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The anesthetic efficacy of eugenol and the essential oils of *Lippia alba* and *Aloysia triphylla* in post-larvae and sub-adults of *Litopenaeus vannamei* (Crustacea, Penaeidae)

Thaylise V. Parodi [a], Mauro A. Cunha [a], Clarissa G. Heldwein [b], Diego M. de Souza [c], Átila Clivea Martins [c], Luciano de O. Garcia [c], Wilson Wasielesky Junior [c], José María Monserrat [d], Denise Schmidt [e], Braulio O. Caron [e], Berta Heinzmann [b], Bernardo Baldisserotto [a,*]

[a] Departamento de Fisiologia e Farmacologia, Universidade Federal de Santa Maria, RS, Brazil
[b] Departamento de Farmácia Industrial, Universidade Federal de Santa Maria, RS, Brazil
[c] Instituto de Oceanografia, Estação Marinha de Aquacultura, Universidade Federal do Rio Grande (FURG), RS, Brazil
[d] Instituto de Ciências Biológicas (ICB), Universidade Federal do Rio Grande (FURG), RS, Brazil
[e] Departamento de Agronomia, Centro de Educação Superior Norte do Rio Grande do Sul, Universidade Federal de Santa Maria, RS, Brazil

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ABSTRACT

The aim of this study was to evaluate the anesthesia induction and recovery times of sub-adult and post-larvae white shrimp (*Litopenaeus vannamei*) that were treated with eugenol and the essential oils (EOs) from *Lippia alba* and *Aloysia triphylla*. Oxidative stress parameters in the hemolymph of this species were also analyzed. The concentrations of eugenol, *A. triphylla* EO and *L. alba* EO recommended for anesthesia were 200, 300 and 750 μL L^{-1} for sub-adults and 175, 300 and 500 μL L^{-1} for post-larvae, respectively. The concentrations studied during the transport of sub-adults were between 20 and 50 μL L^{-1} eugenol, 20–30 μL L^{-1} *A. triphylla* EO and 50 μL L^{-1} *L. alba* EO. For post-larvae, the optimal concentrations for transport were 20 μL L^{-1} eugenol and between 20 and 50 μL L^{-1} *A. triphylla* EO. The white shrimp sub-adults that were exposed to *A. triphylla* EO (20 μL L^{-1}) showed increases in their total antioxidant capacities (150%), catalase (70%) and glutathione-S-transferase (615%) activity after 6 h. *L. alba* EO (50 μL L^{-1}) and eugenol (20 μL L^{-1}) also increased GST activity (1292 and 1315%) after 6 h, and eugenol (20 μL L^{-1}) decreased the total antioxidant capacity (100%). Moreover, concentrations above 30 μL L^{-1} for the EOs of *A. triphylla* and *L. alba* and 20 μL L^{-1} eugenol were effective at inducing anesthesia and improving the antioxidant system against reactive oxygen species (ROS) after 6 h.

1. Introduction

Aquatic animals in culture systems are susceptible to stress caused by capture, handling and transportation, among other stressors. Stress can induce behavioral and physiological changes as well as physical damage and may compromise fish production (Barton, 2002). Anesthetics may be used to reduce the effects of physiological stress in aquatic animals during handling and transport (Di Marco et al., 2011; Pawar et al., 2011).

The signals and responses to pain in shrimp and most crustaceans are not as clearly defined as in vertebrates, but evidence shows that these organisms can experience pain and stress in a manner similar to vertebrates (Elwood et al., 2009). Commonly used drugs do not affect their post-synaptic receptor sites, and they may respond differently to some anesthetics (Ross and Ross, 2008). The topical anesthetic Xylocaine™ reduces the signs of stress in the white shrimp *Litopenaeus vannamei* (Taylor et al., 2004). A few studies have addressed the anesthetic activity of substances of plant origin, such as eugenol (Coyle et al., 2005; Venarsky and Wilhelm, 2006; Akbari et al., 2010) and menthol (Saydmohammed and Pal, 2009), in shrimp. The Pacific white shrimp *L. vannamei* is a tropical species that is naturally distributed along the Pacific coast of Central and South America and is one of the most economically important species cultured worldwide (Zhou et al., 2009).

