Biotechnology Research and Innovation, 2025, 9(2): e2025003. https://doi.org/10.4322/biori.00032025

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Biotechnology Research & Innovation

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RESEARCH PAPERS

In vitro activity of free and nanostructured essential oils of Ocimum basilicum, Cymbopogon citratus and *Eucalyptus globulus* in *Fasciola hepatica* eggs

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Highlights

- Natural products were presented as non-toxic and effective against *Fasciola hepatica*
- Geranium and sour orange essentials were effective inactivating eggs of *F. hepatica*
- Essential oils caused changes in the tegument of all treated specimens

Received 02 June, 2025; Accepted 03 December, 2025.

KEYWORDS
Nanostructured;
Phytotherapy;
Trematode;
Ovicidal;
Alternative control.

Abstract: Fasciolosis is one of the most important parasitic diseases in animal health, primarily affecting ruminants and causing economic and productivity losses. This study aimed to evaluate the *in vitro* ovicidal activity of essential oils from *Ocimum basilicum* (Basil), *Cymbopogon citratus* (Lemongrass), and *Eucalyptus globulus* and their respective nanostructures in *Fasciola hepatica* eggs at concentrations of 0.5, 1, and 1.5%. The eggs were deposited in six-well plates along with the free and nanostructured essential oils at their respective concentrations, packaged in aluminum paper, and incubated at 27°C and 80% humidity in a B.O.D. for 14 days. As a positive control, therapeutic dose albendazole (0.025 mg) was used. For negative control eggs only and water, blank nanostructure was used to control the nanostructured samples. Egg counts for hatched and total eggs were performed at 14 and 21 days using an inverted microscope. For the tests conducted with free and nanostructured essential oils of lemongrass and basil, better results were obtained in all tested concentrations. However, neither free nor nanostructured eucalyptus oil showed proven efficacy against *F. hepatica* eggs in this study.

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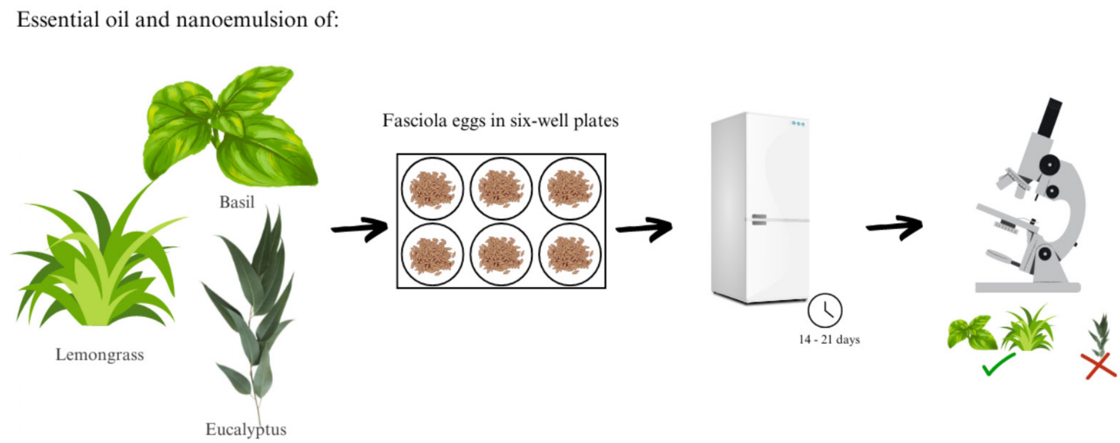
Graphical Abstract



Introduction

Fasciola hepatica (Linnaeus, 1758) is a trematode parasite that infects the liver and bile ducts of ruminants, horses, rabbits, pigs, rodents, and occasionally humans (Ueno et al., 1975). Considered one of the most important helminths in domestic ruminants, causing losses in herd productivity due to interference in animal development, reduced fertility, decreased milk and meat production, liver condemnation in slaughterhouses, and expenses related to parasitic control (Queiroz et al., 2002; Arias-Pacheco et al., 2020).

The spread of the parasite is closely linked to the presence snails of the family Lymnaeidae, the intermediate host of *F. hepatica*. There are ecological factors that are vital and of fundamental importance for fasciolosis, including hosts, climate, temperature, humidity, aquatic flora, soil chemical composition and adequate water supply, all of which favor the development of this pathogen (Queiroz et al., 2002; Carneiro et al., 2013).

