Received: 31 March 2020 | Revised: 10 May 2020 | Accepted: 12 May 2020

DOI: 10.1111/are.14728

ORIGINAL ARTICLE

Aquaculture Research WILEY

Essential oil of *Lippia grata* (Verbenaceae) is effective in the control of monogenean infections in *Colossoma macropomum* gills, a large Serrasalmidae fish from Amazon

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Funding information
Conselho Nacional de Desenvolvimento Científico e Tecnológico, Grant/Award Number: 303013/2015-0

Abstract

This study investigated the efficacy of *Lippia grata* essential oil (EO) against monogeneans of *Colossoma macropomum* and effects on the haematology after immersion baths. In the in vitro assays, the efficacy of 100, 250, 350 and 700 mg/L of *L. grata* EO and two controls were tested, of which one control was with the cultivation tank water and the other was with the tank water and 70% ethyl alcohol. Composition of majority bioactive compounds in the EO was carvacrol (48.12%), p-cymene (24.39%) and γ-terpinene (2.49%). *Anacanthorus spathulatus*, *Notozothecium janauachensis*, *Mymarothecium boegeri* and *Linguadactyloides brinkmanni* obtained of fish gills showed 100% immobilization in the in vitro assays when exposed to 700 and 350 mg/L after 30 min and 2 hr respectively. Fish showed tolerance to 700 mg/L of the EO, which was used in therapeutic in three consecutive baths, which caused lamellar hyperplasia in *C. macropomum* gills. The 700 mg/L of the EO in the therapeutic baths showed an efficacy of 95.1% against the monogeneans and caused decrease in haemoglobin, whereas control fish showed a reduction in mean corpuscular haemoglobin concentration. Results indicated that 700 mg/L of *L. grata* EO is a safe and effective concentration to treat *C. macropomum* infected with monogeneans.

KEYWORDS

aquaculture, haematology, parasites, therapeutic bath, treatment

1 | INTRODUCTION

An increasing global population and economic growth have led to rising demands for animal protein sources to meet human consumption. In 2016, aquaculture contributed 47% of global fish consumption that was met by a production of 54.1 million tons of harvested biomass and has potential for growth. Aquaculture has grown 5.8% between 2001 and 2016 and is projected to be among the major contributing sectors for supplying animal protein to markets (Food and Agriculture Organization (FAO) of the United Nations, 2018; Hoai, 2020). However, expansion of aquaculture activities requires advances in management of parasitic diseases, which is one of the major limitations of fish production.

Infections caused by monogeneans are among the most problematic diseases in fish farming worldwide and may compromise the economic feasibility of productions. Monogeneans are ectoparasitic flatworms of both marine and freshwater fish and can rapidly infect entire fish stocks because of their simple life cycle and rapid transmission between host fish (Alves et al., 2019; Hoai, 2020). Infections of these parasites can negatively affect growth, feed conversion rates and commercial value of the fish, as well as causing economic losses due to epizooties (Hoai, 2020; Soares et al., 2016; Tavares-Dias, 2018;

Tavares-Dias & Martins, 2017). Thus, management requires adequate control and preventative antiparasitic treatments.

Many conventional chemotherapeutics are toxic to the fish, environment and consumers, and often have little or no efficacy (Alves et al., 2019; Soares et al., 2016, 2017), mainly when used of inadequate manner. Many of these products have since become banned for use in aquaculture in many countries. Hence, there exists the need to seek alternative and environmentally friendly therapeutics to control and treat fish diseases (Boijink, Miranda, Chagas, Dairiki, & Inoue, 2015; Lieke et al., 2019), including those caused by monogeneans, which is a current challenge for fish farming.

Medicinal plants have become popular for use in fish farming due to their diversity of bioactive components with different modes of action, including anthelmintic activity against monogeneans in fish (Boijink et al., 2015; Soares et al., 2016; Soares & Tavares-Dias, 2013; Tavares-Dias, 2018). About 4,000 species of medicinal plants with essential oils (EO) are known, including *Lippia* spp., which have high potential to treat fish infected with parasites (Soares et al., 2016, 2017; Tavares-Dias, 2018). Hence, EOs from different medicinal plants have been recommended for treatment against monogeneans in farmed fish (Boijink et al., 2015; Meneses et al., 2018; Soares et al., 2017; Steverding, Morgan, Tkaczynski, Walder, & Tinsley, 2005; Tavares-Dias, 2018), but not yet *Lippia grata* Schauer, a medicinal plant with potential anthelminthic activity. *Lippia grata* is a shrub from Verbenaceae family that is endemic to Brazil and occurs in the Caatinga, Campo rupestrian and Cerrado regions. This medicinal plant is widely used in popular medicine to treat various infections since it has healing, antimicrobial and antiseptic properties (Albuquerque et al., 2007; Melo et al., 2013; Souza et al., 2019).

