Anthelmintic activity of the phytochemical eugenol against the fish parasite Gyrodactylus sp. and acute toxicity in Daphnia pulex

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Abstract: The anthelmintic activity of the eugenol against the fish parasite Gyrodactylus sp. was evaluated. After 1-h exposure to 5 and 10 mg/L eugenol, mortality of the parasites was near 80 and 90%, respectively. The concentrations used in the antiparasitic test were not toxic to Daphnia pulex (LC50 42.5 mg/L).

Keywords: monogenean, zooplankton, toxicity assay, environment, antiparasitic

Resumo: Atividade anti-helmíntica do fitoquímico eugenol contra o parasita de peixes Gyrodactylus sp. e toxicidade aguda em Daphnia pulex. Avaliou-se a atividade anti-helmíntica do eugenol contra o parasita de peixe Gyrodactylus sp. Após 1-h de exposição a 5 e 10 mg/L de eugenol a mortalidade dos parasitas foi próxima de 80 e 90%, respectivamente. As concentrações utilizadas na avaliação antiparasitária não foram tóxicas para Daphnia pulex (CL50 42,5 mg/L).

Palavras chave: monogenético, zooplâncton, ensaio de toxicidade, ambiente, antiparasitário

Various drugs have been used for treating or preventing diseases in aquaculture. Nonetheless, such drugs constitute potential pollutants with negative impact on aquatic systems (Jones et al. 2003). In addition, indiscriminate use of these drugs may select for drug-resistant pathogens. In this context, phytochemicals could represent a feasible alternative to conventional drugs, since their environmental toxicity is quite low compared with that of synthetic pesticides or drugs (Park et al. 2011). The use of phytochemicals as anthelmintic drugs for fish is getting increased attention as a sustainable and environmentally acceptable alternative (Hao et al. 2012).

Eugenol (2-methoxy-4-prop-2-enylphenol) (C10H12O2) is the major component (70-90%) of clove oil (Keene et al. 1998) and it is also found in several other plant essential oils. It is an extremely versatile phenylpropanoid included as an ingredient in cosmetics, perfumes, drinks, food and dental preparations (Kamatou et al. 2012). In aquaculture, eugenol has been used and recommended as an alternative anesthetic for several fish species (Javahery et al. 2012). In addition, the low cost of obtaining eugenol, as well as its antimicrobial (Sutili et al. 2012) and anthelmintic potential (Pessoa et al. 2002) further promote its use in aquaculture.

Monogeneans are a diverse group of parasites with a high specificity to the host (Boeger & Vianna 2006). Diseases caused by these helminths are among the most important in aquaculture, for these parasites feed on the blood and tissues of the host and can act as mechanical vectors for fish pathogens such as viruses and bacteria (Cone 1995). Therefore, based on the need for a therapy against fish monogenean parasites and considering the established use of the phytochemical eugenol as a sedative and anesthetic in aquaculture, the anthelmintic potential of this molecule was...
analyzed for the first time in these parasites. Furthermore, the acute toxicity of eugenol in the freshwater zooplankton *Daphnia pulex* was also assessed.

Due to the very small size of the parasites, it was not possible to perform an intact collection of the specimens through skin scrapings, as suggested by some authors (Buchmann & Bresciani 1999, Pavanelli et al. 2002, Ekanem et al. 2004) or to separate the parasites from the mucus that covered the fish. Therefore, five silver catfish (± 10 g) which were highly parasitized by monogeneans were selected for the assay. Following euthanasia by spinal cord section, the maxillary barbells were carefully excised and cut into smaller fractions (2.0 - 3.0 mm).

The assay was performed in 12-well polystyrene plates (flat bottom). A fragment of the parasitized barbells and 2 mL of the treatment solution were added to each well. The following treatments were tested in triplicate: eugenol (99% Sigma-Aldrich, Brazil) at 5 and 10 mg/L (diluted 1:10 in 95% ethanol); formaldehyde 1:4000 (Pavanelli et al. 2002) and ethanol (at the same concentration used to dilute the highest concentration of eugenol) and water controls. Such concentrations of eugenol were chosen because 20 mg/L is able to promote anesthesia in silver catfish (Cunha et al. 2010). Parasite mortality was evaluated after 1-h exposure with the assistance of a stereomicroscope (10x total magnification). A parasite was considered dead if it did not exhibit any movement after a 2-min observation period. This methodology was approved by the Ethical and Animal Welfare Committee of the Universidade Federal de Santa Maria (Process nº 046/2010).

