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Antiparasitic, physiological and histological effects of the essential oil of *Lippia origanoides* (Verbenaceae) in native freshwater fish *Colossoma macropomum*

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ABSTRACT

This study examined the *in vitro* and *in vivo,* histopathological, anti-parasitic and hematopathological effects of the essential oil of *Lippia origanoides* on *Colossoma macropomum.* Essential oil concentrations of 10, 20, 40, 80, 160 and 320 mg·L^{-1} were tested *in vitro* against monogenoideans (*Anacanthorus spathulatus*, *Notozothecium janauachensis* and *Mymarothecium boegeri*) from the gills of *C. macropomum*. Concentrations of 320 and 160 mg·L^{-1} were 100% effective against these parasites within 20 and 60 min of exposure, respectively. The 80 mg·L^{-1} concentration was approximately 80% effective with 3 h of exposure, reaching 100% with 6 h of exposure. The 40 mg·L^{-1} concentration was also 100% effective with 6 h of exposure. The other concentrations were only weakly effective *in vitro*. Parasite mortality in controls (water or water + alcohol) began after 3 h, with 100% mortality after 8 h. *In vivo* tests, in which fry of *C. macropomum* were placed in baths with 20 mg·L^{-1} of the essential oil for 60 min, and 40 mg·L^{-1} for 30 min, did not lead to reductions in parasite abundances. In addition, the essential oil had an anaesthetic effect on fish, increased total protein levels, increased monocyte and neutrophil numbers, and reduced haematocrit. Slight to moderate and severe damage was observed in the gills of *C. macropomum* fingerling immediately after exposure to the essential oil, and 24 h after the treatments were applied, with no difference between treatments. Histological changes observed in the gills after exposure to concentrations of 20 and 40 mg·L^{-1} of *L. origanoides* essential oil were: hyperplasia and fusion of the lamellar epithelium, capillary dilation, displacement of the lamellar epithelium, and lamellar aneurism and epithelial rupturing with haemorrhaging. Oedema, mucous and chloride cell proliferation, lamellar hypertrophy, congestion and necrosis were less frequently observed. It can be concluded that the essential oil of *L. origanoides* was dose-dependent *in vitro* effect against monogenoidean parasites of *C. macropomum*. Unfortunately, the low concentrations tolerated by the fish in the *vivo* assay (20 and 40 mg·L^{-1}) was not effective.

Statement of relevance: The manuscript represents original research on use of the essential oil of *Lippia origanoides* against ectoparasites of *Colossoma macropomum*, an important finfish of Amazon region. This manuscript includes treatment *in vitro* against monogenoideans, and *in vivo* against protozoans and monogenoideans. Besides, histopathological and hematological analysis of the fish exposed to different concentrations of *L. origanoides*, a medical plant from North, Central and South America, were performed.

1. Introduction

Lippia origanoides Kunth (Verbenaceae), is an aromatic shrub found from Southern North America, through Central America, to the north of South America. In the Brazilian Amazon, *L. origanoides* is an important medicinal plant due to therapeutic and culinary uses. Ethno-botanic studies indicate uses of *L. origanoides* to treat gastrointestinal, urogenital and respiratory problems, and as an anti-malarial (Ribeiro et al., 2014; Soares and Tavares-Dias, 2013; Oliveira et al., 2007; Vásquez et al., 2014). Bioactive products obtained from *L. origanoides* have also antioxidative effects, insecticidal properties against *Aedes aegypti*, antimicrobial and anti-protozoal effects, antigenotoxic properties, and are insect

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Table 1
Chemical constituents of the essential oil of *Lippia origanoides*.

Peak	% content	Retention index	Identification
1	0.5	853	(*E*)-2-hexenal
2	1.2	928	Alpha-thujene
3	0.5	936	Alpha-pinene
4	0.6	977	1-Octen-3-ol
5	2.4	989	Myrcene
6	1.1	1016	Alpha-terpinene
7	13.3	1025	p-Cymeno
8	0.9	1032	1,8-Cyneol
9	4.5	1059	Gamma-terpinene
10	2.8	1096	Linalool
11	0.4	1144	Ipsdienol
12	1.1	1175	Umbelulone
13	0.9	1232	Timil-methyl-ether
14	9.9	1288	Thymol
15	49.7	1298	Carvacrol
16	0.4	1369	Carvacryl acetate
17	1.5	1414	(E)-beta-caryophyllene
18	6.4	1487	Unterminated
19	0.7	1566	Unterminated
20	1.0	1576	Caryophyllene oxide
	Total identified (%): 92.9		

repellents (Oliveira et al., 2007; Escobar et al., 2010; Vicuña et al., 2010; Betancourt et al., 2012; Caballero-Gallardo et al., 2012; Barreto et al., 2014a, 2014b; Sarrazin et al., 2015a, 2015b; Teles et al., 2014; Vera et al., 2014). However, to the best of our knowledge, there are no existing studies exploring the potential of the essential oil of *L. origanoides* as a treatment against parasites in fish.

