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Anthelminthic efficacy of *Cymbopogon citratus* essential oil (Poaceae) against monogenean parasites of *Colossoma macropomum* (Serrasalmidae), and blood and histopathological effects

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ABSTRACT

This study investigated the *in vitro* and *in vivo* anthelminthic efficacy, histopathological and hematological effects of *Cymbopogon citratus* essential oil (EO) in *Colossoma macropomum*. The major compounds of the essential oil were geranial (45.7%) and neral (33.9%). Essential oil was assayed at concentrations of 100, 200, 300, 400 and 500 mg L^{-1} for *in vitro* efficacy against monogeneans *Anacanthorus spathulatus*, *Mymarothecium boegeri* and *Notozothecium janauachensis* of *C. macropomum*. Two controls were considered for *in vitro* assays, one with the use of cultivation tank water and the other with cultivation tank water +70% alcohol. The concentration of 500 mg L^{-1} of the EO *in vitro* assays showed 100% efficacy against the parasites within 5 min of exposure, causing structural damages to their tegument. The 400 and 300 mg L^{-1} concentrations were 100% effective with 10 min and 30 min of exposure, respectively. The 200 mg L^{-1} concentration *in vitro* assays was also 100% effective within 30 min and the 100 mg L^{-1} treatment was 100% effective within 100 min of exposure. Total parasite mortality in both control groups occurred within 7 h. Efficacy was 47.1% after therapeutic baths of 20 min for three consecutive days with 60 mg L^{-1} of this EO. In addition, 60 mg L^{-1} of EO was the highest concentration tolerated by the fish and it had an anesthetic effect, and it caused increase in plasma glucose levels and decrease in leukocytes and lymphocytes number, as well as hyperplasia, lamellar fusion, detachment and aneurysm in *C. macropomum* gills. A low efficacy against the monogeneans was shown perhaps due to the low concentration of this EO tolerated by *C. macropomum* and the strategy used for the therapeutic baths, which may be corrected with a lower concentration of EO and more consecutive days for therapeutic baths.

1. Introduction

Aquaculture is an important economic activity worldwide for its generation of jobs and income and it currently accounts for 50% of global fish consumption (Shah and Mraz, 2019). However, global aquaculture production has suffered economic losses estimated at 1.05 to US$ 9.58 billion/year from problems related to diseases (Shinn et al., 2015), including monogenean parasites in farmed fish.

Monogeneans are parasitic plathelminths that mainly affect the gills and tegument of fish and they are problematic in commercial aquaculture because of their simple life cycle, transmission between host fish, and many species are pathogenic and cause mortality when present in a high abundance (Zhang et al., 2014; Tavares-Dias and Martins, 2017; Morales-Serna et al., 2019). Outbreaks of these ectoparasites have led to losses in the farming of native fish species in the Amazonian countries of Brazil, Peru, Bolivia, Colombia and Venezuela, which produce the tambaqui *Colossoma macropomum* (Centeno et al., 2004; Soler-Jiménez et al., 2017; Tavares-Dias and Martins, 2017; Soares et al., 2017a,b), the second largest scaled fish in the Amazon. Therefore, the maintaining of desirable productions requires an integrated approach of understanding and implementing new strategies to control infections with these parasites. The commercial aquaculture industry is

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seeking technological advances to obtain fish products of better quality while diminishing the use of conventional synthetic chemotherapeutics, many of which are banned in several countries due to risks of toxicity to fish and handlers as well as environmental contamination. Thus, current research is focused on ecofriendly, non-toxic and natural therapeutics as a strategy to increase the overall sustainability of the activity (Soares et al., 2016; Tavares-Dias, 2018; Shah and Mraz, 2019; Morales-Serna et al., 2019).

Since essential oils (EO) also contain chemical substances, when they have therapeutic effects they can also be classified as chemotherapeutics. However, EO are of natural origin and not of synthetic origin, as is the case with most drugs currently used in aquaculture. Essential oils and their bioactive compounds are therefore a new alternative to the use of chemotherapeutics in the control and treatment of fish infected by monogeneans (Soares et al., 2016, 2017a, 2017b; Tavares-Dias, 2018; Meneses et al., 2018; Morales-Serna et al., 2019). *Cymbopogon citratus* (DC) Stapf (lemongrass) is a medicinal plant of the Poaceae family that is widely used in human medicine to treat various diseases (Machado et al., 2012; Avoseh et al., 2015). In addition, its EO has been tested as an anthelmintic in large animals (Matasyoh et al., 2011; Ebani et al., 2018) and has shown potential to control and treat infections of monogeneans in fish farming. Thus, the present study investigated the anthelmintic efficacy (*in vitro* and *in vivo*) of *C. citratus* EO to control and treat *C. macropomum* infected in the gills by monogeneans and evaluate if this EO has adverse effects on this fish species.