The acute toxicity test was conducted in adult specimens of *D. pulex* according to standard methods of the United States Environmental Protection Agency (USEPA 2002) with some modifications. The assay was conducted in plastic containers (50 mL). Temperature was maintained at 20±1 °C under a 16:8 h light:dark cycle. Eugenol was tested at 5, 10, 20, 40, 80 and 160 mg/L (in triplicate, n=15), previously diluted 1:10 in 95% ethanol. The control groups were exposed to water or ethanol (at the same concentration used to dilute the highest concentration of eugenol). Death was assessed 24h after exposure.

Homogeneity of variances between groups in the antiparasitic assay was determined by a Levene test. Comparisons between groups were made using one-way ANOVA and Tukey’s test. The minimum significance level was set at $P \leq 0.05$. The acute toxicity data were evaluated with the SigmaPlot 10.0 software.

Existing methods of monogenetic parasite control rely heavily on synthetic anthelmintic agents. The most effective treatment is the use of formaldehyde baths (1:4000/1h) (Pavanelli et al. 2002). However, the International Agency for Cancer Research, part of the World Health Organization, classifies formaldehyde as carcinogenic to humans (WHO 2006). An *in vitro* test with essential oil of *Ocimum sanctum* (eugenol-rich) and eugenol showed potent anthelmintic activity in the *Caenorhabditis elegans* model. Eugenol exhibited an effective dose (ED$_{50}$) of 62.1 mg/L (Asa et al. 2001). At 0.50% (diluted in Tween 20) concentration *Ocimum gratissimum* (eugenol-rich) essential oil and eugenol showed a maximum eclodibility inhibition in *Haemonchus contortus* eggs, a gastrointestinal helminth parasite of small ruminants (Pessoa et al. 2002). Small lipophilic secondary metabolites, such as terpenoids or phenylpropanoids, found in essential oils of many plants, can dissolve in biomembranes and disturb their fluidity and the function of membrane proteins (Wink 2008).

In the antiparasitic test, formaldehyde killed all parasites after 1-h exposure. In the groups exposed to 5 and 10 mg/L eugenol, parasite mortality was near 80 and 90%, respectively, and differed significantly from 0 h. Parasite survival was approximately 95% in both ethanol and water control groups after 1 h (Fig. 1). These results show that eugenol is effective against the monogenetic parasite *Gyrodactylus sp.* and may be used as an anthelmintic for fish.

The acute toxicity test in *D. pulex* determined eugenol LC$_{50}$ - 24h (the concentration of a substance that is estimated to be lethal to 50% of the test organisms) to be 42.5 mg/L (Fig. 2). There are few studies evaluating eugenol toxicity in aquatic invertebrates, and considerable variation exists within the studies. Acute toxicity effect of clove oil was studied in the tiger prawn *Penaeus semisulcatus*. The 1-hour LC$_{50}$ and 24-hour LC$_{50}$ were determined to be 130 and 30 mg/L, respectively (Soltani et al. 2004). The European Food Safety Authority (EFSA 2012) registered in *D. magna* an EC$_{50}$ (immobilization) of 1.11 mg/L, which is well below the one determined in this assessment. The time of exposure is important in the toxicity
Eugenol effect on *Gyrodactylus* sp. and *D. pulex*

**Figure 1.** Number of live parasites (*Gyrodactylus* sp.) as a function of time after 1-h exposure. (*) indicates significant difference from 0 h as determined by one-way ANOVA and Tukey’s test ($P \leq 0.05$). WC – water control; EC – ethanol control; EU5 – eugenol 5 mg/L; EU10 – eugenol 10 mg/L; FO – formaldehyde.

**Figure 2.** Acute toxicity (24 h) of eugenol in *Daphnia pulex* adult specimens. LC$_{50}$, 50% effective concentration.
response process because effects depend on internal concentration of the substances (Alvarez et al. 2006). Moreover, the Daphnia species are primarily distinguished by their size, which also affects the toxicity (Vesela & Vijverberg 2007).

Eugenol was effective against the fish parasite Gyrodactylus sp. At both concentrations tested, 5 and 10 mg/L. These concentrations are lower than those recorded as anesthetic for silver catfish (Cunha et al. 2010) and several fish species (Javahery et al. 2012). In addition, survival of D. pulex, a zooplanktonic organism that serves as natural food for fish, was higher than 90% at 5 and 10 mg/L in the acute toxicity test. These results strengthen the possibility of using eugenol not only as a sedative or anesthetic for fish but also as a potential therapy for parasitic diseases.

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