Antiparasitic potential of alternative treatments against larval stages of Lernaea cyrpiancea

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Abstract

This study evaluated the potential of alternative treatments against larval stages of *Lernaea cypriancea*. For *in vitro* test, the nanoemulsified oils of *Pinus* sp. acicule and resin were evaluated, along with Biogermex® (commercial product based on citrus biomass). For this, the motility of five larvae of the same stage (nauplii or copepodite) were evaluated in a 96-well microplate. Using the best results, on the *in vivo* test, fries of *Rhamdia quelen* were submitted to a long-term immersion bath (96 h) containing different concentrations of the product diluted directly in the water. It was possible to notice the antiparasitic potential of the resin and the acicule of *Pinus* sp., as well as the citrus biomass extract against the parasites. The nanoemulsified oils successfully inhibited the development of nauplii (10 mg L^{-1} in 24 h) and the fries showed to be tolerant to the presence of the compound (LC_{50} 96h − 16.74 mg L^{-1}). The concentration of 30.5 mg L^{-1} of Biogermex® eliminated the copepodites within 24 h, being more efficient than *Pinus* sp. when tested at the same stage, at the times analyzed. The results obtained indicate a potential use of these compounds as prophylactic agents against *L. cyprinacea*.

Introduction

The silver catfish *Rhamdia quelen* (Quoy & Gaimard, 1824) is a species with a wide geographical distribution, occurring from the central area of Argentina to southern Mexico (Silfvergrip 1996). In the state of Santa Catarina, Brazil, the species is considered promising because of the ease with which it reproduces in captivity, its efficient food conversion due to its carnivorous habits with a tendency towards omnivorous, and its acceptance by the consumer market (Radünz 2004).

The climate of the Neotropical region, of which Brazil is part, allows rapid spread of parasitic diseases and among these agents, Lernaeidae stand out because of their remarkable pathogenic action and economic importance in fish farming (Corrêa et al., 2016). In particular, the parasite *Lernaea cyprinacea* Linnaeus, 1758 is the causative agent of lerneosis and makes it impossible to market these animals because when fixed, it causes severe tegumentary lesions. Besides the body surface, it is common to find the initial life stages of these crustaceans feeding on blood and cellular residues in the gills of its host. The damage caused by the fixation of *Lernaea* spp. frequently serves as an entry point for secondary bacterial and fungal wedge infections, which can lead to massive mortalities in the nursery (Takemoto et al. 2013; Valladão et al. 2014). It is estimated that Brazilian aquaculture loses up to $84 million USD in revenue per year due to parasitosis (Tavares-Dias and Martins 2017).

Conventional treatment against parasitosis is usually done indiscriminately by applying different toxic and sometimes illegal products, which can cause environmental damage and also to the fish farming itself (Samuelsen et al. 2015; Tavares-Dias and Martins 2017). Trichlorfon (dimethyl 2,2,2, trichloro-1-hydroxyethyl phosphonate), one of several organophosphates used in aquaculture, while proven to bioaccumulate in soil and fish tissue, is harmful to human and environmental health, yet are widely used due to their effectiveness in eliminating crustacean parasites such as *Argulus*, *Ergasilus*, and *Lernaea* (Bouboulis et al. 2004; Burridge et al. 2010; Burtle and Morrison 1987; Mabilia and Souza 2006).

The efficiency of conventional treatments is related to their mechanism of action, which inhibits the synthesis of chitin (the main compound of the exoskeleton of ectoparasites), making it impossible for them to achieve their most advanced forms (Eisler 1992; Noga 2011). However, chemical residues that remain in the water can have lethal or sublethal effects on organisms not initially targeted, in addition to damaging the food chain, the biodiversity of the environment, and human health (Egidius and Moster 1987; Pillay 2004). Faced with this problem, the search for alternatives to the use of chemicals such as biodegradable compounds that are less harmful to the environment are important. Several studies have already demonstrated the efficacy of using plant extracts containing compounds with antimicrobial, anti-inflammatory and antiparasitic properties as promoters of the fish immune system (Chakraborty and Hancz 2011; Hashimoto et al. 2016; Valladão et al. 2015; Wang et al. 2015).

Species of pine are widely used in reforestation projects in Brazil. Of the residues generated during harvest, acicule represents up to 6% of the total. Previous studies highlight the antimicrobial and anti-parasitic potential in the composition of the *Pinus* sp., with potential for phytotherapy in farmed fish (Tóro et al. 2003). According to Tóro et. al. (2013), good results were achieved by using separate fractions of *Pinus elliottii* resin (α and β-Pineno) in *in vitro* tests with adult specimens of *L. cyprinacea*. At the time of the assay, both constituents were effective against the parasite at a concentration of 0.5 mg L^{-1}, in addition to establishing the LD_{50} of the raw resin for *Leporinus piau* Fowler, 1941 at 200 mg L^{-1} and confirming its potential as an antiparasitic agent. Although the literature already shows the presence of antiparasitic compounds in plants of this genus, few studies propose to verify its efficacy against parasites of economic importance for aquaculture (Nagnom and Clement 1990; Soile et al. 1990).