Colossoma macropomum Cuvier, 1818 (tambaqui) is an omnivorous fish of the Family Serassalmidae, native to the Amazon. It is an important species in aquaculture, and is cultivated in intensive systems, where high population densities favour parasites transmission (Dias et al., 2015). Such parasites may cause unquantified economic losses, thereby implementation of adequate monitoring and treatments are constant challenges for the farming of this species. Among the most common ectoparasites of farmed *C. macropomum* are the protozoan *Ichthyophthirius multifiliis* Fouquet, 1876 and the monogenoideans *Anacanthorus spathulatus* Kritsky, Thatcher & Kayton 1979, *Notozothecium janauachensis* Belmont-Jégu, Domingues & Martins 2004, *Mymarothecium boegeri* Cohen & Kohn, 2005 and *Linguadactyloides brinkmanni* Thatcher & Kritsky, 1983 (Dias et al., 2015; Martins et al., 2002; Soares et al., 2016). Products derived from natural plant sources are a potential alternative to the chemical products commonly used in aquaculture to treat parasite infestations

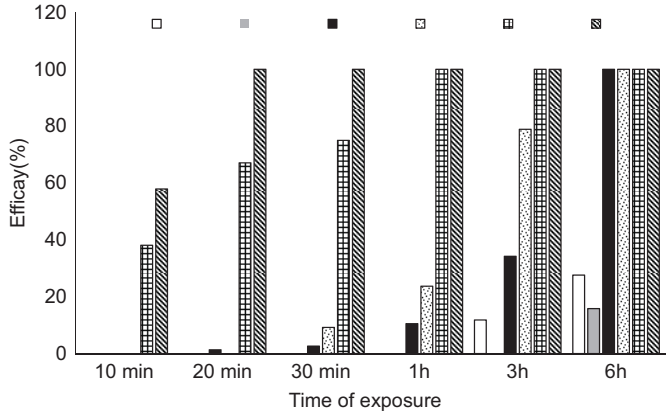

Fig. 1. *In vitro* efficacy of different concentrations of the essential oil of *Lippia origanoides* against monogenoideans of *Colossoma macropomum* 10 mg·L^{-1} 20 mg·L^{-1} 40 mg·L^{-1} 80 mg·L^{-1} 160 mg·L^{-1} 320 mg·L^{-1}.

Table 2
In vitro antiparasitic action of the essential oil of *Lippia origanoides* against monogenoideans of *Colossoma macropomum*, in relation to the concentration and time of exposure.