Lippia grata EO as extracted from leaves has high levels of monoterpenes and sesquiterpenes (Cruz et al., 2013). The main compounds of this EO are thymol, carvacrol, alpha-pinene and p-cymene (Bitu et al., 2015; Franco et al., 2014), which may have antiparasitic action. *Lippia grata* EO has been used effectively in vitro assays against ticks *Rhipicephalus* (*Boophilus*) *microplus* (Costa-Júnior et al., 2016), leishmania (Melo et al., 2013), fungi (Franco et al., 2014) and bacteria (Dantas, Rocha, Medeiros, & Santos, 2010). However, no tests have been carried out to verify the efficacy of *L. grata* EO to control and treat parasitic diseases in fish, including *Colossoma macropomum* (tambaqui), a large fish from Amazon basin that can reach more than 30 kg and 1 m in length. This Amazonian fish is the native species mostly produced in Brazil due to its good growth performance in aquaculture. Thus, the present study evaluated the in vivo and in vitro anthelmintic efficacy of *L. grata* EO in *C. macropomum* naturally infected with monogeneans, as well as its effects on the haematology and gill histology of this fish.

2 | MATERIALS AND METHODS

2.1 | Obtaining and acclimatization of fish

A total of 250 *C. macropomum* fingerlings (30 ± 5 g) were obtained from a commercial fish farm in Macapá (AP) and maintained in the Embrapa Amapá Aquaculture and Fishery Laboratory, Macapá, Amapá State (Brazil). The fish were acclimated for 7 days in 500 L water tanks and fed with commercial feed containing 32% crude protein. The tanks were maintained with constant water renewal and aeration. The following water parameters were monitored daily: mean temperature 28.4 ± 0.1°C, dissolved oxygen 5.5 ± 0.2 mg/L, pH 5.3 ± 0.2, ammonia 0.5 ± 0.2 mg/L, alkalinity 10.0 ± 0.0 mg/L and hardness 10.0 ± 0 mg/L. The tank was siphoned every 2 days to remove accumulated organic matter. The pH, water temperature, dissolved oxygen, electrical conductivity, and hardness were measured using a multiparameter meter (Horiba, Mod U10; Horiba). Total ammonia nitrogen levels were measured using a Benchtop Multiparameter Photometer for Water Analysis (Hanna Model HI 83208, Brazil).

2.2 | Obtaining and chemical composition analysis of *Lippia grata* essential oil

Lippia grata was obtained from a nature preserve area of the Embrapa Meio Norte (03.05'03.6 "S, 41.46'58.8" W), Parnaíba, Piauí state (Brazil). The EO was extracted by hydrodistillation from dried leaves and inflorescences of the plant using a Clevenger apparatus, and yield of EO was 2.8%. The EO was extracted from fresh leaves through hydrodistillation with a Clevenger apparatus for 6. Chemical composition of the EO was determined using gas chromatography–mass spectrometry (GC-MS—Shimadzu QP5050A). The separation was performed using a silica SBP-5 capillary column composed of 5% phenylmethylpolysiloxane (30 m, length × 0.25 mm i.d., 0.25 μm and phase thickness). The sample was dissolved in dichloromethane and analysed according to the following experimental conditions: injection mode split, 1:40; injector temperature, 250°C; carrier gas, helium; flow rate of 1.0 ml/min; oven temperature, 100°C for 5 min and then raised to 260°C at a rate of 4°C/min, ending with an isothermal treatment of 20 min. Mass spectra were acquired in electron ionization mode at 70 eV using a scan range of 40–350 M/*z* and a sampling rate of 1.0 scans/s. The ion source temperature was 200°C, interface temperature was 250°C, and solvent cut time was 2.5 min (Adams, 2007).

2.3 | In vitro assays with the *Lippia grata* essential oil and monogeneans of the *Colossoma macropomum*

Specimens of *C. macropomum* fingerlings (35.0 ± 25.0 g and length of 12.0 ± 3.0 cm) were euthanized by brain section for collection of the branchial arches. Gills of 20 fish were used to evaluate the effective exposure time and concentration of *L. grata* EO to control monogeneans. The assay was carried out with four concentrations of *L. grata* EO (100, 250, 350 and 700 mg/L) with three replicates per concentration. Concentrations of the EO were diluted to a ratio of 1:10 g in 70% alcohol. Two control groups were used, of which one control group was with tank water and the other was with tank water + ethyl alcohol (70%).

