



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 n° 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} - the concentration of a substance that is estimated to cause some toxic sublethal effect on 50% of the test organisms) of 62.1 mg/L (Asha *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

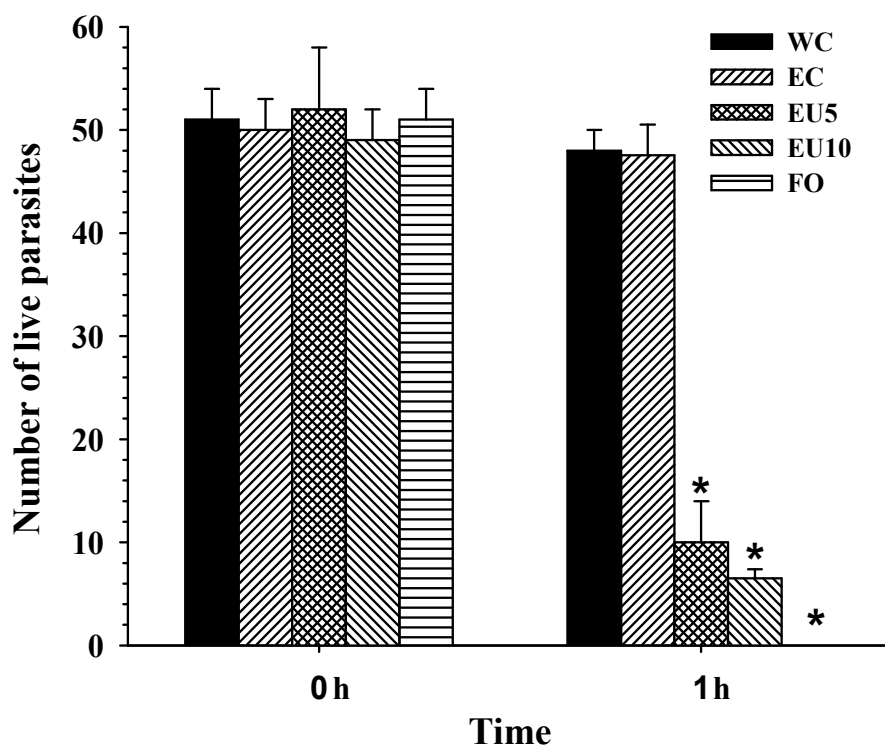


Figure 1. Number of live parasites (*Gyrodactylus* sp.) as a function of time after 1-h exposure. (*) indicates significant difference from 0 h as determined by one-way ANOVA and Tukey's test ($P \leq 0.05$). WC – water control; EC – ethanol control; EU5 – eugenol 5 mg/L; EU10 – eugenol 10 mg/L; FO –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

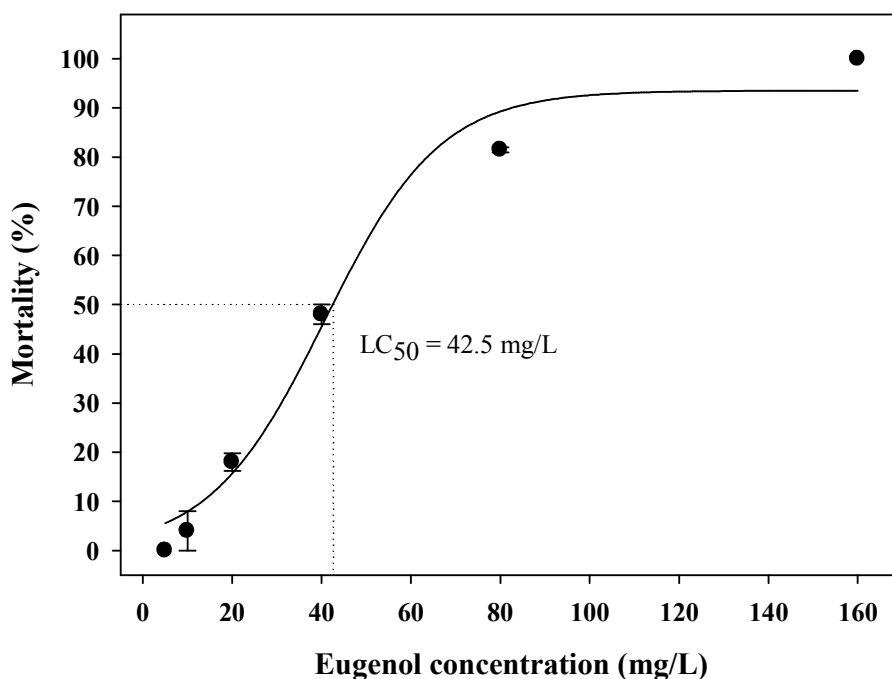


Figure 2. Acute toxicity (24 h) of eugenol in *Daphnia pulex* adult specimens. LC₅₀, 50% effective concentration.