Like all organisms, crustaceans have an array of defense systems that enable them to meet diverse environmental challenges (Zhou et al., 2009), including constant attack from exogenous and endogenous free radicals, which can lead to serious cellular damage. The antioxidant defense system of this organism includes enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione-S-transferase (GST), which are important components of various detoxification, antioxidant and stress-tolerance pathways. Moreover, the integrated antioxidant

* Corresponding author at: Departamento de Fisiologia e Farmacologia, Universidade Federal de Santa Maria, 97105-900, Santa Maria, RS, Brazil. Tel.: +55 3320 9382; fax: +55 3220 8241.
E-mail address: bbaldisserotto@hotmail.com (B. Baldisserotto).

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system is recognized as an essential component of an organism's self-maintenance ability. The equilibrium between GST and CAT, along with non-enzymatic antioxidants, is important for the effective removal of reactive oxygen species (ROS) (Andrew and Mathew, 1989; Guemouri et al., 1991). Some antioxidant enzymes such as CAT and GST are used as biomarkers of general health status, and the levels of these enzymes can discriminate between shrimp cultivated in intensive and extensive systems (Tu et al., 2008).

Previous studies have reported that eugenol, which is the major constituent of *Eugenia caryophyllata* (Myrtaceae) essential oil (clove oil), has anesthetic properties in some shrimp species, including *Macrobrachium rosenbergii* (Saydmohammed and Pal, 2009) and *Penaeus semisulcatus* (Soltani et al., 2004). The plant *Aloysia triphylla* (L'Herit) Britton (Verbenaceae) grows naturally in South America and was introduced in Europe in the late seventeenth century (Carnat et al., 1999). Traditional ethnobotanical applications of this species include its use in folk medicine as a treatment for insomnia and anxiety and as an analgesic and sedative (Valentão et al., 2002). Recently, the essential oil (EO) of this plant has been patented as an anesthetic for aquatic animals (Patent No PI 016090005905). The EO of *Lippia alba* (Mill.) N.E. Brown (Verbenaceae) has been indicated as an anesthetic for two fish species: the silver catfish *Rhamdia quelen* (Cunha et al., 2010) and the seahorse *Hippocampus reidi* (Cunha et al., 2011). Preparations of *A. triphylla* exhibit antioxidant properties, a powerful superoxide radical scavenging activity and a moderate hydroxyl radical scavenging activity (Valentão et al., 2002). In addition, *L. alba* EO improves the redox state of some silver catfish tissues under both hyperoxia and hypoxia (Azambuja et al., 2011).

Therefore, the aims of this study were to determine the optimal concentrations of eugenol and *A. triphylla* and *L. alba* EOs for the induction of anesthesia in *L. vannamei* sub-adults and post-larvae and to evaluate the time required for both anesthesia induction and recovery. In addition, we established the concentrations of these natural products that are suitable for the transport of this species and the effects of the isolated compound and the EOs on oxidative stress parameters in the hemolymph of *L. vannamei* sub-adults.

2. Material and methods

2.1. Animals

L. vannamei sub-adults (15 ± 0.1 g and 10 ± 1 cm) and post-larvae (0.1 ± 0.7 g and 0.5 ± 0.8 cm) were raised at the Marine Station of Aquaculture, Universidade Federal de Rio Grande, Rio Grande do Sul State, Southern Brazil. The animals were collected from rearing ponds that were equipped with biofloc technology and placed in 500 L indoor tanks with continuously aerated clean water at a salinity of 32 ppt, a pH of 8.2 and t 22 °C. The study was conducted in accordance with the ethical committee of animal welfare at the Universidade Federal de Santa Maria, RS, under protocol no. 027176.

2.2. Plant material and essential oil extraction

The plant species *L. alba* and *A. triphylla* were cultivated at São Luiz Gonzaga and Frederico Westphalen, respectively, which are cities in Rio Grande do Sul State, Brazil. The plant materials were identified by the botanist Dr. Gilberto Dolejal Zanetti of the Department of Industrial Pharmacy, UFSM, and voucher specimens (*L. alba* — SMDB no. 10050; *A. triphylla* — SMDB no. 11169) were deposited in the herbarium of the UFSM Department of Biology. The major components of *A. triphylla* EO are Z-citral (29.92%) and E-citral (42.30%), while the predominant compounds in *L. alba* EO are linalool (59.66%) and 1,8-cineole (9.11%). All other constituents occur at concentrations below 5%. Eugenol (99% purity, Odontofarma™, Porto Alegre, Brazil) was purchased commercially.