The assays were performed with an ambient temperature of 20°C. Gill arches of individual *C. macropomum* specimens naturally infected by monogeneans (*Anacanthorus spathulatus*, *Notozothecium janauachensis*, *Mymarothecium boegeri* and *Linguadactyloides brinkmanni*) were collected in Petri dishes and submerged in the different concentrations of *L. grata* EO. After submerging the gills in the EO, each repetition was visualized using stereomicroscopy with a field of view containing at least 20 monogeneans. Live and dead parasites were quantified every 15 min. Dead parasites were considered as those that were detached from the gill tissue and those that were adhered to the gill tissue but with no mobility (Soares et al., 2016). The efficacy of each treatment was calculated using methods described in Zhang et al. (2014). Parasites were collected and fixed formalin (5%) and after preparation in Hoyer's medium were identified using the recommendation of Thatcher (2006).

2.4 | Tolerance of the *Colossoma macropomum* to the concentrations of *Lippia grata* essential oil

Fish tolerance was tested based on the in vitro results to determine the optimal concentration for therapeutic baths with *L. grata* EO. The tolerance assays were carried out with 60 *C. macropomum* fingerlings (12.2 ± 3.1 cm and 35.6 ± 22.0 g). Each treatment consisted of three replicates with five fish per replicate (15 fish per treatment) using 12 tanks of 100 L. Treatments were the different concentrations of the *L. grata* EO (100, 250, 350 and 700 mg/L), and fish were exposed to each concentration for 30 min. Ethyl alcohol (70%) was used to dilute the EO (1:10 g). The tanks used for the tolerance assays were maintained with no water renewal. Changes in fish behaviour and mortality were analysed.

2.5 | *Colossoma macropomum* subjected to therapeutic baths with the *Lippia grata* essential oil

For the therapeutic baths, 90 *C. macropomum* fingerlings (13.9 ± 0.9 cm and 46.2 ± 6.2 g) naturally infected with monogeneans were randomly distributed in 9 tanks of 100 L. Three treatments with three replicates (tanks) were used with 10 fish for each replicate (30 fish per treatment). Two treatments were control groups, of which one control was with tank water and the other was with tank water + 70% ethyl alcohol. The third treatment was with 700 mg/L of *L. grata* EO, which was the highest concentration tested in this study. The baths with the *L. grata* EO were performed for 30 min and for three consecutive days. Ethyl alcohol (70%) was used to dilute the EO (1:10 g).

The fish were maintained without feed and maintained with constant aeration and no water renewal during the therapeutic baths. Means of the water parameters during the exposures to the EO were as follows: temperature 28.3 ± 0.1°C, dissolved oxygen 6.3 ± 0.06 mg/L, pH 5.2 ± 0.09, total ammonia 0.3 ± 0.12 mg/L, alkalinity 10.0 ± 0.0 mg/L and hardness 10.0 ± 0.0 mg/L.

One hour after treatment with the therapeutic baths on the third day, the water of tanks was renewed and fish were euthanized by brain section for collection of the gills of 10 specimens from each repetition (30 fish per treatment) were collected and fixed in 5% formalin for quantification and identification of the monogeneans as described in Eiras, Takemoto, and Pavanelli (2006), and to calculate their prevalence and mean abundance Bush, Lafferty, Lotz, & Shostak, 1997). The efficacy of the therapeutic baths was determined using calculations described in Zhang et al. (2014).

2.6 | Haematological analyses of the *Colossoma macropomum* after therapeutic baths with the *Lippia grata* essential oil

After the third day of the therapeutic baths 700 mg/L of *L. grata* EO, blood samples were collected from the fish, and anaesthetic was used. Five fish from each repetition (15 fish per treatment) were used to obtain blood samples from the caudal vessel with syringes and EDTA (10%). The blood was used to quantify erythrocytes in a haemocytometer, determine haematocrit using the microhaematocrit method and determine the haemoglobin concentration using the cyanometahemoglobin method. These data were used to calculate Wintrobe haematimetric indices of mean corpuscular volume and mean corpuscular haemoglobin concentration (MCHC) as described in Ranzani-Paiva, Pádua, Tavares-Dias, and Egami (2013).

2.7 | Procedures for histopathological analysis of C. macropomum gills after therapeutic baths

After the third day of the therapeutic baths with 700 mg/L of *L. grata* EO, gill arches were collected from three fish from each replicate (nine fish per treatment) for histopathological analysis. The first gill arch on both sides of each fish was collected and fixed in formalin buffer (10%). Gill arches were dehydrated in a gradual series of ethanol (70%, 80%, 90%, 100%) and xylol baths, and embedded in paraffin to obtain consecutive 5-μm sections using an electron microtome (Thermo Scientific™ HM 340E). Histological sections were prepared and stained with haematoxylin–eosin (HE). Images were taken using a common optical microscope (Leica DM 1000) and the software Leica Application Suite 1.6.0 software. Histopathological analyses were performed in a semiquantitative manner using mean assessment values (MAV) (Schwaiger et al., 1997) and the histopathological alteration index (HAI) (Poleksić & Mitrović-Tutundžić, 1994).

