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SHORT COMMUNICATION

Antiparasitic efficacy of essential oils for *Neobenedenia melleni* infecting farmed Lebranche mullet (*Mugil liza*)

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Abstract The present study investigated the phytotherapeutic effects of *Lippia origanoides*, *L. sidoides* and *Mentha piperita* (100, 200, 300, 400, 500, 600 and 700 mg L^{-1}) and the chemotherapeutics formalin (100, 200, 300, 400, 500, 600 and 700 mg L^{-1}), potassium permanganate (1.5, 2.5, 3.5, 4 and 5 mg L^{-1}), hydrogen peroxide (90, 180, 200, 250, 300 and 350 mg L^{-1}) and salinity (0.5 and 10.0 g L^{-1}) on *Neobenedenia melleni* infecting cultivated *Mugi liza*. The parasites were collected from 15 individuals of a first-generation broodstock of aquacultured mullets with average weights and lengths of 900.4 ± 96.99 g and 45.67 ± 1.50 cm, respectively. *In vitro* assessments were conducted in triplicate in experimental units consisting of polystyrene plates with flat bottoms, smooth surfaces and high edges containing 10 parasites in each well. For quantification of dead parasites and behavioral observations, treatments were monitored at different exposure times (5, 10 and 15 min) and then every 30 min. Treatments involving essential oils were effective for ectoparasite immobilization. The treatments with 100% effectiveness in the shortest time were 600 and 700 mg L^{-1} *L. origanoides* (1 min), followed by *L. sidoides* at 700 mg L^{-1} (4 min) and *M. piperita* at 700 mg L^{-1} (9 min). Formalin, hydrogen peroxide, potassium permanganate, and freshwater baths were also effective against *N. melleni*. The present study indicates that sanitary treatments with essential oils and freshwater baths for *N. melleni* infecting the Lebranche mullet *M. liza* are highly sustainable and pose a low risk to the environment.

Keywords Aquaculture . Ectoparasites . Marine fish farming . Sustainability

Introduction

The Lebranche mullet *Mugil liza* is a promising species for use in Brazilian aquaculture (Magnotti et al.

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2020). Research has indicated that *M. liza* is suitable for fish farming because of its euryhaline and eurythermal characteristics, planktivorous eating habits when it is juvenile and iliophagous eating habits when it is adult. Since then, their captive breeding techniques have been studied (Cerqueira et al. 2017; Magnotti et al. 2020).

However, several factors, including high stocking density, inadequate management, poor water quality, and unbalanced nutrition, are responsible for the stress that leads to the immunosuppression of fish and thus increased susceptibility to diseases. Monogenean ectoparasites are among the main disease-causing agents in fish and can cause epithelial and hematological changes in hosts, predisposing them to secondary infections and often leading to high mortality (Huang et al. 2013; Rojas-Garcia et al. 2020).

The *Neobenedenia* genus (Capsalidae family) is composed of marine ectoparasites frequently found in farmed fish (Whittington 2004). This genus comprises species with low host specificity, a direct life cycle, high fertility, and environmentally resistant eggs (Militz et al. 2013; Whittington 2004). Among the therapeutic practices for controlling monogenean ectoparasites in fish are the use of chemotherapeutic agents such as formalin baths at 135 mg L^{-1} (Pahor-Filho et al. 2012), 5-h potassium permanganate baths at 2–20 mg L^{-1} (Umeda et al. 2006), praziquantel at 158.1 mg kg^{-1} body weight (Sitjà-Bobadilla et al. 2006) and 40 mg L^{-1} for 60 min (Forwood et al. 2013) and hydrogen peroxide baths at 50 mg L^{-1} (Bowker et al. 2012), 570 mg L^{-1} for 4 min (Benavides-González et al. 2015) or 75 ppm for 30 or 60 min (Hirazawa et al. 2016).

Phytotherapy based on essential oils has gained prominence for its use in disease control in aquaculture. Herbal medicines often have diverse compositions and are mainly composed of compounds such as terpenoids (monoterpenes and sesquiterpenes) and phenylpropanoids (Calsamiglia et al. 2007). Its use for treating and controlling fish diseases has already been tested *in vitro* and *in vivo* in different species (Tavares-Dias 2018). *In vitro* tests are preliminary procedures in the development of therapeutic protocols, contributing to better *in vivo* applications and results (Cruz et al. 2005; Park et al. 2014). In addition, *in vitro* evaluations are quick, inexpensive, and useful for screening essential oils and their bioactive compounds (Githiori et al. 2006).