2. Materials and methods

2.1. Obtaining and chemical composition of C. citratus essential oil

Cymbopogon citratus was cultivated in the Embrapa Amazon West Medicinal Plants and Vegetables Sector, Manaus (AM), Brazil (1°56′45.7» S- 37° 02′58.5» W). The essential oil was extracted from fresh leaves through hydrodistillation with a Clevenger apparatus for 4 h. Chemical composition of the essential oil was determined using gas chromatography mass spectrometry (GC–MS - Shimadzu QP5050A, Japan). The separation was performed using a silica SBP-5 capillary column composed of 5% phenylmethylpolysiloxane (30 m, length x 0.25 mm i.d., 0.25 μm, and phase thickness). The sample was dissolved in dichloromethane and analyzed 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 was 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 (see Adams, 2007).

2.2. Fish and acclimation

A total of 200*C. macropomum* fingerlings were acquired from a commercial fish farm in the municipality of Macapá (AP), Brazil, and transported to the Embrapa Amapá Aquaculture and Fishery Laboratory, Macapá, Amapá State (Brazil). The fishes were acclimatized for seven days in 500 L tanks with a constant water flux and were fed twice daily with a commercial fish diet containing 36% crude protein (Guabi, Brazil). Water quality parameters of the tanks were monitored daily using a multiparameter probe (Horiba Mod. U52, Japan). Means of the water parameters were: temperature 30.3 ± 0.1 °C, dissolved oxygen 5.4 ± 0.2 mg L^{-1}, pH, 5.3 ± 0.2, total ammonia 0.5 ± 0.2 mg L^{-1}, alkalinity 10.0 ± 0.001 mg L^{-1} and hardness 10.0 ± 0.001 mg L^{-1}. The fish were used for all *in vitro* and *in vivo* assays and the monogeneans were obtained from the fish, which were naturally infected.

All experimental procedures in the present study were approved by the Embrapa Amapá Ethics Committee for the Use of Animals (Protocol N° 016/2019 - CEUA/CPAFAP).

2.3. In vitro assays with C. citratus essential oil against monogeneans of C. macropomum

Seven *C. macropomum* fingerlings (12.5 ± 1.0 cm and 33.7 ± 9.1 g) naturally infected with the monogeneans *Anacanthorus spathulatus*, *Mymarothecium boegeri* and *Notozothecium janauachensis* were used for this assay. Fish were euthanized and the gills were removed for the evaluation of exposure time and *C. citratus* EO concentrations that cause *in vitro* mortality of the monogeneans. The assay was carried out with five concentrations of *C. citratus* EO (100, 200, 300, 400 and 500 mg L^{-1}) with three replicates per concentration. Concentrations of the EO were diluted to a ration 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 cultivation tank water +70% ethyl alcohol (125 μ L^{-1}). These assays were performed with an ambient temperature of 20 °C. Gill arches of individual *C. macropomum* specimens infected by monogeneans were collected in Petri dishes (5.5 cm) and submerged in the different concentrations of *C. citratus* EO. Live and dead parasites were quantified every 5 min using stereomicroscopes. Each replicate was visualized with a field of view containing at least 20 monogeneans. 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 effectiveness of each treatment was calculated using methods described in Zhang et al. (2014).

At the end of these assays, gills of the fish in both control treatments and of those exposed to the different concentrations of *C. citratus* EO were prepared for observation for possible structural damage in the monogeneans using scanning electron microscopy (SEM). Samples were fixed in 2.5% glutaraldehyde solution buffered in 0.1 M sodium cacodylate buffer with a pH of 7.2. The gills were then subjected to three washes at 30 min intervals in buffer solution and were incubated in 4% osmium tetroxide and 0.1 M sodium cacodylate buffer pH 7.2 three times. The gills were incubated in 1% aqueous tannic acid solution for 45 min and gradually dehydrated in 50, 70, 90 and 100% ethanol for 20 min per solution. After dehydration, the samples were subjected to a drying process in a critical point chamber using carbon dioxide and coated with a thin layer of platinum (“Sputtering”, Leica EM SCD 500, German). Samples were visualized with a scanning electron microscope (DSM 940, ZEIZZ, Germany) operated at an acceleration of 15 kV.

2.4. Therapeutic baths of C. macropomum with C. citratus essential oil

Fish tolerance was tested based on the *in vitro* results to determine the concentration of *C. citratus* EO for therapeutic baths. Sixty *C. macropomum* fingerlings (10.5 ± 0.8 cm and 21.2 ± 3.4 g) were distributed in 100 L water tanks with 10 fish per tank. Therapeutic baths were carried out with a different concentration of *C. citratus* EO in each tank (500, 400, 300, 200, 100 and 60 mg L^{-1}). Ethyl alcohol (70%) was used as solvent (1:10 g) for *C. citratus* EO. The tanks were maintained with no water renewal during the assays. The *C. citratus* EO concentration of 60 mg L^{-1} was tolerated by the fish for use in a therapeutic bath.

For the therapeutic baths, 117*C. macropomum* fingerlings (10.7 ± 0.9 cm and 22.4 ± 4.4 g) naturally infected with monogeneans were randomly distributed in 9100 L tanks. Three treatments with three replicates (tanks) were used with 13 fish for each replicate (39 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 60 mg L^{-1} of *C. citratus* EO. The baths with the *C. citratus* EO were performed for 20 min and for three consecutive days. The tanks were maintained with constant aeration and no water renewal. Alterations in fish behavior were

studied during the therapeutic baths.