2.8 | Statistical analyses

All data were evaluated for normality and homoscedasticity using the Shapiro–Wilk and Bartlett tests respectively. No normal distributions were shown for the data, so treatment means were compared using the Kruskal–Wallis test and significant differences were

determined using the Dunn test (Zar, 2010). The significance level was considered when $p < .05$. Statistical analyses were performed on GraphPad 4.03 software.

3 | RESULTS

3.1 | Majority compounds of the *Lippia grata* essential oil

The chemical composition of the *L. grata* EO is shown in Table 1. All chemical components were quantified (97.8%), and carvacrol (48.12%), p-cymene (24.39%) and γ-terpinene (2.49%) were the most abundant compounds in *L. grata* EO.

3.2 | In vitro antiparasitic efficacy of the *Lippia grata* essential oil against monogeneans in gills of the *Colossoma macropomum*

The monogeneans *A. spathulatus*, *N. janauachensis*, *M. boegeri* and *L. brinkmanni* in the gills of *C. macropomum* showed 100% immobilization after exposure to 700 and 350 mg/L of *L. grata* EO at 30 and 120 min respectively. In the concentration of 250 mg/L, 100% immobilization of the monogeneans occurred 4 hr after exposure and 6 hr after exposure with the concentration of 100 mg/L. The onset of immobilization of the monogeneans exposed to the two control groups (cultivation tank water and cultivation tank water + alcohol) occurred 1 hr after exposure and 100% immobilization occurred 9 hr after exposure (Figure 1 and Table 2).

TABLE 1 Chemical composition of the *Lippia grata* essential oil

Peak	Content (%)	Retention index	Identification
1	1.56	937	α-thujene
2	0.54	946	α-pinene
3	0.08	963	Camphene
4	0.17	989	β-pinene
5	3.03	995	Myrcene
6	0.11	1,014	α-phellandrene
7	0.14	1,021	Ω−3-Carene
8	1.42	1,026	α-terpinene
9	24.39	1,034	p-Cymene
10	0.34	1,039	o-Cymene
11	5.19	1,068	γ-Terpinene
12	0.88	1,183	Terpinene−4-ol
13	5.05	1,242	Thymol methyl-ether
14	0.30	1,252	Carvacrol methyl-ether
15	2.49	1,299	Thymol
16	48.12	1,310	Carvacrol
17	2.70	1,433	E-Caryophyllene
18	0.14	1,467	α-humulene
19	0.44	1,528	δ-Cadinene
20	0.11	1,598	Caryophyllene Oxide
Total identified (%): 97.8			

3.3 | Tolerance of the *Colossoma macropomum* to the concentrations of *Lippia grata* essential oil

During the tolerance assays, the fish showed behavioural changes in all concentrations of the *L. grata* EO (100, 250, 350 and 700 mg/L) such as agitation and accelerated opercular beat. Anaesthetic effects were observed at five min of exposure during the 30 min for all concentrations assayed, but no mortality of fish occurred within this time. Anaesthesia of the fish was shown as sedation and falling towards the bottom of the tank. After the onset of continuous water renewal to remove the *L. grata* EO from the tanks, all fish returned to normal swimming and no mortality was shown. Thus, 700 mg/L of the *L. grata* EO was used for the therapeutic baths of 30 min, since the fish showed tolerance to this concentration.

3.4 | Antiparasitic efficacy of the therapeutic baths with *Lippia grata* essential oil against monogeneans in gills of *Colossoma macropomum*

During the therapeutic baths, fish in the control with the cultivation tank water showed no abnormal behaviour, while in the control with the cultivation tank water + alcohol, the fish showed signs of moderate agitation. Fish exposed to 700 mg/L showed agitation and accelerated opercular beat and had sedation, all behavioural changes also observed during the tolerance assays.

The monogeneans *A. spathulatus*, *N. janauachensis*, *M. boegeri* and *L. brinkmanni* were identified in the gills of the *C. macropomum*. The prevalence of *A. spathulatus*, *N. janauachensis* and *M. boegeri* was

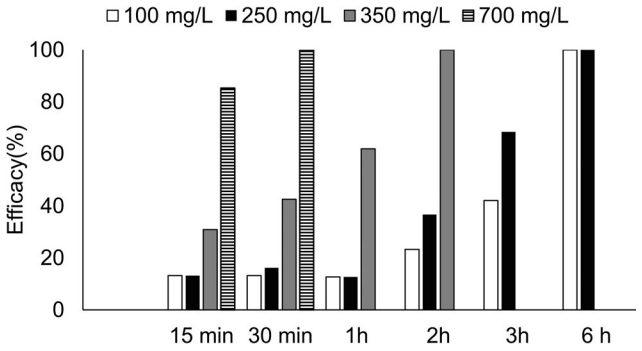

FIGURE 1 In vitro efficacy of different concentrations of *Lippia grata* essential oil against monogeneans of *Colossoma macropomum*