Time (h)	Treatments	No. of live parasites	Mortality (%)
0 h	Water	25.3 ± 4.5	0
	Water + alcohol	22.0 ± 2.6	0
	10 mg·L^{-1}	20.0 ± 0.0	0
	20 mg·L^{-1}	20.7 ± 1.2	0
	40 mg·L^{-1}	20.0 ± 0.0	0
	80 mg·L^{-1}	20.7 ± 1.2	0
	160 mg·L^{-1}	20.7 ± 1.2	0
	320 mg·L^{-1}	20.0 ± 0.0	0
10 min	Water	25.3 ± 4.5	0
	Water + alcohol	22.0 ± 2.6	0
	10 mg·L^{-1}	20.0 ± 0.0	0
	20 mg·L^{-1}	20.7 ± 1.2	0
	40 mg·L^{-1}	20.0 ± 0.0	0
	80 mg·L^{-1}	20.7 ± 1.2	0
	160 mg·L^{-1}	10.7 ± 4.2	48,3
	320 mg·L^{-1}	5.3 ± 2.5	73,5
20 min	Water	25.3 ± 4.5	0
	Water + alcohol	22.0 ± 2.6	0
	10 mg·L^{-1}	20.0 ± 0.0	0
	20 mg·L^{-1}	20.7 ± 1.2	0
	40 mg·L^{-1}	19.7 ± 0.6	1,5
	80 mg·L^{-1}	18.7 ± 2.3	9,7
	160 mg·L^{-1}	3.7 ± 4.7	82,1
	320 mg·L^{-1}	5.3 ± 2.5	73,5
30 min	Water	25.3 ± 4.5	0
	Water + alcohol	22.0 ± 2.6	0
	10 mg·L^{-1}	20.0 ± 0.0	0
	20 mg·L^{-1}	20.7 ± 1.2	0
	40 mg·L^{-1}	19.3 ± 1.2	3,5
	80 mg·L^{-1}	17.7 ± 3.2	14,5
	160 mg·L^{-1}	1.7 ± 2.9	91,8
	320 mg·L^{-1}	0.0 ± 0.0	100
1 h	Water	25.3 ± 4.5	0
	Water + alcohol	22.0 ± 2.6	0
	10 mg·L^{-1}	20.0 ± 0.0	0
	20 mg·L^{-1}	20.7 ± 1.2	0
	40 mg·L^{-1}	19.0 ± 1.7	5
	80 mg·L^{-1}	14.7 ± 2.1	29
	160 mg·L^{-1}	0.0 ± 0.0	100
	320 mg·L^{-1}	0.0 ± 0.0	100
3 h	Water	25.3 ± 4.5	0
	Water + alcohol	20.0 ± 5.0	9,1
	10 mg·L^{-1}	17.0 ± 5.2	15
	20 mg·L^{-1}	20.7 ± 1.2	0
	40 mg·L^{-1}	10.0 ± 2.0	50
	80 mg·L^{-1}	0.7 ± 1.2	96,6
	160 mg·L^{-1}	0.0 ± 0.0	100
	320 mg·L^{-1}	0.0 ± 0.0	100
6 h	Water	4.7 ± 4.6	81,4
	Water + alcohol	12.7 ± 10.7	42,3
	10 mg·L^{-1}	4.3 ± 2.3	78,5
	20 mg·L^{-1}	2.0 ± 1.0	90,3
	40 mg·L^{-1}	0.0 ± 0.0	100
	80 mg·L^{-1}	0.0 ± 0.0	100
	160 mg·L^{-1}	0.0 ± 0.0	100
	320 mg·L^{-1}	0.0 ± 0.0	100
8 h	Water	1.3 ± 2.3	94,9
	Water + alcohol	1.3 ± 1.5	94,1
	10 mg·L^{-1}	0.0 ± 0.0	100
	20 mg·L^{-1}	0.0 ± 0.0	100
	40 mg·L^{-1}	0.0 ± 0.0	100
	80 mg·L^{-1}	0.0 ± 0.0	100
	160 mg·L^{-1}	0.0 ± 0.0	100
	320 mg·L^{-1}	0.0 ± 0.0	100
9 h	Water	0.0 ± 0.0	100
	Water + alcohol	0.0 ± 0.0	100
	10 mg·L^{-1}	0.0 ± 0.0	100
	20 mg·L^{-1}	0.0 ± 0.0	100
	40 mg·L^{-1}	0.0 ± 0.0	100
	80 mg·L^{-1}	0.0 ± 0.0	100
	160 mg·L^{-1}	0.0 ± 0.0	100
	320 mg·L^{-1}	0.0 ± 0.0	100

(Hashimoto et al., 2016; Huang et al., 2013; Soares et al., 2016). While the potential of two plants of the genus *Lippia* as sources of anti-parasitic for fish have previously been studied with *Lippia sidoides* (Hashimoto et al., 2016), and *Lippia alba* (Soares et al., 2016), the present study represents the first evaluation of this potential use of essential oil of *L. origanoides*.

The aims of this study were to investigate *in vivo* and *in vitro* anti-parasitic activity of the essential oil of *L. origanoides* against monogenoideans from the gills of *C. macropomum*, and to evaluate the possible impacts on the blood and gills of the fish as a result of essential oil exposure.

2. Material and methods

2.1. Essential oil extraction and composition

Cultivation of *L. origanoides* and extraction of the essential oil were carried out in the Medicinal Plants and Vegetables sector of Embrapa Western Amazon, in Manaus (03°06′23.04″S and 60°01′ 35.14″W), state of Amazonas, Brazil. Mean altitude is 50 m and mean air temperature is 25.6 °C with annual rainfall of 2200 mm. Plants were collected in the morning and the material processed in the Medicinal Plants and Phytochemistry Laboratory of Embrapa Western Amazon, Manaus (Brazil). The essential oil was obtained from leaves of *L. origanoides* using a Clevenger apparatus. Chemical analysis of the essential oil was carried out by gas chromatography, coupled with a mass spectrometer. The chemical components of the oil used in this study are shown in Table 1.

2.2. Fish

The experiments were conducted in the Aquatic Animal Health Lab of Embrapa Amapá (Macapá, state of Amapá, Brazil). *Colossoma macropomum* fingerling (±30 g) were obtained from commercial fish farms, and were acclimatised over a 7-day period in 500 L water tanks, being fed a diet containing 32% gross protein. The water in the tanks was constantly renewed and the following parameters of the water were monitored: temperature (30.7 ± 0.2 °C), dissolved oxygen (5.6 ± 0.4 mg·L^{-1}), pH (5.3 ± 0.2), ammonia (0.4 ± 0.2 mg·L^{-1}), alkalinity (10.0 ± 0 mg·L^{-1}) and hardness (10.0 ± 0 mg·L^{-1}) using multi-parameters device (YSI, USA). Accumulated organic material was removed from the bottom of the tanks daily.