After treatment with the therapeutic baths, the gills of 10 fish from each replicate (30 fish per treatment) were collected and fixed in 5% formalin for quantification and identification of the monogeneans (Eiras et al., 2006), and to calculate their prevalence and mean abundance (Bush et al., 1997). The efficacy of the therapeutic baths was determined using calculations described in Zhang et al. (2014).

2.5. Blood analysis after therapeutic baths of C. macropomum

Blood samples were collected from the fish after the therapeutic baths with 60 mg L^{-1} of *C. citratus* EO and before removal of the gills. Ten fish from each replicate (30 fish per treatment) were used to obtain blood samples from the caudal vessel with syringes and EDTA (10%). The blood was immediately divided into two aliquots. One aliquot was used to quantify erythrocytes in a homocytometer, determine hematocrit using the microhematocrit method, and determine the hemoglobin concentration using the cyanometahemoglobin method. These data were used to calculate hematimetric indices of mean corpuscular volume (MCV) and mean corpuscular hemoglobin concentration (MCHC) as described in Wintrobe (1934). Blood smears were made and stained panchromically with a May Grünwald-Giemsa-Wright combination (Ranzani-Paiva et al., 2013) for the differential count of leukocytes in up to 200 cells of interest in each extension. The identification and nomenclature of leukocyte counts were performed as described in Tavares-Dias et al. (1999). Blood smears were also used to determine the total number of leukocytes and thrombocytes using indirect method (see Ranzani-Paiva et al., 2013).

The second blood aliquot was centrifuged at 75 G (Centrifuga MCD-2000, Brazil), during 7 min, to obtain plasma and to determine levels of glucose and total protein in the plasma. The glucose concentration was determined by the enzymatic-colorimetric glucose oxidase method using a commercial kit (Biotécnica, MG, Brazil) and spectrophotometer reading at 510 nm. The total plasma protein concentration was determined by the biuret method using a commercial kit (Biotécnica, MG, Brazil) and spectrophotometer reading at 540 nm. The readings were performed on a digital spectrophotometer (Biospectro SP-220, Brazil).

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

After the therapeutic baths with 60 mg L^{-1} of *C. citratus* EO, gills were collected from three fish from each replicate (9 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 ScientificTM HM 340E, USA). Images were taken using a common optical microscope (Leica DM 1000, USA) 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 and Mitrović-Tutundžić, 1994).

2.7. 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 treatments data were compared using the Kruskal-Wallis test and significant differences were determined using the Dunn test (Zar, 2010).

Table 1
Chemical compounds of the *Cymbopogon citratus* essential oil.

Peak	% Content	Retention index	Identification
1	0.6	915	Alpha-pinene
2	0.5	928	Beta-pinene
3	0.5	939	6-methyl-5-hepten-2-one
4	6.6	981	Myrcene
5	0.6	989	6,7-epoxymyrcene
6	1.4	993	Linalool
7	0.5	1095	Ni
8	0.7	1101	Ni
9	0.3	1168	Citronelol
10	33.9	1185	Neral
11	3.0	1231	Geraniol
12	45.7	1244	Geranial
13	1.3	1258	Ni
14	1.1	1341	Ni
15	1.1	1356	Geranyl acetate
16	0.1	1386	Ni
Total identified (%)	97.8		

Ni: Undetermined.

3. Results

3.1. Chemical composition of C. citratus essential oil

The chemical components of *C. citratus* EO are shown in Table 1. Almost all chemical components were quantified (97.8%), of which geranial (45.7%), neral (33.9%) and mircene (6.6%) represented the majority of the compounds in the *C. citratus* EO.

3.2. In vitro antiparasitic efficacy of C. citratus essential oil against monogeneans

The monogeneans *A. spathulatus*, *M. boegeri* and *N. janauachensis* in the *C. macropomum* of the two control groups (culture tank water and tank water + alcohol) showed total immobilization after 7 h. In the control group exposed to tank water + alcohol, *in vitro* immobilization of monogeneans was initiated after 3 h of exposure, whereas in culture tank water group the immobilization was initiated after 6 h of exposure (Table 2). An efficacy of 100% occurred after 5 min for the monogeneans exposed to 500 mg L^{-1} of *C. citratus* EO. Mortality of the monogeneans was observed after 5 min of exposure with the 400 and 300 mg L^{-1} treatments, but 100% efficacy occurred after 10 min with 400 mg L^{-1} and 30 min with 300 mg L^{-1} of *C citratus* EO. Parasite mortality occurred with 10 min exposure in the 200 and 100 mg L^{-1} treatments, and 100% efficacy after exposure was observed at 30 and 100 min for the 200 and 100 mg L^{-1} concentrations, respectively (Fig. 1 and Table 2).

Monogeneans exposed to tank water (control) presented a defined body shape and shallow wrinkles on the tegument surface (Fig. 2A). In contrast, the tegument of monogeneans exposed to 500 mg L^{-1} of *C. citratus* EO showed extensive damage due to perforation caused by this EO (Fig. 2B).