On the other hand, Biogermex® is a commercial, organic, and biodegradable phytotherapic compound produced from citrus biomass, which has inhibitory properties on the development of bacteria, fungi, viruses, and nematodes, and also acts by inducing resistance in animals (Silva et al. 2012). Vitamins P and C—citrus polypeptides— are a variety of fatty acids, palmitic acid, and various sugars that comprise the product. Tsuchiya et al. (1996) explain that the action of bioflavonoids (vitamin P) present in the structure of this product allows the binding of these molecules to the cell walls of bacteria and fungi, causing them to be inactivated by the rupture of their membranes. Later, this fact was proven by Mamprim et al. (2014) and Pares et al. (2017), who verified the reduction of colony forming units (CFU) and the inactivation of the fungus *Beauveria bassiana* in ideal conditions of development. Its main use is in plantations where there is a need to promote the resistance of agricultural crops to the spread of opportunistic pathogens (Vivanco et al. 2005). Despite the potential of the use of Biogermex® on crops, there are no studies about the application of this product in aquaculture.

Thus, considering the harmful potential caused by organophosphates used in the treatment against crustaceans ectoparasites on fish, the objective of this work was to evaluate the *in vitro* efficacy of different alternative treatments against larval forms of *L. cyprinacea* and to verify the median lethal concentration (LC_{50} 96h) of nanoemulsified oil of acicule and citric biomass extract (Biogermex®) in healthy fries of the silver catfish *R. quelen*.

Material And Methods

Biological material and parasite collection

After an outbreak of parasitism by *L. cyprinacea*, infected fish from a total of 13 breeders of silver catfish *R. quelen* (weight 980 ± 160 g, length 42.65 ± 2.32 cm, mean of 192 ± 156 parasites per fish, 40–479) were collected at the Fish Genetic Improvement Unit of the Agricultural Research and Rural Extension Company of Santa Catarina (EPAGRI), located at Itajaí, Santa Catarina, Brazil. The animals were transported alive to the Health of Aquatic Organisms Laboratory (AQUOS, Florianópolis, SC, Brazil) of the Federal University of Santa Catarina (UFSC), where they were acclimatized for 24 h in 1000-L units containing aqueous NaCl (non-iodized) at 3 g L^{-1} and aeration. After that period, the animals were anesthetized with eugenol (75 mg L^{-1}) and euthanized by sectioning the spine for removal and quantification of parasites (CEUA/UFSC PP00928). For this, specimens of *L. cyprinacea* were manually removed from the body surface and gills of the host with sterilized forceps. During the process, 15 adult females of *L. cyprinacea* were collected and fixed in 70% alcohol for confirmation of the species. For this purpose, the authors evaluated specific morphological characteristics that followed the descriptions proposed by Kabata (1979), Robinson and Avenant-Oldewage (1996), and Boxshall et al. (1997).

Hatching of eggs

Adult females of *L. cyprinacea*, collected from the tegument of the *R. quelen* broodstock, were transferred to a Petri dish containing deionized water, and the egg sacs were separated using sterilized histological needles. On the following day, the egg sacs were ruptured in order to release the eggs, and a light stimulus was applied to encourage the natural hatching of the nauplii. To reach the second larval stage, the nauplii were kept in Petri dishes with deionized water at 25°C for two days until they developed into copepodites.

Material collection, extraction, preparation, and determination of bioactive compounds from oils

Samples of *Pinus* sp. acicule and resin were collected from the EPAGRI's Experimental Station of Caçador (26°46'35.3"S 51°00'47.0"W, average altitude 930 m, average temperature 16.3°C, annual rainfall 1707 mm). The material was then packed in plastic bags and transported to the Essential Oils Laboratory, located at the Itajaí Experimental Station, where the preparation of nanoemulsified oils from acicule and resin was performed. From the samples of *Pinus* sp., two essential oils were extracted: one from its raw resin and the other from its acicule, both by hydrodistillation using a Clevenger apparatus. Chemical characterization was determined by gas chromatography coupled to a Shimadzu mass spectrometry detector (GCMS-QP2010) equipped with a ZB-5MS capillary column (30 m x 0.25 mm x 0.25 µm film). The programmed temperature of the injector was 250°C and the flow of the gas charger was established at 1.0 mL min^{-1}. The chromatograph was optimized with an initial temperature of 60°C for 4 min until it reached 210°C, where it remained for another 6 min, for a total of 35 min running time. The oil samples were diluted 200 times in hexane for subsequent injection into the gas chromatography/mass

spectrometry (GC/MS). Afterwards, the mass spectra were compared with the database of the GC/MS NIST libraries. Both oils had a mother solution concentration of 20,000 mg L^{-1}. The initial concentration of the majority compound (α-Pinene), evaluated alone in *in vitro* tests as a positive control, was 14,000 mg L^{-1}. In comparison, the pure concentration of Biogermex® was 1,000,000 mg L^{-1}.

In vitro**test**

Nauplii and copepodites of *L. cyprincea* (Figs. 1A and 1B) were used in independent tests. One hundred microliters of each agent were added to the first well of each row of the 96-well cell culture microplate. After that, 50 µL of distilled water were added to the second well, and a factor 2 series dilution was performed up to the 19th well. Finally, 50 µL of distilled water containing five parasites on the same stage were inserted into each well and larval activity was monitored. For the nauplii tests, the nanoemulsified essential oils of the acicule and resin were evaluated, as well as their major compound (α-Pinene) and the surfactant used as an oil dispersant (vehicle)—Tween at 0.5%—each one in separate tests. The same compounds were used to assess the mortality of copepodites with the addition of the citric biomass extract, Biogermex®.