Fasciolosis, being a disease affecting both humans and animals, is considered an emerging or re-emerging zoonosis in various parts of the world (Villegas et al., 2012). Because it is a zoonosis, fasciolosis is of great veterinary importance, and it can also affect public health (Pile et al., 2001). Infection in humans can occur through the consumption of special foods prepared with raw liver in Asian countries (Schacher et al., 1965). However, human fasciolosis is primarily associated with the ingestion of watercress or other aquatic plants infected with metacercariae (Taira et al., 1997). The disease is characterized by its attraction to the liver and bile ducts, with clinical manifestations ranging from asymptomatic forms to severe disease (Caravedo & Cabada, 2020).

Fasciolicidal drugs are essential for controlling this parasitosis, and they should be easy to administer, cost-effective, non-toxic, leave no residues, and be highly effective against both adult and immature parasite forms. Additionally, acquiring animals with health certification is crucial in endemic regions (Cunha et al., 2007). Misuse of these drugs can lead to the development of resistance, making it necessary to implement usage guidelines and develop alternative control methods, such as vaccines (Fairweather & Boray, 1999). Benzimidazoles are broad-spectrum anthelmintic compounds used in veterinary medicine and in humans to control infections caused by nematodes, cestodes, and trematodes (McKellar & Scott, 1990). Medicinal plants or their bioactive compounds have frequently been used to control diseases caused by viruses, bacteria, fungi, and parasites in humans, as well as in veterinary medicine (Soares & Tavares, 2013; Pandey, 2014).

The search for new treatment alternatives to replace harmful treatments for this trematode justifies the high level of research seeking medicinal plants for the control of gastrointestinal nematodes (Rates, 2001). Recent studies have also demonstrated the potential of alternative control strategies on F. hepatica eggs, such as those by De Mello et al. (2023a, b), Ezeta-Miranda et al. (2024), and Cassani et al. (2025), reinforcing the relevance of natural products and new technologies for sustainable parasitic management. Furthermore, nanotechnology has been studied for the use of nanodrugs, such as nanoemulsions, which are formulations with nanoscale sizes, typically between 20 to 200 nm. They are primarily characterized by their excellent suspension stability due to their reduced dimensions (Solans et al., 2005, Anton et al., 2008). In this context, the objective of this study was to evaluate the *in vitro* activity of free essential oils (EOs) from *Ocimum basilicum* (Basil), *Cymbopogon citratus* (Lemongrass), and *Eucalyptus globulus* (Eucalyptus), as well as their respective nanostructures, on *F. hepatica* eggs.

Materials and methods

Obtaining oils and nanoemulsions

The oils and nanoemulsions used in the research were provided by the Nanotechnology Laboratory of the Franciscana University (UNF) in Santa Maria, RS. The *in vitro* evaluation of the efficacy of the free essential oils from *Ocimum basilicum* (Basil), *Cymbopogon citratus* (Lemongrass), and *Eucalyptus globulus*, as well as their nanoemulsions, on eggs, was conducted at concentrations of 0.5%, 1%, and 1.5%.

Development and characterization of nanoemulsion

The characterization of the oils followed the method described by Hussain et al. (2010) with modifications (Gündel et al., 2018), using the Varian Star 3400CX gas chromatograph (CA, USA). For the qualitative analysis of compounds, a Shimadzu QP2010 Plus gas chromatograph coupled to a mass spectrometer (GC/MS, Shimadzu Corporation, Kyoto, Japan) was used (Gündel et al., 2018).

The oils in nanoemulsions were developed using high-speed homogenization, following the methodologies described by Gündel et al. (2018). The formulations consisted of an oil phase containing oil (5%) and sorbitan monooleate surfactant (2%), while the aqueous phase consisted of polysorbate 80 (2%) and ultrapure water. Both phases were homogenized separately using a magnetic stirrer, and during the aqueous phase, they were placed in the Ultra-Turrax® equipment (IKA, Germany) for 10 minutes at 10,000 rpm. Subsequently, the oil was added to the aqueous phase and kept in the Ultra-Turrax® for 30 minutes at 17,000 rpm, with temperature control. The blank nanoemulsion was developed from a medium-chain triglyceride derived from caprylic and capric acids.