2.3. In vitro trial with L. origanoides essential oil and monogenoideans of C. macropomum

To evaluate the concentrations and exposure times necessary to cause mortality, *in vitro* tests were conducted using monogenoidean parasites collected from the gills of 24 *C. macropomum* fingerling (11.9 ± 2.9 cm e 35.2 ± 25.0 g), in accordance with the methodology used by Soares et al. (2016). For this trial in Petri dish, two control groups were established, one using only water of the fish culture tank, and the other using tank water and absolute ethanol, which was the solvent used to dilute the essential oil in a ratio of 1:10. Three replicates of six treatment groups were also established with concentrations of 10, 20, 40, 80, 160 and 320 mg·L^{-1} of *L. origanoides* essential oil. Based on the *in vitro* results, and after a preliminary test of the fish's tolerance to the essential oil, concentrations to be tested *in vivo* were set as 20 and 40 mg·L^{-1}.

From the *in vitro* results, the concentrations used in the therapeutic baths with the essential oil of *L. alba* were determined, after conducting a tolerance test on fish. All *in vitro* trial were performed at environment temperature of 17 °C and using stereomicroscopes of cold light.

2.4. In vivo trial with C. macropomum exposed to L. origanoides essential oil

Fingerling of *C. macropomum* (13.2 ± 1.1 cm e 42.4 ± 10.1 g), naturally parasitized by *A. spathulatus*, *N. janauachensis* and *M. boegeri*, were randomly distributed in twelve 100 L tanks and maintained in an open water system during 48 h for acclimation. Three replicates for each treatment and two control groups were established: a control with only water, and a control with water and absolute ethanol at a ratio of 1:10, both exposed for 60 min. Two treatments with *L. origanoides* essential oil at concentrations of 20 mg·L^{-1} exposed for 60 min and 40 mg·L^{-1} exposed for 30 min were also used. Each replicate consisted of 20 fish, and the water system was maintained at an mean temperature of 30.7 ± 0.2 °C, dissolved oxygen of 5.6 ± 0.4 mg·L^{-1}, pH of 5.3 ± 0.2, ammonia of 0.4 ± 0.2 mg·L^{-1}, alkalinity of 10.0 ± 0 mg·L^{-1} and hardness of 10.0 ± 0 mg·L^{-1}.

After the required time had passed, the water in the tanks was maintained in continuous flux, and the gills of 10 fish from each replicate were collected and fixed in 5% formalin, for parasite identification and quantification. The parasites were prepared for identification using previous recommendations (Eiras et al., 2006). Based on quantifications, parasite prevalence and mean abundance of infection were calculated (Bush et al., 1997), and the effectiveness of each treatment was calculated (Zhang et al., 2014). The rest of the specimens were used for histopathological analyses.

Blood was collected from the caudal vein of five fish from each replicate (15 fish per control/treatment group), using syringes with 10% EDTA, and divided in two aliquots. One aliquot was used for counting red blood cells, determining haematocrit using the micro-haematocrit method and measuring haemoglobin concentration using the cyanmethaemoglobin method. Based on these data, Wintrobe's haematometric indices – mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) – were calculated. Blood smears were prepared and stained with a combination of May Grünwald-Giemsa-Wright (Ranzani-Paiva et al., 2013), and differential leucocyte counts were conducted in up to 200 cells of interest in each blood smear. Identification and nomenclature of leucocytes followed those suggested by Tavares-Dias et al. (1999). The blood smears were also used to count the total number of leucocytes and thrombocytes (Ranzani-Paiva et al., 2013).

A second aliquot of blood was centrifuged at 75 G to obtain plasma for analysis of total glucose and plasma proteins. The concentration of glucose was determined by the enzymatic colorimetric method, and of plasma proteins by the biuret method, using commercial kits (Biotécnica, MG, Brazil). For both biochemical analyses, the readings were made in a spectrophotometer.

Table 3
Prevalence (P) and mean abundance (MA) of the gill parasites in *Colossoma macropomum* exposed to the essential oil of *Lippia origanoides*.

Species of parasites	Water (n = 30) 60 min		Water + alcohol (n = 30) 60 min		20 mg·L^{-1} (n = 30) 60 min		40 mg·L^{-1} (n = 30) 30 min	
	P (%)	MA	P (%)	MA	P (%)	MA	P (%)	MA
Ichthyophthirius multifiliis	96.3	80.3 ± 47.0^{ab}	96.7	67.2 ± 38.5^{a}	90	108.5 ± 79.9^{b}	82.6	52.7 ± 59.1^{a}
Monogenoidea species	100	341.3 ± 67.3^{a}	100	333.7 ± 86.9^{a}	100	316.2 ± 79.3^{a}	100	352.3 ± 67.3^{a}

Different letters on the same line indicate significant differences by Dunn test ($p < 0.05$).

Table 4
Blood parameters of *Colossoma macropomum* exposed to the essential oil of *Lippia origanoides*.