3.3. Antiparasitic efficacy of therapeutic baths with C. citratus essential oil

The gills of *C. macropomum* were naturally parasitized by three monogenean species (*A. spathulatus*, *M. boegeri* and *N. janauachensis*), which showed variations in prevalence and abundance between different treatments. The mean abundance of *A. spathulatus* was reduced ($p < .05$) after three therapeutic baths with 60 mg L^{-1} of *C. citratus* EO when compared to the control treatments, whereas the mean abundance of *M. boegueri* and *N. janauachensis* were similar ($p > .05$) between treatments. The abundance of *M. boegueri* and *N. janauachensis* in the control treatment with the tank water + alcohol was lower ($p < .05$) when compared to those of the control with only the tank

Table 2
In vitro antiparasitic action of *Cymbopogon citratus* essential oil in monogeneans of *Colossoma macropomum*, in relation to the concentrations and time of exposure.

Time of exposure	Treatments	Live parasites	Mortality (%)
0 h	Water	35 ± 5.3	0
5 min	Water	35 ± 5.3	0
10 min	Water	35 ± 5.3	0
30 min	Water	35 ± 5.3	0
100 min	Water	35 ± 5.3	0
3 h	Water	35 ± 5.3	0
6 h	Water	19 ± 4.3	49.0
7 h	Water	0.0 ± 0.0	100
0 h	Water + alcohol	33 ± 1.3	0
5 min	Water + alcohol	33 ± 1.3	0
10 min	Water + alcohol	33 ± 1.3	0
30 min	Water + alcohol	33 ± 1.3	0
100 min	Water + alcohol	33 ± 1.3	0
3 h	Water + alcohol	31 ± 1.7	5.0
6 h	Water + alcohol	10 ± 5.1	86.0
7 h	Water + alcohol	0.0 ± 0.0	100
0 h	100 mg L^{-1}	31 ± 2.0	0
5 min	100 mg L^{-1}	31 ± 2.0	0
10 min	100 mg L^{-1}	30 ± 1.0	2.0
30 min	100 mg L^{-1}	15 ± 4.0	48.0
100 min	100 mg L^{-1}	0.0 ± 0.0	100
0 h	200 mg L^{-1}	26 ± 2.0	0
5 min	200 mg L^{-1}	26 ± 2.0	0
10 min	200 mg L^{-1}	22 ± 0.0	11.0
30 min	200 mg L^{-1}	0.0 ± 0.0	100
0 h	300 mg L^{-1}	31 ± 6.0	0
5 min	300 mg L^{-1}	22 ± 0.0	27.0
10 min	300 mg L^{-1}	19 ± 1.0	35.0
30 min	300 mg L^{-1}	0.0 ± 0.0	100
0 h	400 mg L^{-1}	25 ± 5.0	0
5 min	400 mg L^{-1}	10 ± 10.0	45.0
10 min	400 mg L^{-1}	0.0 ± 0.0	100
0 h	500 mg L^{-1}	23 ± 1.0	0
5 min	500 mg L^{-1}	0.0 ± 0.0	100

water (Table 3). The therapeutic baths with 60 mg L^{-1} of *C. citratus* showed an efficacy of 47.1%.

Changes in fish behavior were observed during the therapeutic baths with the *C. citratus* EO. The fish showed agitated behavior, increase of the opercular movement and erratic swimming after 5 min of exposure. After 10–12 min, the fish completely lost balance and opercular movements and were going to the bottom of the tank.

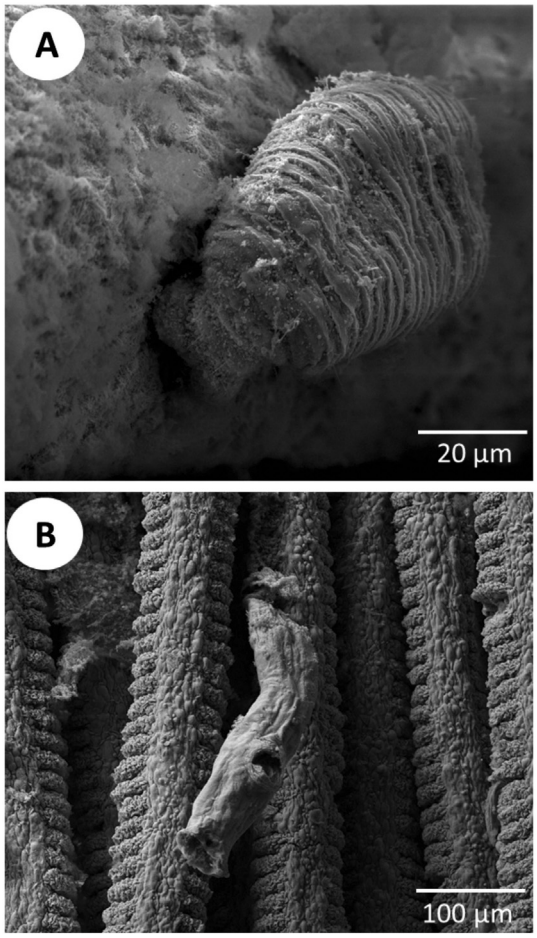

Fig. 2. Scanning electron microscopy (SEM) of monogeneans on *Colossoma macropomum* exposed to *Cymbopogon citratus* essential oil. Untreated parasite (**A**). Parasites exposed to 500 mg L^{-1} *Cymbopogon citratus* essential oil (**B**).