The physical-chemical characterization of the formulation was performed by determining the average droplet size, polydispersity index, zeta potential, and pH. The average droplet size and polydispersity index were determined using dynamic light scattering technique (Zetasizer® equipment, nano-ZS model ZEN 3600, Malvern) after sample dilution (500 times) in ultrapure water. The zeta potential was determined using the electrophoretic mobility technique (Zetasizer® equipment, model nano-ZS ZEN 3600, Malvern) after sample dilution (500 times) in an aqueous solution of sodium chloride (10 mM). The pH was determined using a pH meter (DM-22, Digimed®) previously calibrated with standard solution, and the readings were taken directly from the formulations. Readings were taken in triplicate, and the results are expressed as mean ± standard deviation (Gündel et al., 2018).

Collecting and obtaining eggs of *F. hepatica*

Eggs of *F. hepatica* were collected from livers that tested positive during post-mortem examination at two slaughterhouses in the municipality of Pelotas, Rio Grande do Sul. The collected trematodes were stored in glass vials containing saline solution heated to 30°C to stimulate the release of eggs. The eggs were washed three times with distilled and filtered water through a set of four sieves with mesh sizes of 100, 150, 180, 200, 250, and 300 meshes per inch (Girão & Ueno, 1985). Subsequently, they were stored in labeled glass containers containing sterile distilled water and refrigerated until the preparation of the egg hatching assay (Ueno & Gonçalves, 1998).

In vitro assay

The eggs were deposited in six-well plates based on methodologies (Robles-Pérez et al., 2014; Arafa et al., 2015), along with the free and nanostructured EOs oils according to their concentration. The test was performed in triplicate for each tested concentration. Distilled water and eggs were used as the negative control, while for the nanostructured control, blank nanoemulsion and distilled water were used. Lastly, albendazole at a concentration of 0.025 mg (therapeutic dose) was used as the positive control.

The plates were then wrapped in aluminum foil to prevent the eggs from being exposed to ambient light. Subsequently, the plates were incubated at 28°C with 80% humidity in a B.O.D. incubator (Eletrolab brand, model EL 101/03) in a dark environment for 14 days at a controlled temperature of 27°C. On the fourteenth day of incubation, the plates were exposed to light for 2 hours to stimulate the emergence of miracidia (Robles-Pérez et al., 2014; Arafa et al., 2015). Egg hatching and total egg counts were conducted at 14 and 21 days using an inverted microscope (Brand: Olympus - CKX 41).

Statistical analysis

The efficacy of each tested was calculated using the formula: efficacy (%) = [(average percentage observed in the negative control – average percentage observed in the treated group)/average percentage of the negative control×100. This calculation expresses the relative effectiveness of treatment compared with the untreated control (Wood et al., 1995). For statistical evaluation, a one-way analysis of variance (ANOVA) was applied with a significance threshold of $p < 0.05$, followed by Tukey's post hoc test using GraphPad PRISM® 8 software.

Results

The nanoemulsion formulation used exhibited nanometric size, with the average size of all nanoemulsions ranging from 66 to 127 nm, and a homogeneous size distribution as observed in the polydispersity index. The zeta potential was measured at pH. The nanoemulsions containing *C. flexuosus*, *O. basilicum*, and *E. globulus* had, respectively, an average particle size of approximately 98 ± 0.82, 113 ± 0.78, 66 ± 0.81 nm; a polydispersity index of 0.20 ± 0.007, 0.19 ± 0.007, 0.23 ± 0.004; zeta potential of -7.48 ± 1.02, -8.47 ± 0.98, -7.53 ± 0.72, and acidic pH of 4.00 ± 0.07, 5.37 ± 0.04, 5.90 ± 0.04. The blank nanoemulsion maintained an average particle size of 127 ± 1.02, a polydispersity index of 0.25 ± 0.005, zeta potential of -6.37 ± 0.58, and a pH of 5.15 ± 0.03. The nanoemulsions, when kept under refrigeration, remained stable for 90 days, as the parameters were maintained when compared to the parameters immediately after preparation. Nanoemulsions containing essential oils are dispersed systems made up of immiscible liquids that can be used in low therapeutic concentrations to control and treat *F. hepatica* eggs.

In the gas chromatography carried out, for the essential oil (EO) of *C. flexuosus* used in our study were β-geranial (45.74%), Z-citral (34.42%), and geraniol (6.01%). The components of *O. basilicum* oil were also determined, obtaining the main constituents: ursol (87%), 1,8-cineole (3%) and trans-alpha-bergamotene (2%). In oil the *E. globosus* oil, the main identified components were 1,8-cineole (75.7%); p-cymene (7.5%); alpha-pinene (7.3%) and limonene (6.4%). The results of the assays with the three free and nanostructured essential oils from lemongrass, eucalyptus, and basil tested on *F. hepatica* eggs are described below (Figure 1).