The *A. triphylla* and *L. alba* EOs were obtained from fresh plants using hydrodistillation, which was performed with a Clevenger apparatus (2 h for *L. alba* and 3 h for *A. triphylla*) according to guidelines from the European Pharmacopoeia (2007). The essential oils were stored at −20 °C in amber glass bottles. The densities were approximately 0.8 for the *L. alba* EO, 0.9 for the *A. triphylla* EO and 0.98 for eugenol.

2.3. Analysis of essential oils

GC–MS TIC analysis was performed using an Agilent-6890 gas chromatograph coupled with an Agilent 5973 mass selective detector under the following conditions: HP-5MS column (5%-phenyl–95%-methylsiloxane, 30 m × 0.25 mm × 0.25 μm); EI–MS: 70 eV; operating conditions: split inlet 1:100; temperature program, 40–260 °C; 40 °C for 4 min; ramp rate, 4 °C/min; carrier gas, He; flow rate, 1 mL min^{-1}; injector and detector temperature, 220 °C; interface temperature 250 °C; Databank NIST 2002.

The constituents of the EOs were identified by comparing their mass spectra with a mass spectral library (NIST, 2002) and by comparison of the Kovats retention index with literature data (Adams, 2001).

2.4. Anesthesia induction and recovery

The experiments involving anesthesia induction and recovery were conducted according to the procedure described by Coyle et al. (2005). Shrimp were classified as stage 1 if they demonstrated a partial loss of equilibrium but were still reactive to touch stimuli and as stage 2 if they demonstrated a complete loss of equilibrium and were not reactive to stimuli. Shrimp were considered recovered from anesthesia when they regained control of their equilibrium and attained an upright position on the bottoms of the aquaria.

The essential oils and eugenol were dissolved in ethanol at a ratio of 1:10 before being added to aquaria containing seawater. To evaluate the time required for anesthesia induction, 16 sub-adults and 30 post-larvae were used for each concentration tested. Each animal was used only once. After induction, the sub-adults and post-larvae were transferred to anesthetic-free aquaria to measure the anesthesia recovery time. The controls were added to aquaria that contained only ethanol at a concentration that was equivalent to the highest concentration used in the experimental conditions (9 mL L^{-1}). The concentrations studied were chosen based on preliminary tests.

2.4.1. Concentrations for short-term anesthesia

Sub-adult animals were transferred to 1 L aquaria (2 animals per aquarium), and the post-larvae were evaluated in 250 mL beakers (10 post-larvae per beaker). The sub-adults were exposed to the following concentrations: 250, 500, 750 or 1000 μL L^{-1} *L. alba* EO; 50, 100, 300 or 500 μL L^{-1} *A. triphylla* EO and 50, 100, 200 or 400 μL L^{-1} eugenol. The post-larvae were exposed to the following concentrations: 400, 500 or 600 μL L^{-1} *L. alba* EO; 100, 300, 400 or 500 μL L^{-1} *A. triphylla* EO and 100, 150 or 175 μL L^{-1} eugenol. The maximum observation time in this experiment was 30 min.

2.4.2. Concentrations for transport

To evaluate the anesthetic concentration range suitable for shrimp transport, the anesthetic exposure time was fixed at 6 h. Sub-adult animals and post-larvae were maintained in continuously aerated 1 L aquaria (2 animals per aquarium) or 250 mL beakers (5 post-larvae per beaker). Both the sub-adults and post-larvae had five replicates each. The sub-adults were exposed to eugenol (5, 10 or 20 μL L^{-1}), *L. alba* EO (50, 100, 200 or 250 μL L^{-1}) or *A. triphylla* EO (20, 30 or 40 μL L^{-1}). The post-larvae were also exposed to eugenol (10, 20 or 50 μL L^{-1}), *L. alba* EO (100, 200 or 250 μL L^{-1}) or *A. triphylla* EO (10, 20 or 50 μL L^{-1}). The concentrations chosen were below those that