Mortality assessment

With the aid of a stereomicroscope, the record of motility and mortality of the parasites were made at 30 min, 60 min, 120 min, and 24 h after the start of the tests. The death of the larvae was recorded when the complete absence of motility was noted, even after stimulation with a histological needle. The minimum inhibitory concentration (MIC) was determined as the last dilution of the product where there was total inhibition of the larval forms of the crustacean. All tests were performed in triplicate, including at least one control group. For the test performed with the citric acid based extract, only deionized water was used, while in tests with oils of *Pinus*, two other controls were used in addition water: 1) the dispersant Tween 0.5% (used in the preparation of nanoemulsified oils as a negative control); and 2) α-Pinene (the major compound of oils of *Pinus* sp. as a positive control). The best results found for nauplii and copepodite in the *in vitro* tests, was used for the evaluation of the median LC_{50} 96h of the respective compounds for fish.

LC_{50} 96h (acicule oil and Biogermex®)

For the mean LC_{50} 96h test, a total of 500 fries, approximately 30 days post-hatch (1.13 ± 0.33 g weight and 5.07 ± 0.63 cm length), were collected from the Fish Culture Experimental Field (CEPC/EPAGRI) and transported alive to the AQUOS laboratory. Upon arrival, the animals were equally distributed and acclimatized in two units of 100 L each containing aqueous NaCl (non-iodized) at 3 g L^{-1} and aeration, where they remained for the next 24 hours. A sample of 20 individuals was collected to confirm the health state of the animals. Once this criterion was met, the individuals were distributed to perform LC_{50} 96h.

To determine the LC_{50} 96h of alternative treatments against *L. cyprinacea*, healthy *R. quelen* fries were submitted to long-term immersion in a bath containing different concentrations of the product

(Biogermex® or acicule nanoemulsified oil) diluted in water. The products tested were those that produced the best results for *in vitro* immobilization in the nauplii phase and in the copepodite phase.

Acicule

A total of 240 specimens of *R. quelen* (5.327 ± 0.644 cm length, 1.154 ± 0.349 g weight) were distributed in 16 experimental units (n = 15). The experimental units used were rectangular in shape and had 16.5 L of useful volume, with only 10 L used in the test. For the *Pinus* sp. acicule nanoemulsified oil, the following 16 concentrations were evaluated by dilution in water: 0, 3, 5, 7, 10, 12, 15, 20, 25, 30, 35, 40, 45, 50, 100, and 150 mg L^{-1}. Every six hours the animals were observed, and the dead individuals were removed and quantified to verify the accumulated mortality for each of the evaluated concentrations. Constant and individual aeration was provided for each experimental unit throughout the test period and the animals were not fed.

Biogermex®

Following the same methodology as the acicule LC_{50} 96 h test, a total of 225 specimens of *R. quelen* (4.850 ± 0.561 cm length and 1.05 ± 0.331 g weight) were also distributed in 15 experimental units (n = 15). Here, the concentrations chosen for Biogermex® were 0, 1, 3, 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 75, and 100 mg L^{-1}. The evaluation of mortality as well as the maintenance of the animals during the experiment period followed the same procedures described for acicule. Again, at every six hours, the dead individuals were removed and quantified. Here, the aeration was also provided and the animals were not fed.

Evaluation of water quality parameters

Water quality parameters (temperature, dissolved oxygen, and pH) were measured daily, at the end of each day, using a Hanna® multiparameter probe (model HI 9828). Nitrite, toxic ammonia, and total ammonia levels were monitored every two days using the Alfakit® commercial colorimetric kit.

Data analysis and statistics

The MIC was determined as the lowest dilution of the agent at which there was total inhibition of the larval forms of the crustacean in all of the triplicates. Statistical analysis was performed using Statistica 13.0 software, and the median (LC_{50} 96 h was determined by the *Trimmed Spearman-Karber* statistical regression method according to the methodology described in Hamilton, Russo, and Thurston (1997).

Results

After evaluation with the specialized literature the adult parasites collected from the tegument of catfishes were confirmed as belonging to the taxon *L. cyprinacea*. Of the parasites, approximately 100 eggs (127.70 ± 14.14 µm long and 109.26 ± 12.64 µm wide) per ovarian sac were quantified.

In vitro test

The nanoemulsified oils of *Pinus* sp. acicule and resin were able to inhibit nauplii of *L. cyprinacea* in concentrations ranging from 10 to 19.5 mg L^{-1} (Figure 2). Despite the small range of variation between them, the oil in the resin showed greater consistency by maintaining a stable concentration for the first 24 h of the test. In the first 60 min, the concentration of 10 mg L^{-1} was able to inhibit nauplii of *L. cyprinacea*, a scenario that was maintained for the next 24 h. In contrast, it took 19.5 mg L^{-1} for the same scenario to occur when assessing the nanoemulsified oil of the acicule. After 24 h, however, the acicule attained the same performance as the resin oil, inhibiting the larval forms with a concentration of only 10 mg L^{-1}. The α-Pinene–major compound of acicule and resin oils (Table 1)—obtained a lower performance than the other solutions and inhibited the nauplii in concentrations that varied from 109.5 to 437.5 mg L^{-1}, depending on the time of analysis. Finally, the Tween was not able to stop the motility of the first larval phase of the parasite in any of the concentrations tested and times evaluated. Individuals treated with deionized water only were not affected because the larvae remained active throughout the experimental period.