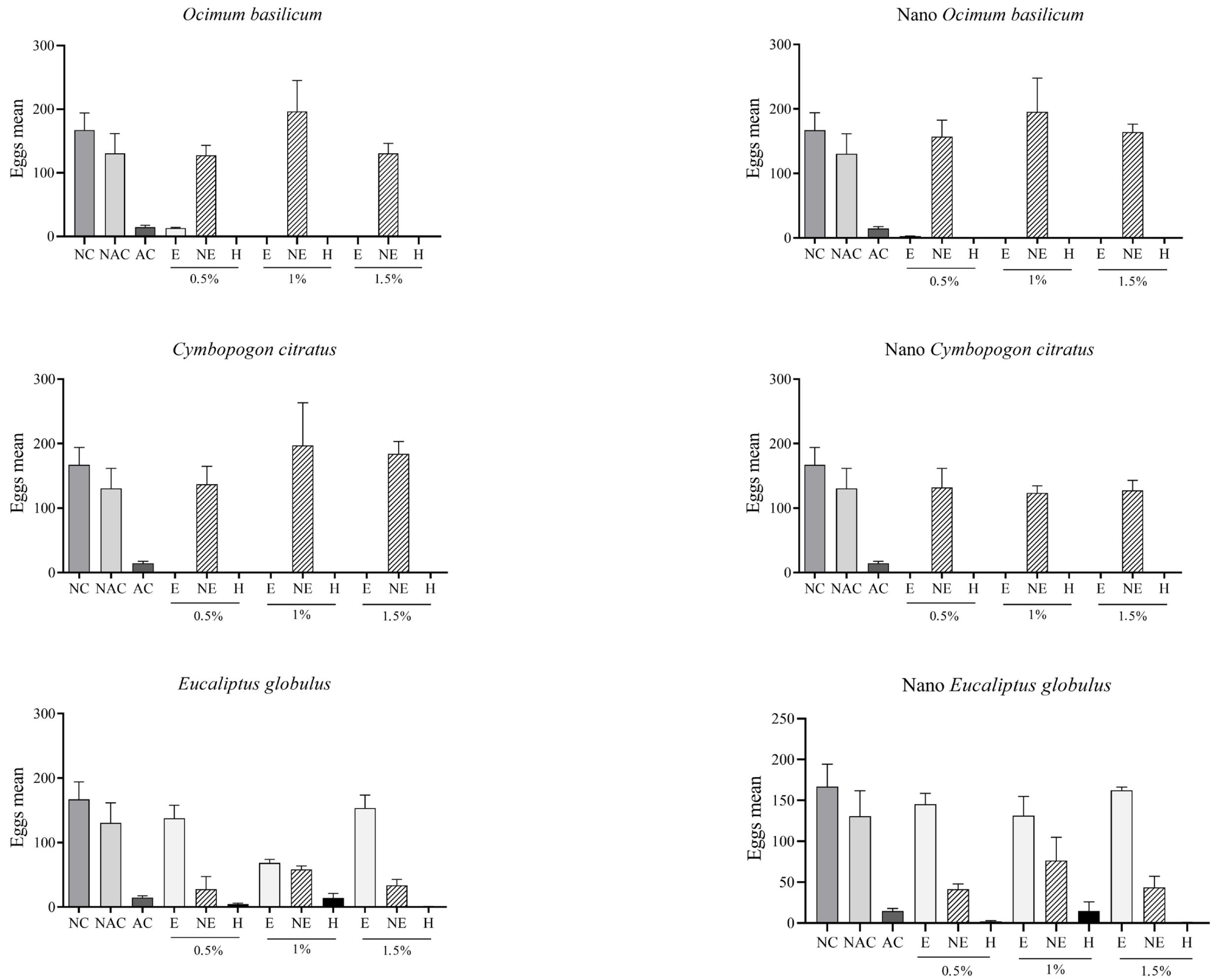


Figure 1. Mean of eggs obtained in the tests with essential oil of *Ocimum basilicum*, *Cymbopogon citratus* and *Eucaliptus globosus* and their respective nanoemulsions. NC – Negative control; NAC – Negative controle nanoemulsion; AC – Albendazol control; E – Embryonated egg; NE – Non-embryonated egg and H – Hatched egg. The controls presented (NC, NAC and AC) represent the average number of embryonated eggs.