Parameters	Water (n = 15)	Water + alcohol (n = 15)	20 mg·L^{-1} (n = 15)	40 mg·L^{-1} (n = 15)
Body weight (g)	42.4 ± 11.5^{a}	40.6 ± 8.5^{a}	41.2 ± 8.9^{a}	39.3 ± 9.0^{a}
Length (cm)	13.6 ± 1.2^{a}	13.1 ± 0.9^{a}	12.9 ± 1.0^{a}	13.0 ± 1.3^{a}
Glucose (g·dL^{-1})	97.5 ± 16.9^{a}	104.2 ± 25.4^{a}	99.8 ± 21.6^{a}	99.4 ± 21.6^{a}
Proteins (mg·dL^{-1})	2.5 ± 0.4^{a}	3.2 ± 0.8^{a}	3.6 ± 1.1^{b}	3.6 ± 0.5^{b}
Erythrocytes (×10^{6}·μL^{-1})	1.07 ± 0.15^{a}	1.13 ± 0.23a	1.18 ± 0.47a	0.96 ± 0.17a
Haemoglobin (g·dL^{-1})	5.3 ± 0.6a	5.6 ± 0.5^{b}	5.0 ± 0.6a	5.0 ± 0.5a
Haematocrit (%)	17.5 ± 1.5^{a}	17.7 ± 2.4^{a}	15.8 ± 1.7^{b}	15.1 ± 1.8^{b}
MCV (fL)	166.0 ± 25.5^{a}	160.9 ± 28.9^{a}	145.9 ± 38.6a	162.2 ± 37.8^{a}
MCHC (g·dL^{-1})	30.4 ± 2.6^{a}	32.3 ± 4.6a	31.7 ± 2.5^{a}	33.2 ± 4.1^{a}
Thrombocytes (μL)	26,144 ± 9993^{a}	22,980 ± 7965^{a}	23,434 ± 10,704^{a}	20,325 ± 5129^{a}
Leukocytes (μL)	10,114 ± 2524^{a}	9702 ± 4541^{a}	12,895 ± 5465^{a}	11,761 ± 3412^{a}
Lymphocytes (μL)	6566 ± 2235^{a}	5695 ± 2398^{a}	4800 ± 2287^{a}	5499 ± 2040^{a}
Monocytes (μL)	1075 ± 338^{a}	1150 ± 549^{a}	2659 ± 1164^{b}	1661 ± 960^{b}
Neutrophils (μL)	2272 ± 1057^{a}	2444 ± 1521^{a}	5197 ± 2031^{b}	4489 ± 2012^{b}
Eosinophils (μL)	27 ± 38^{a}	9 ± 28^{a}	14 ± 32^{a}	22 ± 43^{a}
PAS-GL (μL)	173 ± 306^{a}	392 ± 774^{a}	324 ± 396^{a}	89 ± 77^{a}

Data are expressed as mean ± standard deviation. Different letters in the same line indicate significant difference by Tukey test ($p < 0.05$). MCV: mean corpuscular volume, MCHC: mean corpuscular haemoglobin concentration, PAS-positive granular leukocytes (PAS-GL).

2.5. Histopathological analyses of C. macropomum gills exposed to L. origanoides essential oil

The gills of six fish per treatment/control group (two fish per replicate) were used for histopathological analyses immediately after the end of the experiment. Twenty four hours post treatment, the gills of another six fish per treatment/control group (two fish per replicate) were also analysed, to look for recuperation. The first right gill arch of each fish was collected and fixed in formalin (10%), then dehydrated through a gradual series of ethanol and xylol, embedded in paraffin and cut with a microtome to produce consecutive sections. The histological sections were stained with haematoxylin and eosin (HE) and viewed under a light microscope (Soares et al., 2016). The histopathological analysis was performed semi-quantitatively using the mean assessment values (MAV) (Schwaiger et al., 1997) and the histopathological alteration index (HAI) (Poleksic and Mitrovic-Tutundzic, 1994).

2.6. Statistical analyses

Shapiro-Wilk and Bartlett tests were used to check for normality and homoscedasticity, respectively, and as the data did not meet these assumptions, a Kruskal-Wallis followed by Tukey test were used to compare the medians ($p < 0.05$).

3. Results

3.1. In vitro anti-parasitic action of L. origanoides essential oil against monogenoideans

Concentrations of essential oil of 320 and 160 mg·L^{-1} were 100% effective against *A. spathulatus*, *N. janauachensis* and *M. boegeri* from the gills of *C. macropomum* with 30 and 60 min exposure, respectively. At a concentration of 80 mg·L^{-1}, the essential oil was approximately 80% effective with 3 h of exposure, reaching 100% with 6 h of exposure. The oil was also 100% effective at a concentration of 40 mg·L^{-1}, with 6 h of exposure. The two lower concentrations, of 10 and 20 mg·L^{-1}, were not very effective, failing to reach 40% mortality in 6 h of exposure, and requiring 8 h to reach 100% mortality. In the control groups, mortality began after 3 h (only water) and 1 h (water + alcohol), and in both groups 100% mortality occurred after 8 h (Fig. 1 and Table 2).