For copepodites—a more resistant stage—higher concentrations of the products tested were necessary to inhibit development of the parasite. The acicule concentration, for example, varied between 39 and 78 mg L^{-1} in 24 h and 60 min, respectively, after the start of the tests (Figure 3). The concentration of the resin that inhibited the larval forms in 24 h coincided with that established for acicule in 60 min. However, for the same 60 min, the resin proved to be less effective in ceasing motility of individuals at a concentration of 312.5 mg L^{-1}. The α-Pinene inhibited copepodites at a concentration of 875 mg L^{-1}, which was maintained at this level throughout the evaluation period. The best result, however, came from Biogermex®, which inhibited the second larval phase of *L. cyprinacea* over a range that varied between 30.5 in 24 h and 244 mg L^{-1} in 60 min. after the start of the experiment. Here, none of the concentrations of Tween 0.5% or the negative control (deionized water) were able to stop the motility of copepodites, repeating what was observed in nauplii tests.

In vivo test (LC_{50} 96h)

Statistical analysis of the data indicated that a concentration of 16.74 mg L^{-1} was the LC_{50} 96h of the acicule nanoemulsified oil from *Pinus* sp. for silver catfish fries. During the experiment, the water quality parameters were maintained as follows: water temperature 24.17 ± 0.81 °C, alkalinity 16 ± 4.81 mg L^{-1}, dissolved oxygen 7.28 ± 0.48 mg L^{-1}, pH 6.69 ± 0.52, nitrite 0, and non-ionized ammonia 0.008 ± 0.002 mg L^{-1}, values within the tolerance range for *R. quelen* species (Baldisserotto and Radünz, 2004).

The behavior of the animals in the presence of Biogermex® was similar to that observed for the acicule nanoemulsified oil of *Pinus* sp. Concentrations above 25 mg L^{-1} seemed to be toxic to individuals, repeating the test scenario observed with the oil. Between the concentrations of 15 and 20 mg L^{-1}, mortalities stabilized and after data analysis, a concentration of 12.75 mg L^{-1} was determined to be the LC_{50} 96 h of citrus biomass extract for silver catfish *R. quelen* fries. During the five days of experiment, the water quality parameters remained at 25.01 ± 1.95 °C for temperature, 9.38 ± 0.99 mg L^{-1} for dissolved oxygen, 8.00 ± 0.50 for pH, 0 for nitrite, 2.45 ± 1.04 mg L^{-1} for toxic ammonia, and 0.09 ± 0.08 mg L^{-1} for total ammonia, values within the tolerance range for *R. quelen* species (Baldisserotto and Radünz, 2004).

Discussion

Despite the great economic impact on aquaculture caused by the *L. cyprinacea* parasite, few studies have sought alternatives methods of treatment that are less harmful to animals and consumers, yet equally effective as organophosphates. Among the chemicals widely used to combat the parasite crustaceans are Diflubenzuron and Teflubenzuron, which act by inhibiting chitin synthesis and preventing the early stages of the parasite from reaching more mature stages (Eisler 1992). However, problems caused by the indiscriminate use of these products on fish farms have been reported in the literature (Branson et al. 2000; Burridge et al. 2010; Rodrigues et al. 2001). Even after treatment, the effluent contaminated with these substances may retain high toxicity, such as para-chloroaniline (byproduct of DFB degradation), which has been associated with tumor formation in the spleen, bladder, and liver of rats and mice (Chhabra et al. 1991). Even with its desirable effects (the elimination of crustacean parasites from fishponds), there is a negative impact on both the treated animal and the environment. Shariff et al. (1986) tested the efficiency of different types of organophosphorus insecticides in 58 different ornamental fish species accidentally infected by *L. cyprinacea*. Despite complete elimination of the parasites in three days of treatment, the fish stopped feeding during the whole period and their use was only indicated by the authors due to the ornamental purpose of these individuals. However, the prolonged use of these substances may lead to rapid development of resistance by the pathogen by not killing it anymore, causing an opposite effect to that expected (Hakalahti-Sirén et al. 2008).

Although the antibacterial, antifungal, and antiparasitic actions of the *Pinus* genus have been verified, the antiparasitic actions are noticeably less explored than the others (Leandro et al. 2014; Salem et al. 2016; Silva et al. 2012; Tascioglu et al. 2013). In one of the only recent studies on this subject, Tóro et al. (2003) evaluated the efficiency of the crude resin oil from *Pinus elliottii* against *L. cyprinacea* infection in *Leporinus piau*. The results showed that in 24 h, concentrations between 1 and 10 mg L^{-1} were equally effective in inhibiting half of the parasites used for the *in vitro* tests. In another more recent test, Raghavendra et al. (2018) evaluated the efficacy of *Azadiracta indica* seed extract against nauplii and copepodites of *L. cyprinacea*. The authors verified that 100 mg L^{-1} of the phytotherapic compound was sufficient to stop the motility of the two larval stages and was also tolerated by the target species *Labeo fimbriatus* (Bloch, 1795) for a period not longer than 30 min. This result, still according to the authors,

may have been achieved due to the solubilization process of the extract in hot water, which possibly potentiated the release of free radicals and stimulated the antiparasitic action in the larvae. This process was not applied in this study. However, it is possible that in a new test, where the extract of *Pinus* sp. is heated before use, the result will be further improved.

