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SHORT COMMUNICATION

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In vitro efficacy of two terpenes against ancyrocephalid monogeneans from Nile tilapia

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Abstract Terpenes are naturally produced compounds with a broad range of biological activities. Currently, there is limited information regarding the anthelminthic effect of terpenes against monogenean parasites of fish. The aim of this work was to evaluate the in vitro efficacy of two terpenes [α-terpinene and (+)-limonene oxide] against ancyrocephalid monogeneans found on farmed Nile tilapia (*Oreochromis niloticus*). (+)-Limonene oxide was more effective in killing these parasites than α-terpinene, with 86 and 90% mortality at concentrations of 36 and 55.4 mg/L, respectively, with a 5-h treatment. The estimated 5-h EC_{50} of (+)-limonene oxide was 4.8 mg/L. Even though this compound has the potential to be used as an anthelmintic compound in finfish aquaculture, before in vivo experiments are performed, additional studies are needed to find a more effective concentration, as well as to evaluate other terpenic compounds.

Keywords Parasitic Platyhelminthes · Control · Treatment · Essential oils · Finfish aquaculture

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Introduction

Monogeneans are flatworms (Platyhelminthes) that are primarily ectoparasites of marine and freshwater fish, although they can also be found on other aquatic organisms. These parasites have a direct, typically short lifecycle. In most monogenean species, adult individuals continuously lay eggs that hatch into infectious, free-living larvae known as oncomiracidia that reach the juvenile and adult stages once they settle onto their fish hosts. The attachment and feeding activities of monogeneans on the host generally result in excessive mucus secretion, haemorrhaging, tissue loss and inflammatory reactions, which may provoke the mass mortality of fish in cases of heavy infections (Ogawa 2015). Experimental studies have shown that fish infected with monogeneans are more susceptible to bacterial infections and, consequently, experience increased mortality (Zhang et al. 2015). Severe epizootics caused by monogeneans have been reported around the world, mainly when fish are confined or in crowded conditions, such as those of aquariums and aquaculture (Thoney and Hargis 1991; Ogawa 2015).

Several drugs or chemical agents, such as formaldehyde, hydrogen peroxide, praziquantel and mebendazole, are used to control monogenean infections; however, these agents are not always totally effective and can be toxic to fish, humans and the aquatic ecosystem (Schelkle et al. 2009; Ogawa 2015; Morales-Serna et al. 2018a). Thus, it is necessary to develop new control strategies, including those based on compounds of natural origins, which are supposed to reduce or delay anthelmintic resistance and to be environmentally friendly. Essential oils of aromatic plants are known for their antimicrobial properties. Many plants naturally produce these oils to attract pollinators or to deter pathogens or predators. In particular, terpenes represent one of the largest classes of essential oils, with an enormous structural diversity that ensures a broad range of biological properties, including antiparasitic activity (Kayser et al. 2003; Mafud et al. 2016). However, the antiparasitic efficacy of terpenes on fish parasites has been scarcely evaluated.

Tilapia is among the most important fish in global aquaculture. In Mexico, tilapia is cultivated throughout the country and represents the second largest aquaculture product with 117,806 t years^{-1} (CONAPESCA 2017). Surveys performed on tilapia farms from different regions of Mexico have uncovered that tilapia are typically parasitized with monogeneans, including species of the family Ancyrocephalidae (Aguirre-Fey et al. 2015; Paredes-Trujillo et al. 2016; Morales-Serna et al. 2018b). This parasitism has not been related to fish disease or mortalities; however, it may be possible that monogeneans influence some of the fish health issues usually experienced in tilapia farms. Therefore, the aim of this study was to evaluate the in vitro efficacy of two terpenes [α-terpinene and (+)-limonene oxide] against ancyrocephalid monogeneans found on farmed Nile tilapia (*Oreochromis niloticus*).

Materials and methods

The terpenes α-terpinene and (+)-limonene oxide (mixture of cis and trans) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Both terpenes were dissolved in 70% alcohol. Specimens (n = 50) of Nile tilapia (mean weight, 142.6 g) were obtained from a fish farm located in southern Sinaloa, Mexico. In the laboratory, the fish were sacrificed by spinal severance, then the gill arches were removed and put in a Petri dish to detect monogeneans under a stereomicroscope. The presence of four ancyrocephalid species, *Cichlidogyrus sclerosus* (75%), *C. tilapiae* (17%), *C. dossoui* (7%) and *Scutogyrus longicornis* (1%), was determined based on 178 specimens. The identification of these species required the partial proteinase K digestion of individuals for observation of haptor and copulatory complex, which morphology is showed in Fig. 1. This method is laborious and some species may be confused because of their morphological similarity. Therefore, the experiments were based on ancyrocephalid monogeneans in general, which represents a real situation that occurs in farmed tilapia.