The present study investigated the ideal *in vitro* concentrations of phytotherapeutic essential oils of *Lippia origanoides*, *L. sidoides*, and *Mentha piperita*, as well as the concentrations of the chemotherapeutic compounds formalin, potassium permanganate, hydrogen peroxide, and salinity, for the immobilization of the ectoparasite *N. melleni* infecting the aquacultured *M. liza*.

Materials and methods

The parasite *N. melleni* was collected from a broodstock of first-generation (F1) aquacultured mullets (15 individuals) with average weights and lengths of 900.4 ± 96.99 g and 45.67 ± 1.50 cm, respectively, in the Marine Fish Farming Laboratory (LAPMAR) at the Federal University of Santa Catarina (UFSC). The fish were randomly sampled (two to three individuals each), and the parasites were collected by scraping the integument. The presence of the parasites was confirmed via stereomicroscopy (Leica EZ4 HD, Leica Microsystems, Wetzlar, Germany). The water quality parameters of temperature, dissolved oxygen (YSI 55, Yellow Springs, OH, USA), pH (EcoSense pH 10A, Yellow Springs, OH, USA) and salinity (portable manual refractometer - model Rhs - 28atc) were measured daily. During collection, in the morning (8 am), the water quality variables included a pH of 7.22 ± 0.16, a dissolved oxygen concentration of 6.9 ± 0.58 mg L^{-1}, a temperature of 24 °C ± 0.45, and a salinity of 33.19 ± 0.44‰. These values were within the comfort range for the species (Cerqueira et al. 2017).

After collection, a sample of the parasites was incubated in a water bath at 55 °C and then fixed in 5% formalin. The parasites were placed in Hoyer's medium and mounted between a slide and a coverslip for the observation of specific sclerotized structures.

Chemical composition of the essential oils

The essential oils of *L. origanoides*, *L. sidoides*, and *M. piperita* were obtained from the Brazilian Agricultural Research Corporation (EMBRAPA), Manaus-AM, Brazil. The leaves were collected in the morning and dried in a continuous oven at 45 °C for 48 h, and the essential oils were extracted via hydrodistillation in a Clevenger-type apparatus (Matos 1996; Silveira et al. 2012).

The chemical composition of the essential oils was determined via gas chromatography with Agilent 6890 equipment and an Agilent 5973 N selective mass detector. The separation of the compounds was performed on an HP5-MS capillary column (30 m × 0.25 mm × 0.25 µm) with a temperature ranging from 60 °C to 240 °C and a variation of 3 °C m^{-1}. The identification of the constituents of each essential oil was performed by comparing the calculated retention index of each component with the mass spectra from a spectrum library (McLafferty and Stauffer 2004). The index was calculated by injecting a series of n-alkanes under the same analytical conditions used for other essential oils (Adams 2007).

For the chemical components of *L. origanoides*, 100% were quantified, and 92.90% were identified. The most abundant components were carvacrol (49.7%), thymol (9.9%), γ-terpinene (11.6%), and linalool (2.8%). In *L. sidoides*, 100% were quantified, and 99.28% were identified. The most abundant components were thymol (75.4%), p-cymene (7.3%), e-caryophyllene (4.3%), and γ-terpinene (3%). For *M. piperita*, 100% of the components were quantified, and 99.8% were identified. The most abundant components were menthol (30.5%), menthyl acetate (14.5%), pulegone (14.2%), and menthone (12.9%).

In vitro assay

A pretest of the efficacy of essential oils at concentrations of 20, 40, 60, and 80 mg L^{-1} against monogenean parasites was performed on the basis of similar studies with freshwater species (Malheiros et al. 2016; Soares et al. 2016). However, at these concentrations (20, 40, 60 and 80), it was not possible to determine the effectiveness of the essential oils, considering that after eight hours of exposure, the parasites were still alive. Therefore, the experimental concentrations should be above 80 mg L^{-1}.

The essential oils were diluted in grain alcohol (CH_3CH_2OH, Analytical Reagent, Labsynth Ltd., Diadema, SP) to a 10% stock solution, and then the following concentrations and treatments were prepared: the naïve group with water from the maintenance tank fish (salinity 33.19 ± 0.44 ‰); the control group with grain alcohol; and the essential oils of *Lippia origanoides*, *L. sidoides* and *Mentha piperita* at 100, 200, 300, 400, 500, 600, and 700 mg L^{-1}.