3.2. Antiparasitic action of L. origanoides essential oil in C. macropomum

There was no difference in abundance of *A. spatulathus*, *M. boegeri*, *N. janauachensis* or *I. multifiliis* between control and treatment groups (Table 3), indicating that, at the concentrations used, the essential oil was not effective against these gill parasites. During the application of

Table 5
Mean alteration value (MAV) and histopathological alteration index (HAI) of the gills of *Colossoma macropomum* exposed to the essential oil of *Lippia origanoides*.

Treatments	n	MAV	HAI	Severity of the lesions according to the HAI
After 30 min therapeutic bath				
Water	6	1.3 ± 0.5^{aA}	46.3 ± 49.8^{aA}	Moderate to severe alterations to the gills
Water + alcohol	6	1.2 ± 0.4^{aA}	16.5 ± 7.9^{aA}	Low to moderate alterations to the gills
20 mg·L^{-1} 60 min	6	1.8 ± 0.4^{aA}	87.3 ± 59.6^{aA}	Severe alterations to the gills
40 mg·L^{-1} 30 min	6	1.7 ± 0.8^{aA}	51.2 ± 54.6^{aA}	Severe alterations to the gills
After 24 h of recovery subsequent to therapeutic bath				
Water	6	1.2 ± 0.4^{aA}	16.0 ± 7.8^{aA}	Low to moderate alterations to the gills
Water + alcohol	6	1.2 ± 0.4^{aA}	18.2 ± 5.7^{aA}	Low to moderate alterations to the gills
20 mg·L^{-1} 60 min	6	1.5 ± 0.5^{aA}	52.0 ± 53.7^{aA}	Severe alterations to the gills
40 mg·L^{-1} 30 min	6	1.3 ± 0.5^{aA}	13.7 ± 9.7^{aA}	Low to moderate alterations to the gills

The same lower-case letter in the same column indicates that there were no differences between the treatments, while upper-case letter in the same column indicates differences between the times, according to the Tukey test ($p < 0.05$).

the therapeutic baths, the following behaviours were observed in the fish: normal in the water-only control, moderate agitation in the water + alcohol control, and immobilization and sinking to the bottom of the tank in both essential oil treatments. After the end of the bath, when a continuous water flow was re-established, the fish in the treatment groups gradually returned to normal swimming behaviour, and there was no fish mortality recorded.

3.3. Effects on blood parameters in C. macropomum exposed to L. origanoides essential oil

The level of plasma proteins, and the number of monocytes and neutrophils increased in fish exposed to the essential oil at both concentrations (20 and 40 $mg \cdot L^{-1}$), and haematocrit declined, while other measured parameters showed no change. The 60 min bath in water + alcohol led to an increase in haemoglobin, relative to fish in the other control and treatment groups (Table 4).

3.4. Histopathological effects on the gills of C. macropomum exposed to L. origanoides essential oil

Immediately and 24 h after therapeutic baths, there was no change in MAV or HAI among treatments. Unfortunately, after exposure to the *L. origanoides* essential oil, the severity of gill lesions in treated fish varied from those in the water-treated control group, in accordance with the index of histopathological change, with