In the plates used for the negative control, we obtained 60% of embryonated eggs, 20% non-embryonated eggs, and 20% hatched eggs. In the control plate containing nanowhite, we obtained 70% of embryonated eggs, 29.5% of non-embryonated eggs, and 0.5% hatched eggs. In the positive control plate using 0.025mg of albendazole, we obtained a considerably higher rate of non-embryonated eggs (80%), with only 20% being embryonated and 0% hatched eggs, demonstrating the effectiveness of the substance. In our results, we obtained efficacy of the free EO of *Cymbopogon citratus* (lemongrass) and its respective nanostructured oil with 100% efficacy at all concentrations against *F. hepatica* eggs, with no statistical difference in readings at 15 and 21 days. When analyzing the efficacy of the *Ocimum basilicum* (basil) oil, at a concentration of 0.5%, it showed 95% efficacy, while at the other concentrations, it demonstrated 100% efficacy. Conversely, when assessing the nanostructured oil, the concentration of 0.5% achieved 98% of non-embryonated eggs. At the subsequent concentrations (1 and 1.5%), we achieved 100% non-embryonated eggs.

Finally, in our study, when the plates related to free and nanostructured eucalyptus oil were observed, in comparison to the others used and the fasciolicide medication, they did not yield satisfactory results, presenting more than 50% of embryonated or hatched eggs.

Discussion

Reports of the ineffectiveness of various active principles in controlling various parasitic infections have become recurrent (Molento, 2004; Fissiha & Kinde, 2021). Resistance of *F. hepatica* to the fasciolicides used has already been described for clorsulon, albendazole, and triclabendazole, a first-line medication (Moll et al., 2000; Martínez-Valladares et al., 2014). For this reason, the use of alternative measures in treatment has been gaining prominence in research. The use of essential oils represents another option for phytotherapeutic approaches and is among the classes of substances that have reported antiparasitic activity (Anthony et al., 2005). The major compounds were determined using gas chromatography. The main constituents identified in the EO of *C. flexuosus* were ß-geranial, Z-citral and geraniol, mentioned before. Similar results were found by Pandey et al. (2003), who identified the same major compounds as citral (43.80%) and Z-citral (18.93%).

The components of *O. basilicum* oil were also determined, obtaining the main constituents: estragole, 1,8-cineole and trans-alpha-bergamotene. Falowo et al. (2019) reported that the chemotypes estragole (41.40%), 1,6-octadien-3-ol, 3,7-dimethyl (linalool) (29.49%), bergamotene (5.32%), and eucalyptol (3.51%) were the major extracted components. However, Chenni et al. (2020) reported the variation of 1,8-cineole (6.8% and 7.3%), which is also present in the oil.

The EO *E. globosus*, the main identified components were 1,8-cineole, p-cymene, alpha-pinene and limonene. This is consistent with the study by Boukhatem et al. (2014), which found two similar major compounds, namely 1,8-cineole (51.083%) and alpha-pinene (24.6%). Similarly, Silvestre et al. (1997) identified the same major compounds in their analyses, although the concentrations differed. Other investigations have also reported the activity of essential oils against *F. hepatica*, both in the egg and adult stages, reinforcing the possibility of their use as alternative strategies to chemical fasciolicides (De Mello et al., 2023a, b; Ezeta-Miranda et al., 2024). These studies describe different mechanisms of action, such as interference with egg embryonation, reduction of larval viability, and direct effects on adult parasites (Cassani et al. 2025).

Several studies demonstrate the potential of different essential oils against *Fasciola* spp., such as Jeyathilakan et al., (2010), who evaluated the essential oils of *Cymbopogan nardus* and *Azadirachta indica*. Their results indicate that citronella essential oil showed anti-helminthic activity (100%), while neem oil did not have an effect. Their findings suggested the potential for developing plant-based anthelmintics to control *F. gigantica*. In addition to these, Da Silva et al. (2020) used cumin oil against *F. hepatica* eggs and obtained satisfactory results, where at a concentration of 0.03 mg/ml, it inactivated the eggs with 99% efficacy. Studies were also conducted with *F. hepatica* eggs and adults, testing the essential oil of *Cuminum cyminum* (cumin), where it was effective against the trematode at low concentrations, with 0.031125 mg/ml for eggs and 0.06225 mg/ml for adults (De Mello et al., 2023a). In addition to these, the oils of *Pelargonium graveolens* and *Citrus aurantium* sour were tested against *F. hepatica*, showing satisfactory results against both the trematode's eggs and adults (De Mello et al., 2023b).