The oil of *Pinus* sp. resin was able to inhibit 100% of the nauplii submitted to 10 mg L^{-1} of the product as from 60 min from the beginning of the tests. The same concentration had its effect maintained throughout the following 24 hours of the analysis, suggesting an efficient sustaining of the antiparasitic activity, even when at low concentrations. Also, here, the authors suggested the presence of one or several constituents in the *Pinus* sp. resin oil, which allowed this early and deleterious action to the larvae in the first minutes of evaluation. According to Shields and Tidd (1968), each of the three nauplii phases of *L. cyprinacea* last approximately 30 h, this being its longest period of susceptibility because it is still a free life phase and does not have its complete defense structures. In a real scenario, where the reproduction of the parasite occurs quickly and constantly, the premature action of the product already in the first minutes of application, would cause a negative effect in the first nauplii phase and would make the process of reinfection and dispersion of the disease in the nursery impossible. In this way, a greater efficiency of the resin oil over the other products tested is evidenced, since all the processes were carried out using larval phases with the same post-hatching time.

Similar to Raghavendra et al. (2018), Kone et al. (2013) evaluated a possible *in vitro* treatment using basil oil *Ocimum gratissimum* against adult parasites of *Argulus* spp. collected from *Oreochromis niloticus* Linnaeus, 1758. The authors found lethal concentrations of the extract for the parasite ranging from 194 to 200 mg L^{-1}, values almost 20 times higher than those found in the present study for *Pinus* sp. Again, the result was attributed to the synergistic effect caused by the extract because the tested controls did not result in the parasite's mortality. Although the concentrations of *O. gratissimum* seem high, Kone et al. (2013) revealed that its use would not have significant consequences for tilapia, since the LC_{50} was established at approximately 1270 mg L^{-1}. Despite this, abnormal reactions in the behavior of the animals were noted, in addition to mortalities, as also observed during *in vivo* tests. However, it is important to note that the removal of the parasite from the fish and the deprivation of mobility space for escape (*in vitro* test), may leave the individual more susceptible to the action of the compound (Kirby 1996). In contrast, in the natural environment, the *Argulus* spp. parasite tends to protect itself under the scales and gills of the fish by moving actively over the surface of the host body, a behavior that does not occur with the *Lernaea* parasites. This fact further narrows the results obtained in the *in vitro* tests and those potentially found in *in vivo* situations.

Although few studies have evaluated the use of phytotherapic compounds against crustacean parasites, most agree that synergism among the compounds is the main factor that influences the interruption or reduction of parasite development, as observed here. Tóro et al. (2003) evaluated two of the major compounds of *P. elliottii* oil alone (α and β-Pinene). Both were also found in significant amounts in the oils used in the present study (69.96 and 11.95% for acicule and 45.56 and 37.27% for resin, respectively). However, Tóro et al. (2003) concluded that neither were responsible for parasite inhibition,

which agrees with what was observed for α-Pinene in the present study. Here, when tested alone, a markedly lower performance was verified when compared to the nanoemulsified oil resin, whose efficiency is attributed to the synergistic effect generated by all nanoemulsion compounds. Nevertheless, Tween 0.5% did not interfere in the survival of the larvae, which were alive at all times and concentrations used in this experiment.

The results of the median LC_{50} 96h test revealed a margin of safe use of the acicule nanoemulsified oil for young forms of silver catfish. Ten parts per million of it would be sufficient to prevent re-infection by the parasite and apparently does not cause deleterious effects to *R. quelen* fries (LC_{50} 96h was 16.74 mg L^{-1}), highlighting the potential use of the product as a prophylactic agent. Even so, it is important to point out that early stages of fish development are proven to be more sensitive than advanced stages due to incomplete development of the immune system and lymphoid organs (Ellis 1988; Hrubec et al. 2004; Tatner and Manning 1983), which may mean an even greater margin of use of the product in the treatment against *L. cypriancea*. Barry et al. (1995), for example, evaluated the effects of different post-closure periods on the tolerance of *Melanotaenia fluviatilis* larvae Castelnau, 1878 against exposure to a synthetic insecticide used in crop pest control. By submitting animals between 1 and 90 days post-hatch, the authors found an increasing resistance to the chemical as the individuals matured. While the mean lethal dose (LD_{50} 96h) established for newly hatched animals was 2.32 µg L^{-1}, the tolerance increased significantly to 3.960 µg L^{-1} after 90 days. With this in mind, it is suggested that in future evaluations using adult animals, the resistance of the animals to the presence of the oil should be higher than that observed in this study, offering an effective alternative treatment clearly able to prevent the arrival of the parasite during later stages. Moreover, it should be noted that the residues generated and discarded during the forest harvesting of *Pinus* wood can be used to boost the future development of a commercial product through the reuse of this material. It is also important to comment on the importance of conducting studies that, like this one, verify the feasibility of using phytotherapeutic drugs in the control of parasites of economic importance to fish farms. In this way, the result is beneficial not only for the product's handler, who is often exposed to chemical compounds used on crops, but also for the consumer, who will have a greater amount of fish protein available at more affordable prices.