3.4. Effects on blood parameters of C. macropomum after therapeutic baths with C. citratus essential oil

Colossoma macropomum showed an increase ($p < .05$) in plasma glucose levels and a reduction in the number of leukocytes and total lymphocytes after therapeutic baths with 60 mg L^{-1} of *C. citratus* EO when compared to fish in the control treatments. In addition, the fish

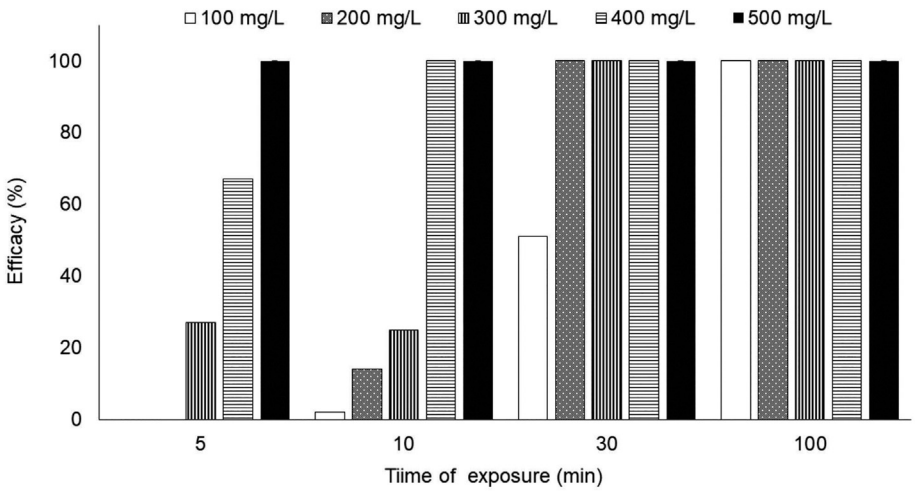

Fig. 1. *In vitro* efficacy of different concentrations of *Cymbopogon citratus* the essential oil against monogenean parasites.

Table 3
Prevalence (P%) and mean abundance (MA) of the parasite monogeneans on gills of *Colossoma macropomum* exposed to *Cymbopogon citratus* essential oil.

Tratamentos	Tank water		Tank water + alcohol		60 mg L^{-1}	
Parasite species	P (%)	MA	P (%)	MA	P (%)	MA
Anacanthorus spathulatus	100	9.3 ± 4.6[a]	100	18.4 ± 16[b]	100	6.3 ± 4.5[c]
Mymarothecium boegeri	50	2.3 ± 2.7[a]	10	0.3 ± 0.8[b]	46.7	0.8 ± 1.0[ab]
Notozothecium janauachensis	46.7	2.4 ± 3.3[a]	3.3	0.1 ± 0.7[b]	23.3	0.2 ± 0.4[ab]

Values express mean ± deviation standard. Different letter, in same line, indicate differences by the Dunn test ($p < .05$).

Table 4
Body and blood parameters of *Colossoma macropomum* exposed to *Cymbopogon citratus* essential oil.

Parameters	Water	Water + alcohol	60 mg L^{-1}
Body weight (g)	21.6 ± 4.9[a]	23.1 ± 4.2[a]	22.9 ± 3.5[a]
Length (cm)	10.5 ± 0.9[a]	10.8 ± 0.9[a]	10.8 ± 0.7[a]
Glucose (g dL^{-1})	101.0 ± 18.5[a]	78.0 ± 23.0[b]	122.1 ± 22.6[c]
Total protein (mg dL^{-1})	4.3 ± 0.6[a]	6.3 ± 9.0[a]	3.8 ± 0.7[a]
Red blood cells (x10^6 μL^{-1})	1.33 ± 0.21[a]	1.39 ± 0.39[a]	1.24 ± 0.21[a]
Hemoglobin (g dL^{-1})	6.5 ± 0.8[ab]	6.8 ± 1.4[b]	5.9 ± 1.1[a]
Hematocrit (%)	22.8 ± 3.0[a]	23.1 ± 2.6[a]	24.1 ± 2.4[a]
MCV (fL^{-1})	177.2 ± 41.1[a]	193.0 ± 117.4[a]	197.9 ± 26.4[a]
MCHC (g dL^{-1})	29.4 ± 6.8[a]	29.6 ± 5.9[a]	24.4 ± 4.1[a]
Thrombocytes (μL^{-1})	15,087 ± 12,364[ab]	6050 ± 2048[a]	19,179 ± 3346[b]
Leukocytes (μL^{-1})	9945 ± 1661[a]	14,595 ± 4300[a]	6187 ± 1079[b]
Lymphocytes (μL^{-1})	4303 ± 2039[a]	6187 ± 472[a]	1206 ± 866[b]
Monocytes (μL^{-1})	385 ± 335[a]	2090 ± 1916[b]	600 ± 377[ab]
Neutrophils (μL^{-1})	4777 ± 0.30[a]	4733 ± 3460[a]	3970 ± 1074[a]
PAS-GL (μL^{-1})	102 ± 165[a]	376 ± 593[a]	149 ± 218[a]
Eosinophils (μL^{-1})	382 ± 224[ab]	1219 ± 908[a]	262 ± 269[b]

