, and 50µL L^{-1}), but loss of mucus and degradation of the calcareous shell were observed. However, as 750µL L^{-1} of both EOs provoked 20-30% mortality within 40min, it is likely that the 100-750µL L^{-1} range would induce 100% mortality. Deleterious effects caused by these EOs may be due to some of their constituents or the synergistic combination of several compounds. These EOs have a larvicidal effect against the Coenagrionidae family and fungitoxic activity *in vitro* against species of pathogenic fungi and wood decay (SILVA et al., 2014; SCHINDLER, 2015). Dillapiole, the major constituent of the EO of *P. gaudichaudianum*, is present in other species of the genus, and its insecticidal activity has previously been described (VOLPE et al., 2016). Natural products, such as glycosides extracted from fresh leaves of *Nerium indicum* (DAI et al., 2011) and fractions of the methanolic extract of seed flour *Camellia oleifera* (KIJPRAYOON et al., 2014), also caused *P. canaliculata* mortality. Molluscicidal effect of the EO *Cymbopogon winterianus* against *Biomphalaria glabrata* (RODRIGUES et al., 2013) and the hexane and ethyl acetate extracts from the aerial parts of *Atriplex inflata* against *Galba truncatula* (HAMED et al., 2015) have also been described.

CONCLUSION

Based on the results obtained, we can conclude that eugenol, as well as the EOs of *O. americanum* and *O. majorana*, have relaxing activity in *P. canaliculata* and can be used for research purposes in this species. The EOs of *H. ringens* and *P. gaudichaudianum* did not promote relaxation, even at high concentrations, and were also not effective as molluscicides at up to 50µL L^{-1} against this species.

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