TABLE 2 In vitro antiparasitic action over time of the *Lippia grata* EO against monogeneans in the gills of *Colossoma macropomum*

Time of exposure	Treatments	Live parasites	Mortality (%)
0 hr	Tank water	21.0 ± 1.7	0
1 hr	Tank water	19.3 ± 1.1	7.9
3 hr	Tank water	16.7 ± 0.6	20.6
6 hr	Tank water	11.7 ± 1.5	44.4
9 hr	Tank water	0	100
0 hr	Tank water + alcohol	22.7 ± 2.5	0
15 min	Tank water + alcohol	22.7 ± 2.5	0
30 min	Tank water + alcohol	22.7 ± 2.5	0
1 hr	Tank water + alcohol	21.0 ± 1.0	7.3
2 hr	Tank water + alcohol	20.0 ± 1.0	11.8
3 hr	Tank water + alcohol	19.0 ± 1.0	16.2
6 hr	Tank water + alcohol	8.0 ± 1.0	64.7
9 hr	Tank water + alcohol	0	100
0 hr	100 mg/L	20.0 ± 0	0
15 min	100 mg/L	19.3 ± 0.6	1.7
30 min	100 mg/L	19.3 ± 0.6	1.7
1 hr	100 mg/L	18.3 ± 0.6	8.3
2 hr	100 mg/L	15.3 ± 1.5	23.3
3 hr	100 mg/L	11.0 ± 1.0	45.0
6 hr	100 mg/L	0	100
9 hr	100 mg/L	0	100
0 hr	250 mg/L	20.0 ± 0	0
15 min	250 mg/L	19.7 ± 0.6	1.7
30 min	250 mg/L	19.0 ± 0	5.0
1 hr	250 mg/L	18.3 ± 0.6	8.3
2 hr	250 mg/L	12.7 ± 0.6	36.7
3 hr	250 mg/L	6.0 ± 1.0	70.0
4 hr	250 mg/L	0	100
9 hr	250 mg/L	0	100
0 hr	350 mg/L	20.0 ± 0	0
15 min	350 mg/L	15.7 ± 4.9	22.9
30 min	350 mg/L	13.3 ± 4.7	36.1
1 hr	350 mg/L	8.0 ± 3.0	60.6
2 hr	350 mg/L	0	100
9 hr	350 mg/L	0	100
0 hr	700 mg/L	20.0 ± 0	0
15 min	700 mg/L	3.3 ± 2.9	83.3
30 min	700 mg/L	0	100
15 min	700 mg/L	3.3 ± 2.9	83.3
30 min	700 mg/L	0	100
9 hr	700 mg/L	0	100

high in the two controls, and mean abundance was higher than in the host fish treated with 700 mg/L of *L. grata* EO (Table 3). The control with the cultivation tank water + alcohol showed a low efficacy against the monogeneans in the fish, whereas 700 mg/L of *L. grata* showed an efficacy of 95.1% (Figure 2).

3.5 | Haematology parameters of the *Colossoma macropomum* after therapeutic baths with the *Lippia grata* essential oil

After therapeutic baths with 700 mg/L of *L. grata* EO, the haemoglobin concentration decreased when compared to the control with the cultivation tank water. In the control group with the cultivation tank water + alcohol, there was a reduction in MCHC when compared to the control group with only the tank water (Table 4).

3.6 | Histopathological alterations in C. macropomum gills after therapeutic baths with L. grata essential oil

After therapeutic baths with 700 mg/L of *L. grata* EO, histopathological analyses of gills showed no significant differences ($p > .05$) between treatments regarding MAV and HAI (Table 5). The HAI of gills of fish exposed to 700 mg/L of *L. grata* EO showed alterations when compared to controls, which were characterized by low-to-moderate lamellar hyperplasia (Figure 3).

4 | DISCUSSION

Essential oils are mixtures of volatile secondary metabolites that generally consist of more than 20 different bioactive compounds and in varying concentrations (Souza et al., 2019; Tavares-Dias, 2018). Hence, the bioactive effects of EOs are frequently due to the synergy of their bioactive compounds (Tavares-Dias, 2018), many of which induce anaesthesia in fish (Souza et al., 2019). In general, the main chemical compounds of *L. grata* EO are terpenes such as thymol, carvacrol, α-pinene and p-cymene (Bitu et al., 2015; Franco et al., 2014; Siqueira-Lima et al., 2019). In the present study, 82.7% of the major compounds in the *L. grata* EO were formed by carvacrol (48.1%), p-cymene (24.4%), γ-terpinene (5.2%) and thymol (5.0%). The terpenes γ-terpinene and p-cymene are precursors of carvacrol, and their synergistic action appears to cause the anaesthetic effects of the *L. grata* OE concentrations (100–700 mg/L) that was observed in the *C. macropomum*. The same effects were shown with the *C. macropomum* when exposed to 100 and 150 mg/L *Lippia alba* (Soares et al., 2016) and 20 and 40 mg/L of *Lippia origanoides* (Soares et al., 2017) with other different terpenes. In addition, the present study showed optimal anaesthesia for the *C. macropomum*, and as described in Silva et al. (2013), which is that the anaesthetic should induce rapid sedation within 3 min or less with minimal stress and have a recovery of 10 min or less. Thymol and carvacrol are positive allosteric modulators of the GABA receptor, which corresponds to one of the main targets of sedatives in phytotherapy (Silva et al., 2013;