The results obtained indicate that plant-derived oils may represent an important tool within integrated parasite control programs, either as complementary or substitute therapies, especially in the face of increasing drug resistance (Da Silva et al., 2020). According to Fairweather (2011), chemical treatment is considered appropriate when there is a reduction of 95% in efficacy. In this study, the count of embryonated eggs demonstrates that Albendazole was effective (80% non-embryonated eggs) at the therapeutic dose.

Cymbopogon citratus is popularly known as lemongrass (Abegaz et al., 1983). Regarding its therapeutic properties, it is known that the essential oil has antibacterial and antifungal action (Carriconde et al., 1996). Both the hydroalcoholic and aqueous extracts exhibit a significant analgesic effect, similar to that of the isolated EO, tested in rats, where they demonstrated the inhibition of pain-inducing substances such as carrageenan and prostaglandin E-2. Citral, the main component of the oil, possesses larvicidal and insect-repelling properties (Plata-Rueda et al., 2020), these studies justify the positive efficacy of this oil.

Sunita et al. (2014) assessed the seasonal variation in the toxicity of citral, the major compound in lemongrass, on sporocysts, rediae, and cercariae of *F. gigantica*, obtaining positive results in the mortality of cercarial larvae, untested stages of *Fasciola* sp. in our studies. Furthermore, there are studies that show the anthelmintic efficacy of the oil, as in the study by Jeyathilakan and collaborators (2010), who tested *Cymbopogon citratus* oil at concentrations of 0.1, 0.5 and 1%, where only the A concentration of 1% was effective against *Fasciola gigantica*, differing in the concentration of effectiveness, since in our study the oil was effective in all concentrations tested (0.5, 1 and 1.5%).

The EO of *O. basilicum* (basil) is already known for its repellent action by various authors (Rao et al., 2000; Prajapati et al., 2005). Several authors (Mwangi et al., 1995; Murugan et al., 2007; Kostic et al., 2008; Del Fabbro & Nazzi, 2008) have evaluated this free essential oil and its isolated compounds, demonstrating insecticidal and acaricidal action. Studies have also reported the activity of lemongrass essential oil on protozoa such as *Leishmania* spp., *Trypanosoma brucei*, and *Plasmodium falciparum*, as well as *Haemonchus contortus* (Machado et al., 2012; Kpoviessi et al., 2014; Macedo et al., 2015). In the study by Alimi et al. (2022) *O. basilicum* oil and its main compounds showed acaricidal and nematocidal activities., in concentrations ranging between 0.73 and 0.97 mg/mL.

On the other hand, the essential oils of *Eucalyptus* spp. are well-known for their anthelmintic properties (Araújo-Filho et al., 2019). The essential oils of *Eucalyptus citriodora* and *Eucalyptus globulus* have been previously described to reduce the hatching of namatode *H. contortus* larvae (98.8% and 99.3%) (Macedo et al., 2009; Macedo et al., 2011), differing from our study. Our results with nanostructured products were different from Carvalho et al. (2012), who demonstrated that the use of eucalyptus essential oil was completely satisfactory for *Haemonchus contortus*, albeit with higher concentrations, suggesting a higher dosage may be needed to potentially make the oil effective against *F. hepatica*.

Conclusion

Based on the findings of this study, it can be concluded that both free and nanostructured essential oils had an effect on *Fasciola hepatica* eggs. Lemongrass and basil essential oils yielded better results at all tested concentrations, with the 0.5% concentration being considered the most effective regardless of the treatment. However, there was no proven efficacy of free or nanostructured eucalyptus oil against *F. hepatica* eggs in the experiment.

Conflict of interests

The authors declare no conflicts of interest.

Funding: The authors thank the Federal University of Pelotas, Coordination for the Improvement of Higher Education Personnel Brazil (CAPES) (Finance Code 001) and PROAP/CAPES.

Acknowledgements

The National Council for Scientific and Technological Development (CNPq), the Coordination for the Improvement of Higher Education Personnel - Brazil (CAPES). This study was partially funded by the Coordination for the Improvement of Higher Education Personnel - Brazil (CAPES) – Finance Code 001. The authors are grateful to the following: Federal University of Santa Maria (UFSM) and Frigorífico Famile for the contribution and support.

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