Values express mean ± deviation standard. Different letter, in same line, indicate differences by the Dunn test ($p < .05$).

exposed to 60 mg L^{-1} of *C. citratus* EO showed a decrease in hemoglobin and increase in number of thrombocytes ($p < .05$) when compared to the fish in the control treatment with the water + alcohol. Fish exposed to the water + alcohol showed an increase ($p < .05$) in monocytes when compared to the other control and an increase in eosinophils when compared to the fish exposed to the *C. citratus* EO. The other parameters evaluated showed no changes between treatments (Table 4).

3.5. Histopathological alterations in C. macropomum gills after therapeutic baths with C. citratus essential oil

After therapeutic baths with 60 mg L^{-1} of *C. citratus* EO, histopathological analyses of gills showed significant differences ($p < .05$) between treatments regarding MAV and HAI (Table 5). The HAI of gills of fish exposed to 60 mg L^{-1} of *C. citratus* EO showed moderate to severe alterations when compared to controls, which were characterized by hyperplasia, lamellar fusion, detachment and aneurysm. The histopathological alterations observed in the gills of control fish exposed to tank water + alcohol were lamellar hyperplasia, fusion and secondary lamella congestion (Fig. 3).

4. Discussion

Essential oils possess different bioactive properties according to their production of secondary metabolites of mainly monoterpenes, sesquiterpenes and oxygenated derivatives, all of which vary in quantity, quality and composition according to the climate, soil composition, part of the plant, age and stage of the plant cycle, as well as chemotype of the plant (Vale et al., 2002; Barbosa et al., 2008; Teixeira et al., 2015; Soares et al., 2016; Tavares-Dias, 2018), as well as chemotype of the. Many essential oils have anesthetic effects and anthelmintic activity, in addition to several other bioactive proprieties that are beneficial in aquaculture (Soares et al., 2016; Soares et al., 2017a, 2017b; Meneses et al., 2018; Tavares-Dias, 2018; Morales-Serna et al., 2019). In the present study, the major compounds of *C. citratus* EO were the monoterpenes geranial and neral, both of which composed nearly 80% of the compounds in this oil. The high occurrence of geraniol and neral as the main compounds in *C. citratus* EO has been shown in previous studies (Barbosa et al., 2008; Teixeira et al., 2015). The combination of the aldehyde isomers of geranial and neral form the monoterpene citral, which is responsible for the anthelmintic property of this EO (Barbosa et al., 2008; Teixeira et al., 2015). Bioactive properties of the essential oil may result from a synergistic effect of the major molecules or high concentrations of certain molecules (Teixeira et al., 2015; Tavares-Dias, 2018) as shown with the presence of citral in several EOs. Citral is the main bioactive compound in several OEs and has sedative and relaxing effects (Vale et al., 2002), which were observed in *C. macropomum* exposed to the *C. citratus* EO in the present study.

In vitro studies are an important preliminary step prior to the evaluation of therapeutic baths when antiparasitic effects of EO are unknown in fish (Tavares-Dias, 2018), thus facilitating the establishment of more appropriate strategies to control infections caused by monogeneans. The different concentrations of *C. citratus* EO (100, 200, 300, 400 and 500 mg L^{-1}) showed 100% efficacy *in vitro* against the monogeneans *A. spathulatus*, *M. boegeri and N. janauachensis*, but the immobilization time of these parasites was concentration dependent. Similar studies with the essential oils of *Lippia alba* (Soares et al., 2016), *Lippia sidoides* (Soares et al., 2017a) and *Lippia organoides* (Soares et al., 2017b) also reported concentration dependent efficacy against monogeneans. However, the modes of action of EO in the treatment of monogeneans are little understood. Scanning electron microscopy

Table 5
Values of histopathological alteration index (HAI) and mean assessment values (MAV) for gills of *Colossoma macropomum* exposed to the essential oil of *Cymbopogon citratus*.