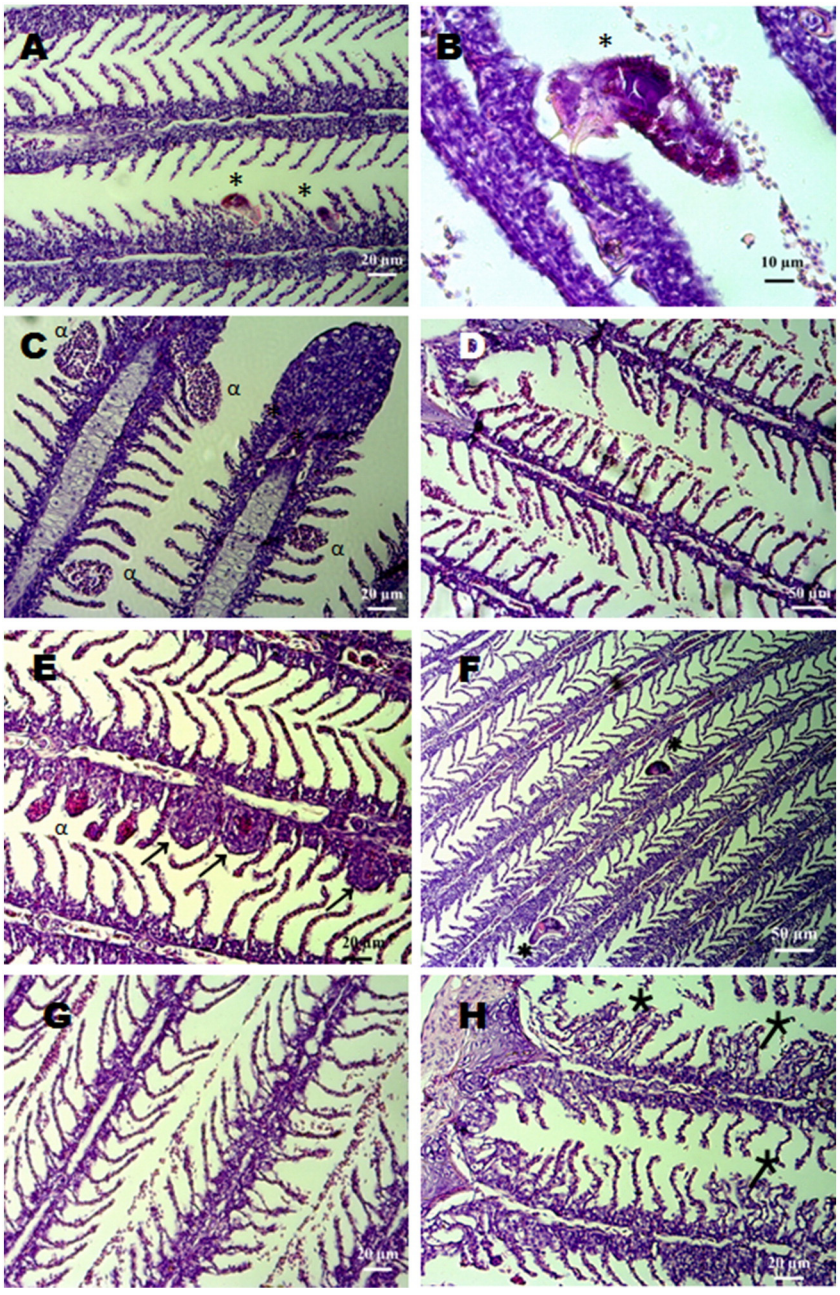

Fig. 2. A–H. Histological alterations on the gills of *Colossoma macropomum* exposed to 20 and 40 $mg \cdot L^{-1}$ of essential oil of *Lippia origanoides*. (A) Monogenoideans (*) and lamellar hyperplasia in gills of fish exposed to water. (B) Monogenoideans (*) in gills of fish exposed to 40 $mg \cdot L^{-1}$ of essential oil. (C) Aneurysm (α) in lamellar extremity of fish gills exposed to water + alcohol. (D) Epithelial disruption with widespread bleeding in the gills of fish exposed to 20 $mg \cdot L^{-1}$ of essential oil. (E) Aneurysm (α) and lamellar hyperplasia (arrow) in gills of fish exposed to 20 $mg \cdot L^{-1}$ of essential oil. (F) Monogenoideans (*), central blood vessel dilation and widespread lamellar hyperplasia in gills of fish exposed to 40 $mg \cdot L^{-1}$ of essential oil, after 24 h. (G) Epithelial disruption with disseminated haemorrhage in gills of the fish exposed to 40 $mg \cdot L^{-1}$ of essential oil. (H) Epithelial displacement (⁎) in gills of the fish exposed to 40 $mg \cdot L^{-1}$ of essential oil after 24 h of recovery.

moderate to severe damage recorded. Slight to moderate damage was recorded on gills of fish in the water + alcohol control group. After the 24 h recuperation period, the gills of fish in the two control groups and the 40 mg·L^{-1} treatment group were slightly to moderately damaged, while the gills of fish in the 20 mg·L^{-1} treatment groups were severely damaged (Table 5). Histological changes (hyperplasia and fusion of the lamellar epithelium, capillary dilation, displacement of the lamellar epithelium, and lamellar aneurism and epithelial rupturing with haemorrhaging) are shown in Fig. 2A–H. Oedema, mucous and chloride cell proliferation, lamellar hypertrophy, congestion and necrosis were less frequently observed.

4. Discussion

Analysis of the *L. origanoides* essential oil used in this study indicated that its major chemical constituents are carvacrol, p-cymene and thymol. Similar results have been reported previously with *L. origanoides* essential oil (Teles et al., 2014; Ribeiro et al., 2014; Sarrazin et al., 2015a, 2015b; Vera et al., 2014; and Vicuña et al., 2010). Thymol and carvacrol have been shown to have antimicrobial, antigenotoxic and anti-protozoal properties, and may therefore be responsible for the bioactive effects of *L. origanoides* essential oil (Nostro et al., 2004; Sarrazin et al., 2015a, 2015b; Vicuña et al., 2010; Escobar et al., 2010). However, bioactivity may also be due to a synergism of the chemical components of the essential oil (Barreto et al., 2014a).