Biogermex® is a compound formed by citric bioflavonoids and ascorbic acid, substances that induce the extrusion of stressing agent cell membranes (Mamprim et al. 2014; Pares et al. 2017; Tsuchiya et al. 1996). Despite these promising scenarios, its use as a prophylactic method in aquaculture has not been evaluated until now. In this study, 30.5 mg L^{-1} of Biogermex® were sufficient to eliminate the second larval stage of *L. cyprinacea* in 24 h, proving to be more efficient than the acicule nanoemulsified oil of *Pinus* sp. when tested in the same stage at the analyzed times. In contrast, *R. quelen* fries was susceptable to small concentrations of citric extract (12.75 mg L^{-1}), which would make its use for early stages of fish development impossible. It is worth mentioning, however, that the copepodite is a phase known to be more resistant than the nauplii, and it has fully developed appendages that will become part of the adult individual after metamorphosis (Kabata 1979). Nevertheless, Lahav et al. (1964) revealed that the moment of greatest susceptibility of *Lernaea* larvae is during molting between the first and

second larval stages. Before and after this process, the larvae proved to be more resistant to the use of chemicals and phytotherapic compounds. Therefore, it is suggested that new tests check the potential for inhibition of *L. cyprinacea* nauplii using Biogermex®, which can effectively stop the development of the parasitic larvae at reduced concentrations.

Conclusion

Based on the results found in this study, the antiparasitic potential of the nanoemulsified oils of resin and acicule of *Pinus* sp., as well as of citric biomass extract (Biogermex®), against nauplii and copepodites of *L. cyprinacea* should be noted. The major compound in both oils (α-Pinene) did not succeed alone in interrupting the life cycle of *L. cyprinacea* at low concentrations, suggesting that synergism between α-Pinene and the other compounds were the real cause of the adverse effects on the parasite larvae. The nanoemulsified oil of *Pinus* sp. acicule successfully inhibited the development of *L. cyprinacea* nauplii (10 mg L^{-1} 24 h), and the silver catfish *R. quelen* fries tolerated the presence of the compound (LC_{50} 96h, 16.74 mg L^{-1}). The nanoemulsified oil from the *Pinus* sp. resin also showed good results in the initial in vitro tests for both nauplii and copepodites. The authors also recommend new tests for median LC_{50} using larger fish, where an even greater tolerance for the oils is expected. Even so, the results obtained so far indicate the potential use of the extracts from acicule and resin of *Pinus* sp. as a prophylactic agent against *L. cyprinacea*.

Declarations

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STATEMENT OF ANIMAL ETHICS

All the animal procedures used in this project was approved by the Ethic Committee on Animal Use of Federal University of Santa Catarina (CEUA/UFSC/PP00928).

AUTHORS CONTRIBUITIONS

WF worked on the assessment, system`s maintenance, processing of the material analyzed, and wrote the first draft of the manuscript. LC, PM, NL and EB were the technical team who assisted during the assessment of the alternative treatments. MM, FB, and NM contribute with the data analysis and article editing. All authors contributed to the article and approved the submitted version.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Tables

Table 1. Chemical characterization of nanoemulsified oils from acicule and resin of *Pinus* sp.

Compounds	CAS	TR	IRc	IRt	Acicule (%)	Resin (%)
α-Pinene	80 - 56 - 8	6,32; 6,25	937; 935	936	69,96	45,56
Camphene	79 - 92 - 5	6,71	951	951	-	0,63
β-Pinene	127 - 91 - 3	7,54; 7,50	980; 979	980	11,95	37,27
β-Myrcene	123 - 35 - 3	7,84	991	992	1,47	
NI	-	8,14	1001	-	-	0,78
α-Terpinene	99-86-5	8,61	1018	1020	-	0,83
β-Cymene	535 - 77 - 3	8,83	1026	1027	-	8,68
Carvomentheno	5502 - 88 - 5	8,96	1030	1032	-	2,75
NI	-	8,99	1032	-	0,69	-
β-phellandrene	555 - 10 - 2	9,05; 9,01	1033; 1032	1033	2,46	0,72
gamma-terpinene	99-85-4	9,79	1060	1060	-	0,91
cis-dihydro-alpha-terpineol	7322-63-6	12,30	1152	1152	-	1,87
NI	-	13,52	1198	-	0,51	-
Caryophyllene	87 - 44 - 5	18,89	1427	1428	2,99	-
epi-bicyclosesquiphellandrene	54324-03-7	20,20	1488	1488	3,75	-
NI	-	20,51	1503	-	2,87	-
δ-cadinene	483 - 76 - 1	20,95	1525	1524	2,47	-
NI	-	23,42	1650	-	0,23	-
NI		23,68	1663		0,65	-
total identified	-	-	-	-	95,05	99,22
total non-identified‡	-	-	-	-	4,95	0,78

NI = unidentified component, TR = retention time (min), IRc = calculated retention rate, IRt = retention rate found in literature (NIST, 2018), % = percentage of component in the essential oil, † = TR and IRc values separated by semicolons refer to the essential oils of acicule and resin, respectively, and ‡ = components differing in interpretation by mass spectra or with a retention rate not consistent with values found in the literature.