TABLE 3 Prevalence (*P*) and mean abundance (MA) of the monogeneans in the gills of the *Colossoma macropomum* after therapeutic baths with *Lippia grata*

Treatments	Tank water		Tank water + alcohol		700 mg/L EO	
Parasites	*P* (%)	MA	*P* (%)	MA	*P* (%)	MA
Anacanthorus spathulatus	83.3	5.6 ± 4.4^{a}	83.3	5.9 ± 4.2^{a}	36.7	0.6 ± 0.8^{b}
Mymarothecium boegeri	100	16.4 ± 12.3^{a}	96.7	10.3 ± 5.0^{a}	33.3	0.4 ± 0.6^{b}
Notozothecium janauachensis	100	14.2 ± 12.0^{a}	96.7	10.1 ± 5.5^{a}	16.7	0.3 ± 0.6^{b}
Linguadactyloides brinkmanni	30.0	0.5 ± 1.0^{a}	6.7	0.1 ± 0.3^{a}	0	0^{a}

Note: Different letters in same line indicate significant differences according to the Dunn test ($p < .001$).

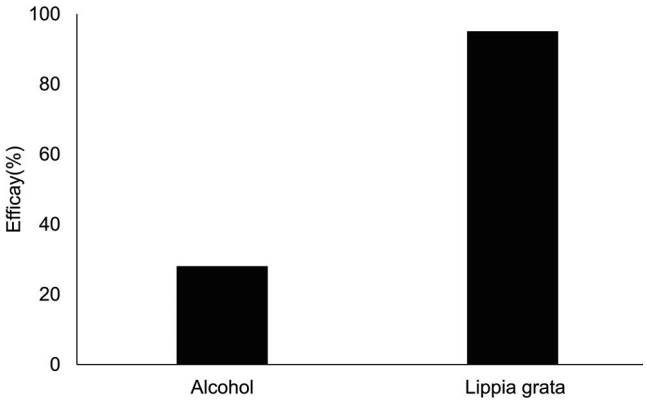

FIGURE 2 Anthelminthic efficacy of therapeutic baths with water + alcohol (control) and 700 mg/L of *Lippia grata* essential oil against monogeneans of *Colossoma macropomum* gills

Siqueira-Lima et al., 2019). P-cymene and carvacrol produce analgesic effects through mechanisms of inhibition of proinflammatory cytokines (IL1, TNF, IL-4, TGF and IL-17), enhancement of anti-inflammatory cytokines (IL- 10) and involvement of the opioid system (Siqueira-Lima et al., 2019).

Cytotoxic properties of EOs are of great importance in relation to parasites, as these phytotherapeutic substances have a direct interaction with cell membrane phospholipids due to the short extension of their carbon chains and high hydrophobicity of their chemical constituents. However, due to the high number of chemical constituents, EOs appear to have no specificity of cell targets (Tavares-Dias, 2018).

The in vitro tests showed that all *L. grata* OE concentrations (100–700 mg/L) had 100% antiparasitic efficacy on *A. spatulathus*, *M. boegeri*, *N. janauachensis* and *L. brinkmanni*, and the time of this efficacy was dose-dependent. Therefore, as *L. grata* EO has therapeutic properties, it demonstrates great potential as an alternative solution for monogeneans control in farmed fish, thus requiring more studies on its in vivo efficacy, concentration and appropriate use strategy. In vitro tests also showed that *L. alba* concentrations (160–2,560 mg/L) had 100% efficacy against *A. spatulathus*, *M. boegeri* and *N. janauachensis*, and was dose-dependent (Soares et al., 2016). On the other hand, Soares et al. (2017) reported that low concentrations (10, 20 and 40 mg/L) of *L. origanoides* EO showed no in vitro efficacy against *A. spatulathus*, *M. boegeri* and *N. janauachensis*, while higher concentrations (80, 160 and 320 mg/L) had a dose-dependent efficacy. Therefore, the efficacy of EOs against monogeneans appears to reflect the concentrations used in in vitro assays.