The *in vitro* test showed that at low concentrations (10, 20, 40 mg·L^{-1}) the *L. origanoides* essential oil was not an efficient antiparasitic against the monogenoideans *A. spatulathus*, *M. boegeri* and *N. janauachensis*, while higher concentrations (80, 160 and 320 mg·L^{-1}) had a dose-dependent efficacy. Soares et al. (2016) also showed a dose-dependent efficacy of the essential oil of *L. alba* against these same parasites, using concentrations of 160, 320, 640, 1280 and 2560 mg·L^{-1}. Essential oil trials with *L. sidoides*, at concentrations of 40, 80, 160 and 320 mg·L^{-1} have also shown that the highest concentrations (160 and 320 mg·L^{-1}) were effective against the monogenoideans *Cichlidogyrus tilapiae*, *Cichlidogyrus thurstonae*, *Cichlidogyrus halli* and *Scutogyrus longicorni*, from the gills of *Oreochromis niloticus* (Hashimoto et al., 2016). Despite being congeneric species, different concentrations and chemical compositions may have influenced the effectiveness of these three essential oils against the parasites *in vitro*.

An anaesthetic effect was observed in the *C. macropomum* during the therapeutic baths with concentrations of both 20 and 40 mg·L^{-1} of the *L. origanoides* essential oil. A similar effect was reported for *C. macropomum* exposed to 100 and 150 mg·L^{-1} of *L. alba* essential oil (Soares et al., 2016) and *O. niloticus* exposed to 40 mg·L^{-1} of *L. sidoides* essential oil (Hashimoto et al., 2016). Furthermore, the baths showed no efficacy against the monogenoideans *A. spatulathus*, *M. boegeri* and *N. janauachensis*, or against *I. multifiliis*. However, therapeutic baths with extracts of *Caesalpinia sappan*, *Lysima chiachristinae*, *Cuscuta chinensis*, *Artemisia argyi*, and *Eupatorium fortunei* have shown efficacy against *Dactylogyrus intermedius* parasitizing *Carassius auratus* (Huang et al., 2013). Ji et al. (2012) also found anthelmintic activity against *D. intermedius* parasitizing *C. auratus* using extracts of *Cinnamomum cassia*, *Lindera aggregata* and *Pseudolarix kaempferi*. The present study represents the first trial of *L. origanoides* as an antiparasitic in fish.

Plasma protein concentrations and the number of monocytes and neutrophils, increased in *C. macropomum* exposed to 20 and 40 mg·L^{-1} of the *L. origanoides* essential oil, whereas haematocrit decreased. These results are similar to those of Soares et al. (2016) who found decreased haematocrit and increased plasma protein concentrations and neutrophil numbers in *C. macropomum* exposed to 100 and 150 mg·L^{-1} of *L. alba* essential oil, and those of Hashimoto et al. (2016) who found increased numbers of neutrophils in *O. niloticus* exposed to 40 mg·L^{-1} of *L. sidoides* essential oil. Therefore, in summary, evidence shows that the essential oils of *Lippia* congeneric species, when used in low concentrations in therapeutic bathing, cause moderate changes in the blood of exposed fish.

Our results show damage to the gills after exposure to *L. origanoides* essential oil. However, these lesions were most likely caused by the parasites themselves as they occurred frequently in all treatments and controls. Indeed, *L. origanoides* essential oil has been previously shown to not be very cytotoxic in rat (Sarrazin et al., 2015b), insect (Caballero-Gallardo et al., 2012) and other mammalian cells (Escobar et al., 2010). Furthermore, similar gill lesions have also been described for *Piaractus brachypomus* (Verján et al., 2001) and *Rachycentron canadum* (Guerra-Santos et al., 2012), infected by different species of parasites. However, severe and irreparable damage to gills has been previously reported for *C. macropomum* exposed to the essential oil of *L. alba* at concentrations of 100 and 150 mg·L^{-1} (Soares et al., 2016). The diluted alcohol used as a control in this study did not cause blood and histopathology alteration in the gills of *C. macropomum*, as have been recorded for some other types of diluents (Hashimoto et al., 2016; Steverding et al., 2005).

5. Conclusions

The essential oil of *Lippia origanoides* possesses antiparasitic properties *in vitro*, with dose-dependent efficacy. However, even at the low concentrations tested here, the oil has an anaesthetic effect on *C. macropomum*, and furthermore, although it causes few histopathological and blood changes, at these concentrations it is not effective against parasites of *C. macropomum*. As such, *L. origanoides* essential oil can not be recommended as a treatment against ectoparasites. However, owing to its *in vitro* effects, *in vivo* trials should now be conducted using the constituent components of *L. origanoides* essential oil to test their efficacy against parasites of this fish species, and their effects on host fish.

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