Figures

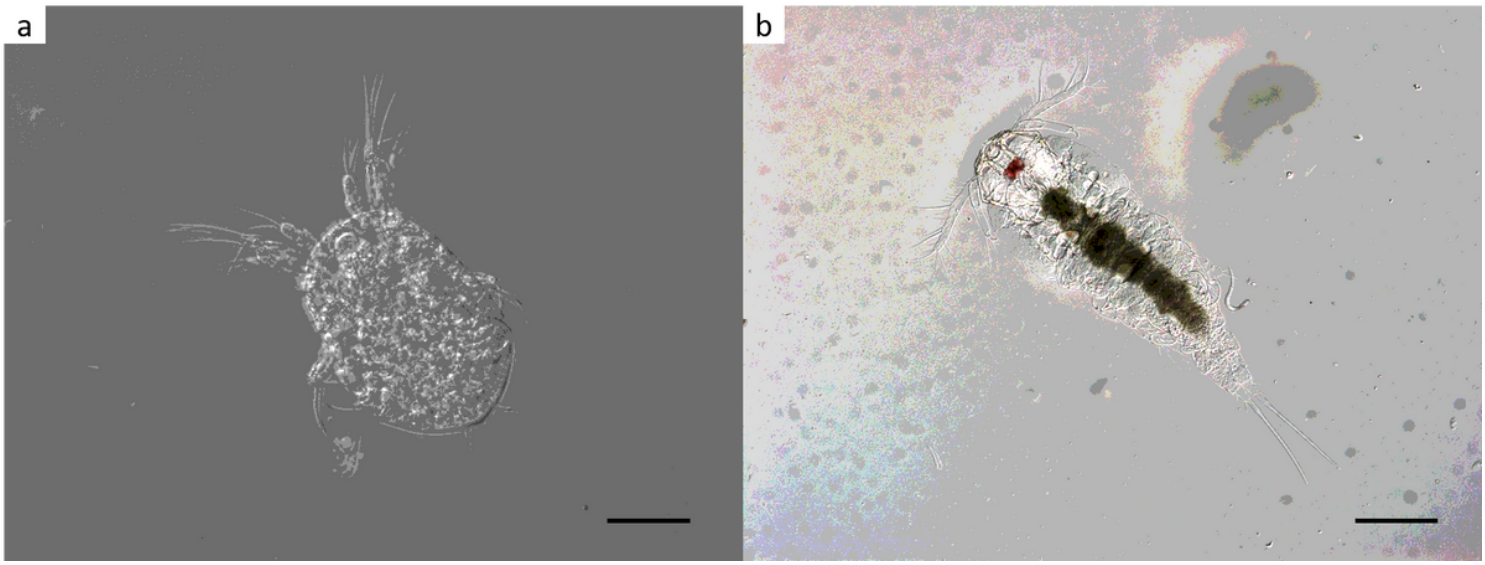

Figure 1

Larval stages of the crustacean parasite Lernaea cyprinacea. a) Nauplii (Scale bar = 50 µm); b) Copepodite (Scale bar = 200 µm).