For the chemotherapeutic groups, formalin (37% stabilized aqueous solution of formaldehyde, Quimidrol Comércio Indústria Import Ltda., Joinville, SC) was diluted with water from the fish maintenance tank to obtain a 10% stock solution. Then, treatments with concentrations of 100, 200, 300, 400, 500, 600 and 700 mg L^{-1} were established. The potassium permanganate ($KMnO_4$, Dinâmica Química Contemporânea, Indaiatuba, SP) was diluted with water from the fish maintenance tank until a 4% stock solution was obtained, and then the treatments were determined at concentrations of 1.5, 2.5, 3.5, 4 and 5 mg L^{-1}. Hydrogen peroxide (35%, hydrogen peroxide 130 volumes, Quimidrol Comércio Indústria Import Ltd., Joinville, SC) was diluted with water from the fish tank, and then the treatments were determined at concentrations of 90, 180, 200, 250, 300, and 350 mg L^{-1}. The control groups were established with water from a fish maintenance tank.

The salinity treatment was prepared after the water from the fish tank was diluted with tap freshwater (the chlorine content was neutralized with sodium thiosulfate). The following concentrations and treatments were then prepared: control with water from the fish tank and salinities of 0‰, 5‰, and 10‰.

The *in vitro* assessment was carried out in experimental units consisting of polystyrene plates for cell and tissue culture with six wells, flat bottom and smooth surface plates and high edge plates (KASVI, k12--006), all in triplicate, containing 10 parasites in each well. For quantification of dead parasites and behavioral observations, treatments were monitored at different exposure times (5, 10 and 15 min) and then every 30 min. Each test lasted eight hours to ensure that the parasites were dead and not anaesthetized by the tested compounds. Monogeneans were considered dead when they did not show movement after stimulation with an insulin needle (13 mm × 0.25 mm).

Statistical analyses

All the data were previously evaluated for normality and homoscedasticity via Levene's test. For normally distributed data, analysis of variance (ANOVA) was used, followed by Tukey's test to compare means. Differences were considered significant when $p < 0.05$. To determine the ideal concentration of each herbal and chemotherapeutic agent, the dependent variable "time until total parasite mortality (in minutes)" was

subjected to regression analysis. Trend lines for graphical representation were used to identify trends, assisting in decision making. All analyses were performed via SigmaStat 4.0 software.

Results

Assays with essential oils

In the *L. origanoides* treatment, mortality at concentrations of 600 and 700 mg L^{-1} was significantly faster ($p < 0.05$) than that at the other concentrations, whereas mortality at a concentration of 200 mg L^{-1} was significantly delayed ($p < 0.05$) than that at the other concentrations. In the *L. sidoides* and *M. piperita* treatments, mortality at a concentration of 700 mg L^{-1} was significantly faster ($p < 0.05$) than that at the other concentrations (Figure 1 and Table 1). When exposed to the essential oil, the parasites were completely immobilized after one min of exposure at the highest concentrations, without any stimulus responses for at least 60 min of monitoring. The parasites detached themselves from the plate and contracted the body so that they became curled or shell shaped and had a whitish color. After 20 min of monitoring, some of the dead individuals returned to their normal shape. The treatments that did not cause mortality were 100 mg L^{-1} *L. origanoides*, *L. sidoides*, and *M. piperita*; 200 mg L^{-1} *M. piperita*; and the control with all concentrations of grain alcohol.

Assays with formalin, potassium permanganate, hydrogen peroxide and salinity

The most significant results were obtained with the three chemicals at the highest concentrations. This could be a concerning result, indicating some resistance of the parasite to chemotherapeutic agents.

Water with a salinity of 0‰ was significantly more effective ($p < 0.05$) than water with other salinities. Compared with the other concentrations, the concentration of hydrogen peroxide used at a concentration of 350 mg L^{-1} was significantly faster ($p < 0.05$), whereas the concentration of potassium permanganate used