Treatments	N	MAV	HAI	Severity of lesions according to the HAI
Water	9	0.3 ± 0.4[a]	0.3 ± 0.4[a]	No alteration in the gills
Water + alcohol	9	0.6 ± 0.8[ab]	5.7 ± 8.4[a]	No alteration in the gills
60 mg L^{-1}	9	1.4 ± 0.4[b]	37.8 ± 38.2[b]	Moderate to severe alterations of gills

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

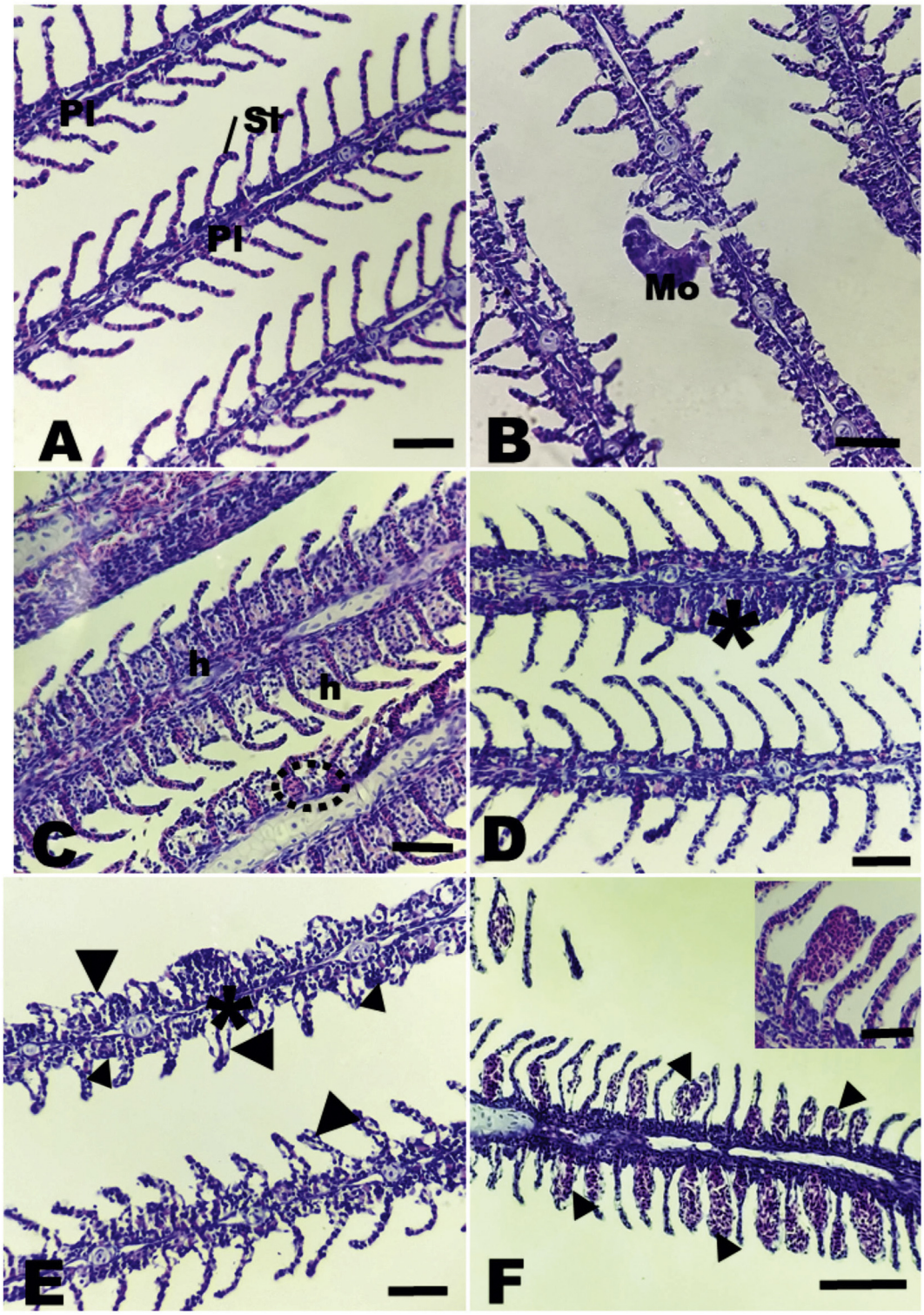

Fig. 3. (**A**) *Colossoma macropomum* gills exposed to water of cultivation tank (control) showing primary (Pl) and secondary lamellas (Sl). (**B**) Monogenean in gills of fish exposed to water of cultivation thank (control). (**C**) Lamellar hyperplasia (h) and congestion of secondary lamellas (circle) in fish exposed to water of cultivation thank + alcohol (control). (**D**) Hyperplasia with fusion of secondary lamellas (*) in fish exposed to water of cultivation thank + alcohol. (**E**) Hyperplasia with fusion of secondary lamellas (*), detachment of lamellar epithelium (triangles) and hyperplasia in gills of fish exposed to 60 mg L^{-1} *Cymbopogon citratus* essential oil. (**F**) Aneurism (triangles) in gills of fish exposed to 60 mg L^{-1} *Cymbopogon citratus* essential oil. Scale bar: 30 μm.

(SEM) in the present study showed that the monogeneans exposed to *C. citratus* EO had perforated tegument after treatment. *Dactylogyrus intermedius* exposed to cinnamaldehyde of *Cinnamomum cassia* EO have been visualized with SEM and were shown with deep wrinkles and other extensive damage to the tegument (Ling et al., 2015). Justino and Barros (2008) observed that nematodes *Contracaecum* sp. exposed to *Cymbopogon* sp. presented an increased body volume, rupture of the tegument cuticle and disintegration of the intestinal wall.