For in vitro toxicity tests, gill filaments holding monogeneans were gently removed and placed in 6-well plates, with each well containing 5 mL of distilled water at 24.5 °C and 10 monogeneans. After initial range-finding tests, five logarithmically separated concentrations (5, 9.1, 16.6, 30.2, and 55.4 mg/L for 5 h, seven replicates each) of both terpenes were selected for definitive tests. Control wells containing only distilled water and monogeneans were also studied. To account for any possible effect of alcohol on the parasites, there were control wells that contained distilled water plus the alcohol volume that was used for the highest concentration of terpenes. Monogeneans were observed under a stereomicroscope and the mortality was recorded after 5 h. Parasites were considered dead if they did not respond to touch and did not show any reaction when being transferred to clean distilled water. Significant differences in mortality were detected between treatments with ANOVA and the a posteriori Tukey test using R Commander (Fox 2005). The median effective concentration (EC_{50}) values with a 95% confidence interval (CI) and the dose–response curves were calculated by non-linear regression using the equation log(agonist) versus response—variable slope (four parameters) in the program GraphPad PRISM 8.2, with the bottom parameter constrained to equal zero and the top parameter constrained to equal 100.

Results and discussion

In the present study, at the concentrations and time point tested, neither of the terpenes was 100% effective in killing monogeneans. The relationships between mortality and concentration of both terpenes are shown in Fig. 2. Average mortality in the water-only control was 1.6% and in the water-alcohol control was 3.3%. The highest concentration (55.4 mg/L) of α-terpinene killed 60% of parasites after 5 h; however, there were no significant differences ($P > 0.05$) between the treatment and control conditions. The estimated value of the 5-h EC_{50} of α-terpinene was 20.6 mg/L (CI 12.6–39.4 mg/L, slope 0.7). Studies on the biological effects of α-terpinene are scarce. Recently, Mafud et al. (2016) evaluated the in vitro anthelminthic activity of α-terpinene and 37 other terpenes against the blood fluke *Schistosoma mansoni*, a trematode responsible for a disease called schistosomiasis in humans. These authors found that of the 38 compounds, only dihydrocitronellol had anthelmintic activity after the maximal screen time of 120 h at 100 µM (= 13.6 mg/L). Likewise, Baldissera et al. (2016) reported that α-terpinene was effective in vitro but not in vivo against the protozoan *Trypanosoma evansi* (the aetiologic agent of the disease known as "surra" in horses). These results suggest that α-terpinene may not be the best candidate to be used as an anthelminthic, at least not if it is used alone.

Although (+)-limonene oxide was not totally effective, its anthelminthic activity was higher than that of α-terpinene. The (+)-limonene oxide concentrations of 36 and 55.4 mg/L killed 86 and 90% of monogeneans, respectively, after 5 h, which resulted in a significantly higher ($P < 0.05$) mortality than that observed in the control conditions. The estimated value of the 5-h EC_{50} of (+)-limonene oxide was 4.8 mg/L (CI 3.2–7.1 mg/L, slope