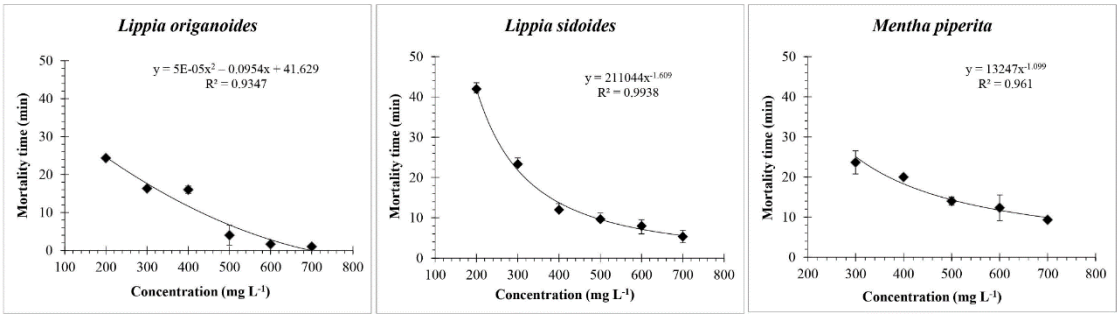

Fig. 1 *In vitro* evaluation of essential oil treatments on *N. melleni* infecting farmed *Mugil liza*
The graphs show the correlations between the effects of different concentrations and exposure times on parasite immobilization.

Table 1 *In vitro* evaluation of the effects of essential oil and chemotherapeutic treatments on *N. melleni* infecting farmed *M. liza*

Phytotherapeutics	Essential oil concentration (mg L^{-1})							
	200	300	400	500	600	700	R	p-value
LO	24.33±0.58^a	16.33±0.58^b	16.00±1.00^b	4.00±2.66^c	1.66±0.58^d	1.00±0.00^d	0.93	<0.001
LS	42.0±1.0^a	23.3±0.6^b	12.0±0.0^c	9.66±0.6^d	8.0±2.0^d	5.33±1.5^e	0,99	<0.001
MP	-	23.66±2.89^a	20.0±0.0^b	14.0±1.0^c	12.33±3.21^c	9.33±0.58^d	0,96	<0.001
Chemotherapeutics	Chemotherapeutics concentration							
F (mg L^{-1})	200	300	400	500	600	700	R	p-value
	188.00±0.00^a	87.33±5.77^b	48.66±1.53^c	45.00±5.20^c	28.33±2.08^d	20.00±2.00^e	0,99	<0.001
PP (mg L^{-1})	-	1.5	2.5	3.5	4.0	5.0	R	p-value
	-	42.00±1.00^a	20.66±1.50^b	10.33±0.58^c	9.66±0.58^c	7.66±0.58^d	0,99	<0.001
HP (mg L^{-1})	-	180	200	250	300	350	R	p-value
	-	21.00±0.00^a	15.00±0.00^b	10.00±0.00^c	9.33±0.58^d	3.66±0.58^e	0,94	<0.001
S (‰)	-	-	0	5	10	35	R	p-value
	-	-	18.66±3.06^d	21.66±1.53^c	59.33±3.06^b	482.33±2.52^a	0,99	<0.001

The data are presented as the time (min) of exposure to different concentrations of different therapeutics. Different letters on the same line indicate a significant difference between different concentrations of the therapeutic agent according to Tukey's test ($p < 0.05$). LO: *L. origanoides*. LS*: L. sidoides*. MP: *M. piperita*. F: Formalin. PP: Potassium permanganate. HP: Hydrogen peroxide. S: Salinity.

at a concentration of 5 mg L^{-1} was significantly faster ($p < 0.05$) in the parasite control. Compared with other concentrations, formaldehyde at a concentration of 700 mg L^{-1} was significantly faster ($p < 0.05$) at immobilizing the parasite (Figure 2 and Table 1).

Discussion

Disease outbreaks caused by parasites remain one of the major challenges in the global development of marine fish farming, and the frequent use of chemotherapeutic agents to control and treat parasitic infestations is an obstacle to sustainable fish production (Tadese et al. 2022). For this reason, the use of phytotherapeutics has gained prominence in aquaculture.

In the present study, *L. origanoides*, *L. sidoides* and *M. piperita* essential oils were effective against *N. melleni* at almost all the concentrations tested. Concentrations of 160 and 320 mg L^{-1} were effective against monogeneans infecting tambaqui (*Colossoma macropomum*). The authors suggested that the mechanism of action of the oil may be associated with the rupture of the cell membrane and subsequent release of liposaccharides, leading to an increase in the permeability to adenosine triphosphate (Soares et al. 2017).

After antiparasitic treatment with *L. origanoides* at 320 mg L^{-1}, Soares et al. (2017) reported 100% mortality in monogeneans infecting tambaqui after 10 min. Thymol and carvacrol inhibit ergosterol synthesis in protozoans, causing an increase in cell permeability and loss of cations such as K+ (Morais et al. 2014), causing the death of the parasite, as observed in the present study.