The negative aspects of chemotherapeutics and legal prohibition of these products for use in aquaculture have led to the need for alternative therapies such as EOs (Lieke et al., 2019; Tavares-Dias, 2018). As several bioactive compounds of the EO have anthelmintic effects, EO of different medicinal plant species has been used to control and treat fish infected with monogeneans. However, the anthelmintic effects of EOs in therapeutic baths can vary according to the chemical composition of the EOs, which influences their majority components and consequently the beneficial effects in fish farming. In the present study, three consecutive therapeutic baths with 700 mg/L of *L. grata* were 95.1% effective against the monogeneans *A. spatulathus*, *M. boegeri*, *N. janauachensis* and *L. brinkmanni* in the *C. macropomum* gills. Soares et al. (2016, 2017) reported

TABLE 4 Erythrocyte parameters of the *Colossoma macropomum* after therapeutic baths with 700 mg/L of *Lippia grata* essential oil

Parameters	Tank water	Tank water + alcohol	700 mg/L EO
RBC ($\times 10^{6}/\mu L$)	1.418 ± 0.312^{a}	1.499 ± 0.247^{a}	1.354 ± 0.331^{a}
Haemoglobin (g/dl)	6.7 ± 0.7^{a}	6.5 ± 0.7^{ab}	5.9 ± 0.5^{b}
Haematocrit (%)	24.1 ± 1.5^{ab}	25.8 ± 2.5^{a}	22.3 ± 2.1^{b}
MCV (fl)	180.3 ± 53.5^{a}	179.5 ± 23.0^{a}	172.4 ± 39.9^{a}
MCHC (g/dl)	27.7 ± 3.1^{a}	25.1 ± 2.0^{b}	26.8 ± 2.2^{ab}

Note: Values are expressed as mean ± *SD*.

Different letters in same line indicate significant differences according to the Dunn test ($p < .05$).

Abbreviations: MCHC, mean corpuscular haemoglobin concentration; MCV, mean corpuscular volume; RBC, red blood cells.

TABLE 5 Values of histopathological alteration index (HAI) and mean assessment values (MAV) for gills of *Colossoma macropomum* exposed to the essential oil of *Lippia grata*

Treatments	N	MAV	HAI	Severity of lesions according to the HAI
Water	9	0.31 ± 0.41^a	0.45 ± 0.42^a	No alteration in the gills
Water + alcohol	9	0.42 ± 0.7^a	0.7 ± 0.63^a	No alteration in the gills
700 mg/L	9	0.47 ± 0.52^a	1.11 ± 2.23^a	Low-to-moderate alterations in the gills

Note: Values express mean ± deviation standard.
Different letters, in same column, indicate differences by the Dunn test ($p < .05$).

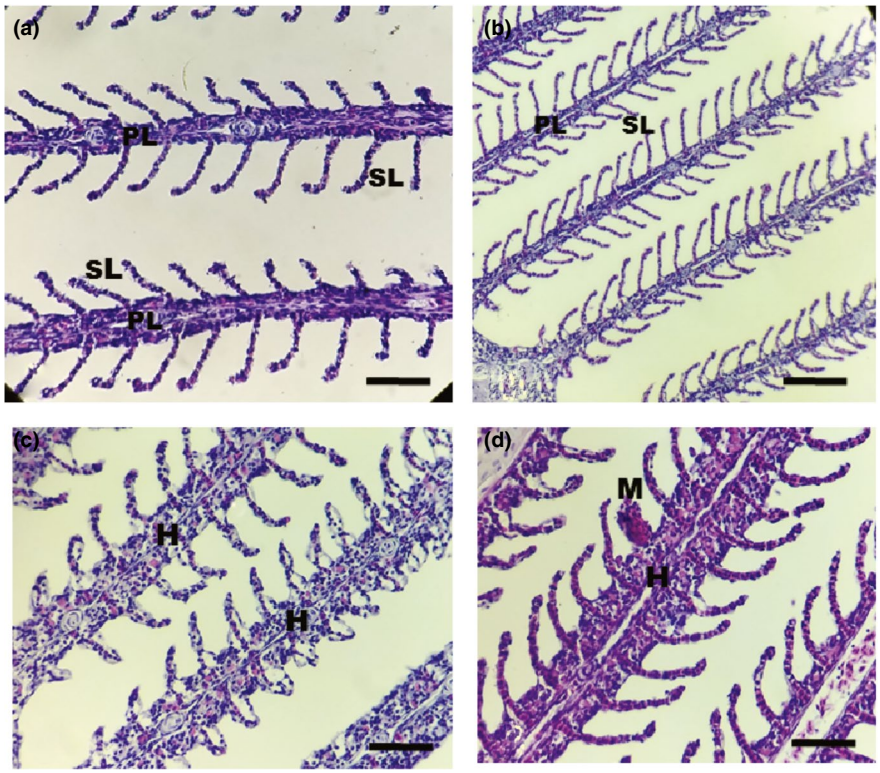

FIGURE 3 (a) *Colossoma macropomum* gills exposed to water of cultivation tank (control) showing primary (PL) and secondary lamellae (SL). (b) Gills of fish exposed to cultivation tank water + alcohol (control) showing PL and SL. (c) Moderate lamellar hyperplasia (H) in fish exposed to 700 mg/L *Lippia grata* essential oil. (d) Lamellar hyperplasia (H) and monogenean (M) in fish exposed to 700 mg/L *Lippia grata* essential oil. Scale bar: 30 μm [Colour figure can be viewed at wileyonlinelibrary.com]