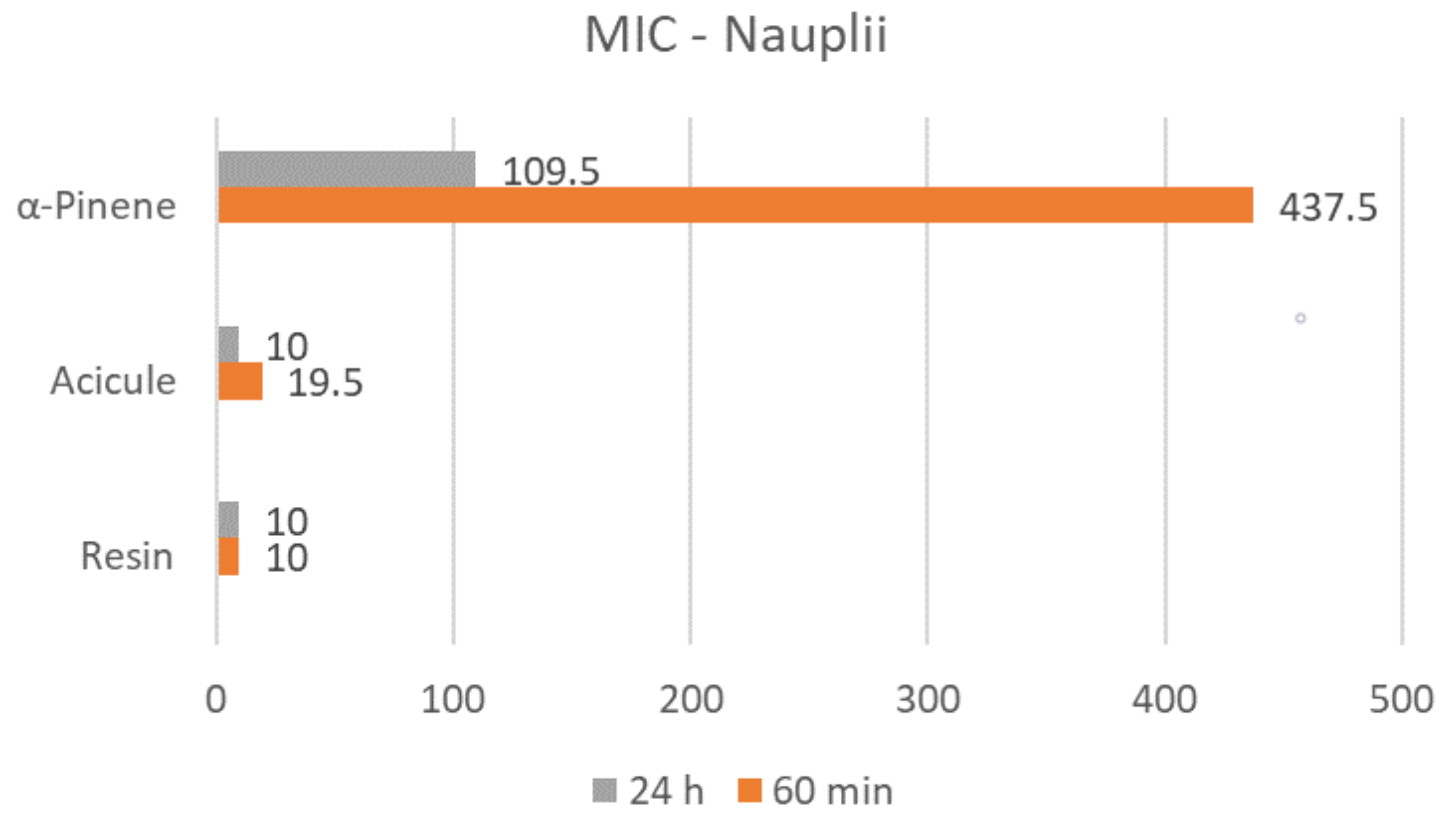

Figure 2

Minimum inhibitory concentration (MIC) of nanoemulsified oils from acicule, resin, and the major compound (α-Pinene) of Pinus sp. (in mg L-1) on the first larval phase of Lernaea cypriancea (nauplii) collected from silver catfish Rhamdia quelen.

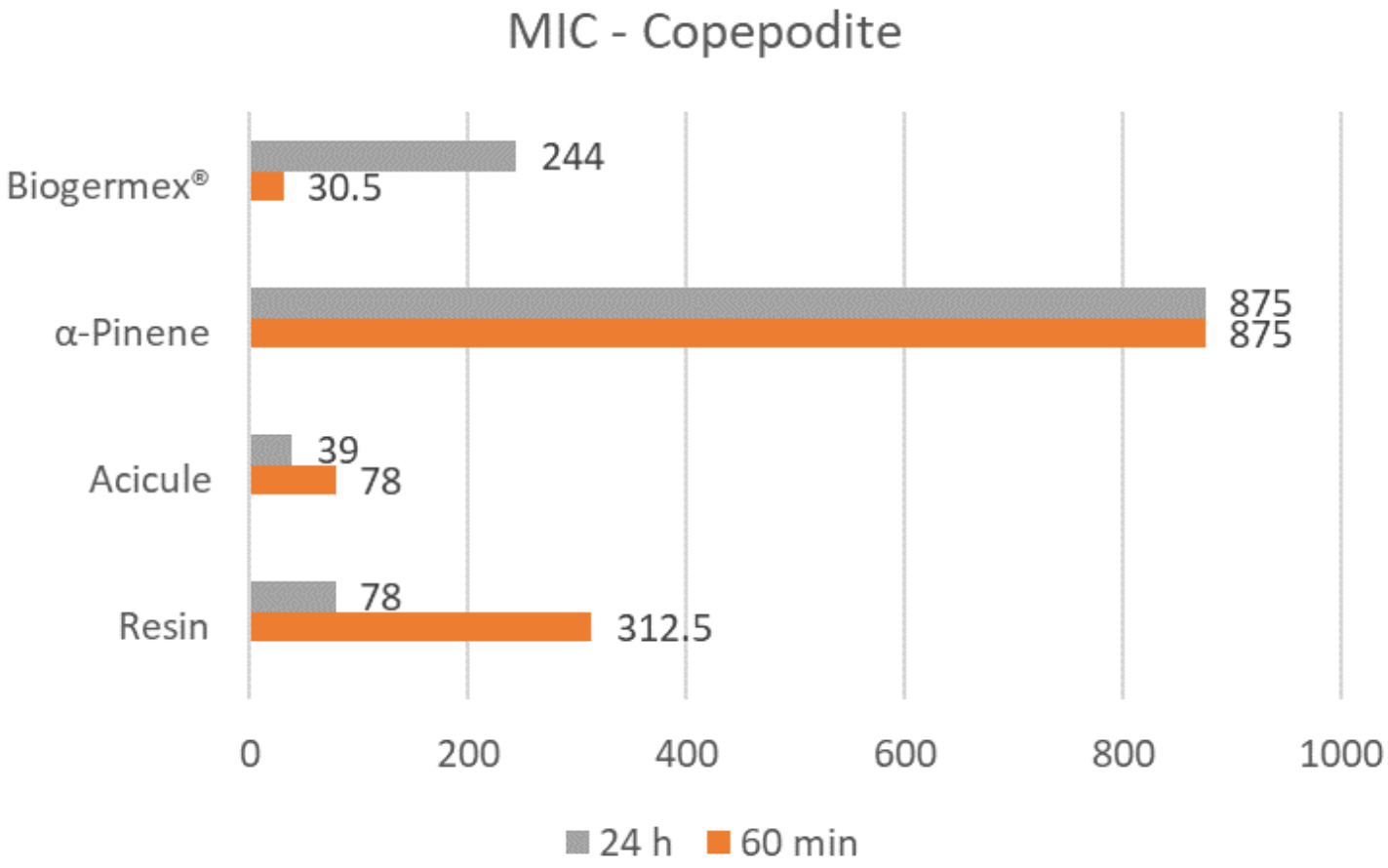

Figure 3

Minimum Inhibitory Concentration (MIC) of Biogermex® and nanoemulsified oils of acicule, resin, and the major compound (α-Pinene) of Pinus sp. (in mg L-1) on the second larval phase of Lernaea cypriancea (copepodite), collected from silver catfish Rhamdia quelen.