With respect to *M. piperita*, Hashimoto et al. (2016) reported that this essential oil at a concentration of 320 mg L^{-1} was 100% effective for controlling monogeneans infecting Nile tilapia. On the other hand, minimal amounts of *Allium sativum* (<16 µL L^{-1}) significantly reduced egg incubation (5%), survival of oncomiracids (100% mortality in two hours), and infection (11%) of *Neobenedenia* sp. in Barramundi *Lates*

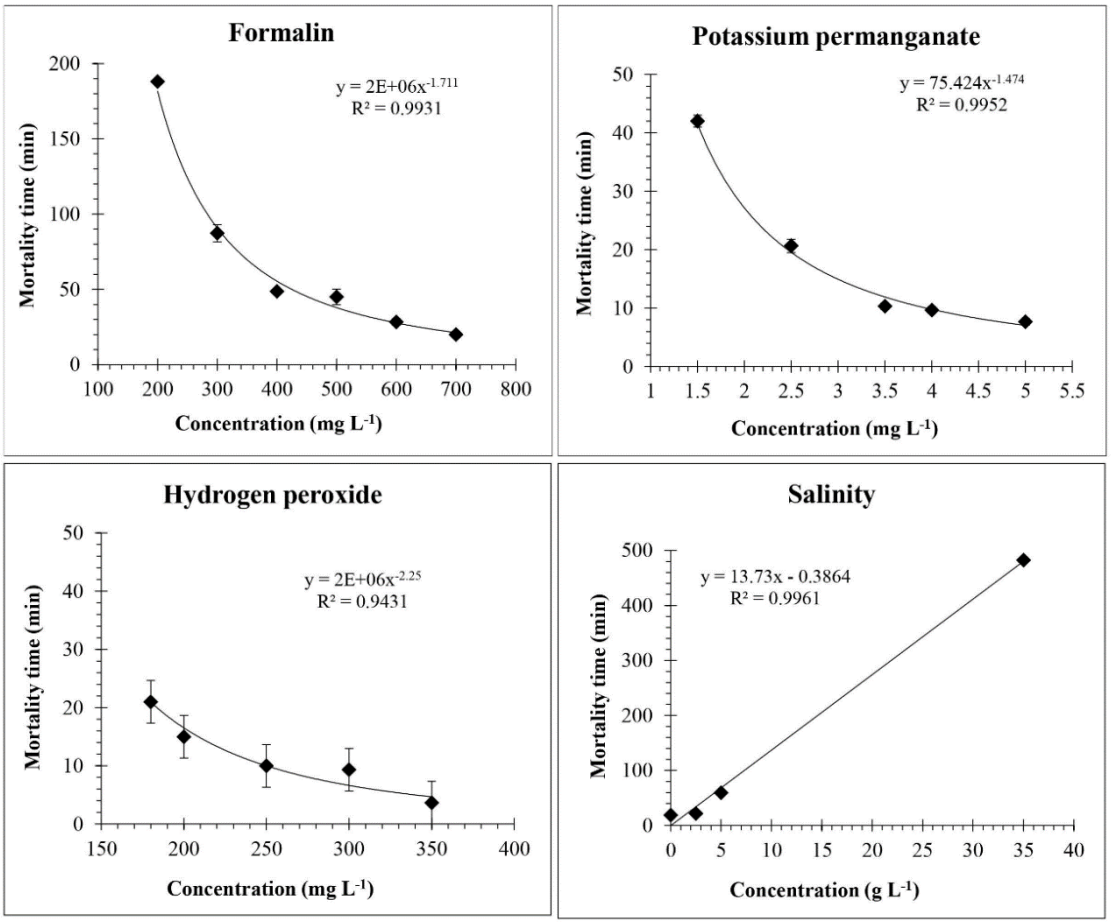

Fig. 2 *In vitro* evaluation of the effects of chemotherapeutic treatments on *N. melleni* infecting farmed *Mugil liza*
The graphs show the correlations between the effects of different concentrations and exposure times on parasite immobilization.

calcarifer (Militz et al. 2013).

Research has indicated that the effects of monoterpenes in the nematode *Caenorhabditis elegans* involve tyramine receptors, which inhibit motility and pump the pharynx (Lei et al. 2010). However, few studies have described the action of menthol and menthyl acetate as anthelmintics for monogenic parasites. Studies with menthol normally demonstrate its anaesthetic properties for some fishes (Spanghero et al. 2019) as it acts on the gamma-aminobutyric acid type A (GABAA) system, an inhibitory neurotransmitter in the central nervous system (Zhang et al. 2008). The stimulation of GABAA by agonist drugs promotes hyperpolarization of the cell membrane, causing depression of the nervous system and consequent body anaesthesia (Guénette et al. 2007). In the present study, menthol may have played an anaesthetic role by inhibiting parasite movement and causing death under deep anaesthesia.