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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References

- Alvarez, O. A., Jager, T., Coloa, B. N. & Kammenga, J. E. 2006. Temporal dynamics of effect concentrations. **Environmental Science & Technology**, 40: 2478-2484.
- Asha, M. K., Prashanth, D., Murali, B., Padmaja, R. & Amit, A. 2001. Anthelmintic activity of essential oil of *Ocimum sanctum* and eugenol. **Fitoterapia**, 72: 669-670.
- Boeger, W. A. & Vianna, R. T. 2006. Monogeneoidea. Pp. 42-116. In: Thatcher, V. E. (Ed.). **Amazon fish parasites**. Sofia: Pensoft Publishers.
- Buchmann, K. & Bresciani, J. 1999. Rainbow trout leucocyte activity: influence on the ectoparasitic monogenean *Gyrodactylus derjavini*. **Disease of Aquatic Organisms**, 35: 13-22.
- Cone, D. K. 1995. Monogenea (Phylum Platyhelminthes). Pp. 289-327. In: Woo, P. T. K. (Ed.). **Fish diseases and disorders - Protozoan and metazoan infections**. Wallingford: CAB International.
- Cunha, M. A., Zeppenfeld, C. C., Garcia, L. O., Loro, V. L., Fonseca, M. B., Emanuelli, T., Veeck, A. P. L., Copatti, C. E. & Baldisserotto, B. 2010. Anesthesia of silver catfish with eugenol: time of induction, cortisol response and sensory analysis of fillet. **Ciência Rural**, 40: 2107-2114.
- Ekanem, A. P., Wang, M., Simon, J. E., Obiekezie, A. I. & Morah, F. 2004. *In vivo* and *In vitro* activities of seed extract of *Piper guineense* Schum and Thonn. against skin and gill monogenean parasites of Gold fish (*Carassius auratus auratus*). **Phytotherapy Research**, 18: 793-797.
- EFSA - European Food Safety Authority. 2012. Conclusion on the peer review of the pesticide risk assessment of the active substance clove oil. **EFSA Journal**, 10(1): 2506.
- Hao, B., Liu, G. L., Hu, X. G. & Wang, G. X. 2012. Bioassay-guided isolation and identification of active compounds from *Semen pharbitidis* against *Dactylogyrus intermedius* (Monogenea) in goldfish (*Carassius auratus*). **Veterinary Parasitology**, 187: 452-458.
- Javahery, S., Nekoubin, H. & Moradlu, A. H. 2012. Effect of anaesthesia with clove oil in fish (review). **Fish Physiology and Biochemistry**, 38: 1545-1552.
- Jones, O. A. H., Voulvoulis, N. & Lester, J. N. 2003. Potential impact of pharmaceuticals on environmental health. **Bulletin of the World Health Organization**, 81: 768-769.
- Kamatou, G. P., Vermaak, I. & Viljoen, A. M. 2012. Eugenol - from the remote Maluku Islands to the international market place: A review of a remarkable and versatile molecule. **Molecules**, 17: 6953-6981.
- Keene, J. L., Noakes, D. L. G., Moccia, R. D. & Soto, C. G. 1998. The efficacy of clove oil as an anaesthetic for rainbow trout *Oncorhynchus mykiss* (Walbaum). **Aquaculture Research**, 29: 89-101.
- Park, H. M., Kim, J., Chang, K. S., Kim, B. S., Yang, Y. J., Kim, G. H., Shin, S. C. & Park, I. L. 2011. Larvicidal activity of myrtaceae essential oils and their components against *Aedes aegypti* acute toxicity on *Daphnia magna* and aqueous residue. **Journal of Medical Entomology**, 48: 405-410.
- Pavanelli, G. C., Eiras, J. C. & Takemoto, R. M. 2002. **Doenças de peixes: profilaxia, diagnóstico e tratamento**. 2ª Edição.

- Maringá: Eduem, 305 p.
- Pessoa, L. M., Morais, S. M., Bevilaqua, C. M. L. & Luciano, J. H. S. 2002. Anthelmintic activity of essential oil of *Ocimum gratissimum* Linn. and eugenol against *Haemonchus contortus*. **Veterinary Parasitology**, 109: 59-63.
- Soltani, M., Marmari, G. H. & Mehrabi, M. R. Acute toxicity and anesthetic effects of clove oil in *Penaeus semisulcatus* under various water quality conditions. **Aquaculture International**, 12: 457-466.
- Suttili, F. J., Kreutz, L. C., Noro, M., Gressler, L. T., Heinzmann, B. M., Vargas, A. C. & Baldisserotto, B. 2014. The use of eugenol against *Aeromonas hydrophila* and its effect on hematological and immunological parameters in silver catfish (*Rhamdia quelen*). **Veterinary Immunology Immunopathology**, 157: 142-148.
- USEPA - United States Environmental Protection Agency. 2002. **Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms**. 5th ed. United States Environmental Protection Agency, Washington, DC.
- Vesela, S. & Vijverberg, J. 2007. Effect of body size on toxicity of zinc in neonates of four differently sized *Daphnia* species. **Aquatic Ecology**, 41: 67-73.
- Wink, M. 2008. Evolutionary advantage and molecular modes of action of multi-component mixtures used in phytomedicine. **Current Drug Metabolism**, 9: 996-1009.
- WHO - World Health Organization. 2006. International Agency for Research on Cancer. **Formaldehyde, 2-butoxyethanol and 1-tert-butoxypropan-2-ol**. IARC Monographs on the Evaluation of Carcinogenic Risks to Humans.

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