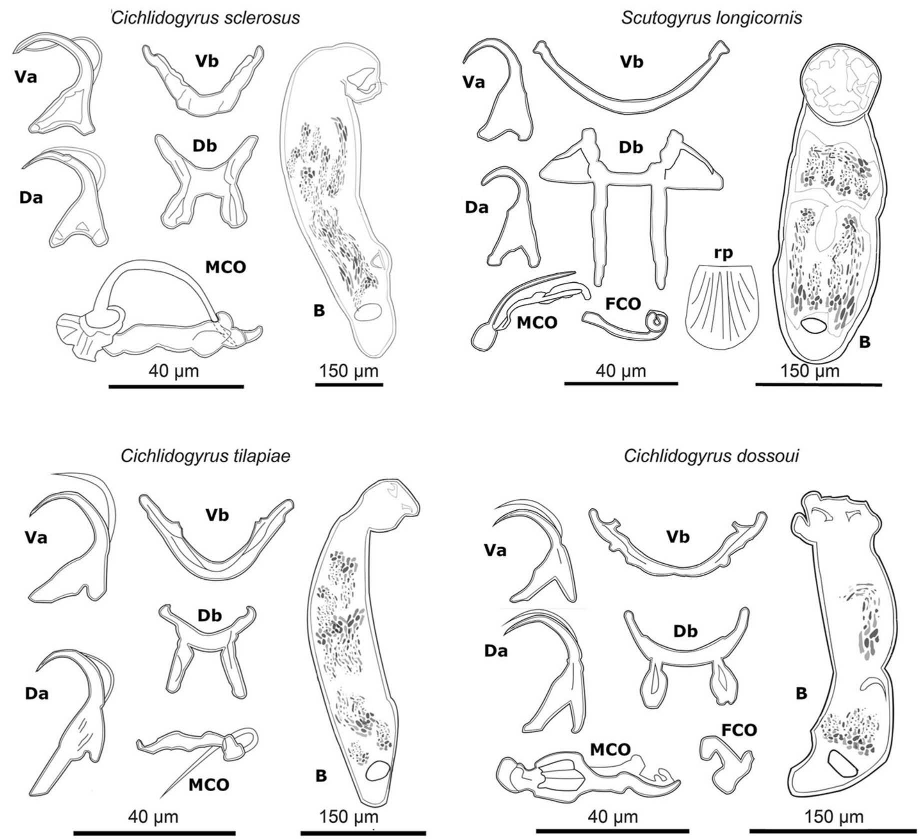

Fig. 1 Body and sclerotized structures of four monogenean species from farmed Nile tilapia (*Oreochromis niloticus*) in Sinaloa, Mexico. *B* entire body, *Da* dorsal anchor, *Db* dorsal bar, *MCO*, male copulatory organ, *FCO* female copulatory organ, *Va* ventral anchor, *Vb* ventral bar, *rp* ribbed portion

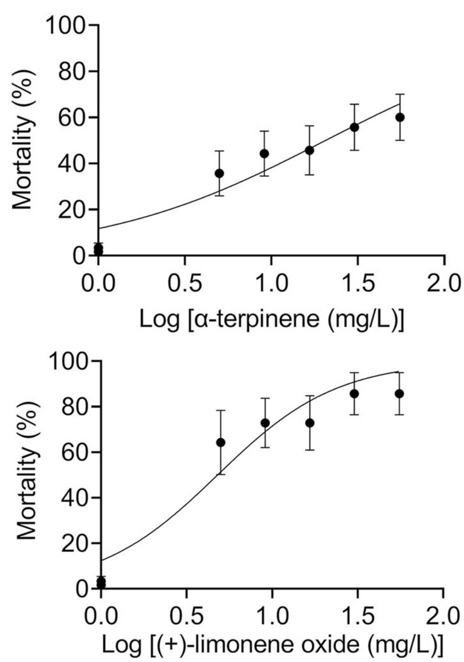

Fig. 2 Relationships between mortality of monogeneans and concentrations of α-terpinene ($R^2 = 0.451$) and (+)-limonene oxide ($R^2 = 0.606$) after 5 h. Error bars indicate SE

1.2). Other studies have also demonstrated the antiparasitic potential of this compound. For instance, at concentrations between 2.5 and 10 mg/L for 48 h, limonene oxide resulted in high lethality to the cattle tick *Rhipicephalus* (*Boophilus*) *microplus*, a parasitic arthropod (Ferrarini et al. 2008).

Other studies have showed that essential oils may be totally effective in killing monogeneans but at higher concentrations than those tested in the present work. For instance, Zoral et al. (2017) showed that 150 g/L for 1 h of the rosemary (*Rosmarinus officinalis*) aqueous extract was completely effective in vitro to kill *Dactylogyrus minutus* monogeneans; however, this concentration was toxic to fish (*Cyprinus carpio*) and lower concentrations did not totally eliminated parasite infections in vivo. Similarly, de Oliveira Hashimoto et al. (2016) reported that 160 mg/L and 320 mg/L of essential oils of pepper rosemary (*Lippia sidoides*) and peppermint (*Mentha piperita*) were, respectively, effective in killing monogeneans just within a few minutes in vitro; however, these compounds were not totally effective in vivo. The mechanism of action of essential oils with anthelmintic activity is unknown. Nonetheless, the tegument of plathelminths may be disrupted by the action of terpenes, such as carvacryl acetate (de Moraes et al. 2013). According to Mafud et al. (2016), to better understand the mechanism of action of terpenes, it is important to know their structure and to identify those features that could underlie the anthelmintic activity.

For the purpose of the present study, we consider that the method (motility and recovery) used to verify monogeneans death was reliable. However, assessing helminth parasite viability through microscopic observations is slow and subjective and is therefore a bottle-neck for high-throughput screening (Panic et al. 2015). Alternative methods for evaluating drug-induced death of helminth parasite of fish have been scarcely investigated. An option could be the fluorometric/colorimetric assays based on indirect measurements of cellular metabolism. For instance, using the trematode *Posthodiplostomum minimum* as a model, Bader et al. (2017) showed that dead metacercariae were stained by propidium iodide and that image analysis software could readily be used to detect live and dead parasites. Going forward, studies are needed to determine if that kind of methodologies may be used in assays dealing with monogeneans.

In conclusion, the anthelmintic activity of (+)-limonene shown in the present work suggests that this compound has a good potential for use in aquaculture. However, to attain a better efficacy, a higher dosage should be tested. Additionally, the anthelmintic activity of other terpenic compounds alone or in combination should be evaluated.

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Compliance with ethical standards

Conflict of interest The authors declares that they have no conflict of interest.

Ethical standards The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals.

Data availability All data generated or analyzed during this study are available from the corresponding author on reasonable request.

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