The expansion of fish farming has led to an increase in infections caused by monogeneans. Hence, the aquaculture industry requires

continuous advancements in technology and innovations to control outbreaks of such parasites, which have threatened productions of several fish species around the world (Centeno et al., 2004; Zhang et al., 2014; Soler-Jiménez et al., 2017; Tavares-Dias and Martins, 2017; Morales-Serna et al., 2019). Various EOs currently have broad applications in fish farming (Shah and Mraz, 2019) and their efficacy in controlling monogeneans vary according to the species of parasite and the concentration of an EO, which depends on fish tolerance (Soares et al., 2016; Soares et al., 2017a, 2017b; Tavares-Dias, 2018) and the strategy used when treating with therapeutic baths. For the *C. macropomum*, the efficacy of therapeutic baths with *C. citratus* EO at 60 mg L^{-1} was low (47.1%) when considering that it reduced < 50% of the monogeneans (*A. spathulatus*, *M. boegeri* and *N. janauachensis*) in the fish gills. Therefore, since the maximum tolerance of *C. macropomum* exposed to *C. citratus* EO was low in the short baths of three days, a longer exposure time of about 6–7 days should be used to improve the efficacy of therapeutic baths with up 60 mg L^{-1} of *C. citratus* EO may be used for controlling monogeneans. In contrast, Meneses et al. (2018) showed that short-term baths of 5 min for 3 consecutive days with 320 mg L^{-1} of *Ocimum gratissimum* EO had an efficacy of 87.7% against the monogenean *Cichlidogyrus tilapiae*, whereas long-term therapeutic baths for 2 h and with 40 mg L^{-1} of the same EO had an efficacy of 65.8%.

Despite the advantages of using EOs in fish farming, some EOs may have adverse effects and toxicity to the target fish species (Soares et al., 2016; Meneses et al., 2018; Tavares-Dias, 2018; Soares et al., 2017b). Therefore, the present study investigated alterations of hematological and biochemical parameters in *C. macropomum* exposed to 60 mg L^{-1} of *C. citratus* EO, as well as histology of the gills to evaluate their tolerance to this therapeutic concentration. *Colossoma macropomum* exposed to 60 mg L^{-1} of *C. citratus* EO showed an increase in plasma glucose levels and a reduction in the number of leukocytes and lymphocytes, hyperplasia, lamellar fusion, detachment and aneurysm in gills after therapeutic baths. In addition, there was a reduction in hemoglobin levels and an increase in the number of thrombocytes when compared to the fish exposed to water + alcohol. This increasing plasma glucose level and decrease in number of leukocytes and lymphocytes is a secondary response to stress in *C. macropomum* caused by the therapeutic baths. An increased glucose level is due to the glycogenolytic and gluconeogenic effects of catecholamines and cortisol, respectively, and has been used to measure acute and chronic stress responses in fish (Barton and Iwama, 1991; Davis, 2006; Soares et al., 2016), including exposure to EO. *C. macropomum* submitted to a 30 min bath with 100 or 150 mg L^{-1} of L. *alba* EO have also been shown with an increase in glucose levels accompanied by a decrease in leukocytes and lymphocytes, and severity of gill lesions was directly proportional to the concentration of this EO (Soares et al., 2016). In the same study, the lesions in the gills were more severe in the fish exposed to 150 mg L^{-1} of *L. alba* EO at 24 h after the bath (Soares et al., 2016). In contrast, one short bath using low concentrations of L. *origanoides* (20 or 40 mg L^{-1}) showed no alterations in glucose levels and in the number of leukocytes and lymphocytes in *C. macropomum*, but lesions in the gills varied in severity with the use of this EO (Soares et al., 2017a). Meneses et al. (2018) reported that *O. niloticus* treated with three short-term therapeutic baths with 320 mg L^{-1} of *O. gratissimum* EO showed no damage in the gills, whereas long-term baths with 40 mg L^{-1} provoked hyperplasia in the gills (Meneses et al., 2018). Therefore, these results indicate that damages in gills of fish exposed to essential oils are dependent on the fish species as well as the phytotherapics species and applied concentrations.

5. Conclusions

Cymbopogon citratus EO possesses *in vitro* anthelminthic efficacy that was concentration dependent, and low concentrations of this EO showed anesthetic effects on *C. macropomum*. Furthermore, this EO had low anthelminthic efficacy against monogeneans of *C. macropomum* gills due to the low tolerance of the fish to the EO and the use of a low concentration for the therapeutic baths, which caused stress that may compromise the fish immune system, as well as moderate to severe changes in gills. We indicate prolonged therapeutic baths with up 60 mg L^{-1} of *C. citratus* EO for controlling infection by monogeneans in *C. macropomum*.

Declaration of Competing Interests

The authors declare no conflict of interest.

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Statement of relevance

The manuscript represents original research on use of *Cymbopogon citratus* essential oil in control and treatment against monogenean parasites of *Colossoma macropomum*. After therapeutic baths, there was low anthelmintic efficacy and stress that may compromise the fish immune system, besides moderate to severe changes in gills of fish. Thus, the strategy better for treatment antiparasitic in therapeutic baths for this Amazon fish was recommended.

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