that a single therapeutic bath, for 30 min, with 100 or 150 mg/L of *L. alba* EO and 20 or 40 mg/L of *L. origanoides* EO showed no efficacy against *A. spatulathus*, *M. boegeri* and *N. janauachensis* of the *C. macropomum* gills. Hashimoto et al. (2016) also found no efficacy against monogeneans in gills of the *Oreochomis niloticus* after three therapeutic baths, for 10 min, with 20 mg/L of *L. sidoides* EO.

Ethyl alcohol used as solvent to *L. grata* EO has effect against monogeneans of *C. macropomum* gills. As EOs are insoluble in water, it is necessary to use solvents such as alcohol, Tween or dimethyl sulphoxide, which may also exhibit some antiparasitic activity at low concentrations (Tavares-Dias, 2018). Essential oils are advantageous as an alternative to chemotherapeutics for having lower toxicity, but high concentrations that are effective in in vitro assays may be inadequate in therapeutic baths due to low fish tolerance (Soares et al., 2016; Tavares-Dias, 2018). In addition, the strategy used in the administration of EO for the control and treatment of monogeneans influences the anthelmintic effects of some phytotherapeutics in fish (Tavares-Dias, 2018). Therefore, it is necessary to evaluate the most appropriate therapeutic strategy to obtain good anthelmintic efficacy of each OE before use in fish farming.

Some EOs used in therapeutic baths and at concentrations below those that induce sedation in fish can reduce stress responses (Souza et al., 2019). After exposure to 700 mg/L of *L. grata* EO, the *C. macropomum* of the present study showed lower haemoglobin levels due to the high concentration of EO used in the three therapeutic baths that can had been stressful for the fish. In addition, after therapeutic baths with 700 mg/L of *L. grata* EO occurred low to moderate lamellar hyperplasia on gills of *C. macropomum*. This histopathological change in gill lamellae increases the water–blood diffusion distance and, consequently, decreases the absorption of toxicant agent (Shiogiri et al., 2012). In contrast, therapeutic baths with 100 or 150 mg/L of *L. alba* EO (Soares et al., 2016) and 20 or 40 mg/L of *L. origanoides* EO (Soares et al., 2017) caused severe damages irreversible on the *C. macropomum* gills. Low concentrations of *L. sidoides* EO (20 or 40 mg/L) caused no changes in the erythrocyte parameters of *C. macropomum* after a single therapeutic bath (Soares et al., 2017). In contrast, Soares et al. (2016) reported that *C. macropomum* showed increased MCHC and reduced the

erythrocytes number and haematocrit after a single therapeutic bath with 100 or 150 mg/L of *L. alba* OE. Unfavourable results in the previous study were likely due to haemorrhages in the gills that showed no return to homeostasis 7 days after the treatment; however, no haemorrhage was not observed in the present study. Therefore, *L. grata* had lower toxicity than *L. alba*, *L. origanoides* and *L. sidoides*.

In conclusion, *L. grata* EO had in vitro antiparasitic activity with a dose-dependent efficacy and a high in vivo efficacy. *Lippia grata* EO also showed anaesthetic effects on *C. macropomum* and without important changes in erythrocytic parameters. Moreover, therapeutic baths with 700 mg/L of *L. grata* EO caused a moderate lamellar hyperplasia on gills of fish. Thus, the present study recommends this concentration of *L. grata* EO for the treatment of fish infected with monogeneans using 30-min therapeutic baths every 24 hr for three consecutive days.

ACKNOWLEDGMENTS

The authors thank the National Council for Scientific and Technological Development (CNPq, Brazil) for the research grant awarded to Dr. M. Tavares-Dias (Grant 303013/2015-0).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICAL APPROVAL

This study was approved by the Ethics Committee on Animal Use of Embrapa Amapá (Protocol N° 016-CEUA/CPAFAP) and was conducted in accordance with the principles of the Brazilian College of Animal Experimentation (COBEA).

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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How to cite this article: Barriga IB, Gonzales APPF, Brasiliense ARP, Castro KNC, Tavares-Dias M. Essential oil of *Lippia grata* (Verbenaceae) is effective in the control of monogenean infections in *Colossoma macropomum* gills, a large Serrasalmidae fish from Amazon. *Aquac Res*. 2020;51:3804–3812. https://doi.org/10.1111/are.14728