Even though essential oils or their isolated compounds have lower toxicity than chemical products do (Huang et al. 2013), high concentrations with efficacy in *in vitro* tests cannot always be used to control and treat parasites in fish due to their toxicity (Hashimoto et al. 2016). In addition, few studies have accurately and clearly described the antiparasitic effects of phytotherapeutics against monogenean parasites in fish species, as well as the underlying mechanisms (Van Doan et al. 2020). The mechanisms of action of certain phytotherapeutics are diverse, and they can exert antiparasitic effects on parasites through diverse spectra of activity. For example, metabolic inhibitors that target diverse organelles and subcellular compartments of parasites, such as mitochondria and the plasma membrane, can exert antiparasitic effects. Thus, the selective targeting of metabolic pathways is a promising antiparasitic tool (Mukherjee et al. 2016).

As expected, in the present study, hydrogen peroxide, potassium permanganate ($KMnO_4$) and formalin were effective against the parasite. Chemotherapeutics are primary tools that are constantly used in the prevention and treatment of parasitic and bacterial infections in aquaculture (Preena et al. 2020). However, these substances are not always effective against all pathogens and can present risks of environmental contamination, as well as high toxicity to fish and humans (Cruz et al. 2005). On the other hand, phytotherapeutics are considered more viable and promising alternatives for aquaculture (Tadese et al. 2022).

The mechanism of action of hydrogen peroxide is based on the formation of hydroxyl radicals, which are highly reactive oxidants that act on membrane lipids, DNA, and other cellular components of parasites, reducing infestation (Martins et al. 2021). This chemical acts through oxidative stress that releases substances that disrupt the cell membrane (Hirazawa et al. 2016).

On the other hand, potassium permanganate ($KMnO_4$) acts as an oxidizer of organic material, transforming it into relatively nontoxic manganese dioxide, which is commonly used to treat diseases of the skin and gills of fish. $KMnO_4$ at concentrations of 2.0, 5.0, 10, and 20 mg L^{-1} affected *Pseudodactylogyrus anguillae* and *P. bini* (Monogenea), with a gradual reduction in movement and death after five hours of exposure (Martins et al. 2021). In the present study, the probable cause of death of the monogeneans might be the toxic and corrosive effects of the product, since the parasite absorbed the product quickly, with changes in color (to bluish-purple) as the concentration increased, and fragmentation occurred when the product was in contact with a needle.

Andrade-Porto et al. (2017) reported that formaldehyde at concentrations above 660 mg L^{-1} was effective for treating Monogenea *Dawestrema ciclancistrum* infecting arapaima (*Arapaima gigas*). This likely occurred because formalin inactivates parasite proteins, DNA and RNA (Martins et al. 2021) which could explain the results obtained in the present study related to this chemotherapeutic.

Like salt (NaCl) is a safe parasiticide for use in freshwater fish farming (Martins et al. 2021) freshwater is already recommended for the treatment of cultured marine fish, usually when it is parasitized by monogeneans (Sanches 2008). This treatment has advantages, especially for species that support great variations in salinity. Sanches (2008) reported that bathing in freshwater for a duration of 10 min was efficacious in releasing *Neobenedenia* sp. from the grouper *Epinephelus marginatus*, whereas freshwater baths lasting 15 minutes were effective in eradicating *N. melleni* in *Epinephelus tauvina* (Jithendran et al. 2005).

Conclusion

The present study indicates that sanitary treatments with *L. origanoides*, *L. sidoides*, and *M. piperita* essential oils and freshwater baths for *N. melleni* infecting the Lebranche mullet are highly sustainable and pose

a low risk to the environment. However, the results presented here will be tested *in vivo* so that these compounds can be used safely and efficiently in the marine fish farming of Lebranche mullet (*M. liza*), which is eventually parasitized by monogeneans.

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Availability of supporting data The data related to this research are available upon prior request.

Ethical Approval All procedures involving the use of fish in this study were performed according to ethical principles in animal experimentation and approved by the Ethics Committee on the Use of Animals (CEUA) of the Federal University of Santa Catarina – UFSC, Florianópolis, SC, Brazil, under protocol number CEUA/UFSC/n° PP00861.

Competing interests The authors declare that there are no competing interests.

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